Blossom Damania
James M. Pipas
Editors

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ISBN: 978-0-387-68944-9 e-ISBN: 978-0-387-68945-6
DOI 10.1007/978-0-387-68945-6

Library of Congress Control Number: 2008939847

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Cover illustration: This cartoon depicting SV40 was created by Stefan A. Welte, University of Tübingen using ProteinExplorer [1] (www.proteinexplorer.org) and the PDB code 1sva [2].

[1] Martz, E. 2002. Protein Explorer: easy yet powerful macromolecular visualization. Trends Biochem.Sci. 27:107–109. [2] Liddington R. C. et al. Structure of simian virus 40 at 3.8-A resolution. Nature 1991 Nov 28:354(6351):278–84.

Printed on acid-free paper

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This book is dedicated to our mothers:
Loretta Margaret Damania and Jean Moore Pipas.

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Introduction: DNA Tumor Viruses

Viruses are as fascinating as they are diverse. Viruses are important because they are disease-causing agents and the prevention of viral infection and the development of antiviral therapies will ease human suffering. However, viruses are also major drivers of modern molecular and cellular biology. They serve as powerful model systems for macromolecular processes such as DNA replication, transcription, and for the assembly of multicomponent structures and machines. Furthermore, because all viruses encode gene products that redirect cellular systems to virus production or antagonize host antiviral defense systems, viruses are also important tools for the discovery and elucidation of cellular pathways that regulate cell proliferation, apoptosis, or the innate and adaptive immune responses. Viruses are even used as therapeutic agents, serving as vectors for gene therapy, or as targeted anticancer agents.

Tumor viruses are viruses that induce cancer in their natural hosts or in experimental animal systems. Tumor viruses comprise a small subset of the 2,000 or so recognized viruses and they have been broadly classified into RNA tumor viruses and DNA tumor viruses based on the nature of the nucleic acid contained within their virion. Some members of both these classes of tumor viruses are associated with human cancer. These include HTLV and Hepatitis virus C for the RNA tumor viruses, and EBV, KSHV, Hepatitis virus B, and specific strains of HPV for the DNA tumor viruses (Table 1).

The mechanisms by which RNA and DNA tumor viruses induce cancer in humans or experimental animals are different but there are also several common themes shared by these viruses, and their study has greatly contributed to our understanding of the molecular events that drive tumorigenesis. This book reviews our current knowledge of the DNA tumor viruses: their genome structure, infectious cycle, and how viral infection contributes to cancer. In this introductory chapter we review current theories of tumorigenesis and the role that DNA tumor viruses play in understanding the molecular mechanisms that drive tumor initiation and progression.

Table 1 Tumor viruses and their association with human cancer

Retroviridae	HTLV-1, 2	T cell 1eukemia
Polyomaviridae	MCPyV	Merkel cell carcinoma?
Papillomaviridae	HPV-16, 18	Cervical carcinoma
Adenoviridae		None
Herpesviridae	EBV	Burkitt's lymphoma
		Hodgkin's lymphoma
		Nasopharyngeal carcinoma
		Post – transplant lymphoproliferative diseases
	KSHV	Kaposi's sarcoma
		Primary effusion lymphoma
		Multicentric Castleman's disease
Hepadnaviridae	Hepatitis B	Hepatocellular carcinoma
Flavivirdae	Hepatitis C	Hepatocellular carcinoma

Molecular Genetics of Cancer

Cancer is one of the most intriguing and vexing diseases that faces mankind. It occurs in nearly all the diverse tissue types present in higher metazoans, and even within a given tissue, cancer often occurs in multiple forms, each presenting unique properties and clinical behavior. Some properties of cancers clearly reflect their tissue or origin. Other properties are the consequence of genetic and epigenetic events that have occurred during tumor initiation or progression. Still others are probably a reflection of the particular cell-type within a tissue from which the cancer originated. One of the challenges of molecular oncology is to discern how all these factors contribute to tumorigenesis and to identify points that present opportunities for clinical intervention.

Epidemiologic and molecular studies suggest that tumorigenesis requires multiple events. Several lines of evidence indicate that genetic mutation is a major mechanism that drives cancer initiation and progression [3]. Most cancers do not result from a single hit to one gene, but from many such events. Thus, cancer arises from multiple genetic changes and most of these are very rare occurrences. Not all mutations lead to cancer and most mutations are likely to be in genes that do not contribute to cancer. However if the mutation occurs in a proto-oncogene or a tumor suppressor, it predisposes that particular cell to tumorigenesis and additional hits may then lead to full-blown cancer. Once the right combination of genetic mutations occur in a single cell, the cell loses its normal growth restriction and begins to proliferate uncontrollably.

The observation that cancers contain mutations affecting many different genes is consistent with these notions. In fact, sequence analysis of multiple breast and colorectal tumors reveals a surprising number of mutations, many of which occur in a subset of the tumor types examined [4]. At present, it is difficult

to discern which of these mutations directly contribute to tumorigenesis and which are merely the consequences of the tumorigenic state. In part, this is because genetic instability is a hallmark of many cancers. At the present time the question as to whether genetic instability contributes to tumor progression remains controversial. Much effort is currently focused on identifying specific mutations that are critical for tumorigenesis and on understanding the role that the affected gene products play in this process.

In a broad sense, the products of genes mutated in cancers fall into three classes: oncogenes, tumor suppressors, and those that affect the fidelity of DNA replication, DNA repair, or chromosome segregation and cytokinesis. Proto-oncogenes, the wild-type version of the genes from which oncogenes arise, are generally components of signal transduction pathways that govern cell proliferation or arrest, cell survival or death, or other cell behaviors that are less well understood. Genetic changes that convert proto-oncogenes to oncogenes usually give rise to amino acid substitutions or small deletions that result in expression of an altered protein. These mutations render the oncoprotein refractory to regulation by upstream signals of the pathway and/or lock the oncoprotein into a constitutively active state. In contrast, tumor suppressors encode proteins that normally function to block cell proliferation and/or enhance cell death. The mutations found in cancer that affect tumor suppressor genes generally lead to a loss of function, sometimes including complete deletion of the gene.

The picture emerging from molecular studies suggests that these mutations contribute to tumorigenesis by misregulating specific signal transduction pathways. Furthermore, since these pathways are regulated at different points along the signaling cascade, the dysregulation can affect any of several proteins that regulate the pathway. An example of this principle is illustrated by the retinoblastoma (Rb) and p53 tumor suppressor pathways (Fig. 1). One critical function of Rb proteins is to regulate the expression of genes needed to enter and progress through the cell cycle. Many such genes require E2F transcription factors for their expression. In growth-arrested cells, E2F-dependent transcription is inhibited by one or more of the Rb-family of tumor suppressor

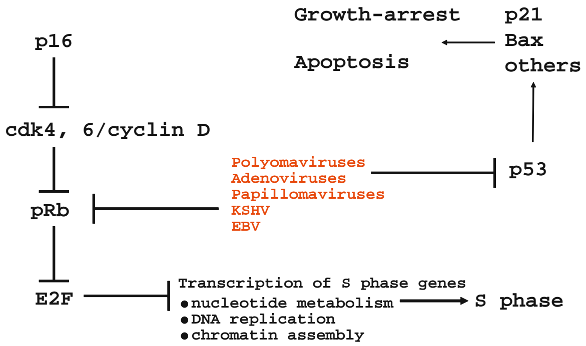

Fig. 1 Diagram of the Rb and p53 pathways showing points where mutations occur in cancer and where viral transforming proteins act

proteins, pRb, p107, and p130. Several upstream signals activate or inhibit cyclin-dependent kinases (cdk4 or 6) that phosphorylate Rb proteins and block their ability to repress E2F-dependent transcription. The Rb-pathway is thought to be dysregulated in almost all cancers. However, this misregulation occurs at different steps of the pathway in different cancers, with some showing deletion of the Rb gene, others showing constitutive activation of the Rb-inactivating kinases, and others having deletions of negative regulators of these kinases. Similarly, most cancers harbor mutations that inactivate the p53 tumor suppressor pathway. Some of these affect the p53 gene directly while others alter proteins that modulate p53 activity [3].

The path from a "normal" cell to an invasive solid tumor is thought to involve progression through several histopathological states. In an idealized case these might include: hyperplasia, an abnormal increase in cell density; dysplasia, indicated by disorganized tissue architecture; benign tumor, a non-invasive neoplastic tissue mass; invasive tumor, where the cancer penetrates adjacent tissues; and metastasis, where tumor cells establish colonies in tissues distant from the site of the primary tumor. Progression from one of these stages to the next is thought to be driven by mutations. Presumably each mutation affects a different signaling pathway, thus altering some aspect of cellular behavior that results in the outgrowth of descendent cells harboring the mutation. Since each pathway is regulated at multiple points, mutations misregulating the pathway can affect any of several targets. Thus, each cancer would be expected to have one of several possible combinations of mutations, or genetic signature. Two of the current challenges in cancer research are to understand how the misregulation of each specific pathway alters cell behavior and thus contributes to tumor progression; and how the misregulation of different combinations of pathways relates to specific tumor properties such as invasiveness and metastasis [2].

Tumor Viruses

Where do viruses fit into this picture and what can they teach us about tumorigenesis?

RNA Tumor Viruses

Specific members from several families of viruses induce tumors in experimental animals. Some of these are associated with human cancer [1]. Three of these are hepatitis B virus, hepatitis C virus, and human T cell leukemia virus (HTLV-1 and 2). In these cases the molecular mechanisms by which they contribute to cancer is unclear. However, much has been learned from the study of animal retroviruses, specifically members of the group *Oncovirinae*.

RNA tumor viruses induce neoplastic transformation by one of two mechanisms, insertional mutagenesis or oncogene capture. The integration of a DNA copy of the retroviral genome into the infected cell's chromosome is an obligatory step of their productive infectious cycle. Since this integration occurs at a random position in the host chromosome, some of the integration events occur within cellular genes resulting in loss of gene expression of the encoded protein's function. For example, integration within a tumor suppressor gene can contribute to tumorigenesis by inactivating tumor suppressor function. Since many of these viruses are not cytotoxic and infection results in substantial viremia, insertional mutagenesis has proved a powerful tool for identifying tumor suppressor genes.

Sometimes aberrant retrovirus replication leads to the capture and altered expression of cellular genes. If a proto-oncogene is captured and becomes mutationally activated during the course of retroviral replication, progeny virus expressing the oncogene will induce neoplasia. This property of RNA tumor viruses has been exploited, primarily in the mouse and chicken but other systems as well, to identify over one hundred oncogenes.

DNA Tumor Viruses

Early studies of murine polyomavirus, simian virus 40 (SV40), and human adenoviruses established that these DNA tumor viruses encode dominantly acting transforming proteins [1]. At present, it appears that this is true of all the DNA tumor viruses. Each of these viruses encodes one or more proteins that contribute to transformation by interacting directly with and altering the activity of cellular targets. In general, the action of these viral proteins is either to activate specific oncogenes or to inhibit tumor suppressors. In fact, the tumor suppressors p53 and pRb were discovered because of their interaction with viral-transforming proteins (Fig. 1). Some of these viruses also encode noncoding RNAs, some of which act on cellular targets. To date, it is not clear if these RNAs contribute to tumorigenesis or are associated with immune evasion or viral productive infection.

What is Transformation and How Does It Relate to Tumorigenesis?

A cell is transformed when it stably acquires the ability to proliferate and survive in an environment that would normally lead to growth arrest or cell death. Consequently, various assays for the transformed phenotype have been developed that monitor the ability of the cell to grow in various types of growth-restrictive conditions. Viral-transforming genes are generally identified by developing stable cell lines that express the gene products and then assessing their ability to proliferate and survive in one of these assays.

Primary cells can grow and divide when placed in culture but their proliferative capacity is limited and they will eventually enter senescence. Immortalized cells are cells whose life spans have been extended beyond what is normal for primary cells. Immortalization is generally thought to occur through a telomerase-dependent pathway in which there is upregulation of telomerase activity in these cells, but can also occur via a telomerase-independent ALT pathway. However immortalized cells do not grow indefinitely in culture. Transformed cells on the other hand can grow indefinitely in culture and have a number of properties including loss of contact inhibition, loss of cell-cycle control, growth in reduced serum conditions, karyotypic instability, and tumor formation when introduced into immunocompromised mice. These properties are unique to transformed cells and are not shared by either primary or immortalized cells. Based on these biological properties, a number of different assays for transformation have been developed. The assays outlined below can be performed to determine (i) whether specific cells are transformed or cancerous and (ii) whether a putative gene is transforming. In case of the latter, the gene is introduced into a particular cell line and the biological properties of the cells expressing this gene are compared to the parental cell line.

(1) *Focus formation*. Many types of cultured cells growth arrest upon contacting their neighbors. In contrast, transformed cells are not growth inhibited when they come in contact with neighboring cells. As a consequence they grow as multiple layers on top of normal cell monolayers. The resulting outgrowth of dense, transformed cells is termed a focus. The focus formation assay is a common method for detecting viral or cellular oncoproteins.
(2) *Morphological and cytoskeletal changes*. Transformed cells can sometimes be distinguished from their counterparts because they do not attach well to surfaces and display altered shapes. These phenomena are primarily due to a disorganization of the cytoskeleton. The microtubules and actin filaments of transformed cells appear to be disorganized rather than being ordered in a highly organized fashion.
(3) *Growth in reduced serum conditions*. Many types of cells require a basal nutrient medium supplemented with serum for growth and survival in culture. Transformed cells can grow in media containing little or no serum. Thus, culture in the absence of serum can be used as both a test for transformation and as a selection for transformed cells.
(4) *Anchorage independent growth*. Some types of cells must adhere to a solid surface, such as coated plastic of a culture dish, in order to proliferate. When such cells are suspended in a semi-solid environment, such as 0.6% agarose, supplemented with nutrient medium and serum, they remain viable as suspended, growth-arrested, single cells for weeks. On the other hand, transformed cells proliferate under these conditions, forming large clonal aggregates that are often visible to the naked eye. The ability of viral

or cellular proteins to induce anchorage-independent proliferation is a common assay for transformation.

(5) *Metabolic changes*. Under aerobic conditions, most cells have a minimal rate of glycolysis and oxidize most of their glucose to carbon dioxide and water in the process called respiration. However, many (but not all) transformed cells have an increased rate of glycolysis, the anaerobic breakdown of glucose to form ATP, even in the presence of all the enzymes required for aerobic respiration. These cells convert large amounts of glucose to lactate, a phenomena known as the Warburg effect. Thus, the overall metabolic requirement of many transformed cells is significantly higher than normal cells.

(6) *Tumors in nude mice*. Viral or cellular genes can be tested for the ability to convert nontumorigenic cells to tumorigenic cells. When injected into immunocompromised mice, such as nude mice or severe combined immunodeficiency (SCID) mice that lack T and B cells, transformed cells form tumors whereas primary and immortalized cells do not.

(7) *Transgenic animals*. An assay used to demonstrate the transforming nature of a suspected oncogene is to construct transgenic mice, which express the putative oncogene in many cells or specific cell types. The transgenic animals are then scored for the development and appearance of neoplasia in the particular cell type or organ.

This collection of assays has been used not only to identify viral-transforming proteins but also to provide the primary experimental arsenal for studying the mechanisms by which oncoproteins function. While it is not clear how each of these assays relates to tumor initiation and progression in a natural setting, the fact that the same gene products that induce transformation in these cell culture assays are also associated with viral tumorigenesis *in vivo*, attest to their usefulness.

How is Tumorigenesis Linked to Viral Infection?

In order for a virus to induce tumors it must first infect the host. However, tumorigenesis does not offer any obvious selective advantage to the virus, and thus is the indirect consequence of viral strategies meant to redirect specific cellular components toward virus production and to escape innate and adaptive antiviral defenses. In their natural hosts, viruses contact many different types of cells, only some of which are susceptible to a complete infectious cycle. Tumorigenesis is thought to be initiated in cells where viral infection is incomplete. Thus, viral tumorigenesis is a rare accident that occurs when viral productive infection fails and the surviving cell continuously expresses a subset of viral genes. An understanding of the viral productive cycle and the various ways different cell types respond to infection is paramount to understanding how DNA tumor viruses contribute to cancer.

There are essentially three types of viral infection: permissive, semi-permissive, and non-permissive. A permissive infection is one in which the virus can infect the cell and produce progeny virions. In this case, the infected cells are said to be permissive to the virus, and the cells are usually lysed resulting in the release of progeny virions. Semi-permissive infection is one in which viral growth is less efficient resulting in low yields of progeny virions. In non-permissive infection, the virus either cannot enter the cell due to lack of receptors or if it does enter the cell, it cannot replicate due to a lack of specific host factors. Progeny virions are not produced under non-permissive conditions.

Upon viral infection, the virus can enter many different phases of its life cycle. The infection may be latent, lytic, transforming, chronic, or persistent. In latent infection, viral replication is either actively suppressed by a latent viral protein or the cell lacks specific factors that enable viral replication. Thus, the virus can persist indefinitely inside the cell. Members of the herpesvirus family are prime examples of viruses that are capable of establishing life-long latent infections in their hosts.

In a lytic infection, the virus can replicate its genome producing multiple virion progeny. In most cases, the cell dies either by apoptosis (programmed cell death) or by necrosis, a process in which membrane integrity is lost and the cytoplasmic contents leak out of the cell. These virus-induced cellular changes are referred to as cytopathic effect or CPE. It is currently thought that necrosis leads to an inflammatory response while apoptosis does not.

In persistent infection, either the virus can infect the cell but not kill it and the virus-infected cell can produce virion progeny continuously or virus production and cell loss are balanced by cell proliferation and tissue regeneration. Hence there is constant viral replication, and the viruses are constantly released. In some cases, the infected host cell remains intact and can still divide. Chronic infection is similar to persistent infection in that the virus can infect the cell but not kill it. In addition, there are also cells that have not been infected by the virus and these cells are continually being infected by the virion progeny produced during the persistent infection. Thus, chronic infection typically represents a dynamic state or situation in which there is constant production of virus and virion shedding. In chronic infection, the host immune system is ineffective in clearing the viral infection.

Finally, another possible outcome of viral infection is viral transformation of the cell. This form of infection is relevant to all the DNA tumor viruses discussed in this book. The virus infects the cell and instead of this resulting in cell lysis or virion production, the virus alters the growth properties of the cell causing the cell to proliferate uncontrollably. Many of the DNA tumor viruses encode viral oncoproteins that are capable of transforming cells. The viral oncogenes encoded by the small DNA tumor viruses like polyoma and papillomaviruses deregulate cell cycle pathways to allow viral replication by

the host cell polymerases. The large DNA tumor viruses like Epstein–Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) come equipped with their own viral DNA polymerase and do not need to push the infected cell into the cell cycle. Instead these large viruses encode genes that deregulate cell signal transduction pathways and subvert the host immune response to avoid detection by the immune system. Generally speaking, non-permissive or semi-permissive infection by the DNA tumor viruses leads to transformation of the cell.

It is unlikely that a tumor virus is sufficient to convert a normal cell to a tumor cell. Rather a combination of the action of viral proteins and cellular gene mutations cooperate to drive tumorigenesis. A key issue is to understand how viral infection fits into the broader picture of tumorigenesis. An understanding of the synergy between viral and genetic mechanisms of tumorigenesis will lead to the identification of new targets for therapeutic intervention and new therapeutic strategies.

Summary

This book focuses on polyomaviruses, papillomaviruses, adenoviruses, and two members of the herpesvirus family, EBV and KSHV. This is a diverse collection of viruses that have very different life cycles and infectious strategies. However, there is much to be learned about cancer by understanding the common threads and unique mechanisms each of these viruses uses to alter cell behavior. These viruses do not create new signaling pathways, but rather they redirect existing cellular pathways toward enhancing virus production and spread. The following chapters will describe an array of transforming mechanisms including: oncogene activation, tumor suppressor inactivation, paracrine stimulation of cell survival and proliferation, and immune system evasion.

There are five broad unifying questions that tie each of these systems together:

1. Which viral proteins contribute to tumorigenesis?
2. What are the cellular targets of these proteins?
3. What do the viral proteins do to the targets?
4. What is the molecular mechanism by which the viral proteins alter the function of their cellular targets?
5. How does this action contribute to tumor initiation and/or progression?

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Chapter 1
Polyomavirus Life Cycle

Ellen Fanning, Xiaorong Zhao, and Xiaohua Jiang

1.1 Introduction

Since the discovery of the polyomavirus of mice (MPy) and simian virus 40 (SV40) nearly five decades ago, these deceptively simple viruses have served as tools to explore the molecular biology and biochemistry of mammalian cells (Acheson 2007; Cole 2001; Tooze 1973, 1980). Studies of the SV40 life cycle contributed greatly to the discovery of transcriptional enhancers, mRNA splicing and polyadenylation, chromatin structure, nucleo-cytoplasmic protein transport, and tumor suppressor proteins p53 and Rb. SV40 and MPy have been particularly useful as models to study DNA replication and oncogenic transformation of mammalian cells (Ahuja et al. 2005; Bullock 1997; Simmons 2000; Stenlund 2003; Sullivan and Pipas 2002).

Polyomavirus hosts include birds, mice, hamsters, monkeys, baboons, and humans, and new polyomaviruses are still being identified (Allander et al. 2007; Johne et al. 2006). In their natural hosts, polyomaviruses generally reproduce at a low level in specific tissues and are controlled by the immune system. Recent work on human polyomaviruses JC and BK, which are associated with disease in immunosuppressed patients, is discussed elsewhere in this volume.

This review will focus on the advances in polyomavirus research over the past 5 years and attempt to link them with new insights into host cell biology and biochemistry. A basic productive polyomavirus life cycle is diagrammed in Fig. 1.1, in which circled numbers denote steps in the productive infectious cycle that are discussed in this review.

1.2 Viral Genomes and Virus Entry

A key feature of these viruses is the small genome ($\sim$5.2 kb) of supercoiled duplex DNA, which is packaged with 24–25 host nucleosomes as a mini-chromosome that is surrounded by a nonenveloped viral capsid (Fig. 1.1).

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_1, © Springer Science+Business Media, LLC 2009

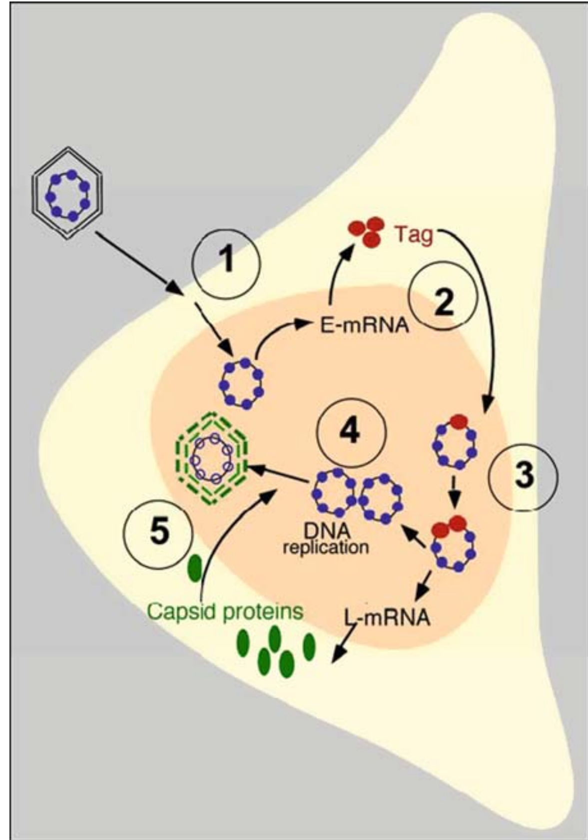

Fig. 1.1 Diagram of the polyomavirus life cycle in a permissive host cell. Circled numbers refer to phases of the life cycle discussed in this chapter. (1) Virus attachment, entry, and intracellular trafficking. (2) T-antigen (Tag) expression, structure, and function. (3) Tag-directed re-programming of the cellular milieu. (4) Viral DNA replication. (5) Virion assembly and release

The structures of the SV40 and MPy virions are known (Stehle et al. 1996; Stehle and Harrison 1997). Each of the 72 capsomeres in the capsid is composed of a VP1 pentamer and one interior VP2/3 that contacts the condensed mini-chromosome. VP1 pentamers are held together through a network of interactions among their flexible C-terminal peptides (Fig. 1.2A). Calcium bound to VP1 also promotes interactions among pentamers that are crucial for virus entry and virion assembly (Li 2003b). The virion attaches through VP1 to host cell surface gangliosides or N-linked sialic acid-containing glycoproteins: e.g., SV40 binds ganglioside GM1 and class I major histocompatibility proteins (Breau et al. 1992; Dugan et al. 2006; Tsai et al. 2003). However, some polyomaviruses recognize other classes of receptors: e.g., the neurotropic human JC virus (JCV), which causes a fatal demyelinating disease in immunosuppressed patients, was recently shown to bind to a serotonergic receptor on human glial cells (Elphick et al. 2004).

Polyomaviruses generally enter host cells through plasma membrane structures called caveolae (Damm et al. 2005; Dugan et al. 2006; Parton and Simons 2007). Caveolae are flask-shaped membrane microdomains rich in cholesterol and sphingolipid. The cytoplasmic face of caveolae is lined by the membrane

protein caveolin. Caveolae appear to operate as cargo-triggered vesicular transporters dependent on dynamin for cargo internalization (Sharma et al. 2004; Tagawa et al. 2005). SV40 and MPy internalized in caveosomes are sorted directly to the smooth endoplasmic reticulum (ER) through a microtubule-dependent pathway, bypassing endosomes and the Golgi, while caveolin is recycled to the plasma membrane (Dugan et al. 2006; Eash and Atwood 2005; Gilbert and Benjamin 2004; Gilbert et al. 2003; Norkin et al. 2002; Norkin and Kuksin 2005; Pelkmans et al. 2001). In contrast with most polyomaviruses, JCV enters host cells through clathrin-dependent endocytosis and is then sorted to caveosomes (Querbes et al. 2006).

Movement of polyomaviruses from perinuclear ER into the nucleus is not well understood. However, the N-terminal myristate of VP2 appears to protrude from the ER-internal virus particles, promoting viral escape into the cytoplasm, reduction of VP1 disulfide bridges and changes in calcium binding, leading to VP3 exposure (Chen et al. 1998; Ishizu et al. 2001; Kawano et al. 2006; Li et al. 2005; Li et al. 2003b). The mini-chromosome, still associated with some capsid proteins, is imported into the nucleus through importin recognition of a nuclear localization signal in VP3 (Nakanishi et al. 2007, 2006, 2002; Stewart 2007).

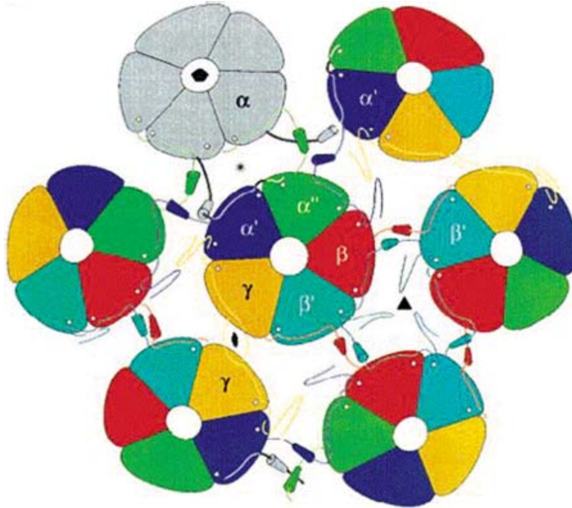

Fig. 1.2A SV40 capsid structure and genomic map. A) The VP1 pentamer is the capsid building block. VP1 C-terminal arms reach out from each subunit, linking it with adjacent pentamers. The icosahedral capsid contains twelve pentamers linked with five others, and 60 pentamers linked with six others. White dots denote calcium binding sites [Reprinted from (Stehle et al. 1996) with permission]. B) The SV40 DNA contains a control region (top) composed of the origin of DNA replication (ori), the early and late promoters, enhancer, and packaging signal. The early transcription unit (left) encodes T antigens, and the late genes (right) encode capsid proteins, and agno protein, in alternatively spliced mRNAs as indicated. A pre-micro RNA expressed late in infection limits immune recognition by reducing early gene expression (Sullivan, Grundhoff, Tevethia, Pipas and Ganem 2005). Nucleotide numbers are diagrammed clockwise around the genome beginning in the origin [Reprinted from (Ahuja et al. 2005) with permission]

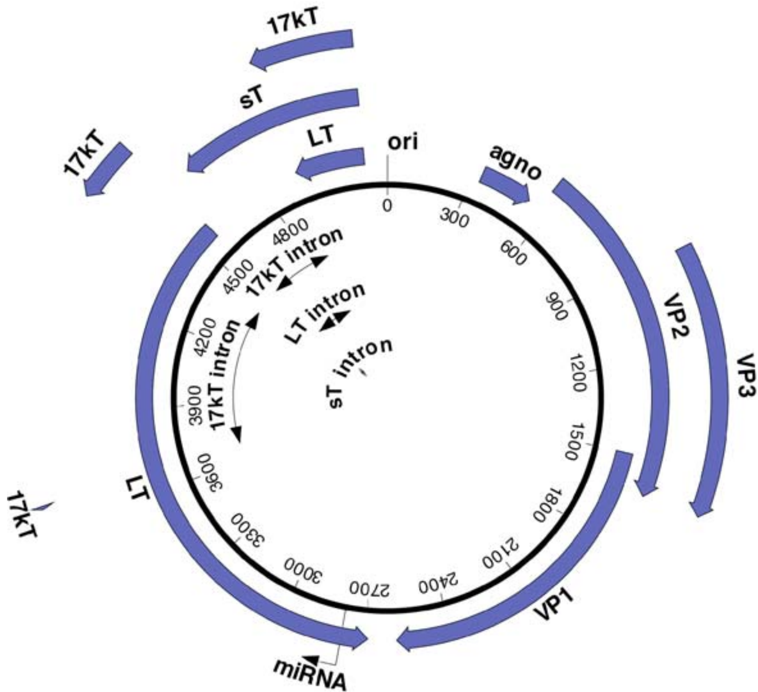

Fig. 1.2B (continued)

In the nucleus, host transcriptional machinery expresses the early genes of the mini-chromosome. The genome contains two diverging transcription units (Fig. 1.2B). One encodes an overlapping set of multifunctional regulatory proteins expressed early in infection. The other encodes three capsid proteins expressed late in permissive cells and, in some polyomaviruses, an agno protein that facilitates virion assembly. The control region between the early and late transcription units contains a bidirectional enhancer, early and late promoters, the viral origin of DNA replication (Fanning and Knippers 1992), and the viral packaging signal (ses), a tandem array of six binding sites for the host transcription factor Sp1 (Gordon-Shaag et al. 2002).

Early polyomavirus gene expression requires that host transcription factors gain access to the viral genome through dissociation of remaining capsid proteins and nucleosome remodeling in the control region. Sp1 and AP1 interact with the SV40 control region to generate a nucleosome-free region (Milavetz 2002), but additional chromatin remodeling factors are also involved. PARP-1 interaction with residual VP1 bound to the control region of infecting MPy mini-chromosomes facilitates VP1 release and is vital for robust early MPy gene expression (Carbone et al. 2006). RNA polymerase II-chromatin-immunoprecipitation experiments on SV40 chromatin from infected cells have recently shown that

histone acetylation patterns in coding regions are dynamic during transcription, with hyperacetylation by histone acetyltransferase p300 as pol II moves into a nucleosome, followed by deacetylation (Balakrishnan and Milavetz 2005, 2006, 2007), much like a model proposed for transcription of host chromatin (Orphanides and Reinberg 2000).

1.3 Polyomavirus T Antigen: Re-programming the Host Cell

Driven by the limited coding capacity of their genomes, polyomaviruses are exquisitely well adapted to their hosts and rely on their multifunctional regulatory proteins (tumor [T, t] antigens) to carry out highly sophisticated re-programming of the host cell milieu for virus reproduction. Genetic and biochemical analyses of SV40 and MPy T antigens have revealed that large T antigen (Tag) is essential to drive quiescent infected cells into S phase, to initiate and complete viral DNA replication, to activate the late viral promoter, and to promote virion assembly (Ahuja et al. 2005; Pipas 1992; Simmons 2000; Sullivan et al. 2002). Furthermore, Tag is necessary and sometimes sufficient to effect cell transformation (reviewed elsewhere in this volume). Other early viral proteins, small t, middle T, and 17 k T, contribute in fascinating ways to the early phase of infection and are vital for cell transformation (Chen et al. 2007; Dilworth 2002; Skoczylas et al. 2004; Skoczylas et al. 2005; Sontag and Sontag 2006). Thus, an understanding of the structure and function of the T antigens is the key to understanding control of the viral life cycle.

The ability of Tag to impact so many diverse biological processes can be explained by its multiple structural domains, which possess distinct biochemical activities. Using SV40 Tag as an example, the polypeptide is composed of six domains (Fig. 1.3A). A J domain structurally and functionally related to DnaJ chaperone proteins (Hennessy et al. 2005; Kim et al. 2001) is connected through an extended linker to the origin DNA-binding domain (OBD), a zinc-binding domain (Zn), an AAA+ (ATPases associated with various cellular activities)/helical domain, and the host range domain (HR). The OBD was the first polyomavirus Tag domain to be characterized structurally (Luo et al. 1996). It is a compact structure with two protruding basic loops that interact specifically with the viral origin region to control transcription and replication, non-specifically with single- or double-stranded DNA, and with two domains of the host single-stranded DNA-binding protein RPA (Arunkumar et al. 2005; Bochkareva et al. 2006; Jiang et al. 2006; Meinke et al. 2006; Meinke et al. 2007). In addition, a different surface of OBD is involved in interactions between Tag hexamers that are required for viral DNA replication (Weisshart et al. 1999). The Zn and AAA+/helical domains of Tag constitute the viral replicative helicase needed to form the Tag hexamer and to unwind parental DNA (Gai et al. 2004; Li et al. 2003a). The HR domain permits the virus to replicate in CV-1 monkey cells and supplies an unknown function needed for adenovirus to replicate in monkey cells, but its structure has not been determined.

Each of the Tag domains also binds to host proteins, modifying their functions, and some of the linker regions bind host proteins as well. Tag domains must interact with one another in a coordinated fashion to direct the infection, but the nature of this coordination remains poorly understood. Lastly, Tag forms oligomers and undergoes post-translational modifications that affect its activities (Fanning 1994; Fanning and Knippers 1992; Poulin et al. 2004).

The ability of polyomavirus Tag to stimulate G1/S cell cycle progression in permissive and nonpermissive cells relies on its well-established ability to bind to the retinoblastoma tumor suppressor (Rb) protein family and on J domain activity (Ahuja et al. 2005; Garimella et al. 2006; Whalen et al. 2005). The Rb family binding site in SV40 Tag resides in residues 103–107 between the J domain and the OBD (Fig. 1.3B). Rb proteins bound to E2F family transcription factors normally inhibit their ability to stimulate the G1/S transition until G1 cyclins phosphorylate Rb, releasing active E2F. In infected cells, this regulation is overcome by the chaperone activity of the Tag J domain on Rb proteins bound in cis (Ahuja et al. 2005; Whalen et al. 2005). The detailed mechanism of this reaction is not yet known.

At least three other regions of Tag contribute independently to its ability to overcome host controls on the G1/S transition: the J domain, the OBD, and AAA+/helical domain (Fig. 1.3A, B) (Dickmanns et al. 1994; Dobbelstein et al. 1992). How the activities in these domains promote premature G1/S

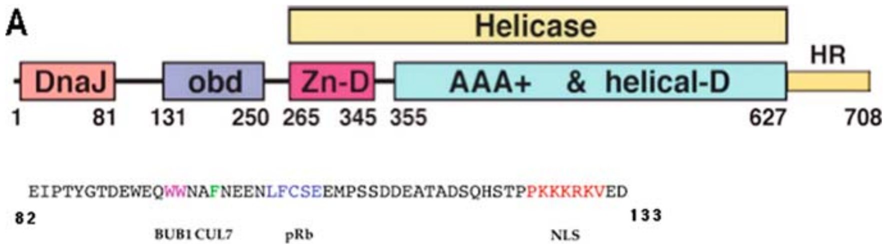

Fig. 1.3A SV40 Tag, the master controller of the infectious cycle. A) Tag structural domains are depicted as colored boxes connected by flexible linkers (straight lines). Residue numbers below refer to the beginning and end of a domain. A blow-up of residues 82-133 containing a loop and helix 4 of the DnaJ domain, binding sites for Bub1, Cul7, and the pRb protein family, functionally important phosphorylation sites (asterisks), and the nuclear location signal appears below. Substitutions in the colored residues weaken Tag interaction with the indicated proteins. See text for details [Adapted from (Gai, Li, Finkielstein, Ott, Taneja, Fanning and Chen 2004a) with permission]. B) Models of Tag structures, clockwise from top left, the DnaJ domain [Reprinted from (Kim et al. 2001) with permission] obd with the basic origin DNA/RPA binding surface circled [Reprinted from (Jiang et al. 2006) with permission] the helicase domain in a top view of the hexamer, and a side view of the helicase monomer [Reprinted from (Li et al. 2003a) with permission] (Luo et al. 1996). C) Confocal micrographs of nuclei in human cells mock-infected or SV40-infected for 48 h, pre-extracted, fixed and stained for activated ATM kinase, Tag, or DNA, as indicated. Merged images appear at the right. (Zhao and Fanning, unpublished)

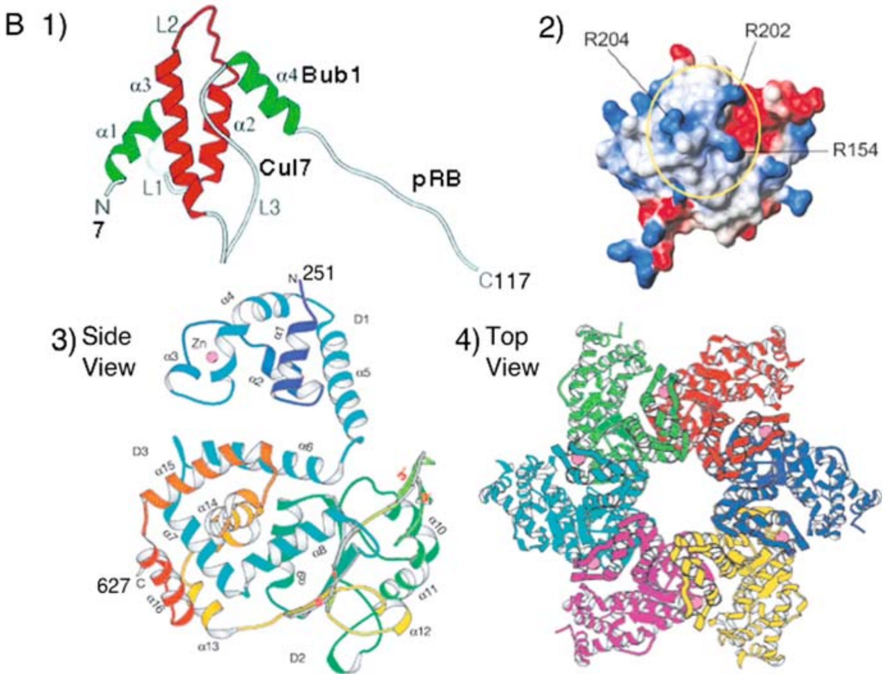

Fig. 1.3B (continued)

progression has remained a puzzle. However, the striking recent observation that viral infection of permissive cells induces an ATM-dependent DNA damage response may be pertinent (Dahl et al. 2005; Shi et al. 2005). ATM is activated through an unknown signal within 3–6 hours after infection, promotes optimal viral DNA replication, and co-localizes in unusually prominent nuclear foci with Tag (Dahl et al. 2005; Shi et al. 2005; Zhao and Fanning unpublished data) (Fig. 1.3C). ATM phosphorylates Tag on serine 120 (Shi et al. 2005), a modification that inhibits SV40 DNA replication in a cell-free reaction (Fanning 1994). On the other hand, mutation of serine 120 to alanine inhibits SV40 DNA replication and virus production in infected cells, implying that phosphorylation of the site is required at the same stage of the viral life cycle in vivo (Schneider and Fanning 1988; Shi et al. 2005). As a possible resolution to this conundrum, we suggest that ATM phosphorylation of Tag on serine 120 early in infection prevents premature activation of viral DNA replication, allowing time for sufficient Tag to accumulate and properly re-program the cellular milieu for efficient virus production. As a corollary to this interpretation, ATM signaling would need to be attenuated as the infection progresses, allowing a fraction of Tag molecules to escape modification on serine 120 and initiate viral DNA replication. Alternatively, Tag active in viral DNA replication might be confined to subnuclear compartments with reduced ATM signaling [see Section 3 below].

ATM signaling in infected cells would be expected to trigger a p53-mediated G1 checkpoint arrest (Kastan and Bartek 2004; Shiloh 2003). In this case, p53 activation of p21 expression would be needed to inhibit cyclin-dependent kinase activity. However, the Tag AAA+/helical domain firmly sequesters the p53 DNA-binding surface (Lilyestrom et al. 2006), blocking its ability to activate p21 or other p53 target genes and compromising checkpoint control of the G1/S transition. Consistent with this model, mutations in Tag (D402N) that weaken Tag binding to p53 cause a reduction in the ability of the AAA+/helical domain to promote the G1/S transition (Dickmanns et al. 1994). The AAA+/helical domain also binds to transcriptional co-activators CBP/p300, probably indirectly through its ability to bind to p53 (Poulin et al. 2004), but it has not been determined whether this interaction is crucial for the viral life cycle.

The role of Tag OBD in promoting the G1/S transition may be linked to its ability to form a complex with the N-terminal region of the ATM-signaling partner Nbs1 (Digweed et al. 2002; Lanson et al. 2000; Wu et al. 2004) (Fig. 1.3A). In normal cells, a tight complex of Nbs1 with Mre11 and Rad50 proteins (MRN) binds rapidly to DNA double strand breaks in host chromatin and activates ATM damage signaling (Bartek and Lukas 2007; Lee and Paull 2005; Stracker et al. 2004). MRN is also thought to participate in repair of DNA double strand breaks (Trenz et al. 2006). As the level of Tag rises early in infection, perhaps its association with Nbs1 gradually limits MRN activation of ATM signaling, overcoming the G1 checkpoint arrest. Further work will be needed to uncover the consequences of Tag–Nbs1 interaction in the viral life cycle.

The Rb/E2F-independent roles of the J domain in preparing the cell to support the infectious cycle remain mysterious. Proteins that bind to the J domain of SV40 Tag include Hsc70 (Beau et al. 2006; Mayer and Beau 2005; Sullivan et al. 2002), the SCF cullin subunit Cul7 (Ali et al. 2004; Dias et al. 2002; Kasper et al. 2006; Kasper et al. 2005; Kohrman and Imperiale 1992; Skaar et al. 2007; Tsai et al. 2000), and the spindle checkpoint protein Bub1 (Cotsiki et al. 2004; Musacchio and Salmon 2007) (Fig. 1.3A, B). Cul7 binding can be weakened or abolished by amino acid substitutions at F98 or F74, deletion of residues 71–74 or 69–83 (Ali et al. 2004; Kasper et al. 2005), suggesting that it binds in helix 4 of the J domain and/or perhaps in the unusually long loop between helices 3 and 4. Substitutions that weaken Cul7 binding appear not to affect Bub1 binding. Substitutions at Tag residues W94 or W95 in helix 4 and deletion of residues 89–97 inactivate Bub1 binding (Cotsiki et al. 2004), localizing the Bub1-binding site very close to that of Cul7. Whether the substitutions in Tag that weaken Cul7 or Bub1 binding also affect its ability to stimulate Hsc70 ATPase and chaperone activity has not been directly addressed.

Hsc70 binds to a conserved loop (HPD) between helices 2 and 3 of the J domain, an interaction required for stimulation of Hsc70 ATPase by all known J domains (Hennessy et al. 2005). The ability of the SV40 Tag J domain to stimulate Hsc70 ATPase activity is essential for viral DNA replication in infected cells (Sullivan et al. 2002). On the other hand, the J domain is dispensable in a cell-free SV40 replication system, implying that it is not directly

involved in the replication mechanism (Campbell et al. 1997; Maulbecker et al. 1992; Sullivan et al. 2002; Weisshart et al. 1996). Interestingly, Hsc70 translocates from the cytoplasm to the nucleus in SV40-infected permissive cells, but does not bind stably to Tag (Sainis et al. 2000). In contrast, Tag–Hsc70 complexes are stable in nonpermissive cells. The inability to detect stable binding of Hsc70 to Tag in permissive cells might be explained by cycles of Hsc70 association and dissociation with the J domain as substrate proteins undergo folding or unfolding. This speculation suggests that J chaperone activity in permissive cells is more active than in nonpermissive cells and that some of its substrate or accessory proteins differ in permissive and nonpermissive cells. On the other hand, Rb-E2F complexes are substrates of the J domain-Hsc70 in both permissive and nonpermissive cells. Taken together, the data suggest that J domain chaperone activity likely re-programs the intranuclear milieu to support the infection, but does not act directly in DNA replication.

Bub1 binding to Tag is important for cell transformation and for the ability of Tag to override the spindle checkpoint, but it is not yet clear how this contributes to the productive viral life cycle (Cotsiki et al. 2004). Bub1 interaction with Tag could be responsible, at least in part, for the ability of SV40 to induce re-replication of the host genome (Lehman et al. 2000; Okubo et al. 2003). Similarly, the role of Tag binding to Cul7 in the viral life cycle is not known. An amino acid substitution F98A in SV40 Tag weakens Cul7 binding and cell transformation substantially, but reduces plaque formation only modestly (Ali et al. 2004; Pipas and Fanning unpublished data). Intriguingly, c-myc is a substrate protein for the Cul7 SCF complex (Koch et al. 2007). One can speculate that Tag interaction with Cul7 might lead to stabilization of c-myc that could enhance cell transformation or damage signaling. Developing a better understanding of how Hsc70, Bub1, and Cul7 interact with the J domain of Tag to coordinate viral infection remains a challenge.

1.4 Viral DNA Replication in a DNA Damage-Signaling Environment

Polyomavirus Tag functions together with host replication proteins to replicate the viral mini-chromosome during S phase in infected cells (Bullock 1997; Stenlund 2003). A minimal set of ten human proteins, together with Tag, is sufficient to replicate SV40 DNA in a cell-free reaction (Waga and Stillman 1994). The orthologs of these host proteins are also essential for genomic DNA replication in Xenopus extracts, and are encoded by genes essential for viability in yeast. This large body of work has led to the widely accepted view that replication of the SV40 mini-chromosome or viral plasmid DNA in vitro closely resembles host DNA replication. On the other hand, several discordant observations have led to doubts about the resemblance, e.g., the multiple rounds of re-replication of SV40 DNA, and the dispensability of DNA polymerase epsilon (Pospiech and Syvaoja 2003). The recent findings that MPy and SV40

induce an ATM-mediated DNA damage response in infected cells (Dahl et al. 2005; Shi et al. 2005), conditions that would ordinarily arrest the cell cycle and stall ongoing replication, make it imperative to re-examine polyomavirus DNA replication in infected cells.

Besides the gross differences between viral and host DNA replication noted above, the two processes differ in more subtle ways. DNA damage-dependent phosphorylation of replication protein A (RPA), the major single-strand DNA-binding protein involved in viral DNA replication and most DNA-processing pathways in the cell, has little effect on its activity in SV40 DNA replication, but inhibits its ability to support host DNA replication (Françon et al. 2004; Olson et al. 2006; Vassin et al. 2004). Phosphorylation of the p68 (or B) regulatory subunit of DNA polymerase alpha-primase (pol-prim) by S-phase cyclin-dependent kinases (S-CDK) inhibits replication of SV40 DNA replication in vitro by blocking primer synthesis on RPA-coated template (Dehde et al. 2001; Ott et al. 2002). If S-CDK-phosphorylated pol-prim also cannot replicate viral DNA in infected cells, ATM-mediated checkpoint signaling to inactivate S-CDK would be predicted to increase the level of hypo-phosphorylated pol-prim available to replicate viral DNA. On the other hand, DNA damage signaling results in ubiquitin-mediated destruction of two subunits of DNA polymerase delta, an enzyme required for SV40 replication (Li et al. 2006; Liu and Warbrick 2006; Podust et al. 2002; Xie et al. 2002; Zhang et al. 2007). Perhaps these subunits of pol delta are dispensable for polyomavirus DNA replication. In sum, these results suggest that SV40 and MPy, like many animal viruses, may exploit host DNA damage signaling and repair pathways in a sophisticated, spatially and/or temporally regulated program to favor viral propagation (Everett 2006; Weitzman et al. 2004).

How SV40 and MPy carry out this program is largely undiscovered. The seminal work of Maul and colleagues demonstrating that replicating SV40 genomes and a subpopulation of Tag localize in subnuclear foci closely associated with PML/ND10 bodies provides a foundation (Ishov and Maul 1996; Tang et al. 2000). As shown in Fig. 1.3C, Tag associates with activated ATM in subnuclear structures in infected cells that resemble those reported by Maul and colleagues. Consistent with the possibility that these sites are important for viral DNA replication, Tag did not localize at such sites in nonpermissive cells (Zhao et al. unpublished data).

Cellular chromatin replication is thought to occur in replication factories that assemble on nuclear matrix for replication and disassemble upon its completion (Kitamura et al. 2006; Meister et al. 2007). Since SV40 chromatin replication also takes place on nuclear matrix (Schirmbeck and Deppert 1987, 1989, 1991), perhaps ND10 bodies provide a structural anchor on the matrix (Everett 2006). Localization of the MPy replication origin on nuclear matrix was recently shown to depend on Runx1 transcription factor binding to its recognition site in the viral enhancer, an element that is also required for MPy DNA replication (Murakami et al. 2007). Runx1 with a defective nuclear matrix-binding region was unable to support viral replication. DNA damage

signaling and repair proteins can also reside in ND10s (Boe et al. 2006), potentially creating an optimal environment for viral DNA replication. The mechanism(s) that recruits the viral origin and Tag to the nuclear matrix and ND10 bodies and how this localization facilitates viral replication is not known.

1.5 The SV40 Replisome: A Dynamic Nanomachine

The basic mechanisms of polyomavirus DNA replication in cell-free systems reconstituted with purified proteins are well established (Bullock 1997; Stenlund 2003). Tag is the core of the viral replisome. It binds specifically to a palindromic arrangement of four pentanucleotides in the viral origin of replication, assembles into a head-to-head double hexamer in the presence of ATP, distorts the origin DNA locally, rearranges into an active helicase, unwinds the parental DNA, and directs the synthesis of primers by pol-prim on the leading and lagging strand templates (Bullock 1997; Simmons 2000; Stenlund 2003).

Recent progress on the structure of SV40 Tag and concomitant biochemical analysis is now leading to an atomic level understanding of the initiation process (Bochkareva et al. 2006; Gai et al. 2004; Gomez-Lorenzo et al. 2003; Kim et al. 2001; Kumar et al. 2007; Li et al. 2003a; Luo et al. 1996; Meinke et al. 2006; Meinke et al. 2007; Reese et al. 2006; Shen et al. 2005; Valle et al. 2006; Wang et al. 2007). Despite this progress, many questions remain incompletely answered, foremost the path of DNA through the double hexamer during unwinding and the coordination of ATP hydrolysis cycles in the two hexamers with Tag movement relative to the DNA during unwinding. One of the current models for binding and assembly of Tag monomers into a double hexamer on the SV40 origin is depicted in Fig. 1.4A. Initially, two Tag OBD domains bind to each half of the palindromic recognition site, nucleating assembly of eight additional Tag monomers when ATP binds to the AAA+ domains. Duplex DNA is melted locally by a beta-hairpin motif in each AAA+ domain that protrudes into the central chamber of each hexamer (Gai et al. 2004; Reese et al. 2004; Shen et al. 2005). The four additional OBD domains in each hexamer interact with the two pentanucleotide-bound OBDs to form an open spiral (Bochkareva et al. 2006; Meinke et al. 2006, 2007). This structure is postulated to contact other nonspecific DNA sequences on each strand as the two strands separate, allowing each hexamer to surround one strand and displace the other strand, yielding the active bidirectional helicase (Bochkareva et al. 2006; Kumar et al. 2007).

The exact destination of the two displaced strands, which become the "rabbit-ears" visualized by electron microscopic snapshots of the Tag double hexamer during unwinding (Fig. 1.4B) (Wessel et al. 1992), is currently unresolved. One possibility is that the displaced strand does not enter the central chamber of the helicase domain, but like papilloma E1 helicase, resides on the exterior surface of the hexamer from which it was excluded (Enemark and Joshua-Tor 2006). However, if we assume a double hexamer is the bidirectionally active helicase, this

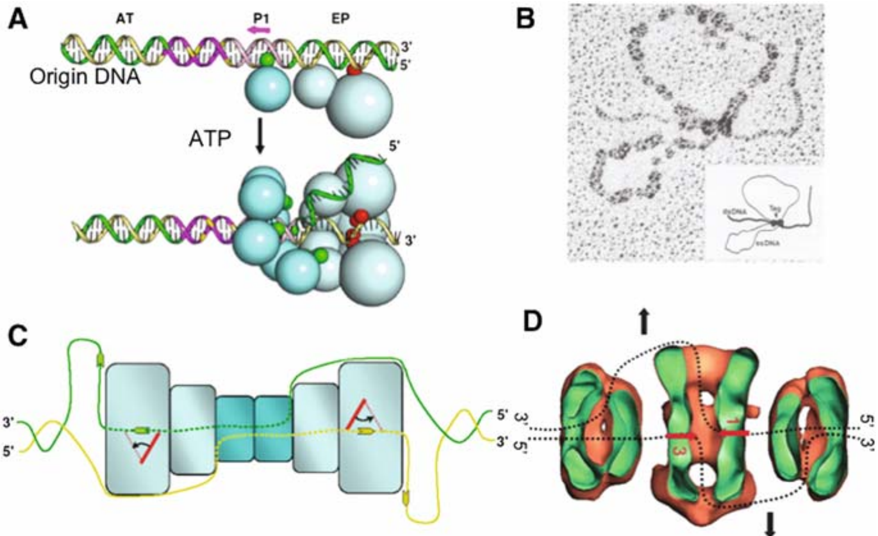

Fig. 1.4 Assembly and activation of the bidirectional SV40 Tag helicase: clues to the puzzle. A) The OBD of a Tag monomer binds specifically to each of four pentanucleotide sites (e.g. P1) in the origin. The beta-hairpin (red dot) in the helicase domain contacts the flanking EP region, and assembles with other monomers in the presence of ATP/ADP into a double hexamer, locally distorting the EP and AT regions [Reprinted from (Kumar et al. 2007) with permission]. B) Electron micrograph of negatively stained Tag double hexamer during bidirectional unwinding of SV40 DNA. The ssDNA loops are coated with bacterial ssDNA-binding protein [Reprinted from (Wessel, Schweizer and Stahl 1992) with permission]. Interactions between the hexamers are essential for bidirectional unwinding and virus propagation in infected cells (Weisshart et al. 1999; Fanning 1994). C) Based on a crystal structure of a related papillomavirus helicase hexamer bound to ssDNA (Enemark and Joshua-Tor 2006), a path for DNA through the activated bidirectionally unwinding SV40 double hexamer was proposed [Reprinted from (Kumar et al. 2007) with permission]. The tip of each beta hairpin (red) binds to DNA in the presence of ATP and translocates ~17 angstroms upon ATP hydrolysis and ADP, Pi release (Gai et al. 2004b; Shen et al. 2005). D) Cryo-electron microscopic image analysis of double hexamers on origin reveals several holes through the structure proposed to serve as entry or exit sites for DNA. A possible path of DNA through the protein is denoted by dotted lines. Black arrows suggest extrusion of template strands [Reprinted from (Valle et al. 2006) with permission]

model would probably require that the Tag or E1 hexamers first disassemble to release the displaced strand and then re-assemble around the strand on which the hexamer translocates. A second model is that the strand displaced upon melting exits the central chamber through hydrophilic “side channels” in the AAA+ domain during the helical rearrangement process (Gai et al. 2004; Gomez-Lorenzo et al. 2003; Li et al. 2003a; Shen et al. 2005; Valle et al. 2006; Wang et al. 2007).

Powered by cycles of ATP hydrolysis, the beta-hairpins bind to ssDNA in the central chamber and translocate it through the protein, producing templates for replication (Gai et al. 2004; Shen et al. 2005). In the model shown in Fig. 1.4C,

each hexamer pulls one parental DNA strand 5′–3′ into the double hexamer, displacing the complementary strand to the exterior (Kumar et al. 2007). This is equivalent to 3′–5′ movement of the hexamer on the strand in the central chamber, and therefore consistent with the crystal structure of E1-ssDNA and the body of evidence in support of coupled bidirectional unwinding by the double hexamer. Figure 1.4D depicts a model analogous to that of an archaeal double hexameric helicase (McGeoch et al. 2005), in which the beta-hairpins on each hexamer pull duplex DNA into the protein, spooling one strand out to be replicated (Gai et al. 2004Cell). However, several other models have also been proposed for the path of the DNA strands through the double hexamer (Bochkareva et al. 2006; Gai et al. 2004; Jiang et al. 2006; Li et al. 2003a; Valle et al. 2006). Additional structural, mutational, and biochemical work will be needed to understand how the double hexamer unwinds DNA bidirectionally.

Given the flexibility of Tag, one can speculate that the path of DNA through a single Tag or E1 hexamer during unidirectional DNA unwinding differs from that through a hexamer assembled in a bidirectionally unwinding double hexamer, perhaps due to differences in the assembly process. Some support for this possibility comes from a mutant Tag with Ala substituted for Thr124 and from the class 4 mutant Tag proteins (Moarefi et al. 1993; Weisshart et al. 1999). These mutations abolish bidirectional origin unwinding and replication of SV40 DNA in vitro and in monkey cells, but have no effect on unidirectional helicase activity. Further work will hopefully reveal more clearly the atomic details of how unwinding takes place and how topoisomerase I activity is coupled with these events (Roy et al. 2003; Simmons et al. 2004).

Interestingly, the assembly process of Tag (and E1) double hexamers may typify a common mechanism used to assemble replication initiators on origins of replication. The spiral structure of six Tag OBD domains resembles the spiral arrangements of Aquifex aeolicus DnaA and Drosophila ORC (Clarey et al. 2006; Erzberger et al. 2006; O'Donnell and Jeruzalmi 2006; Schuck and Stenlund 2005; Speck et al. 2005). Like Tag, these initiator proteins are members of the AAA+ family and bind to their respective origins in an ATP-bound form. Unlike Tag, these initiators have no helicase activity and must recruit and load their cognate helicases at the origin. Whether their cognate helicases also form a spiral intermediate in assembling into an active form is not known. However, consistent with this possibility, archaeal MCM helicase does assemble a double hexamer that structurally resembles Tag (Forsburg 2004; McGeoch et al. 2005; Sclafani et al. 2004).

As bidirectional unwinding of the SV40 origin begins, the single-stranded DNA is first coated by RPA and then serves as a template for primer synthesis by pol-prim. Recent work has provided evidence that Tag orchestrates the recruitment of RPA, effectively coupling DNA unwinding with RPA binding to ssDNA (Jiang et al. 2006). Tag OBD binds to the high-affinity ssDNA-binding domains A and B of RPA70 at a site distal to the ssDNA-binding loops of RPA70AB. In the presence of an 8- to 10-mer ssDNA, RPA70AB binds to both ssDNA and Tag OBD, forming a stable ternary complex. However, in the presence of longer

ssDNA, RPA releases Tag and binds to ssDNA in a higher affinity extended binding mode. This two-step coupling mechanism may better protect the template from hairpin formation and nucleases than diffusion-mediated RPA binding, and would be predicted to stimulate unwinding and replication.

The next step in the initiation process is primer synthesis by DNA polymerase alpha-primase (pol-prim), another process orchestrated by Tag (Arunkumar et al. 2005; Fanning et al. 2006). At least two subunits of the pol-prim hetero-tetramer bind independently to the helicase domain of each Tag hexamer (Huang et al. 1998). RPA-saturated ssDNA is a poor template for pol-prim, but in the presence of Tag, primer synthesis is restored. Tag binding to pol-prim, as well as to RPA70AB and RPA32 C-terminus, appears to be necessary for primer

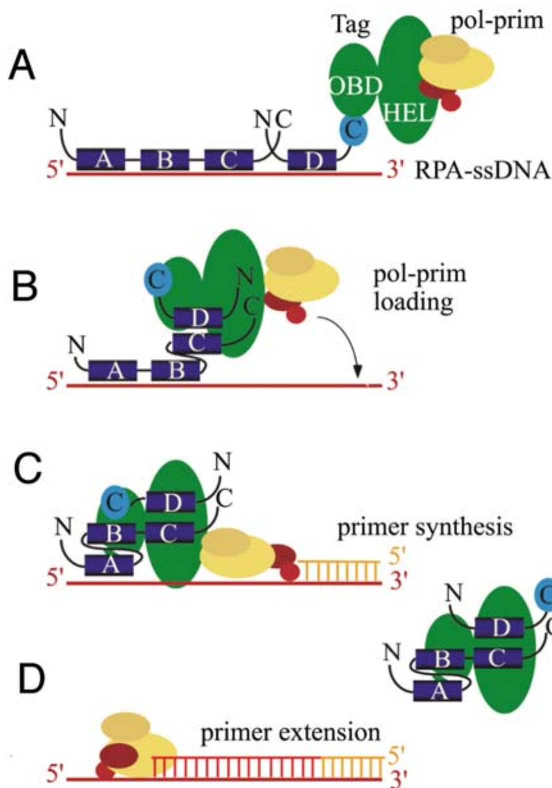

Fig. 1.5 Dynamic protein interactions in initiation of SV40 DNA replication: a model [Reprinted from (Arunkumar et al. 2005) with permission]. A) RPA in an extended binding mode occludes ~30 nucleotides of ssDNA. B) Transient electrostatic interaction of a Tag OBD with the C-terminal winged helix of RPA32 leads to remodeling of RPA into a compact binding mode that occludes 8-10 nucleotides (Fanning, Klimovich and Nager 2006; Jiang et al. 2006). C) Pol-prim associated with the helicase domain of the Tag hexamer is poised to initiate priming on the free ssDNA. D) Pol-prim shifts the primer to the polymerase active site and extends it, presumably displacing RPA and Tag and setting the stage for the polymerase switch (Waga et al. 1994; Yuzhakov, Kelman, Hurwitz and O'Donnell 1999)

synthesis. The current model is that RPA in the extended binding mode on ssDNA is remodeled and stabilized in a compact binding mode upon contact with Tag (Fig. 1.5). This remodeling allows pol-prim that is bound to the Tag helicase domain to gain access to the template for primer synthesis and extension (Arunkumar et al. 2005). In other words, Tag-facilitated cycling of RPA between its extended and compact ssDNA-binding modes is thought to direct the initial steps in initiation of SV40 replication. Similar cycles of protein-mediated remodeling of RPA ssDNA-binding modes may underlie other steps in DNA-processing pathways, including primer extension by pol-prim, and the switch from pol-prim to DNA polymerase delta/RFC/PCNA (Fanning et al. 2006; Yuzhakov et al. 1999). Viral DNA replication is completed through Okazaki fragment processing, the termination of replication, and segregation of daughter DNAs (Bullock 1997; Stenlund 2003; Waga and Stillman 1994).

1.6 Virion Assembly on Viral Chromatin

Host histones assemble into nucleosomes on viral chromatin during replication in the nucleus of infected cells (Alexiadis et al. 2000; Shibahara and Stillman 1999; Shibahara et al. 2000; Vestner et al. 2000). In the cytoplasm, viral capsid proteins VP1–3 assemble soon after their synthesis into capsomeres composed of a VP1 pentamer and one molecule of either VP2 or VP3 (Kasamatsu et al. 2006; Nakanishi et al. 2006). In the absence of viral chromatin, VP2 greatly enhances assembly of SV40 VP1 into spherical particles in vitro (Kawano et al. 2006). The assembled particles are irregular but roughly the size of virions and contain the minor capsid protein in a molar ratio of 1:5 VP1. VP1 pentamers associated with a minor capsid protein are imported into the nucleus, and may assemble in much the same way around each viral mini-chromosome. The SV40 mini-chromosome is thought to serve as a scaffold for virion assembly (Gasparovic et al. 2006; Roitman-Shemer et al. 2007).

Although SV40 capsid proteins bind nonspecifically to DNA, they assemble specifically on viral chromatin in infected cells. Virion assembly begins at a specific site in the viral genome, directed by specific binding of the transcription factor Sp1 to the packaging signal in the viral origin-promoter region (Gordon-Shaag et al. 2002). VP2/3 binds cooperatively to DNA and Sp1 bound to the packaging signal. A separate region of Sp1 contacts VP1, forming an initial VP1 pentamer-VP2/3-Sp1 complex that is thought to nucleate cooperative virion assembly. Contacts among the capsid proteins have been mapped (Kasamatsu et al. 2006; Nakanishi et al. 2006). The Sp1 molecules appear to be excluded from virions, but the mechanism responsible is not known (Roitman-Shemer et al. 2007).

Virion exits from the nucleus of the infected cell occurs through programmed cell necrosis. SV40 VP3 interacts specifically with a poly-ADP-ribose polymerase (PARP) in the nucleus, stimulating its enzymatic activity (Gordon-Shaag et al. 2003). Depletion of ATP leads to loss of plasma membrane integrity and swelling

of the ER and nuclear membranes (cytopathic effects), which facilitates virion release. Inhibitors of PARP activity reduce ATP depletion and consequently viral yield without reducing viral DNA replication or capsid protein expression. In addition, SV40 VP2 and VP3 have the general capacity to permeabilize membranes, as shown by the ability of the recombinant proteins to permeabilize, or even lyse (VP3), bacterial membranes (Daniels et al. 2006). Nuclear membranes become leaky late in infection, as evidenced by detectable levels of nuclear proteins in the cytoplasm. Virions assembled in the nucleus accumulate late in infection at the nuclear membrane, poised for release when the membranes are disrupted (Daniels et al. 2006). A third activity that promotes both virion entry and exit from infected cells is the myristylation of VP2, observed in MPy and JCV (Gasparovic et al. 2006; Mannova et al. 2002; Sahli et al. 1993).

1.7 Summary and Perspective

Significant recent advances in cell biology have begun to reveal the detailed mechanisms of polyomavirus trafficking in host cells. Structural biology has illuminated our understanding of polyomavirus packaging mechanisms and virion structure, and is beginning to enable dissection of the structural basis of the dynamic protein interactions in the viral replication machinery. The growing evidence that polyomaviruses induce DNA damage signaling to promote viral replication has exciting potential implications, raising the possibility that polyomaviruses may replicate their genomes by hijacking host replication fork restart pathways. Re-priming of stalled forks is well established in bacterial cells, but has not yet been described in eukaryotic cells (Lambert et al. 2007; Heller and Marians 2006). Notably, the proteins that re-prime stalled replication forks in bacteria were discovered as replication factors for bacteriophage φX174 DNA, providing a precedent for this speculation and suggesting that polyomaviruses may continue to be useful models to understand host cell biology.

Acknowledgments We thank Dr. Kun Zhao for editorial assistance, present and former members of the Fanning Lab, Jeff Brodsky, Walter Chazin, Xiaojiang Chen, Jim Pipas, and Tim Peters for stimulating discussion, Kevin Harvey (EMD BioScience) for anti-phospho-ATM, and the NIGMS (GM52948), NCI (P30 CA068485 to Vanderbilt-Ingram Cancer Center), HHMI Professors Program (52003905), and Vanderbilt University for research support. We apologize to the many colleagues whose work could not be cited directly due to space constraints.

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Chapter 2
Transformation by Polyomaviruses

Dweepanita Das and Michael J. Imperiale

Abstract Polyomaviruses are small DNA tumor viruses that encode tumor antigens which interfere with the two major tumor suppressor pathways, pRB and p53. The viral early proteins have been utilized by researchers as model systems to decipher mechanisms relating to oncogenesis and tumorigenesis. Investigation of molecular mechanisms of these early proteins has contributed significantly to our understanding of cell cycle regulation, cell proliferation and growth, and signal transduction pathways. This chapter discusses the oncoproteins of polyomaviruses and how they induce cellular transformation.

2.1 Introduction

2.1.1 Discovery

Polyomaviruses are small, icosahedral non-enveloped DNA viruses that infect a large number of vertebrates (reviewed in Imperiale and Major, 2007). They were first discovered by Gross in 1953 while studying leukemia in mice (Gross, 1953). Subsequent work demonstrated that polyomavirus induced tumor formation at a variety of sites when injected into mice; hence the name "polyoma" (Stewart et al., 1958). Depending on the type of host cell infection, polyomaviruses can either induce cellular transformation/tumorigenesis or produce infectious virions with subsequent cell lysis. These viruses encode proteins that oncogenically transform cells in culture, induce tumor formation in infected and transgenic mice, and have been reported to be associated with human cancers. The ability of these oncoproteins to interact with and regulate host cell proteins has been exploited extensively by researchers to understand mechanisms of cell immortalization and transformation, cell cycle regulation, and signal transduction.

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_2, © Springer Science+Business Media, LLC 2009

The biology of the polyomaviruses has been reviewed recently, and the reader is referred to this review for additional detail (reviewed in Imperiale and Major, 2007).

Simian virus 40 (SV40) was isolated in 1960 by Sweet and Hilleman from rhesus monkey kidney cells used to produce poliovirus vaccine, while screening for the presence of contaminating viruses (Sweet and Hilleman, 1960). The two known human polyomaviruses, JCV and BKV, were isolated from immunocompromised hosts in 1971. Padgett et al. isolated JCV from the brain tissue of a patient with Hodgkin's lymphoma and Gardner et al. isolated BKV from the urine of a renal transplant recipient (Gardner et al., 1971; Padgett et al., 1971). Genus Polyomavirus members have been categorized into the family *Polyomaviridae* because they share common physical and biological characteristics. So far, 14 members of the polyomaviridae have been isolated from various mammalian and avian species, of which 12 have been fully sequenced (Pipas, 1992). Polyomaviruses can replicate most efficiently in the host cells of the species from which they originate, causing lytic cell death. There are other cell types in which polyomaviruses cannot replicate their genomes, and therefore in which continuous expression of early-gene products causes cellular transformation or oncogenesis (reviewed in Imperiale and Major, 2007).

2.1.2 Genome Structure

The circular viral genome is organized into three parts: the early region, which encodes proteins that are expressed before DNA replication ensues; the late region, which encodes proteins that are expressed after the start of DNA replication; and the regulatory region, otherwise known as the non-coding control region (NCCR), which consists of a single origin of DNA replication and contains promoters for early and late transcription. The non-overlapping coding sequences of the early and the late regions are transcribed from the opposite strands of the viral genome.

All the polyomaviruses known to date encode at least two early mRNAs which are produced by alternative splicing and are translated into large tumor antigen (TAg) and small tumor antigen (tAg). The early proteins are known as tumor antigens because of their recognition by antisera from animals harboring virally induced tumors (Habel, 1965). The mouse and hamster polyomaviruses encode a third tumor antigen known as middle T antigen (mTAg), which is produced from a third alternatively spliced mRNA. Some of the polyomaviruses encode additional early mRNAs that differ in their splicing patterns. SV40 and mouse polyomavirus (Py) produce an additional mRNA that codes for 17kT and tiny t, respectively (Riley et al., 1997; Zerrahn et al., 1993). JCV produces three alternatively spliced mRNAs which encode proteins known as T′135, T′136, and T′165, and BKV appears to produce one additional mRNA that encodes a T′ antigen (Bollag et al., 1989; Trowbridge and Frisque, 1995).

The late mRNAs code for the three capsid proteins VP1, VP2, and VP3 along with a fourth protein known as the agnoprotein.

This chapter will focus on the early proteins of polyomaviruses, their transforming characteristics, and how they induce oncogenesis and tumorigenesis through interactions with host cell proteins. Although the early proteins have been reported to interact with numerous host cell proteins, only those cellular proteins whose involvement in transformation has been well-established will be discussed.

2.1.3 Life Cycle

There are two possible outcomes to a polyomavirus infection: (1) a permissive host to viral replication allows viral DNA amplification, which results in the production of progeny virions and subsequent cell lysis, and (2) a nonpermissive host to viral replication does not allow viral DNA replication, which leads to an abortive infection, and this may result in cell transformation through the continued expression of the T antigens (small, large, middle, and truncated forms).

Polyomaviruses initiate infection of cells by binding to a cell surface receptor. This is followed by endocytosis of the viral particle and subsequent transport of the DNA genome to the nucleus. In the nucleus, the viral mini-chromosome, which is comprised of the genome in complex with cellular histones, is transcribed by RNA polymerase II to produce early mRNAs. TAg recruits the host DNA polymerase α/primase complex to the viral origin of DNA replication, where it unwinds the DNA to allow initiation to occur. TAg also inhibits early gene transcription and stimulates late gene expression. After DNA replication and the expression of late structural proteins, new progeny virions are assembled and are released from the cell.

The ability of SV40 to grow easily in cell culture has been utilized by researchers extensively to understand mechanisms of cell transformation and oncogenesis induced by viral oncogenes (reviewed in Ahuja et al., 2005; Sáenz-Robles et al., 2001). The characteristics exhibited by a transformed cell are as follows: (1) immortalization, which is indefinite growth in culture; (2) growth in low serum; (3) ability to overgrow a monolayer to form foci; (4) anchorage-independent growth, which is proliferation in the absence of contact to a substrate; and (5) induction of tumors in animals.

2.2 Genetics of Transformation

2.2.1 TAg

Large T antigen of SV40, the best investigated of the polyomavirus T antigens, is a modular nuclear phosphoprotein which can be divided into four major functional domains: an N-terminal J-domain, a retinoblastoma protein

(pRB)-binding domain, a central DNA-binding domain, and a C-terminal domain which interacts with p53 and exhibits helicase activity. It induces transformation through the J-domain, the pRB-binding domain, and the p53-binding domain (Chen et al., 1992; Peden et al., 1989; Peden et al., 1998; Pipas et al., 1983; Srinivasan et al., 1989; Zhu et al., 1992). The pRB-binding domain has a conserved LXCXE motif, through which it interacts with pRB and its family members, and the J-domain contains a HPDKGG motif that bears homology to the DnaJ family of chaperone proteins, through which it associates with heat shock cognate (Hsc) 70 co-chaperone protein (reviewed in Sullivan and Pipas, 2002). Py TAg shares the J- and pRB-binding domains with its primate counterparts, but does not interact with p53 (reviewed in Dilworth, 1990).

2.2.2 tAg

Small tAg is a cysteine-rich protein that shares its amino terminal sequence, including the J-domain, with TAg. It cooperates with TAg to transform both mouse and human cells (Bikel et al., 1987; Rundell et al., 1998). It is found both in the nucleus and in the cytoplasm of the cell (Ellman et al., 1984; Zhu et al., 1992). The LXCXE motif-containing domain in TAg is not present in tAg. The unique C-terminus of tAg binds to protein phosphatase 2A (PP2A), a serine/threonine phosphatase, through which it normally mediates signal transduction, apoptosis, and cell cycle regulation (Janssens and Goris, 2001; Pallas et al., 1990; Sontag, 2001). Interaction of tAg with PP2A is essential for large T-dependent transformation of human cells (Chang et al., 1985; de Ronde et al., 1989; Hahn et al., 2002; Porras et al., 1996; Yu et al., 2001).

2.2.3 mTAg

Middle TAg is a plasma membrane-associated phosphoprotein, has a J-domain like the other two T antigens, and also has the ability to bind to PP2A. It utilizes its unique C-terminal domain to induce cell transformation by promoting interactions with various proteins involved in the signal transduction pathway such as Src, Shc, and phosphatidylinositol 3-kinase (PI3K) which normally stimulate cell growth through activated tyrosine kinase receptors (Bolen et al., 1984; Campbell et al., 1994; Courtneidge and Smith, 1983; Dilworth et al., 1994; Whitman et al., 1985). Although it resides mainly at the plasma membrane there are reports of its existence in the perinuclear compartments of the cell (Dilworth et al., 1986; Ito et al., 1977). The schematics of the T antigen transformation domains are shown in Fig. 2.1.

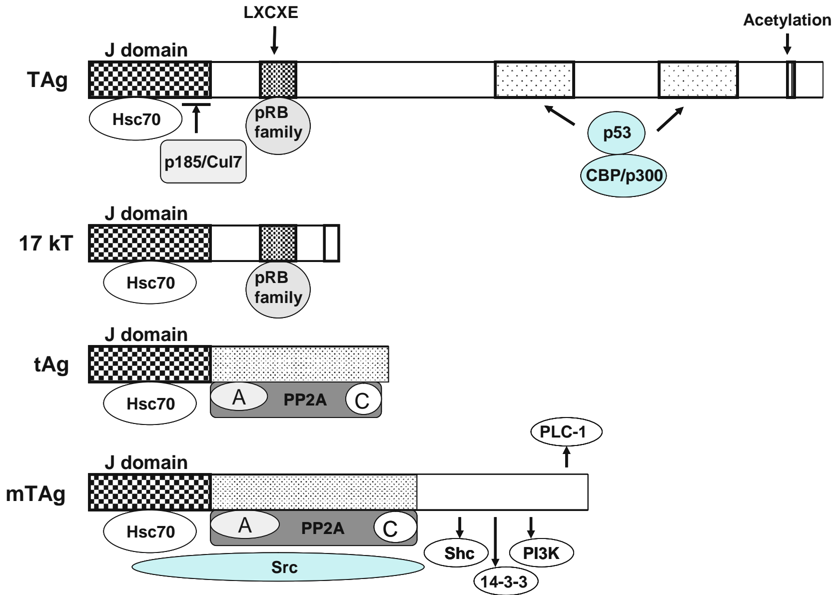

Fig. 2.1 Transformation domains of T antigens. The major domains that contribute to transformation by the polyomavirus T antigens are depicted, along with the cellular proteins that interact with the T antigens in these domains

2.3 Mechanisms

2.3.1 TAg

The large T antigens of the primate viruses mediate oncogenic transformation or establish lytic infection through the inactivation of p53 and the pRB family of tumor suppressor proteins. The pRB family and p53 proteins play major roles in controlling cell cycle progression. p53 guards the G2-M and G1-S transition check points, whereas pRB exerts its effect on the G1-S transition (reviewed in Brown et al., 2007; Van Dyke, 2007).

Polyomaviruses are dependent on the DNA replication machinery of the host cell for viral genome replication. Since the viruses infect quiescent (non-dividing) cells, the outcome of an infection is dependent on the ability of the virus to induce the host cell to enter S-phase in the absence of mitogenic signals. The interaction between the TAg and the pRB family proteins results in the activation of the E2F family of transcription factors, which induce expression of cellular genes essential for entry into S phase and DNA synthesis. T antigen also inactivates the p53 tumor suppressor to promote the transition of cells from G1 to S and to prevent apoptosis. In a permissive host, this leads to progeny virion production followed by cell lysis and death. In a non-permissive host or if rearrangements in the viral chromosome occur which interfere with

replication, it can result in oncogenesis, since T antigens are being continuously expressed but the viral life cycle cannot go to completion. A third potential mechanism of TAg-mediated oncogenesis is the induction of chromosomal damage, but the exact mechanism of how polyomaviruses initiate this event is still not understood (Lazutka et al., 1996; Ray et al., 1990; Theile and Grabowski, 1990; Tognon et al., 1996; Trabanelli et al., 1998). It is tempting to speculate here that chromosomal damage induced by TAg could be mediated through the interaction with Bub1, a mitotic spindle checkpoint protein (Cotsiki et al., 2004).

2.3.1.1 pRB Family

RB1, first identified as a tumor suppressor gene in which homozygous null mutations in humans causes tumors in the retina, plays a major role in cell cycle control and in tumor progression (reviewed in Genovese et al., 2006; Giacinti and Giordano, 2006). Its protein product, pRB, guards the major G1/S checkpoint and inhibits S-phase entry. It does this by interacting with the transactivation domain of E2F. The E2F family of transcription factors regulates genes involved in DNA synthesis and cell cycle progression including cyclins A and E, ribonucleotide reductase, DNA polymerase α, thymidine kinase, and proliferating cell nuclear antigen (PCNA) (reviewed in DeGregori and Johnson, 2006; Nevins, 2001). The retinoblastoma family is made up of three members, pRB, p107, and p130, which are collectively known as the "pocket proteins" because the crystal structure of these proteins indicates a pocket which forms the binding site for viral oncoproteins like adenovirus (Ad) E1A, TAg, and human papillomavirus (HPV) E7, as well as E2F, all of which contain the consensus LXCXE peptide motif (reviewed in Felsani et al., 2006).

The interaction between polyomavirus T antigens and pRB family members has been very well demonstrated both in vivo and in vitro (Dyson et al., 1990, 1989a; Harris et al., 1996; Howard et al., 1998; Krynska et al., 1997). Utilizing multiple assays, several studies have demonstrated that mutations in the LXCXE motif impair the ability of TAgs to induce transformation (Chen and Paucha, 1990; Kalderon and Smith, 1984; Rutila et al., 1986; Srinivasan et al., 1997; Zalvide and Decaprio, 1995). This requirement of the LXCXE sequence to promote transformation by viral oncogenes was first shown with the E1A oncoprotein and subsequently demonstrated for SV40 TAg (DeCaprio et al., 1988; Whyte et al., 1988a). Dyson et al. extended this work to show that TAgs of Py, monkey polyomavirus, baboon polyomavirus, JCV, and two different strains of BKV were able to form complexes with pRB (Dyson et al., 1990). SV40 TAg binds to pRB in G1 when it is hypophosphorylated, dissociates when pRB becomes hyperphosphorylated at the G1/S boundary, and then associates again with the hypophosphorylated form of pRB after the completion of mitosis (Ludlow et al., 1989, 1990). The interaction between TAg and pRB is not sufficient for the activation of E2F, which also requires the J-domain as has been demonstrated by extensive mutational analysis (Harris et al., 1998b; Pipas

et al., 1983; Sheng et al., 1997; Srinivasan et al., 1997; Zalvide et al., 1998). TAg mutants containing in-frame deletions in the J-domain are defective for dense foci formation in a monolayer overgrowth assay, in spite of retaining the ability to interact with the tumor suppressor proteins, pRB and p53. The J-domain and the LXCXE motif act together to disrupt the pRB/E2F complex to transactivate E2F. Truncated T antigen mutants that contain only the LXCXE motif and the J-domain have been shown to promote foci in many established lines and also contribute to hyperplasia in transgenic mice (Fromm et al., 1994; Kim et al., 1994; Srinivasan et al., 1989; Tevethia et al., 1997).

The inactivation of the pRB family members, p107 and p130, is also essential for Tag-induced transformation. In pRB-negative mouse fibroblasts, mutation of TAg in the LXCXE motif completely inactivates the transforming activity of TAg, suggesting that the interaction of p107 and/or p130 with the conserved pRB binding domain is essential for cell transformation (Christensen and Imperiale, 1995). It has also been demonstrated that wild-type mouse embryonic fibroblasts (MEFs) require the J-domain of TAg to grow to high density or under low serum conditions, but the J-domain is not necessary for MEFs lacking both p107 and p130 (Stubdal et al., 1997). Additionally, the expression of the alternatively spliced SV40 third early region product, 17kT, which has the J-domain and the pRB-binding domain, can complement J-domain mutations in TAg and restore transformation of primary human fibroblasts (Boyapati et al., 2003). All the alternatively spliced JCV T antigens (T′135, T′136 and T′165) contain a J-domain and an LXCXE motif and bind to the pRB family with different affinities (Bollag et al., 2000). Although Py TAg can also interact with the pRB family through the LXCXE motif and the J-domain, it has the ability to only immortalize cells in vivo, likely due to the absence of p53-binding function (Dyson et al., 1990; Freund et al., 1992; Holman et al., 1994; Larose et al., 1991; Pilon et al., 1996; Sheng et al., 1997). The proposed chaperone model for how TAg targets pRB/E2F is as follows: TAg interacts with pRB through the LXCXE motif and this leads to the J-domain-mediated recruitment of Hsc70, which utilizes ATP to dissociate E2F from pRB, allowing E2F to transactivate its target genes (Sullivan et al., 2000b,a). Using in vitro assays it was shown that an E2F fragment containing the pRB-binding site was a potent substrate for Hsc70 (Garimella et al., 2006). This study supports the hypothesis that the ATPase activity of the chaperone could provide free energy to dissociate E2F from the pRB family. In addition, SV40 TAg induces p130 degradation as does 17kT (Boyapati et al., 2003; Zalvide et al., 1998). BKV TAg appears to induce degradation of all the pRB family members (Harris et al., 1998b).

2.3.1.2 p53

The tumor suppressor protein, p53, was first discovered as a cellular protein which coimmunoprecipitated with TAg in SV40-transformed mouse cells (Lane and Crawford, 1979; Linzer and Levine, 1979). p53 is inactivated in greater than 50% of all human cancers reported to date (reviewed in Soussi and Lozano,

2005). It is found in a normal resting cell at very low levels and its expression is elevated in transformed cells. Activation of p53 occurs in response to DNA damage, hypoxia or radiation, and this leads to the induction of genes involved in cell cycle control and apoptosis (reviewed in Coutts and La Thangue, 2007). To execute repair of damaged DNA, p53 causes growth arrest by regulating the expression of p21 (cyclin-dependent kinase inhibitor), GADD45, or 14-3-3σ proteins. p53 promotes apoptosis by inducing expression of the genes encoding Apaf1 and Bax. p53 levels in a cell are primarily regulated by the oncoprotein murine double minute (Mdm2), which is an ubiquitin ligase and lowers p53 levels by mediating ubiquitin-dependent proteasomal degradation (reviewed in Coutts and La Thangue, 2007). In fact p53 initiates its own degradation by activating Mdm2 expression (reviewed in Brown et al., 2007; Van Dyke, 2007).

Oncogene-induced activation of p53 is mediated by the ARF (Alternate Reading Frame product of the p16/INK4A locus; $p14^{ARF}$ humans; $p19^{ARF}$ mouse) tumor suppressor (reviewed in Sherr, 2006). It upregulates the expression of p53 to counteract oncogenic stress signals. The expression of ARF is regulated by E2F. Therefore, ARF is a critical tumor suppressor that connects p53 to the pRB pathway (Fig. 2.2). This could lead to p53-dependent cell cycle arrest or apoptosis, but the p53 is inactivated by the polyomavirus oncoproteins (Reviewed in O'Shea, 2005a,b, 2005).

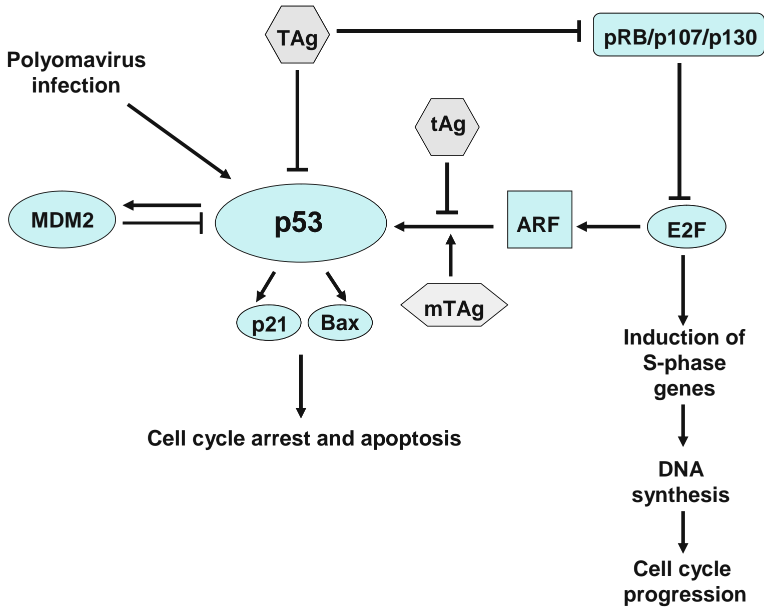

Fig. 2.2 Effects of T antigens on the pRB-p53-ARF pathways. Diagram indicating the cross-talk among the pRB, p53, and ARF pathways, and how the viral T antigens interact with them. Arrows indicate activation while T-shaped lines indicate inhibition. See text for details

Utilizing SV40 mutants, it has been demonstrated that the interaction between p53 and TAg elicits cell transformation (Peden et al., 1989, 1998). This interaction abrogates the ability of p53 to interact with DNA because TAg binds to the p53 DNA-binding domain, thereby inhibiting its transactivation functions (Bargonetti et al., 1992; Jiang et al., 1993). It is interesting to point out here that while viral oncoproteins like HPV E6 or Ad E1B/E4 target p53 for degradation, TAg expression leads to the accumulation of p53 by stabilizing it (Deppert et al., 1989; Oren et al., 1981; Tiemann and Deppert, 1994). The X-ray crystal structure of the SV40 TAg helicase domain bound to p53 DNA-binding domain was reported by Lilyestrom et al (Lilyestrom et al., 2006). This is the first crystal structure of a p53-viral oncoprotein complex. Interestingly, TAg interacts with Arg 248 and Arg 273 located in the DNA-binding domain of p53, which are also the residues that are frequently mutated in human cancers (Cho et al., 1994; Hollstein et al., 1991).

It has also been shown that TAg can block the p53 pathway using a mechanism that is independent of p53 binding; an SV40 TAg mutant containing the J-domain and the pRB-binding region, but lacking the p53-binding domain, can still inhibit p53-mediated growth suppression (Michael-Michalovitz et al., 1991; Quartin et al., 1994).

Interestingly, the binding of SV40 TAg to mouse p53 abolishes p53 transactivation completely, but the interaction with human p53 still allows transactivation of apoptosis and growth arrest genes (Sheppard et al., 1999). It is tempting to speculate here that the observed poor transformation efficiency of human cells by SV40 or, by analogy, human polyomaviruses, could be due to the inability of the TAg to completely block the tumor suppressor functions of p53 and pRB.

Similar to SV40 TAg, JCV and BKV TAgs also interact with p53 and prevent upregulation of p21 in response to DNA damage (Bollag et al., 1989; Harris et al., 1998a; Krynska et al., 1997). None of the T antigens expressed by Py directly bind to p53 and inactivate its function (reviewed in Dilworth, 1990). mTAg prevents transformation of both primary mouse cells and rat REF52 (Fisher rat embryo cells) cells by promoting the activation of the p53 pathway through the induction of ARF (Mor et al., 1997). On the contrary, mTAg can mediate transformation of rat REF52 and primary mouse cells if there is inactivation of either p53 or ARF, or if TAg and tAg are co-expressed (Cuzin, 1984; Mor et al., 1997; Rassoulzadegan et al., 1982). A report by Lomax and Fried explained this finding by demonstrating that in spite of the induction of ARF by mTAg, p53 levels are not elevated in the presence of tAg and/or TAg (Lomax and Fried, 2001). Furthermore, this observation has been supported by a recent report which shows that tAg is the Py protein which downregulates p53 expression (Moule et al., 2004). It is the PP2A-binding domain of tAg which blocks ARF-initiated p53 activation, demonstrating a role for PP2A in the regulation of ARF-p53 pathway.

2.3.1.3 Other Host Proteins

Although it has been very well demonstrated that the J-domain and pRB and p53-binding domains on TAg are the crucial regions on the protein that elicit cell transformation and oncogenesis, several genetic studies point to the presence of additional domains on TAg through which it can mediate interactions with host cell proteins that could possibly contribute to oncogenesis (Cavender et al., 1995; Sachsenmeier and Pipas, 2001; Wei et al., 2003).

CREB-binding protein (CBP)/p300 are transcriptional coactivator proteins that regulate several cellular functions which promote cell transformation and tumorigenesis (reviewed in Goodman and Smolik, 2000). p300 was first discovered as a binding partner for the E1A oncoprotein and it has been demonstrated that this association is important for adenovirus transformation (Egan et al., 1989, 1988; Whyte et al., 1988b). SV40 TAg is capable of complementing transformation by CBP/p300-binding mutants of E1A (Yaciuk et al., 1991). Though several early reports have demonstrated the interaction between CBP/p300 and T antigen, the nature of interaction and its functional significance as it relates to transformation have begun to be unraveled only recently (Avantaggiati et al., 1996; Eckner et al., 1996; Lill et al., 1997). TAg increases the global CBP histone acetyl transferase (HAT) activity (Valls et al., 2003). Two subsequent reports demonstrated that TAg is acetylated on K697, which is conserved in JCV, BKV, and SA12, by CBP in a p53-dependent manner (Borger and DeCaprio, 2006; Poulin et al., 2004). Shimazu et al. reported that overexpression of CBP HAT-acetylated TAg on K697, and additionally demonstrated that acetylation could occur independently of p53, but was further enhanced in the presence of p53 (Shimazu et al., 2006). TAg interacts with both HAT and HDAC enzymes, suggesting that TAg might regulate both its acetylation and its deacetylation depending on the cell environment (Avantaggiati et al., 1996; Borger and DeCaprio, 2006; Eckner et al., 1996; Poulin et al., 2004; Shimazu et al., 2006; Valls et al., 2007, 2003). Py TAg has also been shown to bind to CBP both in vitro and in vivo, and mutants in CBP binding can transform rat embryo fibroblasts but are not able to induce tumor formation in newborn mice, suggesting that this interaction may be important for tumorigenesis (Cho et al., 2001). A recent report proposed a two-step mechanism for the transactivation of E2F by Py TAg, in which TAg not only inactivates the pRB family to release E2F but may also acetylate histones by recruiting CBP/p300 (Nemethova et al., 2004).

The association of SV40 TAg with a member of the cullin family of E3 ubiquitin ligases was first reported by Kohrman and Imperiale, while studying TAg/host protein interactions in transformed mouse cells (Kohrman and Imperiale, 1992). This TAg-interacting host protein was named p185. In a subsequent independent study, Tsai et al. reported the interaction of p193, a Bcl-2 domain containing, apoptosis-inducing protein, with TAg, in a cardiomyocyte tumor cell line (Tsai et al., 2000). Both p185 and p193 were later shown by DeCaprio and colleagues to be Cul7, a member of the cullin family of E3

ubiquitin ligases which execute ubiquitination-dependent proteosome degradation (Ali et al., 2004). p185/Cul7 binding to TAg was shown to be independent of pRB and p53 inactivation and also viral replication. It was also demonstrated that p185/Cul7 could specifically bind to the RING finger protein, Rbx1/Roc1, Skp1, and F box protein Fbw6 [Skp, Cullin, F-box (SCF) containing complex] through the N-terminal region of TAg (Ali et al., 2004). It was demonstrated that TAg residues 69–83 interact with Cul7 and that TAg-induced transformation is dependent not only on the inactivation of pRB family and p53 but also on the inactivation of Cul7 (Kasper et al., 2005). These data suggest that in the presence of TAg, Cul 7 may act as a tumor suppressor and TAg induces transformation by inactivating the growth inhibitory functions of Cul7.

SV40 TAg also interacts with Fbw7 tumor suppressor, the substrate recognition component of the ubiquitin ligase SCF^{Fbw7}, which targets proteins such as cyclin E for degradation (Welcker and Clurman, 2005). Fbw7 binds to TAg in the C-terminus through T701, which resembles a consensus Cdc4-phospho degron (CPD) motif found in Fbw7 substrates. This association with Fbw7 causes TAg to enhance cyclin E-associated kinase activity and also prevents acetylation of TAg (Valls et al., 2007).

TAg has been shown to regulate the insulin-like growth factor I (IGF-I)-signaling pathway by interacting with and stimulating components in this pathway. The binding of IGF-I to its receptor (IGF-IR) induces autophosphorylation of the receptor, which subsequently leads to tyrosine phosphorylation of multiple substrates, including insulin receptor substrate-1 (IRS-1) (reviewed in Laviola et al., 2007). SV40 TAg has been reported to induce the expression of IGF-I, and it has been shown that signaling through IGF-IR is essential for transformation by SV40 (DeAngelis et al., 2005; Porcu et al., 1992; Sell et al., 1993). The activation of IGF-IR by IGF-I is essential for TAg to induce growth in low serum (Valentinis et al., 1994). The ability of TAg to transform MEFs and other cell lines depends on tyrosine phosphorylation of IRS-1 (DeAngelis et al., 2005). In the absence of the IGF-IR receptor, TAg is able to induce tyrosine phosphorylation of IRS-1 and promote transformation (DeAngelis et al., 2005). Additionally it has been demonstrated that TAgs of both SV40 and JCV associate with IRS-1 and this association facilitates its translocation to the nucleus (Fei et al., 1995; Lassak et al., 2002). A dominant negative IRS-1 mutant inhibits growth and anchorage independence of JCV TAg-transformed cells (Lassak et al., 2002). It has been suggested that the facilitation of nuclear translocation of IRS-1 by JCV TAg could lead to the attenuation of faithful DNA repair by inducing interactions between Rad51 and IRS-1 at sites of DNA damage (Reiss et al., 2006; Trojanek et al., 2006).

JCV TAg has also been reported to interact with β-catenin, a major effector of the Wnt-signaling pathway that regulates both development and oncogenesis (Enam et al., 2002; Gan and Khalili, 2004; reviewed in Polakis, 2007). Accumulation of β-catenin at high levels due to an increase in protein stability during Wnt signaling leads to its translocation to the nucleus, where it interacts with the T-cell factor (TCF)/lymphoid-enhancing factor (Lef) transcription factor

and induces transactivation of c-myc, cyclin D1 and many other genes involved in cell proliferation (reviewed in Peifer and Polakis, 2000). The interaction between JCV TAg and catenin also leads to an increase of β-catenin levels, translocation of β-catenin to the nucleus, and stimulation of expression of c-myc and cyclin D1 genes (Enam et al., 2002; Gan et al., 2001; Gan and Khalili, 2004).

The interaction between Bub1, a mitotic spindle checkpoint kinase, and SV40 TAg was demonstrated by using coimmunoprecipitation and yeast two-hybrid analysis (Cotsiki et al., 2004). Genetic analysis revealed that the interaction is not necessary for immortalization but may be required for transformation. Additionally it was demonstrated that a tryptophan-rich motif, WEXWW, which is conserved among SV40, JCV, BKV, and bovine polyomavirus T antigens, was essential for efficient binding. Cells arrested in mitosis by a microtubule-disrupting drug are allowed to progress to the spindle assembly checkpoint with TAg expression. This led the authors to speculate that TAg may transform cells by promoting aneuploidy and genetic instability. As mentioned above, TAg has been reported to induce chromosomal instability in human cells by deregulating mitotic checkpoints (Chang et al., 1997). Interestingly, Bub1 is frequently mutated in human cancers, and a Bub1 mutant has been shown to promote errors in chromosome segregation and induce aneuploidy (Cahill et al., 1998; Ru et al., 2002; Shichiri et al., 2002).

SV40 TAg associates with Nbs1, the Nijmegen breakage syndrome protein, which together with meiotic recombination 11 homolog (Mre11), and Rad50 mediates faithful double-strand break repair. TAg-expressing cells exhibit chromosomal hyperreplication, which is dependent on the interaction between Nbs1 and TAg (Wu et al., 2004). There are also reports of upregulation of Nbs1 protein in SV40 TAg-immortalized cardiomyocytes, and it was proposed that TAg induces immortalization of these cells by deregulating the Mre11/Rad50/Nbs1 pathway (Lanson et al., 2000). SV40 TAg also interacts with TEF-1 (transcription enhancer factor), a transcription factor that induces expression of the viral early genes (Berger et al., 1996; Gruda et al., 1993). An SV40 TAg mutant (S189N) within the DNA-binding domain cannot bind to TEF1, and this same mutant has been shown to be inefficient in both mediating cell transformation and stimulating DNA synthesis in quiescent cells (Berger et al., 1996; Dickmanns et al., 1994). These observations suggest that the interaction between TAg and TEF1 could be important in cell transformation.

2.3.2 tAg

tAg mediates two major functions in the cell, interacting with and inhibiting PP2A activity, and stimulating viral and cellular promoters. PP2A is a heterodimer composed of a catalytic C subunit, a scaffolding A subunit, and several types of regulatory B subunits (Janssens and Goris, 2001; Sontag, 2001). The

A/C heterodimer subunit of PP2A interacts with the regulatory B subunits to yield a PP2A holoenzyme. PP2A activity is very crucial for cell survival, regulation of cell cycle, response to DNA damage, and embryonic development (Janssens and Goris, 2001; Sontag, 2001). tAg binds to the A and C subunits and causes displacement of the B subunit, which results in inhibition of PP2A activity (Pallas et al., 1990; Walter et al., 1990; Yang et al., 1991; Chen et al., 2004; Ruediger et al., 1992; Sontag et al., 1993). Mutations in the unique C-terminus of SV40 tAg led to the identification of residues 97 and 103 as being crucial for the interaction with PP2A (Mungre et al., 1994; Porras et al., 1996; Yu et al., 2001). A tAg mutant containing only the PP2A inactivation domain (aa 88–174) can induce transformation efficiently, indicating that tAg mediates transformation primarily by targeting PP2A activity (Hahn et al., 2002).

tAg-mediated targeting of PP2A leads to the stimulation of growth factor-signaling pathways involving the mitogen-activated protein kinases (MAPK), extra cellular signal-regulated kinases (ERK), and stress-activated protein kinases (SAPK), and tAg mutants that lose the ability to bind are unable to affect these pathways (Sontag et al., 1993; Watanabe et al., 1996). Inhibition of PP2A activity by tAg leads to the stabilization of c-Myc since c-Myc is a direct substrate for PP2A, and in tAg-dependent transformation assays a stable c-Myc mutant can replace tAg expression (Yeh et al., 2004). It has also been demonstrated that tAg activates Akt, a serine/threonine protein kinase, through PP2A inactivation, leading to cell transformation (Yuan et al., 2002; Zhao et al., 2003). PP2A also regulates the mammalian target of rapamycin (mTOR) pathway by interacting with $p70^{S6K}$, which regulates nutrient signaling, thereby allowing cells to proliferate in low-nutrient medium (Ballou et al., 1988; Chen et al., 2004; Hahn et al., 2002; Westphal et al., 1999). All the above reports point to the critical importance of regulation of PP2A activity by tAg in inducing transformation. The tAgs of JCV and BKV are not very well characterized, although tAg of both viruses contain two conserved cysteine motifs (CXCXXC) which are involved in the interaction with PP2A (Mateer et al., 1998; Mungre et al., 1994; Pipas, 1992). Additionally, it has been shown that BKV tAg is able to coimmunoprecipitate two cellular proteins which may be subunits of PP2A (Rundell et al., 1981). tAg also utilizes the J-domain for chaperone activity and to transactivate the cyclin A promoter (Porras et al., 1996; Srinivasan et al., 1997).

The involvement of tAg in cellular transformation attained additional prominence when it was determined that the cooperation of tAg with TAg was essential for the transformation of human cells. In rodent cells, tAg mediates transformation by stimulating cell proliferation, which can be substituted by high levels of TAg expression (Bikel et al., 1987). In comparison to rodent cells, human cells are more difficult to transform (Chang et al., 1985; Sager et al., 1983). Human cells expressing TAg alone exhibit normal cell characteristics, whereas TAg- and tAg-expressing cells exhibit morphological changes very similar to tumor-derived cell lines (Chang et al., 1985; de Ronde et al.,

1989; Sager et al., 1983). Additionally, some human cells with both TAg and tAg expressions exhibit anchorage-independent growth in short-term assays (Chang et al., 1985; Yu et al., 2001).

2.3.3 Role of T Antigen in Cell Immortalization and Transformation of Human Cells

Although the expression of TAg can allow transformation of most cells in culture, the ability of TAg to accomplish immortalization and transformation depends both on the species and on the cell type used (reviewed in Ahuja et al., 2005 and Ali and DeCaprio, 2001; Zhu et al., 1992). TAg alone is sufficient to transform primary and established rodent cells (Brown et al., 1986; Kriegler et al., 1984). In primary mouse cells, loss of p53 or ARF is sufficient to induce immortalization in most cell types (reviewed in Levine, 1997; Sherr, 1998). Therefore, TAg expression in primary MEFs is sufficient to cause cell immortalization (Zhu et al., 1991). However, simultaneous inactivation of the p53 and the pRB pathway by TAg is unable to immortalize human cells, suggesting that human cell immortalization is more complex (Hahn and Weinberg, 2002).

There are several notable differences observed between human and rodent cells for cell immortalization (reviewed in Chen and Hahn, 2003). Hayflick and Moorhead demonstrated that normal mammalian cells have a limited lifespan in culture (Hayflick and Moorhead, 1961). Immortalization, which is the ability of the cell to replicate indefinitely, is a necessary step for cell transformation to occur. However, primary human cells have to overcome two proliferative barriers, the "mortality stage" and the "crisis stage," to obtain cell immortalization (Newbold and Overell, 1983; Newbold et al., 1982). The mortality stage occurs when cells are arrested near the G1/S checkpoint and can be overcome by the stimulation of DNA synthesis, and the "crisis stage" is when cells fail to undergo cell division and ultimately die. TAg expression allows human cells to overcome replicative senescence, which is irreversible cell cycle arrest in the G1 state, and these cells are able to proliferate until the "crisis stage" (Jha et al., 1998; Kim et al., 1998; Ozer, 2000; Ozer et al., 1996; Ryan et al., 1992). Therefore TAg alone is not sufficient for human cell immortalization. Co-expression of the catalytic subunit of telomerase, hTERT, with TAg allows cells to overcome the crisis stage and attain immortality, but even this combination is unable to transform most human cells (reviewed in Chen and Hahn, 2003). For full transformation of human cells, co-expression of tAg is essential along with TAg, hTERT, and an activated oncogene such as a mutated *Ras* allele (Hahn et al., 1999).

The studies of SV40 infection of human mesothelial cells are of particular interest because of the suggested role of SV40 in the development of human mesotheliomas (reviewed in Rizzo et al., 2001). Interestingly, human mesothelial cells can be easily infected by SV40 and importantly, tAg cooperates with

TAg to induce telomerase activity in these cells, stimulates Notch activity which exerts its effect through the extracellular signal-regulated kinase (ERK) pathway, and activates the autocrine loop of hepatocyte growth factor (HGF) receptor (Met) (Bocchetta et al., 2003; Cacciotti et al., 2001; Foddis et al., 2002; reviewed in Skoczylas et al., 2004). These molecular studies, together with the ability of SV40 to transform primary human mesothelial cells, in the presence of asbestos fibers, support a role in human mesotheliomas (reviewed in Carbone and Bedrossian, 2006).

2.3.4 mTAg

Middle T antigen is a phosphoprotein that is responsible for many of the transforming functions of Py. It is a potent oncoprotein that has the ability to transform several established cell types in culture and can induce a variety of tumors in animals, independent of TAg and tAg expression (reviewed in Gottlieb and Villarreal, 2001; Ichaso and Dilworth, 2001). However, for the transformation of primary fibroblasts, mTAg is dependent on TAg or tAg (Cuzin, 1984; Land et al., 1983; Rassoulzadegan et al., 1982).

mTAg associates with host cell membranes through a stretch of hydrophobic amino acids located in its C-terminal end, and using mutational analysis it has been demonstrated that this domain is essential for transformation (Dilworth et al., 1986; reviewed in Ichaso and Dilworth, 2001). The N-terminus of mTAg interacts with PP2A and mutations that prevent its association with PP2A inhibit its tyrosine phosphorylation and abolish the ability of mTAg to activate growth signal transduction pathways (Pallas et al., 1990; Walter et al., 1990). Although mTAg has been reported to interact with Hsc70, deletion of the HPDKGG motif in the mTAg J domain has no effect on the mTAg-induced focus formation, indicating that this interaction is not required for transformation of cultured cells (Campbell et al., 1995; Glenn and Eckhart, 1995).

Membrane-bound mTAg also interacts with Src family members ($pp60^{c\text{-}src}$, $pp62^{c\text{-}yes}$, $pp59^{c\text{-}fyn}$) and analysis of mTAg mutants suggests that association with Src is necessary but not sufficient to elicit cell transformation (Bolen et al., 1984; Grussenmeyer et al., 1987; Courtneidge, 1985; Markland et al., 1986; Cheng et al., 1986). This association of mTAg with Src leads to the phosphorylation of several crucial tyrosine residues on mTAg, mainly Y250, Y315, and Y322 (Dilworth et al., 1986; reviewed in Ichaso and Dilworth, 2001). It has also been reported that mutants that cannot bind PP2A are unable to associate with Src (Glover et al., 1999).

Phosphorylation of mTAg on Y250 serves as a platform for binding by the Shc family of cellular proteins, which then promotes cell proliferation (Campbell et al., 1994; Dilworth et al., 1994). This association causes mTAg to induce tyrosine phosphorylation of Shc (Fig. 2.3). The mTAg/Shc complex then interacts with the Grb2 adaptor molecule, which associates with the Sos guanine

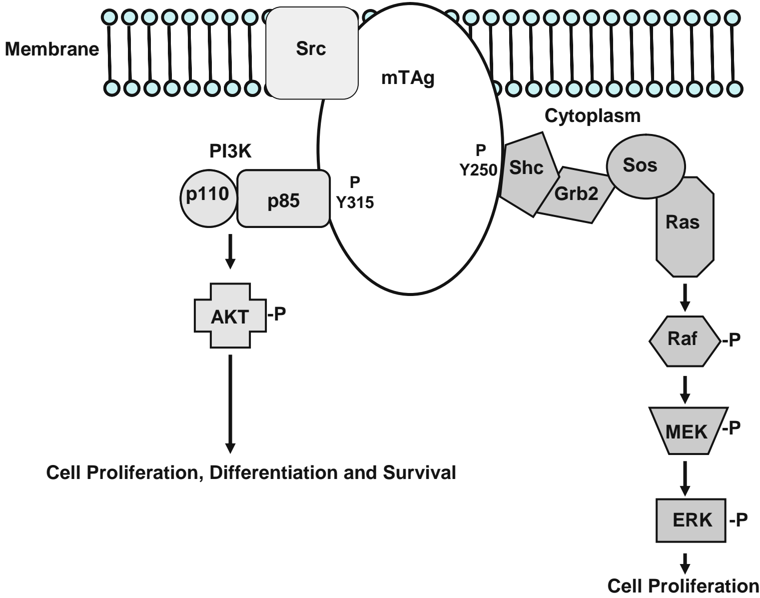

Fig. 2.3 Transformation mechanisms of middle T antigen. The two major signaling pathways that are engaged by mTAg, PI3K and Shc, are shown

nucleotide exchange factor and induces activation of Ras. The activation of Ras ultimately leads to activation of the Raf-MAP kinase pathway, which phosphorylates MEK and ERK (reviewed in Gottlieb and Villarreal, 2001; Ichaso and Dilworth, 2001). Deletion of the Shc-binding site on mTAg dramatically reduces transformation efficiency, indicating that the stimulation of the MAP kinase cascade is an important factor in mTAg-mediated tumorigenesis (Druker et al., 1992; Markland et al., 1986). Additionally, *rac*1 and CDC42, which are downstream elements of Ras, have been shown to be required for mTAg transformation (Chen et al., 1999; Urich et al., 1997).

mTAg phosphorylated at Y315 associates with the PI3K regulatory subunit, p85, leading to the activation of PI3K activity, which phosphorylates several cellular targets (Fig. 2.3). Deletion mutants of mTAg that lack PI3K binding are severely defective in transformation-inducing ability (Markland and Smith, 1987). Interestingly, it has been reported that mTAg transformation activates PKC. This may be mediated by PI3K since its products are involved in the activation of PKC family members (Marcellus et al., 1991). Extensive studies with receptor signaling and mTAg point to the existence of crosstalk between the Shc-MAPK and the PI3K pathways (reviewed in Gottlieb and Villarreal, 2001; Ichaso and Dilworth, 2001). When a mTAg mutant that is unable to bind Shc is co-expressed with a mutant that is defective in its ability to bind to PI3K, induction of transformation is low indicating that

both binding sites have to be on the same molecule for functionality (Chen et al., 1999; Urich et al., 1997).

mTAg also associates with phospholipase Cγ-1 (PLCγ-1) (Su et al., 1995) through phosphorylation on Y322. This induces tyrosine phosphorylation of PLCγ-1, which stimulates its enzymatic activity (Su et al., 1995). Additionally mTAg utilizes its phosphorylated S257 residue to interact with some members of the 14-3-3 dimeric family of proteins, and it has been proposed that this association may promote multimerization of mTAg complexes (Pallas et al., 1994). Although removal of S257 does not reduce transformation efficiency of cells in culture, a S257 mTAg mutation significantly reduces salivary gland tumor formation in mice, indicating that this association may be crucial in certain cell types (Cullere et al., 1998).

2.4 Conclusions

The transformation mechanisms of polyomaviruses have contributed significantly to our understanding of molecular pathways leading to oncogenesis. Early investigations of these viral T antigens led to seminal discoveries about the p53 and pRB pathways. In recent years, new mechanisms for inactivation of other cellular targets have begun to emerge, and exploring these additional pathways will certainly continue to be beneficial for elucidating mechanisms involved in tumorigenesis. The incidence of acute disease caused by the human polyomaviruses, BKV and JCV, is on the rise in immunocompromised and immunosuppressed individuals. While much of the interest in these viruses in the past has been due to their oncogenic potential, the lessons that have been learned from these experiments will undoubtedly be important as scientists attempt to understand how they cause disease in the host and move toward development of effective therapies.

Acknowledgments We thank the members of our laboratory for valuable suggestions and helpful comments. This work was supported by NIH grant CA118970.

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Chapter 3
Polyomaviruses and Disease

Jaime M. Kean and Robert L. Garcea

The first recognized member of the *Polyomaviridae* family, murine polyomavirus, was isolated in 1953 by Ludwig Gross while studying leukemia development in mice. Simian vacuolating virus (SV40) was isolated in 1960 by Sweet and Hilleman from primary monkey kidney cell cultures used to cultivate both the Salk and Sabin poliovirus vaccines (Sweet and Hilleman 1960). The human polyomaviruses, BKV and JCV, were discovered in 1971 and are ubiquitous in humans, infecting over 80% of the population by adulthood. Polyomaviruses express T-antigen proteins that circumvent cell cycle controls in order to replicate efficiently. The ability of polyomaviruses to promote cell proliferation and establish persistent infections in their hosts has implicated them in malignancy. Here we will review diseases caused by the polyomaviruses in animals and compare to those in man in order to identify general features of their pathobiology.

3.1 Animal Polyomaviruses

Polyomaviruses have been identified in many hosts including humans, birds, monkeys, and hamsters; each virus exhibits a relatively narrow host range. Murine polyomavirus was named for its ability to induce tumor formation in a wide variety of tissues in mice. Some strains of murine polyomavirus are highly tumorgenic in mice while infection with others results in a lower incidence of tumor formation. The highly tumorgenic strain, PTA, forms large plaques in culture, while the less pathogenic strain, RA, forms small plaques. The virulent LID strain, also characterized by a large plaque phenotype, spreads rapidly, resulting in immediate mortality to its murine host. While both large plaque and small plaque variants recognize unbranched sialoligosaccharides, large plaque variants bind branched sialoligosaccharides with

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_3, © Springer Science+Business Media, LLC 2009

much lower affinity compared to small plaque variants, resulting in differential adsorption of these variants by host cells. This phenotype has been mapped to a single amino acid in the VP1 protein (Freund, Calderone et al. 1991). It has been suggested that the small plaque variants are slow to spread, resulting in containment by the host immune response. In contrast, variants exhibiting large plaque phenotypes spread rapidly, overwhelming the host, leading to overt disease and rarely death.

Resistance to polyomavirus-induced tumors is mediated by polyomavirus-specific CD8+ cytotoxic T lymphocytes (CTLs) (Moser, Altman et al. 2001; Moser and Lukacher 2001). In one mouse strain, C3H/BiDa, the intrathymic deletion of CTLs by an endogenous mouse mammary tumor virus (MMTV) results in polyomavirus-induced tumor susceptibility in these mice due to deletion of polyomavirus tumor-specific T-cell precursors (Lukacher, Ma et al. 1995). PE mice, free of endogenous MMTV, are also highly susceptible to polyomavirus-induced tumors and, when crossed with the resistant BR mouse strain, transmit this susceptibility as a dominant or codominant trait to their offspring (Velupillai, Yoshizawa et al. 1999). Differences in the immune response of the resistant BR mouse strain compared to the susceptible PE strain have implicated immune differences in polyomavirus-induced tumor susceptibility. A Th2 response is observed in the susceptible PE strain, while a Th1 response is observed in resistant strains. The Th1 response in BR mice results in a sustained CTL response, allowing tumor suppression by the host immune system (Velupillai, Carroll et al. 2002; Velupillai, Garcea et al. 2006).

Transmission of murine polyomavirus may parallel that of the human polyomaviruses. Wild mouse populations are typically infected by horizontal transmission, and pups are protected from early infection by maternal antibodies. In contrast, vertical transmission from infection of naïve mothers results in the death of pups (McCance and Mims 1977). Murine polyomavirus sequences in unborn fetuses have been detected in over 80% of litters from acutely infected pregnant mice, supporting possible transplacental transmission (Zhang, McNees et al. 2005).

Hamster polyomavirus (HaPV) was discovered as the causative agent of skin epitheliomas of the Syrian hamster (Graffi, Schramm et al. 1967). Spread of HaPV likely occurs through the urine of infected animals. Diseased animals exhibit weight loss and palpable lumps in the abdomen and trichoepitheliomas, visible as wart-like nodules on the skin (Scherneck, Ulrich et al. 2001).

Bird polyomaviruses frequently cause acute disease. The first acute disease associated with a polyomavirus, budgerigar fledgling disease, was identified in psittacine birds (e.g., parrots). Budgerigar fledgling polyomavirus (BFPV) may cause sudden death or clinical manifestations such as abdominal distention, hemorrhage under the skin, and reduced formation of feathers. Animals infected with BFPV may also develop neurological signs characterized by ataxia and tremors of the head and neck several days before dying. The mortality rate is high, ranging from 25% to 100% (Krautwald, Muller et al. 1989). Goose hemorrhagic polyomavirus (GHPV) induces hemorrhagic “nephritis enteritis”

in geese, a potentially fatal disease (Guerin, Gelfi et al. 2000), with high morbidity and mortality rates in geese 4–10 weeks old. Disease induced by GHPV is characterized by edema of subcutaneous tissues, gelatinous ascites, inflammation of the kidneys, and hemorrhagic enteritis (Schettler 1980). Such pathology in birds may indicate that the virus has been introduced into a naïve, highly susceptible population.

3.2 Lymphotropic Polyomavirus (LPV)

LPV was first isolated in 1979 from a B-lymphoblastoid line derived from an African green monkey (zur Hausen and Gissmann 1979). LPV is tropic for lymphoid cells, including human lymphoid cells in culture (Pawlita, Lenoir et al. 1987). This tropism is in part mediated by interactions of the major capsid protein, VP1 with surface glycoproteins (Keppler, Herrmann et al. 1994; Herrmann, Oppenlander et al. 1995), as well as by the viral transcriptional enhancer that encodes for three 63-base pair repeats containing *Pu* boxes that restrict expression to lymphoid cells (Mosthaf, Pawlita et al. 1985; Petterson and Schaffner 1987; Erselius, Jostes et al. 1990). LPV transforms hamster embryo cells (Takemoto and Kanda 1984; Kang and Folk 1992). The transformed hamster embryo cells induce solid tumor formation after inoculation into hamsters. Expression of the LPV early region in transgenic mice causes lymphoproliferative disorders and tumors of the choroid plexus (Chen, Neilson et al. 1989). The LPV large T-antigen forms complexes with p53 (Symonds, Chen et al. 1991). Because the LPV large T-antigen shares 45% nucleotide identity with the T-antigens of both SV40 and mouse polyomavirus, it is likely that the T-antigen of LPV also interacts with pRb family members, although it has not yet been documented (Symonds, Chen et al. 1991).

Although the LPV genome is overall very similar to that of SV40 and BKV (Kanda, Yoshiike et al. 1983), the VP1 major capsid protein of LPV is antigenically distinct. Human sera positive for BKV and JCV VP1 do not cross-react with LPV VP1 (Viscidi and Clayman 2006). In contrast, the large T-antigens of SV40, BKV, JCV, and LPV are immunologically similar.

There is a wide distribution of antibodies to LPV among primates. Serologic evidence also originally suggested that LPV infection may occur in humans (Brade, Muller-Lantzsch et al. 1981; Takemoto and Segawa 1983; Viscidi and Clayman 2006) and more recent assays confirm that 15–25% of humans are seropositive for LPV-specific antibodies, indicating that exposure to LPV or a virus antigenically similar to LPV occurs in the human population (Viscidi and Clayman 2006). However, while LPV or a virus antigenically similar to LPV may exist in the human population, age-specific exposure to LPV has not been elucidated. Furthermore, although LPV large T-antigen has transforming capabilities, a role for LPV infection in human malignancy has not yet been established. Because LPV infection is not ubiquitous, LPV-specific seroepidemiology may be used to evaluate correlations with specific disease populations.

3.3 SV40 and Human Disease

Simian virus 40 (SV40) was first isolated from rhesus monkey kidney cells used to cultivate vaccine strains of poliovirus in the 1960 s (Sweet and Hilleman 1960). SV40 is transmitted through urine, respiratory, oral, and subcutaneous routes (Shah and Nathanson 1976) among Asian macaque monkeys and causes disease in both African green monkeys and rhesus monkeys (Horvath, Simon et al. 1992; Ilyinskii, Daniel et al. 1992; Lednicky, Arrington et al. 1998).

SV40 has been isolated from the brains and kidneys of macaque monkeys (Ilyinskii, Daniel et al. 1992) and from the brain and peripheral blood mononuclear cells of SIV-infected, immunocompromised rhesus monkeys (Lednicky, Arrington et al. 1998). SV40 causes PML in SIV-infected rhesus monkeys (Horvath, Simon et al. 1992), and in situ DNA hybridization for SV40 DNA demonstrates viral replication in the kidney and brain tissue of these animals. A fatal renal disease caused by a slow-growing SV40 variant in New World monkeys also has been observed (Zdziarski, Sarich et al. 2004).

SV40 transforms cells from species that are nonpermissive for virus replication, including rats, mice, hamsters, cattle, and guinea pigs (Vilchez, Kozinetz et al. 2003). Subcutaneous injection of SV40 into hamsters results in solid tumor formation (Eddy, Borman et al. 1962), while intravenous inoculation induces lymphoma, leukemia, and osteosarcoma (Diamandopoulos 1972). Hamsters injected intracranially with virus develop choroid plexus and ependymoma tumors (Kirschstein and Gerber 1962), while mesotheliomas develop in hamsters injected intrapleurally (Cicala, Pompetti et al. 1993). Transgenic mice expressing the early region of SV40 under the control of the SV40 early region promoter develop tumors of the choroid plexus (Brinster, Chen et al. 1984; Messing, Chen et al. 1985).

The inadvertent administration of SV40 in contaminated polio vaccines to over 100 million people in the United States between 1955 and 1963 stimulated interest in determining the potential of this virus to cause disease in the human population. Humans may support low-level SV40 replication (Melnick and Stinebaugh 1962). However, while rodent cells are immortalized by SV40 infection, human cells exhibit an extended life span (Bryan and Reddel 1994; Vilchez, Kozinetz et al. 2003). Although epidemiologic studies have been flawed to varying extents, no association has been found between vaccine administration and cancer (Stratton, Alamario et al. 2003).

SV40-like DNA sequences have been detected in a variety of human tumors. SV40 sequences were first detected by PCR in tumors of the choroid plexus and ependymomas (Bergsagel, Finegold et al. 1992). Infectious SV40 particles were subsequently isolated from a human choroid plexus tumor (Lednicky, Garcea et al. 1995). In addition to tumors of the choroid plexus, SV40 DNA sequences have been detected in medulloblastomas using PCR, with concurrent T-antigen immunohistochemistry (Huang, Reis et al. 1999).

SV40-like sequences have been detected in up to 30% of bone tumors (Carbone, Rizzo et al. 1996; Gamberi, Benassi et al. 2000; Yamamoto, Nakayama et al. 2000) and 43% of non-Hodgkins lymphomas (Shivapurkar, Harada et al. 2002; Vilchez, Lednicky et al. 2002). However, a lack of association between polyomaviruses and human lymphomas has also been reported (Hernandez-Losa, Fedele et al. 2005).

SV40 DNA sequences have been found in 40–50% of mesotheliomas (Shivapurkar, Wiethege et al. 1999; Testa and Giordano 2001; Klein, Powers et al. 2002), with some geographic variability (Hirvonen, Mattson et al. 1999; Emri, Kocagoz et al. 2000). Mesothelioma is a complex disease and exposure to asbestos is a known risk factor for its development. Human mesothelioma cell lines are semi-permissive for SV40 infection and undergo cell transformation in response to SV40 infection (Bocchetta, Di Resta et al. 2000). Cell exposure to asbestos has a synergistic effect on transformation (Bocchetta, Di Resta et al. 2000). Given these data and the numerous studies which have described the association of SV40 with mesothelioma, SV40 infection has been postulated as a cofactor in the development of this malignancy. However, while many studies have reported an association between mesothelioma and SV40 infection, others have failed to detect SV40 sequences in mesothelioma samples (Vilchez, Kozinetz et al. 2003).

The debate continues over whether SV40 is present in the human population and contributes to human malignancies. While SV40 DNA has been isolated from choroid plexus neoplasms, ependymomas, mesotheliomas, and bone tumors, there are several reasons why an association between SV40 and human malignancy remains controversial. First, viral copy number has been estimated at one copy per 1000 tumor cells. Second, many PCR cycles are needed to detect SV40 sequences in tissues and there have been reports of PCR contamination with laboratory plasmids, contributing to the inconsistencies in the detection of SV40 sequences in tumor samples between laboratories. Immunohistochemistry for T-antigen is inconclusive because current reagents cannot distinguish between the BKV, JCV, and SV40 proteins. Finally, SV40 seropositivity in humans is extremely low ($< 3\%$) (Carter, Madeleine et al. 2003) and epidemiologic evidence has not linked the limited seroprevalence with any specific malignancy.

3.4 BKV and Human Disease

BKV was first isolated in 1971 from the urine of an immunocompromised renal transplant patient with the initials B. K. (Gardner, Field et al. 1971). BKV is serologically ubiquitous in the human population, with an initial infection occurring during early childhood without apparent symptoms. Viremia may occur during the initial infection, allowing the virus to disseminate to distant organs, establishing a persistent infection in renal and uroepithelial cells, and possibly

in lymphocytes (Dorries, Vogel et al. 1994; Imperiale 2000). Southern blot analyses, as well as virus isolation, have established the kidney as the main site of BKV latency in healthy individuals (Barbanti-Brodano, Martini et al. 1998).

The natural route of BKV transmission has not been resolved, although it likely occurs both horizontally and vertically. Passively acquired antibody is often present at birth, but declines during the first 3 months of life (Gardner SD 1973). BKV infection incidence peaks between 2 and 5 years of age, supporting horizontal transmission (Brown, Gardner et al. 1984). BKV DNA has been detected in tonsils and associated with upper respiratory tract infections in children, suggesting the potential for respiratory transmission (Goudsmit, Wertheim-van Dillen et al. 1982). BKV reactivation and low-level replication accompanied by asymptomatic viruria can occur in approximately 5% of healthy individuals (Hirsch and Steiger 2003). Therefore, both respiratory transmission and spread via the urine-oral route likely contribute to the horizontal transmission of BKV.

BKV may also be vertically transmitted. BKV-specific IgM antibodies have been found in children less than 2 weeks of age (Rziha, Bornkamm et al. 1978) and in approximately 8% of umbilical cord blood serum samples (Taguchi, Nagaki et al. 1975; Rziha, Belohradsky et al. 1978), suggesting prenatal transplacental infection with BKV likely from a naïve infected mother. BKV DNA sequences have also been detected in the placenta, brain, and kidney of aborted fetuses (Pietropaolo, Di Taranto et al. 1998). Although both vertical and horizontal transmission of BKV occurs, the seroepidemiology of BKV suggests that horizontal transmission is the primary mode of spread.

In immunologically impaired hosts BKV reactivation may occur. For example, BKV has emerged as a significant pathogen in kidney transplant recipients causing polyomavirus-associated nephropathy (PVAN) in 1–8% of renal allografts (Binet, Nickeleit et al. 1999; Howell, Smith et al. 1999; Nickeleit, Hirsch et al. 1999; Hirsch, Knowles et al. 2002), leading to allograft dysfunction and loss in >50% of PVAN cases (Purighalla, Shapiro et al. 1995; Ramos, Drachenberg et al. 2002; Hirsch and Steiger; Hirsch 2005). BKV-associated nephritis has a bimodal distribution with respect to time after kidney transplantation. Fifty percent of BKV-associated nephritis occurs 4–8 weeks after transplantion, while the remainder of patients develop the disease months to years post-transplantion (Arthur and Shah 1989). Allograft failure has been observed in approximately 45% of BKV nephropathy patients (Mathur, Olson et al. 1997) and is associated with widespread viral replication in tubular epithelial cells leading to tubular necrosis (Nickeleit, Hirsch et al. 1999). In addition to nephropathy, BKV-associated ureteral stenosis has been reported in 3% of renal transplant recipients (Coleman, Mackenzie et al. 1978; Hogan, Borden et al. 1980; Gardner, MacKenzie et al. 1984), occurring between 50 and 300 days post-transplantion.

Hemorrhagic cystitis, characterized by urinary tract pain as well as hematuria, is a complication of BKV reactivation in about 10% of bone marrow

transplant recipients (Bedi, Miller et al. 1995; Kondo, Kojima et al. 1998; Nevo, Swan et al. 1998; Seber, Shu et al. 1999; Peinemann, de Villiers et al. 2000). BKV-associated hemorrhagic cystitis is distinguished from chemotherapy-related hemorrhagic cystitis by a relatively late-onset, occurring more than 10 days after kidney transplantion. Hemorrhagic cystitis has been reported in 50% of hematopoetic stem-cell transplant patients with persistent BKV shedding (Bedi, Miller et al. 1995). BKV replication does not always correlate with symptoms (Cotterill, Macaulay et al. 1992), and 50% of stem-cell transplant patients may shed BKV in their urine without developing hemorrhagic cystitis (Hirsch and Steiger 2003). Rearrangements in the noncoding regulatory region (NCRR) of BKV may occur in association with clinical disease. The presence of a single rearranged BKV NCRR sequence has been reported to dominate sequences isolated from the CSF and brain of a leukemia patient with meningoencephalitis (Stoner, Alappan et al. 2002). BKV NCRR rearrangements have also been reported in renal biopsies taken from patients suspected of kidney allograft rejection (Chen, Wen et al. 2001). These data support a role for NCCR rearrangement in tissue tropism and/or spread of BKV.

Both cellular and humoral host immune responses are important in host immune responses against BKV. IgG, IgM, and IgA neutralizing and IgG subtype-specific antibodies have been detected against the VP1 major capsid protein (Knowles 2006). However, anti-BKV VP1 antibodies do not prevent viral reactivation in immunocompromised individuals (Flaegstad, Traavik et al. 1988). Cellular immunity may play a more significant role in suppressing BKV replication. (Kahan, Coleman et al. 1980; Hogan, Padgett et al. 1983; Gardner, MacKenzie et al. 1984; Markowitz, Thompson et al. 1993; Sundsfjord, Flaegstad et al. 1994). CTLs may control BKV replication in renal transplant recipients with PVAN (Chen, Trofe et al. 2006). Cellular immunotherapy may aid in the management of BKV-associated renal pathology in high-risk patient populations (Comoli, Basso et al. 2003).

Transplant patients can be monitored for BKV disease using a variety of diagnostic tools. The presence of decoy cells and BKV PCR have been used to detect BKV in urine. However, the use of urine cytology and PCR in tandem has a poor predictive value for determining BKV-associated disease (Pahari and Rees 2003). PCR amplification of BKV sequences from plasma has been shown to be both a sensitive and a specific means to determine BKV-associated nephropathy in adults (Nickeleit, Klimkait et al. 2000). Viral load, assessed using real-time PCR, has also been used with some success to monitor the progression of the infection (Limaye, Jerome et al. 2001). A definitive diagnosis of BKV-induced nephropathy often requires renal biopsy.

Therapy for BKV-associated diseases has been problematic. Unlike replication of large DNA viruses (e.g., herpesviruses), polyomavirus replication is dependent on host cell factors and does not encode virus-specific drug targets (Hirsch 2005). Therefore, no specific antiviral drugs are currently available, and treatment is symptomatic. Pain relief, bladder irrigation, and selective urosurgical intervention are utilized based on the severity and duration of symptoms

(Hirsch 2005). In kidney transplant recipients, BKV reactivation is often treated by reduction of immunosuppressive therapy, allowing the host immune system to suppress viral replication (Brennan, Agha et al. 2005). However, this strategy must balance transplant rejection by the host.

Cidofovir, a broad-spectrum antiviral, which inhibits host DNA polymerase and therefore viral replication (De Clercq and Holy 2005), has been used in treating patients with BKV-induced late-onset hemorrhagic cystitis. However, high doses of cidofovir may be contraindicated in kidney transplant recipients due to kidney toxicity (Gorczynska, Turkiewicz et al. 2005; Izzedine, Launay-Vacher et al. 2005; Ortiz, Justo et al. 2005). At present, cidofovir treatment for BKV-associated disease is unsupported by clinical trials.

The antibiotic ciprofloxacin also has been used to treat BKV-associated disease. Ciprofloxacin is a bacterial DNA gyrase inhibitor and, at standard doses, has been found to reduce urinary BKV load in bone marrow transplant recipients (Leung, Chan et al. 2005). Because BKV viruria has been associated with the development of late-onset hemorrhagic cystitis, treatment with ciprofloxacin may reduce the risk for the development of BKV-induced hemorrhagic cystitis (Bogdanovic, Priftakis et al. 2004; Leung, Chan et al. 2005). However, efficacy remains to be tested in larger clinical trials.

BKV has been associated with human malignancies, although a cause–effect relationship has not been established (Vivaldi, Pacini et al. 2003; Das, Shah et al. 2004; Fioriti, Videtta et al. 2005). BKV T-antigen transforms embryonic fibroblasts, as well as cells cultured from the kidney and brain tissue of mice, rats, hamsters, rabbits, and monkeys (Vivaldi, Pacini et al. 2003; White and Khalili 2004). BKV large T-antigen induces both hepatocellular carcinomas and renal tumors in transgenic mice (Small, Scangos et al. 1986; Dalrymple and Beemon 1990; Imperiale 2000). However, despite BKV induction of transformation and tumors in animal model systems, transformation of human cells by BKV is inefficient and often abortive (Shah and Nathanson 1976; Portolani and Borgatti 1978; Vivaldi, Pacini et al. 2003).

BKV DNA sequences have been detected by PCR in a wide range of human malignancies, including bone, pancreas, kidney, urinary tract, and some brain tumors (Caputo, Corallini et al. 1983); Corallini, Pagnani et al. 1987; Dorries, Loeber et al. 1987; Knepper and diMayorca 1987; Negrini, Rimessi et al. 1990; De Mattei, Martini et al. 1995); Monini, de Lellis et al. 1995; Flaegstad, Andresen et al. 1999). More recently, BKV DNA sequences and expression of BKV proteins have been detected in neoplastic prostate tissue (Das, Shah et al. 2004). However, BKV DNA sequences found in diseased tissue have also been detected in normal brain, bone, and peripheral blood cells (De Mattei, Martini et al. 1995), and the prevalence of BKV DNA between neoplastic and non-neoplastic urinary tract samples is similar. Other studies have found no evidence for the presence of BKV in brain tumors, urothelial carcinomas of the bladder, and of the renal pelvis, medulloblastomas, meningiomas, and ependymomas (Arthur, Grossman et al. 1994; Weggen, Bayer et al. 2000; Knoll, Stoehr et al. 2003).

3.5 JCV and Human Disease

JC virus (JCV) was first isolated in 1971 from the brain of a patient with progressive multi-focal leukoencephalopathy (PML) (Padgett, Walker et al. 1971). Like BKV, JCV infects over 80% of the human population. Seroepidemiological evidence indicates that primary infection with JCV occurs in late childhood (Walker and Padgett 1983; Knight, Hyman et al. 1988), in contrast to early childhood infection typically seen for BKV. It has been proposed that infection with JCV results in viremia, allowing dissemination to the kidneys, where JCV establishes a persistent infection. The presence of JCV in the urine of immunocompetent, healthy individuals (Chesters, Heritage et al. 1983; Kitamura, Aso et al. 1990; Yogo, Kitamura et al. 1990) suggests that the kidney is the primary site for JCV latency. Additionally, replicating JCV DNA has been found in B lymphocytes from the peripheral blood and the spleen, implicating B lymphocytes as an additional site for JCV persistence (Monaco, Atwood et al. 1996; Eash, Tavares et al. 2004).

The route of transmission for JCV remains controversial. Unlike BKV, at present there is little evidence for transplacental transmission of JCV, which may be a rare event (Pietropaolo, Di Taranto et al. 1998). However, JCV may be found in the urine of pregnant women (Kahan, Coleman et al. 1980; Gibson, Field et al. 1981; Markowitz, Eaton et al. 1991; Chang, Tsai et al. 1996). Horizontal transmission of JCV is likely the primary route of spread given the epidemiologic evidence suggesting that exposure to JCV occurs in late childhood. Horizontal transmission of JCV likely occurs through the oral or respiratory routes. JCV infects tonsillar lymphocytes and stromal cells in vitro (Monaco, Atwood et al. 1996), and JCV DNA has been detected in both pediatric and adult human tonsil tissue (Monaco, Jensen et al. 1998; Monaco, Shin et al. 1998). Primary infection with JCV may also occur through ingestion of contaminated food, water, or fomites (Bofill-Mas, Formiga-Cruz et al. 2001; Bofill-Mas and Girones 2001).

Once infected with JCV, the virus establishes a persistent infection, and JCV-specific antibodies can be detected throughout life. JCV expression is modulated by cell-mediated immunity, and impairment of the Th1-type response has been associated with the reactivation of JCV from latency (Weber, Goldmann et al. 2001). JCV-specific CTL activity can be detected in JCV T-antigen-stimulated peripheral blood mononuclear cells of PML survivors, but not in PML patients who rapidly progress to death (Du Pasquier, Clark et al. 2001; Koralnik, Du Pasquier et al. 2001). More recently, JCV-specific CTLs have been detected in immunocompetent individuals, further suggesting a role for cell-mediated immunity in the protection against viral reactivation (Du Pasquier, Schmitz et al. 2004). When reactivation of JCV occurs in the setting of immune suppression, JCV can cause the fatal demyelinating disease PML (Padgett, Walker et al. 1971). PML results from the death of virus-infected oligodendrocytes, myelin-producing cells, which can cause focal areas of

progressive demyelination. The replication and dissemination of JCV in PML causes the death of oligodendrocytes; however, the exact mechanism of cell death in PML is unknown. In addition, it is not understood whether PML is a result of initial entry of JCV into the central nervous system from infected circulating lymphocytes in the blood (Houff, Major et al. 1988), or whether PML is a consequence of localized reactivation of a latent infection (White, Ishaq et al. 1992; Corral, Quereda et al. 2002).

While there have been cases of PML reported in patients without any underlying disease (Safak and Khalili 2003), the majority of PML cases have been in patients with cancer, organ transplantation, or HIV infection (Berger, Chauhan et al. 2001). PML occurs in up to 5% of all HIV-infected patients (Berger and Concha 1995). PML in the HIV-positive population may be attributed to several factors. First, the degree and duration of immune suppression may be greater in HIV patients compared to other disorders resulting in immune suppression (Berger, Chauhan et al. 2001; Berger 2003; Seth, Diaz et al. 2003). Second, HIV/AIDS may alter the blood brain barrier (Power, Kong et al. 1993), facilitating entry of B lymphocytes infected with JCV into the brain (Gallia, Houff et al. 1997). Finally, HIV proteins may promote JCV gene expression (Chowdhury, Taylor et al. 1990; Tada, Rappaport et al. 1990). Studies have also demonstrated synergy between HIV and JCV replication (Chowdhury, Kundu et al. 1993; Krachmarov, Chepenik et al. 1996).

In HIV-positive populations, highly active antiretroviral therapy (HAART) has increased survival of patients with PML from 4 to 10.5 months (Berger, Levy et al. 1998; Clifford, Yiannoutsos et al. 1999; De Luca, Giancola et al. 2000; Cinque, Pierotti et al. 2001). While the mean survival for PML patients seems to have increased in the HAART era, there are also some data that suggest initiation of HAART after PML diagnosis may be associated with worse outcomes due to the rapid immune system reconstitution initiated by HAART (Cinque, Pierotti et al. 2001; Cinque, Bossolasco et al. 2003). The host inflammatory response initiated by HAART must be balanced against its benefits in cases of JCV-induced PML.

While PML has historically been associated with patients exhibiting impaired cell-mediated immunity, more recently, there have been reports of PML cases in patients being treated for multiple sclerosis (MS) (Kleinschmidt-DeMasters and Tyler 2005; Langer-Gould, Atlas et al. 2005). Natalizumab, a monoclonal antibody to the α_4-integrin receptors of peripheral blood lymphocytes, is a novel new therapy for MS based on inhibiting entry of mononuclear cells into the brain. Impaired lymphocyte trafficking into the brain due to Natalizumab treatment may be a contributing factor for the development of PML by preventing access of JCV-specific T-lymphocytes to the site of infection.

PML is often diagnosed using magnetic resonance imaging (MRI), where multi-focal areas of demyelination are detected. Postmortem histopathological findings consistent with JCV-induced PML include multiple foci of myelin loss,

eosinophilic, enlarged oligodendroglial nuclei, and enlarged astrocytes with hyperchromatic nuclei. Electron microscopy has been used to visualize JCV viral particles within inclusion bodies of oligodendrocytes, a feature characteristic of JCV-induced PML (Khalili, Gordon et al. 2006).

Specific antiviral treatment for JCV-associated PML is unavailable. None of the current antiviral agents have been shown to slow or halt disease progression (Hall, Dafni et al. 1998; Gasnault, Kousignian et al. 2001; Geschwind, Skolasky et al. 2001; Marra, Rajicic et al. 2002; Royal, Dupont et al. 2003). Studies assessing the use of cidofovir in the treatment of PML have been largely inconclusive (Haider, Nafziger et al. 2000; Portilla, Boix et al. 2000; Houston, Roberts et al. 2001; Salmaggi, Maccagnano et al. 2001; Roberts, Carmichael et al. 2003). PML is typically treated by attempting to reverse the predisposing condition which has caused the immune suppression (Berger, Levy et al. 1998; Clifford, Yiannoutsos et al. 1999; Weber, Goldmann et al. 2001; Koralnik, Du Pasquier et al. 2002; Du Pasquier, Kuroda et al. 2003). Recently, the serotonergic receptor $5HT_{2A}$ has been identified as a receptor for JCV (Elphick, Querbes et al. 2004). Therefore, serotonin receptor antagonists may be a rational approach to PML treatment in the future.

JCV has been associated with a variety of malignancies. Intracerebral inoculation of syrian golden hamsters with JCV causes neuronal or glial-origin tumors (Walker, Padgett et al. 1973), while infected rats develop primitive neuroectodermal tumors (Ohsumi, Motoi et al. 1986). Both owl and squirrel monkeys infected in adulthood with JCV develop glial neoplasias (London, Houff et al. 1983; Miller, McKeever et al. 1984), and JCV remains the only human virus to induce solid tumor formation in non-human primates (Khalili, Del Valle et al. 2003). Transgenic mice expressing JCV T-antigen develop adrenal neuroblastomas and primitive neuroectodermal tumors (Small, Khoury et al. 1986; Small, Scangos et al. 1986; Franks, Rencic et al. 1996; Krynska, Otte et al. 1999).

JCV DNA has been detected in many human malignant and nonmalignant tumors that originate from cells of the central nervous system (Khalili, Del Valle et al. 2003). JCV DNA sequences and T-antigen immunoreactivity have been detected in a variety of human brain tumors, including tumors of glial origin and medulloblastomas (Caldarelli-Stefano, Boldorini et al. 2000; Del Valle, Gordon et al. 2001; Enam, Del Valle et al. 2002). In one report nearly 77% of medulloblastomas contained JCV DNA, and 36% were positive for T-antigen by immunohistochemistry (Khalili, Del Valle et al. 2003). JCV agnoprotein has also been detected in tumor samples (Del Valle, Gordon et al. 2002). JCV DNA has also been found in both the upper and lower human gastrointestinal tracts (Ricciardiello, Laghi et al. 2000); Ricciardiello, Chang et al. 2001), including over 80% of colon adenocarcinomas (Laghi, Randolph et al. 1999; Enam, Del Valle et al. 2002). However, the ubiquity of JCV has prevented any cause–effect association with human cancer.

3.6 Conclusion

Polyomaviruses have found replicative niches in hosts from birds to man. Although animal models often do not directly translate to humans for assessing pathobiology, it is likely that the biological strategies of polyomaviruses share similarities despite host differences. Overarching themes include (1) a primary infection that is regulated by antibodies (e.g., passive maternal antibody protects neonates) occurring horizontally either by fecal-oral or respiratory routes, (2) a life-long persistent infection regulated by cellular immune functions, with a predilection of the virus to "hide" in renal tubule cells and perhaps blood mononuclear cells, (3) the potential for vertical transmission that may have untoward consequences (e.g., fetal demise), (4) the expression of viral T-antigen proteins that bind p53 and pRb with varying affinities thus dysregulating the cell cycle, (5) tissue tropism and viral spread influenced by subtle mutations in the VP1 capsid protein, and possibly viral enhancer sequence evolution within the host during persistence/reactivation, (6) host determinants that may alter disease profiles, and (7) the "reactivation" of virus production under conditions of host immune suppression (e.g., pregnancy, chemotherapy, immunodeficiency).

The immunological and molecular epidemiology of these viruses has been problematic. For example, all immunohistochemical staining to date has utilized antibodies incapable of distinguishing between the various polyoma T-antigens. However, with improved PCR methods, the development of specific T-antigen monoclonal antibodies, and recombinant VP1 VLP-based ELISAs (Sehr, Muller et al. 2002; Viscidi and Clayman 2006) more precise serologic and molecular methods for differentiating between these polyomaviruses are now becoming available. These new assays have already provided insight. Human sera immune for SV40 sometimes reacts with BKV VP1 protein (false positive) and cannot be distinguished as specific for SV40 unless competition assays are performed (Kean, unpublished results).

To differentiate between association and causation when assigning an etiologic agent to human malignancy, the Bradford-Hill criteria may be considered. These criteria include (1) strength of association between the agent and the disease, (2) consistency in findings between studies, (3) specificity of disease, (4) temporal association between tumor induction and infection, (5) association between viral load and severity of disease, (6) biological plausibility inferred from molecular and animal model data, (7) biological coherence, (8) experimental evidence which supports a role for viral infection in specific malignancy, and (9) analogy to similar diseases induced by viruses (Hill 1965). SV40, BKV, and JCV all express large T-antigen, an established oncogenic protein, implicating a biologically plausible role for these viruses in human malignancy. Both *in vivo* and *in vitro* experiments support a role for these viruses in cell transformation and tumor induction, frequently of the types seen in humans. However, epidemiological evidence remains controversial. While there have been reports

of SV40, BKV, and JCV DNA sequences in many tumors, other studies have disputed these findings. This lack of consistency between studies may be based on methodologic issues such as PCR contamination and specificity of T-antigen staining. Serologic studies, although preliminary, have not shown a prevalence of SV40 greater than 3% in the populations tested, and there are no positive correlations as yet between specific tumors and SV40 serology. In contrast, the ubiquity of BKV and JCV infection and seropositivity confounds correlations with tumor causation. Thus the Bradford-Hill criteria remain unfulfilled.

Certainly, we are not at the end of studying polyomaviruses and their related diseases. There are likely new human viruses to be discovered and characterized, as evidenced by two novel polyomaviruses, WUV and KIV, detected in respiratory tract specimens, and one in Merkel cell carcinomas (Merkel cell polyomavirus or m the latter of which may be the First polyomavirus to have a strong association with malignancy (Allander, Andreasson et al. 2007; Gaynor, Nissen et al. 2007; Feng, Shuda et al. 2008). Effective therapeutics are urgently needed for the treatment of PML, PVAN, and hemorrhagic cystitis in immunocompromised patients. The VLP ELISA has unexpectedly uncovered a prevalence of 20% seropositivity in humans for LPV, a finding that warrants investigation. Finally, standardization of serologic assays and PCR techniques in prospective clinical studies hopefully will clarify the role of polyomaviruses in human malignancies.

Acknowledgments The authors would like to acknowledge institutions providing financial support. Robert L. Garcea was supported by NIH/NCI grant CA37667 and Jaime M. Kean by NIH/NIAID grant T32 AI52066.

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Chapter 4
The Life Cycle of Human Papillomaviruses

Cary A. Moody and Laimonis A. Laimins

Abstract The life cycle of human papillomaviruses (HPV) is dependent on epithelial differentiation and is regulated by a number of cellular proteins. HPVs infect keratinocytes in the basal layer of the epithelium, and upon cell division, one daughter cell migrates to upper layers while undergoing differentiation. Differentiation triggers the productive phase of the viral life cycle, including viral DNA amplification, late gene expression and virion assembly. Through the actions of viral proteins, infected cells remain active in the cell cycle, creating an environment conducive to late viral events and the production of progeny virions.

4.1 HPV and Cervical Cancer

Human papillomaviruses are small DNA viruses that target epithelial cells for infection. More than 120 different types of HPV have been identified and are categorized into two groups based on the site of infection (de Villiers et al. 2004). Mucosotropic HPVs primarily infect the mucous epithelium of the oropharynx and anogenital tract, whereas cutaneous HPVs predominantly target the skin. Approximately one-third of HPV types infect epithelial cells of the genital tract and are further subdivided as low or high risk depending on their association with cancer (de Villiers et al. 2004). The low-risk types, such as HPV 6 and HPV 11, induce benign proliferation of epithelial cells, often resulting in the development of genital warts (condylomata accuminata). In contrast, high-risk HPV types are the etiological agents of cervical cancer, with 99% of cervical squamous cell carcinomas containing these viral DNAs (Walboomers et al. 1999). Four high-risk HPV types are found in over 70% of cervical cancers, with HPV 16 accounting for about half of the cases, while types 18, 31 and 45 account for an additional 25–30% (Clifford et al. 2006; Parkin and Bray 2006). Over 15 other HPV types are associated with the remaining cancers. High-risk HPV types are also detected in 70% of anal carcinomas, 25% of oropharynx carcinomas and 10–35% of

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_4, © Springer Science+Business Media, LLC 2009

eosophagus carcinomas (Parkin and Bray 2006). Infection by high-risk HPVs is thus a major risk factor for the development of multiple human cancers.

HPVs induce the most common sexually transmitted diseases. Within 2 years of initiating sexual activity, greater than 50% of women acquire HPV infections of the genital tract (Koutsky 1997). Although the lifetime risk of contracting a genital HPV infection is greater than 80%, only a minority of high-risk infections progress to cervical cancer (Baseman and Koutsky 2005). Natural history studies indicate that the majority of high-risk HPV infections are transient and that most regress spontaneously within 12–36 months. This regression is likely due to the activation of an immune response (Ho et al. 1998; Holowaty et al. 1999). A small number of women fail to clear HPV infections and are at risk for development of cervical cancer. Persistent infection with high-risk HPVs is considered the major risk determinant in progression to cervical cancer.

Cervical cancer develops from non-invasive pre-malignant lesions and progresses gradually from mild cervical intraepithelial neoplasia (CIN1) to more severe degrees of dysplasia (CIN2 and CIN3) which can ultimately induce invasive malignant disease (Kurman et al. 1991). The development of cervical cancer typically occurs several decades following initial infection (Wallin et al. 1999; Zielinski et al. 2001). Low-grade squamous intraepithelial lesions are highly prevalent in younger individuals, demonstrating that while HPV infection is necessary for development of CIN disease, additional cellular changes are necessary for malignant progression (Bosch et al. 2002). Cervical cancer is the second-most common malignancy among women worldwide. The implementation of screening tools such as the Papanicolaou (Pap) smear to detect atypical cells in the transformation zone of the cervix has reduced cervical cancer incidence by approximately 80% in the United States and Europe (Singer 1995). The impact of cervical cancer is felt the most among socioeconomically disadvantaged women in developing countries, as well as in countries that do not have effective screening programs in place. In 2002, approximately 470,000 women were diagnosed with cervical cancer world-wide with approximately 230,000 deaths (Wiley and Masongsong 2006). Eighty percent of cervical cancer cases occur in developing countries, and it is one of the leading causes of cancer deaths of women in many regions (Parkin et al. 2006).

4.1.1 HPV Vaccines

In 2006, Merck received FDA approval for a prophylactic vaccine that targets the two HPV types most commonly detected in cervical cancer, HPV 16 and HPV 18, as well as the low-risk types HPV 6 and HPV 11, which cause 90% of genital warts. This vaccine consists of HPV L1 capsid proteins expressed in yeast where they spontaneously assemble into virus-like particles (VLP) that resemble intact virions (Zhou et al. 1991). The L1 VLP vaccine produces high titers of neutralizing antibodies following injection (Kirnbauer et al. 1992). A second vaccine, developed by Glaxo Smith Kline, is currently in clinical trials and

contains L1 VLPs of HPV types 16 and 18. Initial studies indicate that the L1 VLP vaccines are highly effective at blocking infection with HPV 16 and HPV 18 (Harper et al. 2004, 2006; Villa et al. 2005). Since cervical cancers develop over several decades, there will be a significant lag before this vaccine impacts the number of new cervical cancers. In addition, these vaccines will not prevent infection by other HPV types that are responsible for 30% of cervical cancers. It is anticipated that efforts will be made to distribute this vaccine to populations in developing countries, as well as to include other HPV types in the formulations.

4.2 Overview of the Life Cycle of HPV

In this review, we will first provide an overview of the differentiation-dependent life cycle of human papillomaviruses and the activities of the viral proteins. In subsequent sections, we will discuss in detail each phase of the viral life cycle and how it is regulated.

4.2.1 Genome Organization

The genomic structure and organization of papillomaviruses are highly conserved between the different genotypes. All papillomaviruses contain double-stranded, circular DNA genomes in a non-enveloped, icosahedral capsid. Although there is slight size variation between HPV types, all genomes are approximately 8 kb and encode for roughly eight open reading frames. A diagram of the genomic organization of HPV 31 is shown in Fig. 4.1. HPV genomes can be divided into early

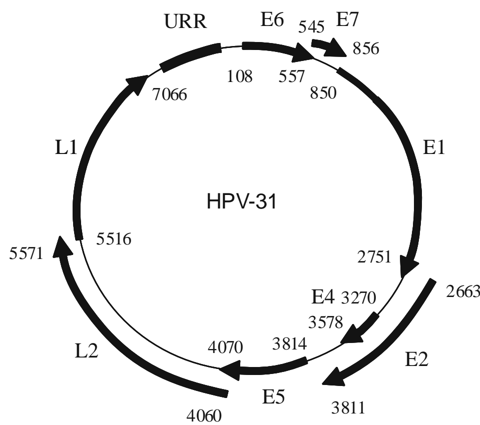

Fig. 4.1 Genomic organization of high-risk HPV 31. Open reading frames are indicated by arrows and their nucleotide positions in the genome are shown. The upstream regulatory region (URR) contains sequences for transcriptional regulation, as well as the origin of replication and is located between the end of the L1 gene and the beginning of the E6 gene

and late regions based on the timing of gene expression during the viral life cycle. A third region, designated the upstream regulatory region (URR) or the long control region (LCR), does not encode any open reading frames but does contain multiple sites for transcription factor binding, as well as the origin of replication. High-risk HPVs initiate transcription from two major promoters (early and late) that are active in different phases of the life cycle (Fig. 4.2). Transcription from both promoters occurs from only one strand of the viral genome and yields polycistronic messages that undergo alternative splicing. Translation is thought to occur by a leaky ribosomal scanning mechanism, which along with splicing,

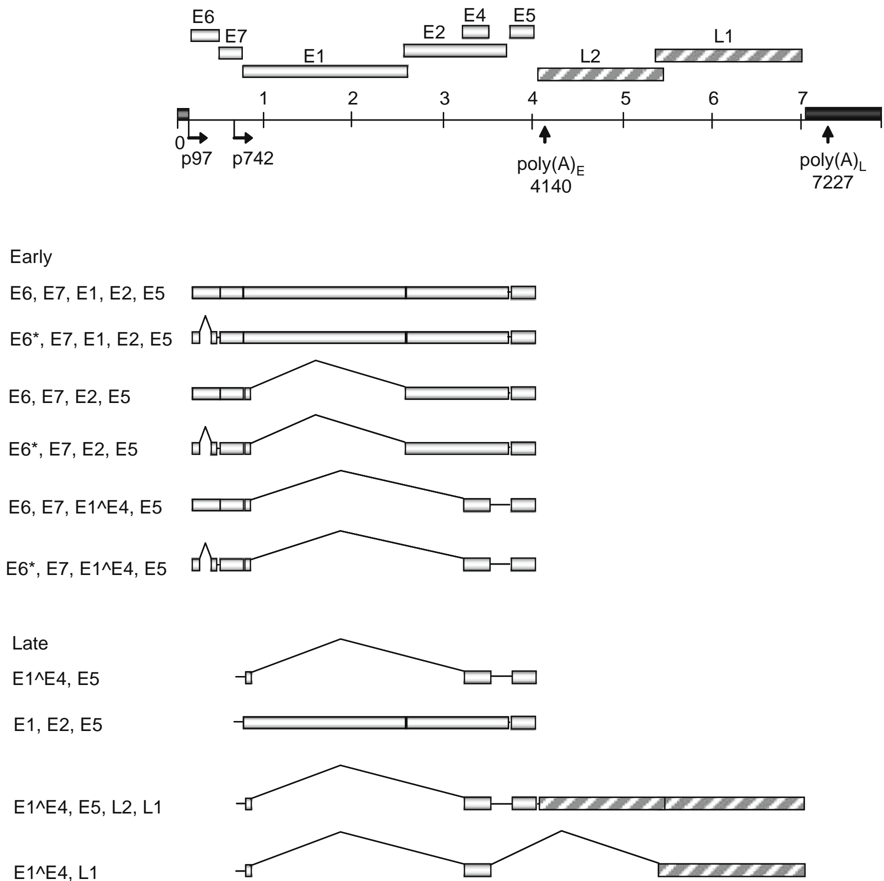

Fig. 4.2 Major viral transcripts expressed during the HPV 31 life cycle. The early open reading frames are indicated in *gray* and the striped boxes designate the open reading frames for the late genes L1 and L2. Transcripts encoding E6, E7, E1, E2, E5 and E6 are expressed from the early promoter p97 or the late promoter p742 depending on the stage of the viral life cycle. Upon differentiation, the capsid genes are expressed from the late promoter, which is accompanied by a switch in polyadenylation site usage from the early site (poly$(A)_E$) to the late site (poly$(A)_L$)

contributes to differential regulation of gene expression throughout the viral life cycle (Remm et al. 1999).

The early promoter is located just upstream of the E6 open reading frame and is active early in infection, prior to productive replication (Fig. 4.2). For HPV 31 and HPV 16, this promoter is designated as p97, and for HPV 18 as p105 (Hummel et al. 1992; Thierry et al. 1987). In undifferentiated cells, polycistronic transcripts encoding E1, E2, E4, E5, E6 and E7 are expressed from the early promoter (Doorbar et al. 1990; Hummel et al. 1992; Ozbun and Meyers 1998a) (Fig. 4.2). While E4 and E5 are expressed in these transcripts, they are the third and fourth open reading frames and likely not translated efficiently. The HPV E1 and E2 open reading frames encode proteins that are essential for viral replication. E1 is a phosphoprotein that exhibits ATPase, as well as helicase activity (Hughes and Romanos 1993; Raj and Stanley 1995). E2 forms a complex with E1 and directs it to the viral origin of replication where it binds with high affinity (Frattini and Laimins 1994a; Mohr et al. 1990). In addition to its role in viral replication, E2 is also a viral transcriptional regulator that can both positively and negatively modulate expression of the early genes (Cripe et al. 1987). E6 and E7 proteins of high-risk HPVs function as oncoproteins and facilitate deregulation of the cell cycle through the targeted degradation of the tumor suppressors p53 and pRb, respectively (Chellappan et al. 1992; Dyson et al. 1989; Dyson 1998; Huibregtse et al. 1991; Scheffner et al. 1990). E6 has also been shown to activate transcription of hTERT, the catalytic subunit of telomerase (Klingelhutz et al. 1996; Veldman et al. 2001), as well as a series of over 12 different cellular factors that may contribute to various aspects of pathogenesis (Hebner and Laimins 2006).

The productive phase of the life cycle is triggered when infected cells are induced to differentiate. Transcripts from the late promoter dramatically increase upon differentiation and initiate from a heterogenous group of start sites clustered around nucleotide 742 of the HPV 31 viral genome, located in the E7 ORF (Grassmann et al. 1996; Hummel et al. 1992; Ozbun and Meyers 1998b) (Fig. 4.2). Activation of the late promoter leads to high levels of transcripts encoding for E1, E1^E4, E5, as well as the capsid proteins L1 and L2 (Hummel et al. 1992; Ozbun and Meyers 1997). The E1^E4 fusion protein and E5 are thought to play important roles in regulating late events in the viral life cycle, including viral genome amplification and late gene expression, although their exact roles are unclear (Fehrmann et al. 2003; Genther et al. 2003; Wilson et al. 2005). L1 and L2 are only expressed very late in the life cycle and constitute the viral capsid (Ozbun and Meyers 1997).

4.2.2 HPV and Differentiation

The life cycle of HPV is regulated by the differentiation status of its host cell, the keratinocyte (Stubenrauch and Laimins 1999) (Fig. 4.3). Infection is

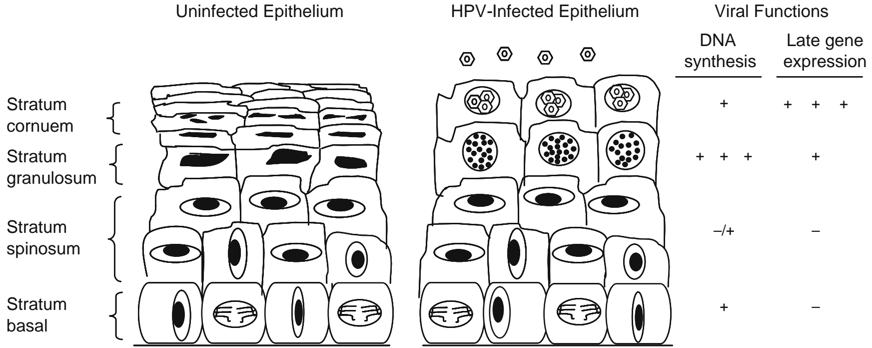

Fig. 4.3 Schematic of uninfected stratified epithelium (*left*) and HPV-infected epithelium (*right*) showing the various differentiated layers. Genome amplification, late gene expression and virion production occurs in the suprabasal layers

thought to occur in stem cells or transient amplifying cells in the basal layer of the epithelium that become susceptible to infection through microlesions. Upon entry, the viral DNA is established in the nucleus as extrachromasomal elements, and the early promoter is activated. As a result of increased expression of the replication proteins E1 and E2, viral genomes are quickly amplified to 50–100 episomal copies per cell. Following this initial establishment phase, copy number is subsequently maintained constant, and replication occurs in synchrony with that of cellular DNA. As HPV-infected cells divide, one daughter cell migrates away from the basal layer and begins a program of differentiation. The other daughter cell remains in the basal layer and provides a continual reservoir of undifferentiated cells that latently maintain viral DNA. As HPV-infected cells differentiate, the late promoter is activated and the productive phase of the viral life cycle is triggered, which leads to amplification of the viral genome to thousands of copies, late gene expression and virion assembly (Fig. 4.3). Paradoxically, these events occur in cells that normally would have exited the cell cycle and ceased proliferation upon differentiation. Largely through the activities of the E6 and E7 proteins, HPV alters cell cycle regulation and maintains a subset of infected cells active in the cell cycle throughout differentiation, ensuring the expression of cellular factors necessary for replication of viral DNA (Cheng et al. 1995; Flores et al. 2000; Garner-Hamrick et al. 2004).

4.3 Mechanisms Regulating the HPV Life Cycle

In the following sections, a detailed description is provided of the various stages of the HPV life cycle and the factors that regulate these activities.

4.3.1 Viral Entry

The non-enveloped capsids of papillomaviruses consist of two proteins, the major capsid protein L1, which is the primary structural component of the virion, and the minor capsid protein L2 (Florin et al. 2002a; Modis et al. 2002). It has been difficult to investigate the mechanisms by which HPVs enter cells to establish a productive infection due to difficulty in producing large quantities of virus in vitro and in vivo. However, high-level expression of L1 and L2 in cells results in the spontaneous assembly of icosahedral structures, termed virus-like particles (VLPs) (Zhou et al. 1991). L1, as well as L1/L2, VLPs can be generated in large amounts in heterologous expression systems and have provided important insights into the initial interaction between papillomaviruses and cell surfaces. Pseudovirions, which carry a reporter plasmid instead of the genome, have been instrumental in providing information concerning later steps in the infectious entry pathway. Papillomaviruses can bind to cells derived from many tissues and species, indicating that the initial binding receptor is widely distributed (Muller et al. 1995; Roden et al. 1994), and several receptor candidates have been proposed. Heparan sulfate proteoglycans (HSPGs) have been shown to be the initial binding molecule for HPV 6, HPV 11 and HPV 16 VLPs, as well as HPV 16 and HPV 33 pseudovirions (Joyce et al. 1999; Shafti-Keramat et al. 2003; Combita et al. 2001; Giroglou et al. 2001). It is likely, however, that additional cellular proteins contribute to entry. One such molecule is $\alpha 6$ integrin, which was suggested as the binding receptor for HPV 6 VLPs (Evander et al. 1997; McMillan et al. 1999); however, it was found to be dispensable for infection of other papillomaviruses, including bovine papillomavirus (BPV) 4 and HPV 11 (Giroglou et al. 2001; Joyce et al. 1999; Shafti-Keramat et al. 2003; Sibbet et al. 2000). The search for the cellular receptor continues to be an area of high interest.

Following binding, most papillomaviruses, including HPV 11, 16, 18, 31 and 33, enter cells through a clathrin-dependent pathway that requires acidification of endosomes and intact microtubules (Bousarghin et al. 2003; Day et al. 2003; Roden et al. 1994; Selinka et al. 2002; Volpers et al. 1995). Internalization of papillomaviruses occurs with delayed kinetics, suggesting the potential involvement of secondary receptors. An L2 peptide that can bind a variety of cell lines has recently been identified, and mutation of this sequence significantly impairs infectivity of pseudo typed virions, suggesting that L2 may interact with cell surface proteins to facilitate infection (Yang et al. 2003). Interestingly, this binding motif is masked in virions and is only exposed upon virus binding to the cell surface. Initial binding of L1 to heparan sulfate may be necessary to induce a conformational change in the capsid, allowing the L2 motif to be displayed on the capsid surface to interact with a secondary receptor, leading to internalization.

Upon endocytosis, papillomavirus particles disassemble and uncoat their genomes into endosomes. Egress of the viral genomes into the cytoplasm is an

essential step in the establishment of a productive infection, and the L2 protein is thought to play a critical role in this process. L2 must be cleaved by the cellular protease furin to facilitate the release of viral genomes into the cytoplasm (Richards et al. 2006). In addition, Kamper et al. demonstrated that L2 is necessary to destabilize endosomal membranes to mediate release of viral genomes from endosomal compartments (Kamper et al. 2006). Furthermore, L2 facilitates transfer of the viral genomes to specific nuclear domains (ND10 bodies) to establish infection (Day et al. 2004), suggesting that L2 is important at multiple steps of the infection process.

4.3.2 Early Viral Gene Expression and Replication

Upon entry into the nucleus, viral genomes are rapidly established as multicopy episomes and are stably maintained upon subsequent cellular divisions. The establishment and maintenance phases of the viral life cycle require the expression of the transcripts from the early promoter, which encode for the oncoproteins E6 and E7, as well as the replication proteins E1 and E2 (Fig. 4.2) (Howley 1996). Transcription from the early promoter is controlled through the binding of transcription factors to recognition sequences in the long control region, or upstream regulatory region (URR). The URR contains binding sites for the transcription factors Sp1 and TFIID, as well as AP-1, GRE, KRF-1, Oct-1, Sp1, TEF-1 and YY1, which contribute to both the positive and negative regulations of HPV transcription (Butz and Hoppe-Seyler 1993; Gloss et al. 1987; Hubert et al. 1999; Ishiji et al. 1992; Kyo et al. 1997; O'Connor and Bernard 1995). The differential binding of these cellular factors to viral enhancer sequences likely contributes to the restriction of HPV infection to cells of epithelial origin (Chong et al. 1991; Cripe et al. 1987; Gloss et al. 1987).

The expression of early viral genes, including those of the viral replication factors, contributes to viral copy number control in undifferentiated cells (Stubenrauch et al. 1998). The chief mediator of this activity is the E2 protein, which functions as a viral transcriptional regulator in addition to its role in replication (Cripe et al. 1987; Gloss and Bernard 1990; Soeda et al. 2006; Stubenrauch et al. 1998). E2 proteins are composed of three distinct functional domains: an amino terminal transcriptional activation (TA) domain, a carboxy-terminal DNA binding and dimerization domain and a flexible, non-conserved hinge region which links the other two regions (Giri and Yaniv 1988). E2 binds as a dimer to the palindromic sequences $ACCN_6GGT$, which are designated as E2-binding sites (E2BS) (Androphy et al. 1987; Dostatni et al. 1988; Hawley-Nelson et al. 1988). Four E2-binding sites are present in the URR of all genital HPVs, and the relative position of these sites is conserved (Dostatni et al. 1988; McBride et al. 1989; Stubenrauch et al. 1998). Two of these sites are located just upstream of the E6 transcription start site, and the third and fourth sites are located approximately 150 and 550 bp upstream. At

low levels, E2 has been shown to activate early transcription, whereas at high levels, E2 represses early transcription, possibly through the displacement of the TATA box-binding protein (TBP) and Sp1, whose binding sites are located proximal to E2-binding sites (Demeret et al. 1997, 1994; Dong et al. 1994; Tan et al. 1994). The binding of E2 to E2BS to modulate transcriptional regulation also affects replication of the viral genome. Expression of the replication proteins is increased at low E2 concentrations and repressed at high E2 concentrations (Stubenrauch et al. 1998). The regulation of E1 and E2 levels may therefore provide a mechanism for copy number control in undifferentiated cells.

The replication of viral DNA is mediated by E1 and E2 (Ustav and Stenlund 1991), which act together with cellular DNA replication proteins. The E1 proteins are highly conserved among papillomaviruses and act as initiator proteins (Stenlund 2003). E1 exhibits site-specific DNA-binding activity, DNA-dependent ATPase activity and 3?–5? helicase activity (Hughes and Romanos 1993; Raj and Stanley 1995; Ustav et al. 1991). The viral origin of replication is located in an A/T-rich region of the URR, adjacent to the start-site of the late promoter and consists of binding sites for both E1 and E2 (Del Vecchio et al. 1992; Frattini and Laimins 1994b; Ustav et al. 1991). E1 can bind to the viral origin of replication, but does so with low affinity. Through a complex interaction with E2, E1 and E2 bind to the origin with high affinity and in a cooperative manner (Frattini and Laimins 1994a; Mohr et al. 1990; Sedman and Stenlund 1995). E2 is subsequently released from the origin in a process that requires hydrolysis of ATP (Sanders and Stenlund 1998, 2000). E1 binds to and recruits DNA polymerase α-primase to the origin, which is necessary for initiation of both leading and lagging strand DNA replication (Masterson et al. 1998; Park et al. 1994). Through interactions with topoisomerase 1 and the single-stranded DNA-binding protein RPA (Clower et al. 2006; Loo and Melendy 2004), E1 also facilitates localized melting and unwinding of the supercoiled DNA template which occurs bi-directionally from the origin (Sanders and Stenlund 2000). Viral genomes are replicated in synchrony with the cellular DNA during S-phase and partition equally to daughter cells during mitosis. E2 is thought to play a critical role in tethering the viral genomes to cellular chromosomes during mitosis to facilitate partitioning. Several cellular factors have been postulated to play roles in this process, including Brd4 and ChlR1, though additional factors may also play a role (Baxter et al. 2005; Parish et al. 2006; You et al. 2004).

4.3.3 Viral Protein Function in Undifferentiated Cells

The E1 and E2 proteins are not the only viral proteins required for stable maintenance of episomes in undifferentiated cells. The expression of E6 and E7 is also necessary to facilitate stable maintenance of extrachromasomal

elements (Thomas et al. 1999). High-risk E6 and E7 are both multi-functional proteins that interact with many cellular proteins, and their roles as oncoproteins are well-characterized. Interestingly, the role of these proteins in maintenance of viral episomes also extends to the low-risk HPV types (Thomas et al. 2001).

The HPV E7 proteins of both high- and low-risk types are nuclear proteins that consist of about 100 amino acids divided into three conserved regions: an amino-terminal domain (CR1), a central domain containing an LXCXE-binding site for pRb (CR2) and a carboxy-terminal domain that contains two zinc finger-like motifs (CR3) (Gage et al. 1990; Phelps et al. 1988). The ability of E7 to bind and degrade members of the Rb family is an important function of the high-risk proteins. The low-risk E7 proteins also bind to pRb, but with reduced affinity (Munger et al. 1989). Rb family members (pRb, p107, p130) are instrumental in the control of cell cycle progression largely through regulation of the E2F family of transcription factors (Dyson et al. 1989; Dyson 1998; Nevins 1992). The binding of E7 to hypophosphorylated Rb results in the release of E2F factors that are necessary for transcription of genes involved in proliferation and cell cycle progression.

Interestingly, E7 interactions with Rb do not appear to be as important for stable maintenance of episomes as other interactions. Longworth et al demonstrated that binding of HPV 31 E7 to class I histone deacetylases (HDACs) is necessary for long-term maintenance of episomes (Longworth and Laimins 2004). HDACs are transcriptional co-repressors that deacetylate N-terminal tails of core histone proteins, resulting in the formation of heterochromatin and transcriptional repression. Rb family members repress E2F-dependent promoters through recruitment of HDAC complexes (Brehm et al. 1998). The HPV 16 and HPV 31 E7 proteins have been shown to interact indirectly with HDACs through Mi2β, a member of the NURD chromatin-remodeling complex, in a Rb-independent manner (Brehm et al. 1999). Genomes containing mutations in HPV 31 E7 that abrogated binding to class 1 HDACS (HDAC1 and 2) were severely impaired in episomal maintenance capability (Brehm et al. 1999; Longworth and Laimins 2004). This indicates that binding to HDACs is important in facilitating HPV maintenance in undifferentiated keratinocytes. Impaired episomal maintenance was also observed when mutations were made in E7 in the context of the low-risk HPV 11 genome (Oh et al. 2004). It is presently unknown if low-risk E7 proteins bind to HDACs and whether this is required for its role in episomal maintenance. E7 interactions with HDACs may have a direct effect on modulation of episomal replication, but may, in addition, indirectly alter the spectrum of cell cycle regulating factors, allowing for the stable maintenance of viral genomes in undifferentiated cells. The maintenance of extrachromosomal elements, such as double minute chromosomes, is not well tolerated by normal cells (Kuttler and Mai 2007). Only cells that are immortalized are able to tolerate these elements, and similar effects may contribute to episomal maintenance in HPV-positive cells.

The most well-characterized activity of E6 is its ability to bind and enhance the degradation of p53 through the ubiquitin–proteasome pathway (Scheffner et al. 1990; Werness et al. 1990). This results in a compromised ability of the host cell to engage cell cycle checkpoints and apoptotic responses. While it is uncertain if the low-risk HPV types bind to p53, it is clear that they do not mediate p53 degradation (Crook et al. 1991; Li and Coffino 1996). However, E6 proteins of both high-risk and low-risk HPVs have been shown to disrupt the transactivation potential of p53. The abrogation of p53 transcriptional activity may be accomplished by interfering with the binding of p53 to its DNA recognition sites (Lechner and Laimins 1994), or for high-risk E6, through interaction with the transcriptional coactivators p300/CBP (Patel et al. 1999; Thomas and Chiang 2005; Zimmermann et al. 1999). p300/CBP-mediated acetylation of p53 enhances the binding of p53 for its cognate DNA-binding sites, leading to transcription of p53-dependent genes. By interacting with p300, E6 is thought to mask the acetylation activity of p300, preventing the activation of p53 target genes. High-risk and low-risk E6 proteins have also been shown to play an important role in the stable replication of viral genomes. HPV 31 genomes harboring a point mutation in E6 that abrogated degradation of p53 could not be maintained episomally, suggesting a role for p53 inactivation in stable replication (Park and Androphy 2002; Thomas et al. 1999). Interestingly, genomes that contain both a mutation in E7 that reduced binding to Rb and a mutation in E6 that was defective for p53 degradation were retained (Park and Androphy 2002). E7 has been shown to increase p53 levels in the absence of E6, which can lead to apoptosis (Demers et al. 1994; Jones and Munger 1997). Therefore, the degradation of p53 by E6 may be necessary to balance the functions of E7, allowing for the long-term maintenance of viral genomes as episomes. The expression of low-risk E6 is also critical for maintenance of viral episomes, as HPV 11 genomes containing a translation termination mutant of E6 were unable to be maintained episomally (Oh et al. 2004). Genomes containing mutations in amino acids conserved between high-risk and low-risk E6 proteins were significantly impaired in episomal maintenance, but did not completely inhibit this activity (Oh et al. 2004). High-risk E6 proteins facilitate accelerated turnover of p53 through interaction with the ubiquitin ligase E6-AP (Huibregtse et. al 1991; Scheffner et al. 1993). A recent study suggests that low-risk E6 proteins also bind to E6-AP and that this activity is important for maintenance of episomes (Brimer et al. 2007). However, since low-risk E6 proteins do not degrade p53, it is likely that degradation of other cellular factors is important in maintaining viral genomes as episomes. Another factor targeted by E6 that is important in episomal maintenance is E6TP1 (Lee et al. 2007). E6TP1 is a ras-GAP regulator and its binding to E6 likely contributes to alteration of the cellular environment to allow for episomal maintenance. Recently, the interaction of high-risk E6 with proteins containing PSD-95/ discs large/ZO-1 (PDZ) domains was shown to contribute to long-term stable maintenance of episomes (Lee and Laimins 2004). PDZ proteins are important in many cellular processes, including cell signaling and cell adhesion (Fanning

and Anderson 1999; Harris and Lim 2001), and E6 proteins of HPV 16, HPV 18 and HPV 31 have been shown to bind and target PDZ proteins for degradation (Glaunsinger et al. 2000; Handa et al. 2007; Kuhne et al. 2000; Lee and Laimins 2004; Lee et al. 2000). Lee et al. demonstrated that HPV 31 genomes containing mutations in the E6 PDZ-binding domain displayed reduced growth, as well as a gradual loss of episomes (Lee and Laimins 2004). Since the ability to maintain episomes was only lost with time, this function does not have an immediate effect on viral genome maintenance.

4.3.4 Late Gene Expression and Genome Amplification

The productive phase of the viral life cycle occurs in the terminally differentiated layers of the stratified epithelium, where viral particles are assembled and shed. Differentiation of infected cells induces a dramatic increase in late gene expression that results in genome amplification and synthesis of virions (Fig. 4.4) (Stubenrauch and Laimins 1999). Transcripts expressed from the early promoter remain unchanged upon differentiation, allowing for continued low-level expression of E6 and E7 throughout all epithelial layers (Hummel et al. 1995). Upon differentiation, late transcription is induced from the differentiation-dependent late promoter (p742 in HPV 31 and p670 in HPV 16), located at the 3′ end of the E7 open reading frame (Fig. 4.2) (Grassmann et al. 1996; Hummel et al. 1992). These transcripts initiate at a heterogenous set of sites that extend over a region of more than 100 bp (Ozbun and Meyers 1998b). The late transcripts terminate either at the end of the E5 gene or the L1 gene through the action of polyadenylation sequences that are conserved among papillomaviruses (Terhune et al. 1999) (Fig. 4.2). Late messages encode for E1^E4 and E5, as well as L1 and L2. The E1^E4/E5 transcripts are polyadenylated at the end of the early region, whereas transcripts encoding L1 and L2 are transcribed through the early polyadenylation sequences to utilize the poly A site at the end of L1 (Terhune et al. 1999).

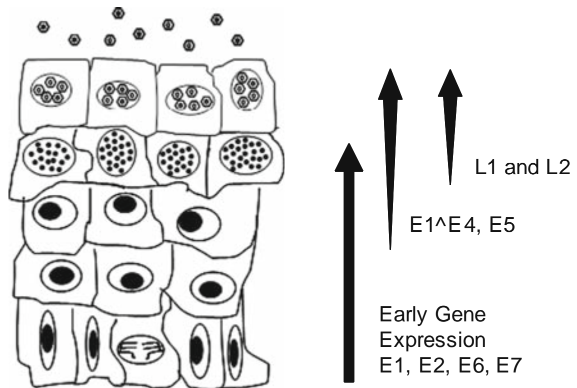

Fig. 4.4 Viral protein expression in differentiating epithelium

The mechanism by which differentiation controls the productive phase of the viral life cycle is an active area of study. Upon differentiation, transcription of E1 and E2 shifts from the early promoter, which is tightly regulated by E2, to the late promoter, which functions independently of E2 (Steger and Corbach 1997). This switch in promoter usage leads to increased expression of E1 and E2 and subsequent amplification of viral genomes to thousands of copies per cell (Klumpp and Laimins 1999). It has been suggested that the activation of the late promoter is dependent on genome amplification, however two recent studies demonstrated that these processes are not linked (Bodily et al. 2006; Spink and Laimins 2005). These studies showed that for HPV 31, increased copy number serves to amplify the magnitude of late gene transcription by providing more templates, but is not required for activation of the late promoter. In addition, induction of late gene expression occurs concomitantly with a differentiation-dependent increase in DNase hypersensitivity around the HPV 31 p742 late promoter, which is suggestive of chromatin remodeling and increased transcriptional activity (del Mar Pena and Laimins 2001). Recently, elements in the URR were identified that positively and negatively regulate the late promoter (Bodily et al. 2006; Spink and Laimins 2005). The profile of transcription factors binding to these elements changes upon differentiation, raising the possibility that induction of the late promoter relies on the binding of differentiation-specific transcription factors (Sen et al. 2004). One prominent candidate is C/EBPβ whose activity correlates with late gene expression (Gloss et al. 1987).

In addition to transcriptional control, late gene expression is also regulated at the post-transcriptional level. At least four papillomaviruses (BPV 1, HPV 16, HPV 1 and HPV 31) have been shown contain *cis*-acting inhibitory RNA elements located in the 3' UTR downstream of L1 (Cumming et al. 2002; Furth and Baker 1991; Tan and Schwartz 1995). Negative RNA elements have also been identified inside the HPV 16 L1 and L2 open reading frames (Kennedy et al. 1991; Oberg et al. 2003; Sokolowski et al. 1997; Tan et al. 1995). Although initially thought to destabilize late transcripts, more recent studies indicate that almost all these elements regulate RNA polyadenylation or RNA splicing rather than directly affecting RNA stability (Zheng and Baker 2006). While expression of late transcripts can be influenced at the level of splicing and polyadenylation, codon usage is also postulated to affect the efficiency with which the capsid genes, L1 and L2, are translated. The capsid genes of HPVs contain very few "consensus" mammalian codons (Zhao et al. 2003). It has been suggested that the amino-acyl transfer RNAs (tRNAs) necessary for efficient translation of L1 and L2 are limiting in undifferentiated cells, but become abundant upon differentiation (Zhou et al. 1999). It has been reported that mouse keratinocytes alter the spectrum of tRNAs upon differentiation (Zhao et al. 2005), suggesting that differentiation-associated changes in the tRNA profile may contribute, along with splicing and polyadenylation, to the restriction of capsid gene expression to only cells that are undergoing terminal differentiation.

4.3.5 Role of E7 in Differentiation-Dependent Late Viral Events

Normally, keratinocytes withdraw from the cell cycle as they migrate from the basal layer to the suprabasal layer and initiate a terminal differentiation program (Webb and Kaur 2006). However, HPVs utilize the cellular replication machinery for the productive replication of viral genomes. The E7 protein functions to create a cellular environment favorable to the productive phase of the viral life cycle. E7 maintains differentiating cells active in the cell cycle through binding to and mediating the degradation of Rb, as well as the related proteins p107 and p130 (Felsani et al. 2006). The abrogation of Rb function by E7 in post-mitotic cells facilitates their re-entry into S-phase, allowing for productive viral replication to occur in differentiating suprabasal cells (Chellappan et al. 1992). This is often associated with the retention of nuclei, which normally are degraded upon differentiation. Although the E7 proteins of high-risk types bind Rb family members with higher affinity than the low-risk types, low-risk HPVs can also induce DNA synthesis in differentiating cells (Cheng et al. 1995). Recently, the E7 protein of HPV 6 was shown to bind and target p130 for ubiquitin-mediated degradation, resulting in delayed differentiation (Zhang et al. 2006). Since HPV 6 E7 was unable to bind and degrade Rb or p107, the ability of both high-risk and low-risk types to degrade p130 may be critical for allowing DNA synthesis to occur in differentiating cells.

While E7-mediated degradation of Rb is necessary for the release of E2F transcription factors, recent studies indicate that E7 may modulate E2F activity in differentiating epithelia by other mechanisms. As mentioned previously, high-risk E7 proteins bind indirectly to HDACs though sequences that are distinct from Rb binding (Brehm et al. 1999; Longworth and Laimins 2004). This interaction was found to be essential for HPV 31 episomal maintenance and indicated that viral replication depends on the ability of E7 to regulate the actions of both Rb and HDACs (Longworth and Laimins 2004). In addition, it was demonstrated that the sequestration of class I HDACs by HPV 31 E7 induced a specific increase in the transcription of the activator E2F, E2F2, upon differentiation (Longworth et al. 2005). Transfection of HPV 31-positive cells with E2F2-specific siRNAs resulted in diminished viral genome amplification upon differentiation, suggesting a possible mechanism by which E7 targeting of Rb and HDACs promotes viral replication. Similar HDAC-binding sites are present in HPV 16 and HPV 18, as well as in low-risk HPV 6 and HPV 11, raising the possibility that increased E2F2 expression may be a general mechanism for facilitating replication in differentiating cells.

E7 has also been shown to bind directly to cyclin A/CDK2 complexes and indirectly to cyclin E/CDK2 complexes through p107 (McIntyre et al. 1996; Ruesch and Laimins 1998; Tommasino et al. 1993). E7 proteins of both high-risk and low-risk HPV types can increase the activation of cdk2 (He et al. 2003), which may contribute to E7's ability to induce S-phase entry by disengaging the

G1/S checkpoint. In addition, multiple cdk inhibitors are induced during keratinocyte differentiation, including p21 and p27, which leads to inhibition of cdk2 activity and growth arrest (Sherr and Roberts 1995). E7-expressing cells exhibit enhanced p21 expression as a consequence of increased protein stability (Ruesch and Laimins 1997). However, E7 has been shown to bind and abrogate the inhibitory activities of both p21 and p27 on cyclin A and cyclin E-associated kinase activity (Funk et al. 1997; Jones et al. 1997; Zerfass-Thome et al. 1996). Inhibition of these cdk inhibitors is likely to be important for E7 to mediate uncoupling of cellular differentiation and proliferation, thereby maintaining an S-phase environment in cells that would normally have withdrawn from the cell cycle.

4.3.6 E6 and E7 Activate Caspases Upon Differentiation

E6 and E7 have been shown to modulate both pro- and anti-apoptotic pathways, depending on the cell type and experimental conditions (Finzer et al. 2002; Garnett and Duerksen-Hughes 2006). Recent studies indicate that the E6 and E7 proteins of high-risk HPV 31 activate caspases of the intrinsic apoptotic pathway during the productive phase of the viral life cycle (Moody and Laimins, unpublished). The activation of caspase-9, caspase-3 and caspase-7 was observed in HPV 31 cells induced to differentiate in organotypic raft cultures, as well as by suspension in methylcellulose and exposure to high calcium media. In contrast, normal keratinocytes exhibited little to no caspase activation. Interestingly, caspase activation occurred in the absence of morphological features of apoptosis and was accompanied by an anti-apoptotic response. Several studies have demonstrated a role for caspases in many cellular processes that are independent of inducing cell death, including cell cycle progression, proliferation and differentiation (Launay et al. 2005). Treatment of differentiating HPV 31 positive keratinocytes with either a general caspase inhibitor or an inhibitor specific for caspase-3 greatly diminished viral genome amplification, indicating that caspase activity may play a role in facilitating the viral life cycle. A conserved caspase-3/-7 consensus cleavage site was identified in the amino-terminus of the HPV 31 E1 protein, and in vitro caspase cleavage assays demonstrated that E1 is a substrate for both caspases. Keratinocytes that stably maintain HPV 31 genomes containing mutations in the E1 caspase cleavage site exhibited a significant decrease in viral genome amplification upon differentiation, suggesting that caspase activity and cleavage of E1 are necessary for the productive viral life cycle. The DxxD caspase cleavage motif in E1 is conserved among the high-risk and low-risk viruses, suggesting that caspase activity may be a common mechanism utilized by HPVs to promote efficient replication of viral genomes in differentiating cells.

4.3.7 E1^E4 Modulates Activation of Late Viral Functions

Activation of the productive phase of the HPV life cycle coincides with high-level expression of E1^E4 (Doorbar et al. 1997). The E4 protein is translated from spliced mRNAs containing a fusion of the first five amino acids from the E1 open reading frame and the entire E4 open reading frame (Nasseri et al. 1987). The E1^E4 protein is the most abundantly expressed viral protein and it accumulates in the cytoplasm of cells located in the upper layers of the stratified epithelium. Upon differentiation, E1^E4 is modified by proteolysis and phosphorylation, which is believed to regulate the function of E1^E4 at different stages during the life cycle (Knight et al. 2004; Roberts et al. 1994). Increased expression of E1^E4 occurs concomitantly with genome amplification and can be detected prior to the expression of the capsid proteins L1 and L2 (Doorbar et al. 1997) (Fig. 4.4). Although the contribution of E1^E4 to the viral life cycle is unclear, mutational analysis of HPV 16, 18 and 31 genomes demonstrated that abrogation of E1^E4 expression results in an inability to efficiently amplify viral genomes, as well as inhibition of L1 and L2 expression (Nakahara et al. 2005; Wilson et al. 2005, 2007). This indicates a role for E1^E4 in modulating viral DNA synthesis and late gene expression. HPV 16 E1^E4 has been shown to bind to a RNA helicase of the DEAD box protein family (E4-DP) that is implicated in the regulation of translation by affecting splicing, ribosome biogenesis, RNA turnover and mRNA export (Doorbar et al. 2000). The expression of L1 and L2 is largely controlled at the level of splicing and stability (Zheng and Baker 2006). A potential interaction between E1^E4 with E4-DP could indicate a role for E1^E4 in post-transcriptional regulation of gene expression, perhaps allowing for stable expression of L1 and L2 and subsequent virion assembly in terminally differentiated cells.

Overexpression studies of E1^E4 have also provided evidence to support a role in regulating late viral functions. Expression of HPV 16 E1^E4 from heterologous promoters in human keratinocytes induces the collapse of cytokeratin networks (Doorbar et al. 1991; Wang et al. 2004). This activity of E1^E4 has been suggested to play a role in the release of newly synthesized virions by altering cellular integrity. The E1^E4 proteins have also been shown to associate with cell cycle regulators, which may contribute to DNA synthesis in suprabasal cells. Over-expression of HPV 16 E1^E4 in transient assays induces a G2 cell cycle arrest by re-localizing active Cdk1/cyclin B1 complexes to cytokeratin filaments in the cytoplasm, preventing nuclear entry and the initiation of mitosis (Davy et al. 2005). Interestingly, E1^E4-expressing cells exhibited greater than a 4 N DNA content, indicating that DNA synthesis continues even though cell division does not. HPV 16 E1^E4 has also been shown to bind cyclin A complexes and sequester them in the cytoplasm (Davy et al. 2006). G2 arrest appears to be a function conserved among E1^E4 proteins of other HPV types, including HPV 18 (Nakahara et al., 2002), HPV 11 (Davy et al. 2002) and HPV 1 (Knight et al. 2004). Targeted inhibition of both cyclin B/cdk and

cyclin A/cdk complexes during the G2 phase of the cell cycle may allow for efficient blocking of cell cycle progression, perhaps stimulating DNA synthesis and creating an environment optimal for viral DNA replication. However, most of these studies have been performed in transient assays, and the investigation of E1^E4 interactions during the productive life cycle in differentiating cells will likely provide the most useful information concerning its role in viral pathogenesis.

4.3.8 HPV E5 Proteins

Another HPV protein that is primarily expressed in differentiating cells is the E5 protein. The E5 proteins are small, extremely hydrophobic proteins that are detected mainly in the Golgi apparatus, endosomal membranes and nuclear membrane (Conrad et al. 1993; Disbrow et al. 2003). Upon differentiation, E5 is expressed in the majority of late transcripts as the second ORF (Ozbun and Meyers 1997) (Fig. 4.2); however, it is difficult to determine the abundance of E5 protein in differentiated cells, as no effective antibodies are available. For bovine papillomavirus (BPV), E5 is responsible for the major transforming activity of the virus (DiMaio 1991; Schiller et al. 1986). In contrast, HPV E5s exhibit weak transforming activity, but can augment transformation by E6 and E7 (Bouvard et al. 1994; Valle and Banks 1995). Mutation of E5 in the context of the complete HPV 31 or HPV 16 genome resulted in reduced viral genome amplification and transcription of late genes, suggesting that E5 primarily plays a role in the productive phase of the viral life cycle (Fehrmann et al. 2003; Genther et al. 2003). The mechanisms by which E5 mediates its effects are an active area of study.

Several in vitro studies have suggested a link between E5 and the epidermal growth factor receptor (EGFR), a transmembrane receptor protein found on all epithelial cells. Transient expression of HPV 16 E5 and HPV 6 E5 resulted in increased proliferation of mouse fibroblasts, as well as human keratinocytes in response to EGF, suggesting that E5 may modulate EGFR-signaling pathways (Leechanachai et al. 1992; Straight et al. 1993). The expression of E5 has also been reported to delay internalization and degradation of the EGF receptor, and increased levels of EGFR have been detected on the surface of E5-expressing keratinocytes (Straight et al. 1993). It is postulated that E5 alters receptor recycling through interaction with the 16 kD subunit of the vacuolar ATPase, which normally acts to acidify the endosomal environment (Conrad et al. 1993). E5 has been shown to inhibit acidification of endosomes (Disbrow et al. 2005; Straight et al. 1995), which could allow for an increased number of receptors at the surface and prolonged activation of the receptor upon ligand binding. These in vitro studies suggest a synergistic relationship between E5 and EGFR signaling; however, the significance of EGFR modulation by E5 in the viral life cycle is not well defined. In the context of the high-risk HPV 31 complete viral genome,

abrogation of E5 expression by mutation did not significantly affect the levels of EGFR, or its phosphorylation in undifferentiated or differentiated cells, indicating that EGFR is not a target of HPV 31 E5 (Fehrmann et al. 2003).

In tissue culture models, HPV 16 E5 has been shown to inhibit, but not completely abolish, the transport of MHC (HLA) class I complexes to the cell surface due to retention in the Golgi apparatus (Ashrafi et al. 2005). While downregulation of MHC class I molecules would efficiently inhibit cytotoxic T lymphocyte (CTL)-mediated immune clearance, the total absence of surface MHC class I would render infected cells more susceptible to natural killer (NK) cell attack. NK cells interact with certain MHC class I molecules (HLA-C and -E) which inhibit NK-mediated cell lysis. E5 appears to selectively downregulate HLA-A and HLA-B molecules on the cell surface, rather than HLA-C and E, protecting infected cells from both CTL and NK cell-mediated immune responses (Ashrafi et al. 2005). Expression of HPV 16 E5 has also been shown to decrease surface expression of MHC class II (Zhang et al. 2003). It is possible that the ability of E5 to disrupt several components of cell-mediated immune response is necessary for the establishment and persistence of HPV infection. It is however not clear how these effects would be mediated if E5 is indeed expressed primarily in differentiated cells.

4.3.9 Viral Assembly and Release

Synthesis of progeny virus occurs in the most terminally differentiated layers of the epithelium. The L1 and L2 capsid proteins are expressed in these layers following the onset of viral genome amplification (Ozbun and Meyers 1997). The newly replicated viral genomes associate with histones and form chromatin-like complexes that are packaged into icosahedral capsids consisting of 360 copies of the major capsid protein L1 and approximately 12 copies of the minor capsid protein L2 (Chen et al. 2000; Modis et al. 2002). In terminally differentiated cells, the expression and nuclear translocation of L2 precedes the expression and nuclear import of L1 (Florin et al. 2002a).

Most of our knowledge about virion morphogenesis has been derived from studies using virus-like particles (VLPs) composed of the two viral structural proteins L1 and L2. L1 assembles into capsomeres in the cytoplasm, which in turn assemble into virions in the nucleus (Florin et al. 2002a). The L2 protein localizes to nuclear substructures termed PML oncogenic domains or nuclear domains 10 (ND10), which are postulated to be sites of HPV replication, as well as viral assembly (Day et al. 2004, 1998). L2 induces the reorganization of these nuclear domains, leading to the degradation of the transcription factor Sp100 and the subsequent recruitment of L1, as well as E2 (Day et al. 1998; Florin et al. 2002b). While the L2 protein of some papillomaviruses can interact directly with DNA (Fay et al. 2004), E2 has been postulated to be responsible for the recruitment of viral genomes to ND10 bodies due to its ability to bind specific

DNA sequences within the viral genome (Day et al. 1998). Although papillomavirus particles can assemble in the absence of L2, its presence is thought to contribute to efficient packaging of viral DNA and is essential for virion infectivity (Okun et al. 2001). For HPV 31, VLPs lacking L2 demonstrated a 10-fold reduction in the amount of DNA encapsidated and over a 100-fold decrease in infectivity (Holmgren et al. 2005). Papillomavirus capsids are thought to undergo a lengthy maturation process whereby inter-L1 disulphide linkages serve to increase the physical stability of the capsid prior to release from the epithelium (Buck et al. 2005). Once mature, papillomavirus particles are shed from the surface of the epithelium, although the exact mechanism by which this occurs is unclear.

4.4 Conclusions

HPV remains a major public health concern due to its ubiquitous nature and association with various malignancies, most notably cervical cancer. The development of genetic systems, along with efficient methods to differentiate epithelial cells in tissue culture, has been instrumental in the study of HPV biology and has yielded important insights into the viral life cycle. However, many questions remain unanswered. The search for the cellular receptor(s) utilized for HPV entry remains an active area of research. Also important is developing a more complete understanding of the innate and adaptive immune responses to HPV infection, as well determining how the virus alters these responses to escape immune surveillance and establish a persistent infection. One of the most important outcomes of HPV research has been the development of preventative HPV vaccines. These prophylactic vaccines have shown a high degree of efficacy and may provide a means of decreasing the incidence of cervical cancer worldwide. However, these vaccines offer little protection to those already infected with HPV, and the development of effective therapeutics to treat HPV-associated diseases remains an important undertaking.

Acknowledgments This work was supported by grants to LAL from the NCI and NIAID. CAM was supported by a fellowship from the American Cancer Society (PF-06-177-01-MBC) and a NIH post-doctoral training grant (5T32 AR007593).

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Chapter 5
Papillomavirus Transformation

Kimberly Johung and Daniel DiMaio

Abstract Papillomaviruses are small DNA tumor viruses that induce benign and malignant epithelial tumors. The most prevalent malignant tumor associated with human papillomaviruses (HPV) infection is cervical cancer. The oncogenic potential of papillomaviruses is reflected in their ability to immortalize and transform cells growing in culture. Analysis of these activities has identified three viral oncogenes: E5, E6, and E7. The E5 gene induces transformation by modulating the activity of cell membrane proteins such as growth factor receptors, whereas the E6 and E7 genes target nuclear tumor suppressor proteins such as p53 and the retinoblastoma protein. Studies of these interactions have provided important insights into cell cycle control and signal transduction and may suggest novel strategies to combat papillomavirus-induced cancer.

5.1 Introduction

Papillomaviruses are small non-enveloped viruses that contain a circular double-stranded DNA genome of approximately 8000 bp within an icosahedral capsid. These viruses infect basal keratinocytes of the cutaneous or mucosal squamous epithelium, and viral gene expression induces proliferation of their host cells, which is necessary to support high-level viral DNA replication as the cells differentiate and migrate away from the basement membrane. The replicated viral DNA is packaged in capsids, and virions are shed from the upper layer of the epithelium (reviewed in Zheng and Baker 2006). Because host cells are stimulated to re-enter the cell cycle, papillomavirus infection often leads to epithelial hyperplasia and the formation of benign epithelial tumors called papillomas or warts. In addition, infection with specific papillomavirus types is associated with the development of certain malignant tumors, most notably cervical cancer in women.

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_5, © Springer Science+Business Media, LLC 2009

The infectious etiology of human and animal warts was first demonstrated in the early 1900s (reviewed in Chapter 2, Garcea and DiMaio 2007). The carcinogenic potential of papillomas in rabbits caused by infection with the cottontail rabbit papillomavirus was observed in the 1930s (Rous and Beard 1935), while the carcinogenic potential of human papillomaviruses (HPVs) was initially suggested by the detection of specific HPV types in the skin carcinomas of patients with the rare disease epidermodysplasia verruciformis (Orth et al. 1979). In the 1980s, HPV DNA was isolated from cervical cancer biopsies and cell lines in the laboratory of Harald zur Hausen (Boshart et al. 1984; Durst et al. 1983; Gissman et al. 1983). Over a hundred HPV types have since been identified. The low-risk mucosal types (including HPV types 6 and 11) cause benign anogenital and laryngeal warts, while persistent infection of keratinocytes with the high-risk types (most commonly types 16 and 18) is required for the development of cervical cancer, a leading cause of cancer death in women, particularly in the developing world (Walboomers et al. 1999). HPV infection is also associated with the development of additional cancers (zur Hausen 2002), including other anogenital malignancies, certain head and neck squamous cell carcinomas, and non-melanoma skin cancer in immunosuppressed individuals.

The transforming capacity of high-risk HPV genomes in cultured cells and transgenic mouse models has been demonstrated in numerous experiments. Introduction of high-risk HPV DNA can immortalize primary human fibroblasts and keratinocytes (Durst et al. 1987; Kaur and McDougall 1989; Pirisi et al. 1987), and co-expression of the high-risk E6 and E7 genes is sufficient for these activities (Hawley-Nelson et al. 1989; Hudson et al. 1990; Kaur et al. 1989; Munger et al. 1989a). The E6 and E7 genes can independently immortalize normal cells, though with decreased efficiency (Band et al. 1991; Halbert et al. 1991). Furthermore, introduction of high-risk HPV DNA into NIH 3T3 cells is sufficient to induce transformation (Hudson et al. 1990; Tsunokawa et al. 1986; Yasumoto et al. 1986). When grown in raft cultures to induce epithelial stratification, HPV16-immortalized primary keratinocytes undergo altered differentiation similar to the dysplastic precursor lesions of cervical cancers (McCance et al. 1988). Transgenic mice expressing HPV16 E6 and E7 in the stratified epithelium develop cervical carcinoma following chronic estrogen treatment (Arbeit et al. 1996), and expression of HPV16 E6 and E7 in the skin of transgenic mice triggers the development of locally invasive skin tumors (Lambert et al. 1993).

Progression of HPV-infected cervical lesions to cervical cancer is often associated with integration of the viral genome into the host cell genome (Schneider-Maunoury et al. 1987; Schwarz et al. 1985), which may confer a growth advantage on infected cells (Jeon et al. 1995). Integration also typically disrupts the viral E2 gene. The E2 protein is a transcription factor that binds to sites in the HPV major early promoter and represses expression of the E6 and E7 oncogenes (Bernard et al. 1989; Thierry and Yaniv 1987). Therefore, loss of E2 expression upon integration results in continuous expression of E6 and E7.

In cervical cancers in which the HPV genome is not integrated, elevated E6 and E7 expression is achieved by other means, such as mutations in the early promoter that prevent binding of E2 (May et al. 1994). Reintroduction of the E2 gene from several papillomavirus types into cervical cancer cell lines and HPV-immortalized keratinocytes effectively represses expression of the E6 and E7 oncogenes and induces a dramatic growth inhibition consistent with the irreversible growth-arrested state of senescence (Dowhanick et al. 1995; Francis et al. 2000; Goodwin and DiMaio 2000; Goodwin et al. 2000; Hwang et al. 1993; Lee et al. 2002a; Naeger et al. 1999; Wells et al. 2000). Antisense inhibition of HPV E6/E7 expression in cervical carcinoma cells also inhibits their proliferation (von Knebel Doeberitz et al. 1988). Thus, continuous expression of the E6 and E7 oncogenes is required to maintain the proliferative state of cervical cancer cells, suggesting that inhibiting the expression or action of the E6 and E7 proteins may be useful for treating HPV-associated cancer. Although E6 and E7 are the two major transforming proteins of human papillomaviruses, E5 is the major transforming protein of bovine papillomavirus type 1 (BPV).

Productive viral replication in differentiated suprabasal keratinocytes requires the E6 and E7 oncogenes (Flores et al. 2000; Thomas et al. 1999), which reprogram host cells to support viral DNA replication (Cheng et al. 1995). The interactions between the viral oncoproteins and the host cellular proteins that promote viral replication also induce cellular transformation and will be the focus of the remainder of this chapter.

5.2 Papillomavirus E6 Protein

The E6 protein consists of approximately 150 amino acids and contains 4 Cys-X-X-Cys motifs that form 2 zinc-binding domains (Mantovani and Banks 2001). In addition, high-risk E6 proteins contain four C-terminal residues (ETQV) that function as a binding site for proteins containing PDZ domains (Kiyono et al. 1997; Lee et al. 1997). Expression of high-risk E6 inhibits terminal differentiation of primary keratinocytes (Sherman et al. 2002), and as mentioned previously, high-risk E6 proteins exhibit transforming properties in cell culture and transgenic mouse models. E6 can also immortalize primary human cells (Band et al. 1991), cooperate with activated oncogenes to transform cells (Liu et al. 1994; Storey and Banks 1993), and is sufficient to induce malignant skin tumors in transgenic mice (Song et al. 1999).

E6 proteins from high-risk HPV, but not low-risk E6 proteins or the BPV E6 protein, associate with and induce degradation of the p53 tumor suppressor (Scheffner et al. 1990; Werness et al. 1990). p53 is a transcription factor that regulates the expression of genes involved in cell cycle arrest or apoptosis in response to cellular stress such as DNA damage. High-risk E6 proteins bind to the E6AP E3 ubiquitin ligase, which allows interaction between E6AP and p53, thereby targeting p53 for ubiquitin-mediated proteasomal degradation

(Scheffner et al. 1993). Interactions between E6 and other cellular proteins serve to enforce the inactivation of p53. For example, high-risk E6 proteins can bind directly to transcriptional co-activators of p53-mediated gene expression such as CBP and p300 and thus inhibit p53-responsive transcriptional activity independent of p53 degradation (Patel et al. 1999; Pim et al. 1994; Zimmermann et al. 1999).

Inactivation of the p53 pathway by E6 expression inhibits p53-triggered growth arrest in response to DNA-damaging agents (Foster et al. 1994; Kessis et al. 1993). In addition, expression of E6 in a transgenic mouse model inhibits apoptosis in both a p53-dependent and a p53-independent fashion (Pan and Griep 1995). The E6-mediated resistance to stress-induced apoptosis and growth arrest promotes the accumulation of cellular mutations and genomic instability. Furthermore, continuous expression of the E6 protein in cervical cancer cells is required to maintain the p53 pathway in an inactive state and prevent apoptosis (DeFilippis et al. 2003; Horner et al. 2004). However, not all of the effects of E6 are mediated by p53 degradation. For example, E6 mutants defective for p53 degradation maintain the ability to immortalize human mammary epithelial cells (Liu et al. 1999). Therefore, p53 inactivation is not required for at least some of the transforming activities of the E6 protein.

In fact, the E6 protein binds to numerous additional cellular-binding partners, and interactions with these proteins may contribute to the oncogenic properties of HPV (Mantovani and Banks 2001). HPV16 E6 binds to E6TP1, a Rap GTPase-activating protein. The ability of HPV16 E6 mutants to induce the degradation of E6TP1 strongly correlates with their ability to immortalize human mammary epithelial cells (Gao et al. 2001). In addition, the BPV E6 protein displays weak transforming activity in mouse C127 cells (Schiller et al. 1984), even though it does not bind to p53 (Werness et al. 1990). The p53-independent ability of BPV E6 to transform cells may involve complex formation between BPV E6 and cellular binding partners such as paxillin or reticulocalbin 2 because binding to these proteins correlates with the transforming ability of the BPV E6 protein (Chen et al. 1995; Tong and Howley 1997).

p53 is not the only cellular protein targeted by the high-risk E6 proteins for E6AP-mediated degradation. For example, high-risk E6 proteins bind to PDZ domain-containing proteins, including putative tumor suppressors, and induce their degradation in an E6AP-dependent manner (Favre-Bonvin et al. 2005; Glaunsinger et al. 2000; Kiyono et al. 1997; Lee et al. 2000; Nakagawa and Huibregtse 2000). The ability of the E6 protein to bind and trigger proteasome-mediated degradation of PDZ proteins is associated with its ability to transform cells in culture (Favre-Bonvin et al. 2005; Kiyono et al. 1997), and interaction between high-risk E6 proteins and PDZ domain-containing proteins is required for E6-mediated induction of epithelial hyperplasia and promotion of skin carcinogenesis in vivo (Nguyen et al. 2003). Therefore, inactivation of PDZ proteins contributes to the transforming activity of the E6 protein.

High-risk E6 also activates telomerase (Klingelhutz et al. 1996), a ribonucleoprotein reverse transcriptase that allows complete replication of DNA ends

by adding telomeric repeats to the 3′ ends of chromosomes. This reaction prevents the progressive shortening of telomeres, which otherwise limits the replicative potential of normal somatic cells. Expression of the human telomerase catalytic subunit (hTERT) is required for immortalization of primary human keratinocytes (Dickson et al. 2000; Kiyono et al. 1998). Thus, the ability of E6 to activate telomerase may contribute to its immortalizing activity. The mechanism of telomerase activation involves upregulation of hTERT transcription (Oh et al. 2001; Veldman et al. 2001) via the activity of c-myc-containing complexes at E-box-binding elements in the hTERT promoter (Liu et al. 2005; Veldman et al. 2003). E6-mediated induction of hTERT expression requires expression of E6AP and may involve E6AP-mediated degradation of a repressor of the hTERT promoter (Gewin et al. 2004; Liu et al. 2005).

5.3 Papillomavirus E7 Protein

The high-risk HPV E7 oncoprotein (reviewed in Munger et al. 2001) is a nuclear protein of approximately 100 amino acids, which contains three conserved regions, CR1, CR2, and CR3. CR1 is located at the N-terminus, CR2 contains an LXCXE motif required for binding to the members of the retinoblastoma (Rb) tumor suppressor family and a conserved phosphorylation site for casein kinase II, and CR3 contains two Cys-X-X-Cys zinc finger motifs. Sequences within CR1 and CR2 are conserved with the adenovirus E1A and simian virus 40 (SV40) large tumor antigen (T) genes, while the C-terminal metal-binding domain may play a role in dimerization of the E7 protein.

Through its ability to interact with and modify the activities of cellular proteins, the E7 protein functions in cellular transformation. E7 expression is sufficient to extend the life span of primary human cells (Foster and Galloway 1996; Halbert et al 1991) and can cooperate with E6 to immortalize human keratinocytes. In addition, HPV E7 induces transformation of rodent fibroblast cell lines (Bedell et al. 1989; Phelps et al. 1988; Vousden et al. 1988) and cooperates with the ras oncogene to transform primary rodent fibroblasts (Matlashewski et al. 1987; Phelps et al. 1988). Transgenic mice expressing HPV16 E7 develop cervical cancer in response to chronic estrogen treatment and locally invasive skin tumors (Herber et al. 1996).

The transforming activities of the HPV E7 protein are largely due to interaction between E7 and the retinoblastoma tumor suppressor protein, p105^{Rb}, as well as the other members of the "pocket protein family[11], p107 (RBL2) and p130 (RB2) (Dyson et al. 1989). These interactions are mediated by an LXCXE-binding domain in CR2 of the E7 protein that is conserved with adenovirus E1A and SV40 T antigen. The high-risk E7 proteins bind Rb with higher affinity than the low-risk E7 proteins (Gage et al. 1990; Munger et al. 1989b), and the high-risk but not low-risk E7 proteins stimulate the degradation of p105^{Rb} and p107 via a proteasome-dependent pathway (Berezutskaya et al. 1997; Boyer

et al. 1996), although both high-risk and low-risk E7 proteins can destabilize p130 (Zhang et al. 2006). The efficiency of binding and degrading $p105^{Rb}$ correlates with the transforming capacity of the E7 oncoproteins (Heck et al. 1992). A single amino acid difference between the Rb-binding domains of high- and low-risk E7 proteins is responsible for the differences in Rb-binding affinity and transforming activity in certain assays (Barbosa et al. 1991; Heck et al. 1992). Inactivation of Rb function by E7 requires Rb degradation and not simply Rb binding (Gonzalez et al. 2001). N-terminal residues within CR1 distinct from LXCXE are critical for degradation of Rb and cellular transformation (Gonzalez et al. 2001; Helt and Galloway 2001; Jones et al. 1997b; Phelps et al. 1992).

The Rb family inhibits tumorigenesis by regulating key growth regulatory processes, including cell cycle progression, senescence, differentiation, and apoptosis. Rb family members regulate the G1/S transition of the cell cycle primarily by binding and modulating the activity of members of the E2F family of transcription factors, which regulates the expression of genes required for S phase (Stevaux and Dyson 2002). In the G0 and early G1 phases of the cell cycle, $p105^{Rb}$ is hypophosphorylated. This active form of Rb forms a complex with repressor E2F family members and inhibits the expression of E2F target genes by binding to the transactivation domain of E2F and recruiting chromatin remodeling proteins including histone deacetylases (Harbour and Dean 2000). Phosphorylation of $p105^{Rb}$ by complexes of cyclins and cyclin-dependent kinases at the G1/S phase transition of the cell cycle leads to dissociation of Rb/E2F complexes, enabling free E2F to activate the expression of genes required for cell cycle progression (Stevaux and Dyson 2002). E7 binding to the hypophosphorylated form of $p105^{Rb}$ leads to the disruption of Rb/E2F complexes (Chellappan et al. 1992) and proteolytic degradation of Rb family members (Berezutskaya et al. 1997; Boyer et al. 1996). In addition, E7 inhibits the function of cyclin-dependent kinase inhibitors (Funk et al. 1997; Jones et al. 1997a); Zerfass-Thome et al. 1996), such that Rb family members exist predominantly in their inactive hyperphosphorylated form. Thus, expression of HPV E7 induces expression of E2F-responsive genes essential for cell cycle progression and viral DNA synthesis (Cheng et al. 1995). Repression of E7 expression in cervical cancer cells is sufficient to induce Rb-dependent cellular senescence (DeFilippis et al. 2003; Johung et al. 2007; Psyrri et al. 2004).

Although inactivation of the Rb pathway is critical for E7-mediated transformation, degradation of the Rb family does not entirely account for the transforming functions of E7. Several studies suggest that the C-terminal domain of the E7 protein, which is not involved in Rb inactivation, may contribute to the immortalization and transformation activities of E7 (Edmonds and Vousden 1989; Jewers et al. 1992) and to the ability of E7-expressing cells to bypass growth arrest induced by DNA damage (Helt et al. 2002; Helt and Galloway 2001). Certain E7 mutants that are still able to bind and degrade $p105^{Rb}$ are unable to transform cells (e.g., Banks et al. 1990; Barbosa et al. 1990; Helt and Galloway 2001; Edmonds and Vousden 1989;

Jewers et al. 1992). Therefore, inactivating the Rb pathway is not sufficient for E7-mediated transformation, and interaction with other cellular-binding partners may be required. Indeed, E7 mutants defective for Rb binding can cooperate with E6 to immortalize primary human keratinocytes (Jewers et al. 1992). These results suggest that inactivation of the Rb pathway may not be absolutely required for the transforming functions of E7.

Rb-dependent and Rb-independent functions of the E7 protein in vivo have been described. Transgenic mice expressing HPV16 E7 in the basal layer of the stratified squamous epithelium develop epithelial hyperplasia, skin tumors, and cervical cancer in response to chronic estrogen treatment (Herber et al. 1996). Inactivation of $p105^{Rb}$ expression in murine skin using a Cre recombinase approach largely reproduces the phenotype of E7 expression in the epidermis, suggesting that E7 acts predominantly via inactivation of the Rb pathway (Balsitis et al. 2003). However, Rb-independent effects of E7 expression have also been observed in vivo. Surprisingly, E7 was able to induce cervical cancer following estrogen treatment independently of $p105^{Rb}$ inactivation, and furthermore, $p105^{Rb}$ inactivation alone was not sufficient to trigger cervical carcinogenesis in this mouse system (Balsitis et al. 2005). Therefore, interactions between E7 and cellular proteins other than $p105^{Rb}$ may also be critical for cervical carcinogenesis. However, microarray analysis indicates that the entire transcriptional response to E7 repression in cervical carcinoma cells is dependent on Rb activation (Johung et al. 2007).

While the ability of the HPV E7 protein to bind and inactivate Rb family members has been well characterized, the E7 protein also binds to numerous other cellular proteins (Munger et al. 2001), and these interactions may contribute to its oncogenic properties. In many cases, interaction with these cellular-binding partners does not depend on the interaction between E7 and Rb. The E7 protein can bind directly to cyclin A/cdk2 complexes (Tommasino et al. 1993) and as noted above binds and inactivates the cyclin-dependent kinase inhibitors, thereby promoting cell cycle progression. HPV16 E7 also interacts with the p600 Rb-associated protein. This association is independent of the ability of E7 to bind Rb family members but appears to contribute to the ability of HPV16 E7 to transform cells (Huh et al. 2005).

The E7 protein binds to several transcription factors, including Skip, interferon regulatory factor-1, the forkhead transcription factor MPP2, members of the AP1 family of transcription factors including c-Jun and c-Fos, E2F1, and transcriptional co-activators such as TATA-binding protein (TBP), the p300 transcriptional co-activator, and the pCAF acetyltransferase (reviewed in Munger et al. 2001). For the most part, the functional consequences of these interactions are poorly characterized. The E7 protein also interacts with several chromatin remodeling proteins, including histone deacetylases (HDACs) and the BRG1 component of SWI–SNF complexes, and these interactions play a role in the growth-promoting activity of the E7 protein (Lee et al. 2002b; Longworth and Laimins 2004). The interaction between E7 and HDACs is essential for HPV-mediated extension of keratinocyte life span and for stable

maintenance of viral episomes (Longworth and Laimins 2004). The E7 protein also interacts with the Mi2beta component of NURD histone deacetylase complexes, an interaction that is critical for bypassing Rb-dependent growth arrest (Brehm et al. 1999). Finally, HPV16 E7 is able to associate with DNA methyltransferase (Dnmt1) in vitro and in vivo. This interaction occurs via the C-terminal zinc finger domain of E7 and upregulates the methyltransferase activity of Dnmt1 (Burgers et al. 2006). Aberrant methylation triggered by the E7 protein may contribute to carcinogenesis via silencing of tumor suppressor genes.

5.4 Papillomavirus E5 Proteins

In addition to the major E6 and E7 transforming proteins, many papillomaviruses encode very small, hydrophobic E5 proteins that exert various growth stimulatory effects by modulating the activity of cellular membrane proteins. The bovine papillomavirus type 1 (BPV) E5 protein is by far the best understood E5 protein, in large part because it efficiently transforms cultured fibroblasts. The HPV16 E5 protein has also been studied in some detail, but there is no significant sequence similarity between the BPV and the HPV E5 proteins other than their overall hydrophobicity.

5.4.1 The BPV E5 Protein

The 44-amino acid BPV E5 protein has a very hydrophobic central segment and exists in cells as a transmembrane dimer of two identical subunits localized largely to intracellular organelles, such as the endoplasmic reticulum and Golgi apparatus (Burkhardt et al. 1989; Schlegel et al. 1986; Surti et al. 1998). The BPV E5 protein efficiently induces focus formation and tumorigenic transformation of immortalized rodent fibroblasts, primarily via activation of the endogenous platelet-derived growth factor (PDGF) β receptor, a transmembrane receptor tyrosine kinase (Lai et al. 2005; Petti et al. 1991). The essential role of the PDGF β receptor in transformation by the BPV E5 protein was demonstrated most clearly in gene transfer experiments in which cells lacking the receptor are resistant to E5-mediated signaling and transformation, unless an exogenous PDGF β receptor gene is introduced (Drummond-Barbosa et al. 1995; Goldstein et al. 1994; Nilson and DiMaio 1993). The BPV E5 protein is able to bind to or activate PDGF β receptor mutants unable to bind PDGF (Drummond-Barbosa et al. 1995; Goldstein et al. 1992; Staebler et al. 1995), indicating that E5-mediated activation of the PDGF β receptor is ligand independent. In addition, R. Schlegel and colleagues have presented evidence that the BPV E5 protein may also utilize other pathways to transform cells (Sparkowski et al. 1996; Suprynowicz et al. 2002; Suprynowicz et al. 2000).

The BPV E5 protein forms a stable complex with the PDGF β receptor (Goldstein et al. 1992; Petti and DiMaio 1992). This interaction results in the dimerization of two receptor molecules, autophosphorylation of the receptor on tyrosine, and binding to signal transduction proteins, thereby generating a sustained mitogenic signal (Drummond-Barbosa et al. 1995; Lai, Henningson, and DiMaio 1998; Petti et al. 1991). Mutation analysis and molecular modeling indicate that complex formation between the E5 protein and the PDGF β receptor is largely driven by specific interactions involving the transmembrane domains of the two proteins (Cohen et al. 1993; Drummond-Barbosa et al. 1995; Goldstein et al. 1992; Klein et al. 1999; Klein et al. 1998; Meyer et al. 1994; Nappi and Petti 2002; Petti et al. 1997; Staebler et al. 1995). Indeed, the hydrophobic segment of the E5 protein is thought to interact directly in an anti-parallel fashion with the transmembrane domain of the PDGF β receptor (Surti et al. 1998). Mutations that disrupt dimerization of the E5 protein also prevent complex formation with the PDGF β receptor and interfere with cell transformation and receptor activation (Horwitz et al. 1988; Mattoon et al. 2001; Nilson et al. 1995), suggesting that dimerization of the E5 protein creates binding sites for two molecules of the PDGF β receptor resulting in receptor dimerization and activation (Surti et al. 1998). The interaction between the E5 protein and the PDGF β receptor appears quite specific, in that at normal levels of expression the E5 protein does not bind or activate other growth factor receptors (Goldstein et al. 1994).

5.4.2 HPV Type 16 E5 Protein and its Cellular Targets

The HPV E5 proteins have relatively weak biological activity compared to the BPV protein. These activities have been best characterized for the 83-amino acid HPV16 E5 protein, which like the BPV E5 protein is very hydrophobic and localized largely to intracellular membranes (Conrad et al. 1993). The HPV16 E5 protein can induce transformation of rodent fibroblasts and keratinocytes and stimulate DNA synthesis in primary human keratinocytes (Bouvard et al. 1994; Leechanachai et al. 1992; Leptak et al. 1991; Pim et al. 1992; Straight et al. 1993). In addition, expression of the HPV E5 protein in transgenic mice can induce epithelial hyperplasia (Genther Williams et al. 2005).

The HPV16 E5 protein activates a variety of cytoplasmic signal transduction pathways and induces the expression of several nuclear transcription factors (e.g., Bouvard et al. 1994; Gu and Matlashewski 1995; Leechanachai et al. 1992). It has also been reported that the HPV16 E5 protein can inhibit cellular gap junction communication, modulate apoptosis, and interfere with the cell surface expression of major histocompatibility (MHC) antigens (see Chapter 9 in Garcea and DiMaio 2007). Because MHC antigens are required for antigen presentation and immune recognition, the ability of the E5 protein to inhibit MHC expression may be a strategy of viral immune evasion.

Analysis of viral mutants in animals and stratified keratinocyte cultures suggests that the high-risk HPV E5 protein may play a role in generating the

optimal permissive host cell for virus replication (Fehrmann et al. 2003; Genther et al. 2003). However, because some papillomaviruses lack an E5 gene, E5 function is not absolutely required for papillomavirus replication. Unlike the HPV E6 and E7 oncogenes, the E5 gene can be lost upon integration of the viral genome into cellular DNA during carcinogenic progression (e.g., Chang et al. 2001). Thus, the E5 protein does not appear to be required for maintenance of cancer, although it may be involved in the initial stages of carcinogenesis.

The biochemical mechanisms of HPV16 E5 action are not understood in detail, but stable association with a variety of transmembrane proteins including the EGF receptor and the vacuolar H^+-ATPase (V-ATPase) is likely to be required for its activities (Conrad et al. 1993; Hwang et al. 1995). Cells expressing the HPV16 E5 protein display enhanced tyrosine phosphorylation of the EGF receptor in response to EGF treatment (Crusius et al. 1997; Crusius et al. 1998; Straight et al. 1993), and E5-expressing cells are more sensitive to EGF stimulation, suggesting that the HPV16 E5 protein enhances ligand-dependent signaling from the EGF receptor (Leechanachai et al. 1992). In normal cells, cell surface EGF receptor is rapidly tyrosine phosphorylated, internalized, and degraded in lysosomes in response to EGF treatment. In cultures of human keratinocytes expressing the HPV16 E5 protein, ligand-activated EGF receptor is not degraded but rather recycled back to the cell surface where it can continue to signal (Straight et al. 1993; Tomakidi et al. 2000). Impaired degradation of the EGF receptor may be due to the ability of the HPV16 E5 protein to disrupt the association of the receptor with c-Cbl, an E3 ubiquitin ligase that normally targets activated EGF receptor for proteasomal degradation (Zhang et al. 2005).

The E5 proteins of all tested human and animal papillomaviruses form a stable complex with the 16 kDa transmembrane pore-forming subunit of the V-ATPase (Conrad et al. 1993; Faccini et al. 1996; Goldstein et al. 1991). The V-ATPase pumps hydrogen ions into intracellular organelles, thereby lowering luminal pH, and it has been reported that organelle acidification is impaired in cells expressing the HPV16 and BPV E5 proteins, presumably as a consequence of E5-mediated inhibition of V-ATPase activity (Disbrow et al. 2005; Schapiro et al. 2000; Straight et al. 1995). This activity may account for reduced degradation of activated EGF receptor, which normally occurs in acidic endosomes and lysosomes. However, there are conflicting reports in the literature regarding the effect of E5 proteins on V-ATPase activity and endosome acidification (Adam et al. 2000; Ashby et al. 2001; Ashrafi et al. 2000; Briggs et al. 2001; Disbrow et al. 2005; Thomsen et al. 2000).

5.5 HPV Oncogenes and Genomic Instability

HPV-immortalized keratinocytes form tumors in animals only in combination with the expression of additional oncogenes (Durst et al. 1989; Pei et al. 1998) or after extended culture (Pecoraro et al. 1991; Pirisi et al. 1988), suggesting that

the accumulation of cellular mutations is required for tumorigenic progression. The E6 and E7 proteins independently promote genomic instability by inactivating normal cell cycle checkpoints and allowing the replication of cells with damaged DNA and the accumulation of chromosomal abnormalities (Demers et al. 1994; Hickman et al. 1994; Kessis et al. 1993; Reznikoff et al. 1994; Slebos et al. 1994; Song et al. 1998; White et al. 1994). Transgenic mice expressing either HPV16 E6 or E7 in the epidermis display a loss of DNA damage-induced cell cycle arrest following treatment with ionizing radiation (Song et al. 1998). In addition, expression of HPV16 E6 and E7 independently triggers mitotic abnormalities, including abnormal chromosome numbers and anaphase bridge formation (Duensing and Munger 2002), and E6 and E7 can abrogate the mitotic checkpoint (Thomas and Laimins 1998; Thompson and Belinsky 1997) to induce numerical and structural chromosomal abnormalities (Duensing and Munger 2002).

Inactivation of Rb and p53 function undoubtedly contributes to these effects. In addition, high-risk E7 proteins induce abnormal centrosome synthesis leading to multipolar mitoses and aneuploidy (Duensing et al. 2001a; Duensing et al. 2001b; Duensing et al. 2000). This HPV16 E7 activity is independent of its ability to inactivate the Rb family (Duensing and Munger 2003; Southern et al. 2004). Finally, high-risk HPV has been shown to induce genetic instability by impairing DNA repair pathways (Rey et al. 1999). Thus, expression of the viral oncogenes promotes the accumulation of mutations that advance carcinogenic progression.

5.6 Summary and Conclusions

The tumorigenic potential of papillomaviruses in animals and humans is reflected in the ability of these viruses and their genes to induce transformation of cells growing in culture. The papillomaviruses encode multiple oncogenes. The nuclear E6 and E7 proteins of the high-risk HPV types have received the most attention because they can immortalize cervical keratinocytes and their continued expression is required to maintain the proliferative state of cervical cancer cells. Like the major oncogene products of other small DNA tumor viruses, these viral proteins inactivate cellular tumor suppressor pathways and cause genomic instability. The small E5 proteins can also transform cells, but they utilize unusual biochemical mechanisms that involve transmembrane growth factor receptors and possibly the vacuolar ATPase. The ability of papillomaviruses to transform cells and ultimately cause cancer reflects the biochemical roles of these proteins during normal virus replication. Further studies of papillomavirus transformation and the viral oncogene products required for this process will provide new insights into cell growth control, determinants of genome stability, signal transduction, virus replication, and human carcinogenesis and may provide new strategies to prevent and treat cancer.

Acknowledgments Work carried out in the authors' laboratory is supported by grants from the NIH (CA16038 and CA37157). K.J. was supported by the MSTP training grant to Yale University. We thank J. Zulkeski for assistance in preparing this manuscript.

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Chapter 6
Papillomaviruses: Biology, Diversity, and Pathogenesis

Hans-Ulrich Bernard

6.1 Introduction

The term “papilloma” refers to a wart, i.e., a benign epithelial tumor. The papillomaviruses received their name since the first isolates were detected in wart-like lesions of the skin of rabbits (cottontail rabbit papillomavirus, CRPV) and cattle (bovine papillomavirus type 1, BPV-1) and in flat warts of the foot soles (human papillomavirus type 1, HPV-1) and common warts of the hands and face of humans (HPV-2). The lesions that are caused by these four specific viruses are often large in size and a rich source of viral particles. Therefore, it was possible to readily isolate the first papillomavirus genomes, which subsequently could be used to search with DNA hybridization protocols for related viruses in various types of neoplastic and normal, cutaneous, and mucosal epithelia of humans and other mammals. Once data emerged that certain gene sequences of different papillomavirus genomes were highly conserved, strategies for the search of new papillomavirus genomes expanded to polymerase chain reaction (PCR) protocols, with primers mimicking homologous segments of remotely related viruses. As a consequence of the combined efforts of numerous laboratories, papillomaviruses have been found in all carefully studied mammals and occasionally in birds, in benign and malignant lesions of cutaneous and mucosal epithelia.

Humans have been particularly well studied subsequent to the discovery of specific papillomaviruses in anogenital carcinomas by Harald zur Hausen and his colleagues nearly 30 years ago (Gissmann et al., 1982), which led to the discovery of HPV-6, 11, 16, and 18. Today, the genomes of more than 100 human papillomavirus types (HPVs) have been isolated and formally described (de Villiers et al., 2004; Chen et al., 2006), and PCR-based data point to the existence of at least another 100 types (Antonsson et al., 2000; 2003). About 18 of these HPV types are referred to as “high-risk” HPVs as they are very

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_6, © Springer Science+Business Media, LLC 2009

frequently associated with malignant lesions, most often of the cervix and less frequently of other anogenital sites (Munoz et al., 2003). HPV-16 and HPV-18 are the most common and best studied of these high-risk types. Two "low-risk" HPV types, HPV-6 and HPV-11, while only rarely associated with carcinomas, are nevertheless of great medical importance as the cause of genital and laryngeal warts. About 70% of all described HPV types are associated with various skin lesions. All HPV types, including those associated with medically important benign and malignant lesions, exist frequently in perfectly normal (i.e., not neoplastic) mucosal and cutaneous human epithelia. This fact points to a yet poorly understood dual nature of these viruses, namely the fact that papillomaviruses typically exist as commensalic episomes and only a subset of all infections has a propensity to progress to benign or malignant tumors. The very terms "latency" or "asymptomatic infection", though frequently used, remain molecularly only poorly defined in the pathogenesis associated with this virus group (see for example Boxman et al., 1999; Castle et al., 2006).

6.2 Fundamentals of Papillomavirus Biology and Pathogenesis

Cellular divisions of all epithelia are restricted to stem cells in the basal layer, and while suprabasal cells continue to differentiate, they do not divide any more and are eventually shed from the epithelial surface. It is generally believed that in order to establish a stable infection, papillomavirus genomes have to infect mitotic cells of the basal layer, where they are stably maintained. In suprabasal epithelial cells, which are normally mitotically inactive, papillomavirus gene products trigger a reentry into the cell cycle in order to create an environment favorable for their own replication, and as a by-product of this mechanism the infected cell population expands, resulting in neoplasia.

The papillomavirus particles have a structure similar to that of polyomaviruses, and both virus groups share the property of having supercoiled circular double-stranded DNA genomes, a unique property among all human pathogenic viruses. However, since polyomaviruses and papillomaviruses do not have any significant amount of genomic similarity (for a minor exception, see Clertant and Seif, 1984; Rebrikov et al., 2002), the two groups of viruses form separate taxonomic families (de Villiers et al., 2004). Papillomavirus particles consist of non-enveloped capsids, built from 72 capsomers, each of which is composed of 5 subunits of the L1 protein. The particles can infect all types of cells indiscriminately by binding to a variety of glycoproteins and integrins, followed by endocytosis and transportation of the viral genome to the cellular nucleus (Selinka et al., 2002). Transcription and replication of the virus occur in the nucleus, and dependence on the unique transcription factor composition of epithelial cells determines the epithelial tropism, subsequent to the promiscuous infection process (Gloss et al., 1987). Papillomavirus transcription and replication activity is low in basal epithelial layers, but increases suprabasally, leading

eventually to a massive expression of late proteins, formation of particles with encapsidated viral genomes, and release of the mature particles as part of the normal desquamation of dead cells at the epithelial surface (Stoler et al., 1992).

6.3 Genome Organization and Gene Functions

All papillomaviruses have circular DNA genomes, with sizes ranging normally from 7.5 to 8 kb. Most papillomaviruses have the typical gene organization shown in Fig. 6.1. A long control region contains most *cis*-responsive elements for transcription and replication, and the principal early promoter (or E6 promoter) is followed in counterclockwise direction (as shown in Fig. 6.1) by the genes E6, E7, E1, E2, E5, L2, and L1. An open reading frame E4 is embedded within E2 and translated in a reading frame different from E2. There is no E3 gene due to some misnomer during an early sequencing effort. This sequence organization is conserved in more than 90% of all papillomaviruses, but some aberrant papillomavirus types can miss the E6, E7, and/or E5 genes or have them replaced by unrelated genes (Chen et al., 2006; Terai et al., 2002).

The products of the E6 and E7 genes have pleiotropic functions that affect the cell cycle and initiate cellular transformation and carcinogenic processes. This extensive and well-studied field of research is discussed in another chapter of this book.

The E1 protein, an ATP-dependent DNA helicase, binds to and thereby functionally defines the viral replication origin, an A + T-rich segment that spans the nucleotide which by definition has the genome position 1, about 100 bp upstream of the E6 promoter. E1 has poor sequence recognition capabilities (Chen and Stenlund, 2001), and its specificity is enhanced by formation of a complex with the E2 protein, which recognizes a precise site, ACCGNNNNGCCT, close to the

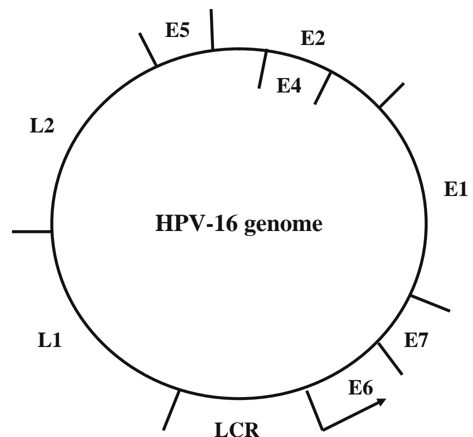

Fig. 6.1 The genomic organization of human papillomaviruses, exemplified at the type HPV-16

E1 target (Chiang et al., 1992). Bound to the replication origin, E1 recruits cellular replication proteins to the papillomavirus origin of replication, including DNA polymerase alpha and topoisomerase I, and thereby initiates the bidirectional mechanism of replication via a theta structure.

The E2 protein, with a size of about 370 amino acids, folds into 3 domains, an N-terminal transcription activation domain, a central hinge, and a C-terminal DNA-binding domain. Aside from the interaction with E1, E2 protein modulates transcription and attachment of papillomavirus genomes to cellular chromosomes during mitoses (McPhillips et al., 2005; Parish et al., 2006).

In the 1980s, the E2 protein was studied in the bovine papillomavirus and was found to be an activator of transcription. Two proximal E2-binding sites occupied by this activator function as an enhancer and activate transcription even of remote promoters (McBride et al., 1991). While the E2 protein of human papillomaviruses can also function as an activator when targeted at E2-dependent enhancers, it rather represses HPV transcription in the natural context, where two E2-binding sites overlap with essential promoter elements (see below).

The E2 coding region contains an E4 gene that is transcribed in the form of a spliced mRNA including some few amino acids from the N-terminus of E1 and which is translated in a different reading frame from E2. Although the E4 gene appears to have an indispensable function, as it is conserved among remotely related papillomaviruses, its functions remain somewhat enigmatic. Functional analyses of the E4 protein reported involvement in viral DNA replication in the productive viral life cycle, capsid protein expression, virion assembly and release, reorganizations of the cytokeratin network and of nuclear ND10 domains, and effects on cell cycle regulators. E4 is considered a late protein due to the co-expression with L2 and L1 and its high expression in differentiating cells (Doorbar et al., 1991; Doorbar, 2005).

The E5 gene is encoded on the 3′ side of the E2 gene, and its absence of the genomes of some papillomavirus types suggests that it does not encode a protein that is important for functional idiosyncrasies shared by all papillomaviruses. This is further supported by the divergent size of E5 proteins in different papillomaviruses (for example, 83 amino acids in HPV-16 and 44 amino acids in BPV-1) and the lack of sequence homology among such proteins (Suprynowicz et al., 2005). However, all E5 proteins are very hydrophobic, which seems to be a prerequisite for localization to the cell membrane. E5 proteins are pleiotropic, and data support several functions, including (i) association with the 16 k subunit of the vacuolar H^+-ATPase, which has an impact on tyrosine kinase receptors stability, (ii) physical interaction with tyrosine kinase receptors such as the epidermal growth factor and platelet-derived growth factor receptors and downregulation of major histocompatibility complex class I proteins (Freeman-Cook and DiMaio, 2005).

The L2 protein is considered a “minor” capsid protein as it is part of the viral particle in a substoichiometric relationship to L1. Most of the L2 is not exposed at the capsid surface, but this protein rather forms a capsid internal complex

with the viral chromatin. Many important functions of L2 can be observed subsequent to the endocytotic uptake of the papillomavirus particle, when L2 seems to "guide" the viral DNA to the nucleus and to specific nuclear compartments (Florin et al., 2006).

L1 protein is sufficient to form pentameric capsomers, and 72 of these capsomers comprise the viral particle. A particle composed only of L1 protein does not differ electronmicroscopically from a natural particle with L2 protein and viral chromatin. Particles that consist of nothing but L1 are the foundation of the commercial prophylactic vaccines introduced by Merck Inc. in 2006 and GlaxoSmithKline in 2007 (Lowy and Schiller, 2006).

6.4 Gene Expression of Papillomaviruses

The transcriptional strategies of remotely related papillomaviruses such as BPV-1 and HPV-16 differ significantly. Only the latter one will be briefly introduced here, although data have pointed to many similarities between the HPV-16 transcription biology and that of the other HPV types involved in anogenital carcinogenesis (for a review, see Bernard, 2002).

Most elements responsible for the initiation of transcription are located in the long control region (LCR), an 850 bp DNA segment downstream of L1 and upstream of E6. Most transcripts that initiate in the LCR are derived from the E6 promoter, the transcription start located only 6 bp 5′ of the E6 ATG. This promoter has a classical structure with a TATA box and a single element bound by a promoter factor, Sp1 (Apt et al., 1996). TATA box and Sp1 site overlap with two binding sites for the viral factor E2 and are repressed by displacement of TFIID and Sp1 by increased E2 concentrations (Tan et al., 1994; Demeret et al., 1997). The formation of a complex between E2 and the cellular factor Brd4 appears to support the repressor function (Wu et al., 2006). E2 is expressed from the E6 promoter via polycistronic mRNAs encoding both E6 and E2 (Baker and Calef, 1995). An increased transcription of these mRNAs results in increased concentrations of E2, and its increased binding to the promoter sequences reduces promoter activity by interfering with Sp1 and TFIID function. Consequently, this arrangement of promoter elements results in a perfect negative feedback loop as it stabilizes low HPV-16 transcription and is one of several examples that portray papillomaviruses as being "streamlined" for a latent or subclinical rather than fulminant viral infection or for aggressive carcinogenicity. The interruption of this negative feedback and the concomitant increased oncogene expression is one of three consequences of the recombination of papillomavirus genomes with chromosomal DNA, a frequent if not even necessary tumor progression event (Daniel et al., 1995; Arias-Pulido et al., 2006). The impact of the interruption of the negative feedback is not fully understood as the recombined HPV genomes are also transcriptionally

activated by the nuclear matrix (Stünkel et al., 2000) and frequently generate mRNAs with increased stability (Jeon and Lambert, 1995).

The activity of the HPV-16 promoter is modulated by two additional elements, a silencer and an enhancer, centered about 100 and 300 bp, respectively, upstream of the promoter elements (Bernard, 2002).

The enhancer contains about 20 *cis*-responsive elements that bind about half a dozen different transcriptional activators. These transcription factors raise the basal activity of the E6 promoter in an epithelial-specific manner due to the cell type-specific composition of these factors (e.g., AP-1, NFI) (Thierry et al., 1992; Apt et al., 1993). Other transcription factors appear to couple HPV transcription with the host cell physiology (glucocorticoid and progesterone receptor) and signaling downstream of physiological signals (AP-1) (Chan et al., 1989 and 1990). The silencer contains multiple binding sites for the two factors CDP and YY1, which couple HPV transcription and epithelial differentiation through availability of high levels of these repressors in undifferentiated and low levels in differentiated epithelial layers (Parker et al., 1997; Pattison et al., 1997; Ai et al., 1999 and 2000; O'Connor et al., 1996 and 2000). CDP and YY1 may also be involved in many epigenetic regulatory mechanisms (such as histone modifications and DNA methylation), which appear to be super-regulators of the transcription biology of papillomaviruses with paramount importance for the productive life cycle, latency, and cancer progression (Stünkel and Bernard, 1999; Zhao et al., 1999; Stünkel et al., 2000; DelMar-Pena and Laimins, 2001; Kim et al., 2003; van Tine et al., 2001; Kalantari et al., 2004).

The E6 promoter generates a large variety of mRNAs that can encode all genes, including L2 and L1, through differential splicing. Efficient expression of these late genes correlates, however, with the activity of another "late" promoter, located 3′ from the LCR inside the E7 gene (Ozbun and Meyers, 1997, 1998), whose regulation is still poorly understood. It is also clear that differential splicing, transcription termination sites, and transcript stability (Stacey et al., 2000; Collier et al., 2002; Oberg et al., 2003; Tang et al., 2006) make major contributions to the differential expression of HPV-16 genes in different biological contexts (mucosal vs. cutaneous epithelia, latent vs. neoplastic infection, basal layer specific and suprabasal layer transcription, etc.), although an understanding of the precise orchestration of such multivariate systems remains still somewhat elusive.

6.5 Genomic Diversity of Papillomaviruses and Taxonomy

Papillomaviruses are traditionally described as "types", and a new papillomavirus type was historically identified as an 8 kb (and therefore presumably full-length) DNA clone that does not cross-hybridize under defined conditions with any of the previously known types. More recently, papillomavirus taxonomy

was based on nucleotide sequence comparisons (Bernard et al., 1994; Chan et al., 1995). Aside from large databases such as GenBank, the National Institutes of Health had sponsored for the establishment and availability of a database that contains not only the genomic and protein sequences of all known papillomaviruses but also a large body of sequence alignments, interpretations, and reviews (discussed in Farmer et al., 1995). Unfortunately, at the time of this writing, this Web site was not functioning due to funding problems, but ongoing efforts will hopefully reinstate this valuable information source.

Founded on these comprehensive sequence databases a papillomavirus type became redefined as a full-length DNA clone whose L1 gene has less than 10% nucleotide sequence identity to that of any other papillomavirus types (de Villiers et al., 2004). Complete genome clones are required for formal description, although partial sequences based on PCR amplification can be proposed as "presumed novel types", but they would not qualify to be named and numbered. The term "type" explicitly refers to genotypes, not to serotypes, as elsewhere in virology, because there is no well-developed research on papillomavirus serology (due to their inconsistent immunogenicity). There is little doubt, however, that most papillomavirus types would also be serotypes, if the serological properties of their L1 capsids would be investigated in animal systems or as part of vaccination studies.

The nomenclature of papillomaviruses follows a system where the abbreviation "PV" follows one or two letters which identify the English or the Latin name of the host species, for example, HPV-16 for human papillomavirus type 16 or MnPV for Mastomys natalensis papillomavirus (isolated from an African rodent). If more than one papillomavirus type is known from any particular host, it is numbered following the historic sequence of isolation.

The limit of 10% sequence identity as a criterion to establish papillomavirus types had been proposed arbitrarily, but it was clearly a fortunate choice as the vast majority of all papillomavirus isolates show either a much greater or a much smaller divergence. Even relatively closely related HPV types, for example, the carcinogenic types HPV-16, 31, 33, and 35, most often differ by 20–30% of their L1 sequence. In contrast, most independent isolates of the same HPV type differ by only 1–2% and have been called variants (Deau et al., 1993; Ho et al., 1993; Ong et al., 1993). Papillomavirus genomes that show sequence dissimilarity in between these two extremes have been termed "subtypes" and are relatively rare (for discussions, see de Villiers et al., 2004; Calleja-Macias et al., 2005a).

Papillomavirus types are natural taxa, and there are no extant genomes that link these types. In analogy with biological taxa elsewhere, it would therefore be appropriate to consider a papillomavirus type a papillomavirus species. Unfortunately, this reasoning is not acceptable to the International Council on Taxonomy of Viruses as the rules of this institution require the identification of biological differences in order to recognize species. Many papillomavirus types are so similar to one another that no biological or pathological differences are known (for example, HPV-16 vs. 31 and HPV-6 vs. 11). In order to resolve

this conflict, it was agreed that the species concept is applied to groups of relatively closely related papillomaviruses. For example, HPV-16 and HPV-31 and five additional related HPV types form one such species (for a complete listing of all papillomavirus types and species, see de Villiers et al., 2004). A recently published phylogenetic tree, which formed the basis for the official taxonomy, is reproduced as Fig. 6.2. While papillomavirus taxonomy is now in harmony with the taxonomy of all other viruses, it is likely that the molecular and medical literature will continue to concentrate on the properties of individual types.

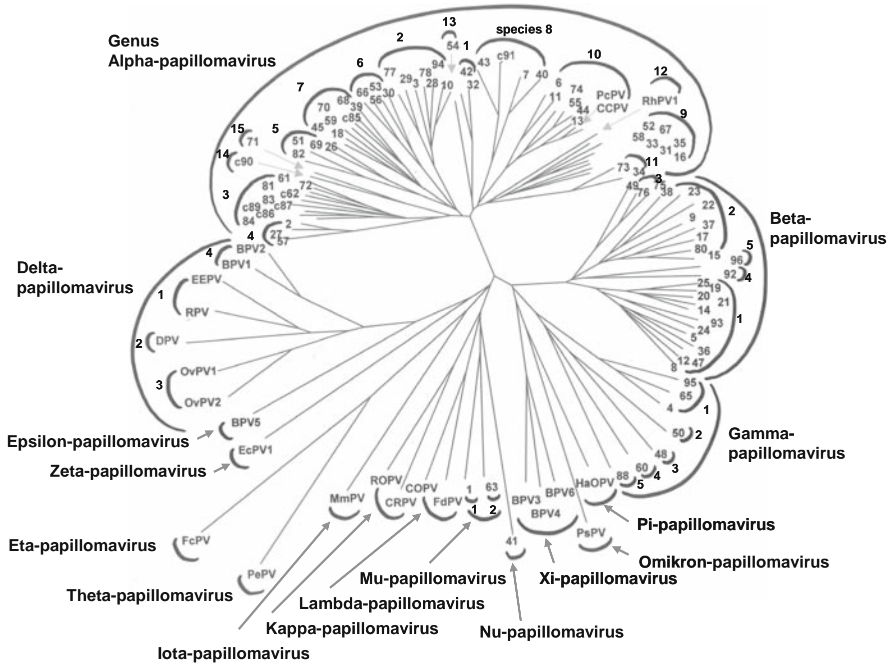

Fig. 6.2 Phylogenetic tree of 118 papillomavirus types. All numbers refer to HPV types and c-numbers to candidate types, i.e., HPV genomes isolated as PCR amplicons. All abbreviations including letters refer to animal papillomaviruses. BPV, bovine PV; COPV, canine oral PV; CRPV, cottontail rabbit PV; DPV, deer PV; EcPV, Equus caballus or horse PV; EEPV, European elk PV; FcPV, Fringilla coelebs or chaffinch PV; FdPV, Felis domesticus or cat PV; HaOPV, hamster oral PV; MnPV, Mastomys natalensis or African rat PV; OvPV, ovine or sheep PV; PePV, Psittacus erythacus or African Grey Parrot PV; PsPV, Phocoena spinipinnis or porpoise PV; RPV, reindeer PV; ROPV, rabbit oral PV. The outermost semicircular symbols identify papillomavirus genera, for example, the genus alpha-papillomavirus. The inner semicircular symbols identify papillomavirus species. For example, the HPV species 8 within the genus alpha-papillomaviruses lumps the HPV types 7, 40, 43, and cand91. Reprinted from "Classification of papillomaviruses" by de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., and zur Hausen, H. Virology, 324, 17–27 (2004), with permission from Elsevier

On a higher taxonomic level, remotely related clusters of papillomavirus types (i.e., species) have been lumped into genera, which are named by Greek letters. Most medically important HPV types (including HPV-6, 11, 16, 18, and 31) fall into the genus alpha-papillomaviruses. In addition, many (but not all) HPV types that infect the skin form the genera beta-, gamma-, mu- and nu-papillomaviruses. There are nearly 20 additional other genera containing papillomaviruses isolated from mammals and birds (de Villiers et al., 2004; Rector et al., 2004; Rehtanz et al., 2006). On the highest taxonomic level, papillomaviruses from the family Papovaviridae separate from the polyomaviruses, and the unifying taxon “Papovaviridae” has become abandoned.

6.6 Genomic Diversity and Evolution of Papillomaviruses

Quantitative comparisons of homologous nucleotide sequences of genes and viruses are visualized by phylogenetic trees. The topology of such trees not only serves as foundation of a taxonomy but can also be interpreted as a reflection of evolutionary pathways, i.e., the split of extinct taxa into extant taxa. The phylogenetic study of papillomaviruses (van Ranst et al., 1992; Chan et al., 1995; de Villiers et al., 2004; Rector et al., 2004; Rehtanz et al., 2006) has come a long way to go beyond topology and taxonomy and extract interpretations of the phylogenetic trees of papillomaviruses.

Papillomaviruses are strictly host species specific (with the exception of certain domestic hoofed animals) probably due to the perfect co-evolution between the molecular properties of papillomavirus proteins and those of their host species. Most notably, no human papillomavirus has ever been observed in any mammal species, and humans clearly never acquired the virus by interspecies transfer as so many other viruses. The relative position of nodes in the phylogenetic tree is often reminiscent of the phylogeny of the host, for instance, papillomavirus types extracted from the lesions of remotely related hoofed animals are members of the same phylogenetic branches, and even the only two known bird papillomaviruses (from an African Grey Parrot and a European Chaffinch) are closely related to one another than either of them is to a mammal papillomavirus (Terai et al., 2002). The tree therefore likely reflects linked evolution between host and virus since the evolutionary divergence between mammals and birds more than 140 million years ago, and the principal organization of papillomavirus genomes has not changed during that time. Even individual branches are quite old, as suggested by the fact that monkey and ape papillomaviruses are embedded among human viruses in the genus alpha-papillomavirus (Chan et al., 1997; de Villiers et al., 2004). This tells us on the one side that this papillomavirus genus originated several ten million years ago (before the split between different primate groups), but also that the five genera containing human papillomaviruses split long before the origin of humans. On a more recent scale, the minor genomic diversity between the

variants of individual HPV types (1–2%) appears to be at least as old as the human species, i.e., several hundred thousand years (Ho et al., 1993; Ong et al., 1993; Stewart et al., 1996; Yamada et al., 1997; Calleja-Macias et al., 2005b). Since that time, all HPV types have always been with humans, as the phylogenetic pattern of their evolution has similarities to the evolution of humans and their spread around the world (Bernard, 1994).

6.7 Genomic Diversity, Gene Functions, and Pathogenesis

Sequence comparisons of all papillomaviruses support that the E1, E2, L1, and L2 proteins, and, where present, also E6 and E7, are homologous, as indicated by a high fraction of conserved amino acid sequences. As any sufficiently informative alignment of these genes can be used to establish the relationship between the viruses, papillomaviruses appear to have diversified into genomically new but biologically similar taxa, i.e., a new clade, once a virus had evolved into a new niche. Another conclusion of the similar topologies of phylogenetic trees of papillomaviruses is that the viral genomes must have evolved as contiguous genomes and normally did not recombine. While the present taxonomy was based on sequence comparison of the L1 genes, minor discrepancies of "official" L1 trees to trees based on E6, E7, E1, or E2 have been noted. They have been variably interpreted either as a reflection of different evolutionary pressure on different genes or as a record of ancient and very rare recombination events (van Ranst et al., 1992; Narechania et al., 2005; Varsani et al., 2006).

Most medically important papillomaviruses are united in the genus alpha-papillomaviruses. The 18 HPV types associated with anogenital cancer are often called "high-risk" HPV types (Munoz et al., 2003), but form not a single but three phylogenetic clusters (species) within this genus based on L1 sequences. It is not known whether these viruses evolved toward pathogenic similarity by convergence of the early genes of these three separate branches (which were, of course, originally of monophyletic origin within the alpha-papillomaviruses) or whether the closer relationship of these HPV types based on the sequences of E6, E7, E1, or E2 (van Ranst et al., 1992; Narechania et al., 2005) reflect a monophyletic origin and differential diversification of L1. Among the high-risk papillomaviruses, different types have a differing propensity to induce progression to cancer (Munoz et al., 2003). The same applies even to variants within specific high-risk types (Xi et al., 1997; Villa et al., 2000; Da Costa et al., 2002).

Outside the groups of the 18 intensely researched high-risk HPV types, alpha-papillomaviruses evolved several additional and quite divergent types of pathogenicity. HPV-6 and 11 and several related types are the most common papillomaviruses in genital and laryngeal warts and must have developed the propensity to infect and pathogenically alter a variety of mucosal as well as cutaneous epithelia, extending the preference of the high-risk types for

pathogenic activity in mucosal epithelia. Yet other alpha-papillomaviruses are specialized to target only cutaneous epithelia (skin), notably the group (species) formed by HPV-2, 27, and 57, the three principal viruses found in common warts of hands and the face.

While types like HPV-6 and 11 viruses are obviously widespread and constitute a significant inconvenience, they are conceived as a minor public health problem and have therefore been addressed by much few molecular studies than the high-risk types. In fact, HPV-2, 27, and 57 and other HPV types that cause common warts are practically completely unstudied. Strangely, these non-carcinogenic viruses are often included in molecular experiments merely as negative controls. For instance, during the analysis of transforming functions it was noted that the E6 and E7 proteins of low-risk types do not target p 53 and RB proteins in the same way as observed for the homologous proteins of high-risk types (Munger et al., 1989; Scheffner et al., 1990). This is somewhat puzzling as the proteins of low-risk types must have substantial molecular effects on the infected cell, since the resulting neoplasia often exceeds in mass the barely detectable precursor lesions of anogenital malignancies. E6 and E7 are the proteins with the least similarity between different types (often less than 20% of the amino acid sequence) and, although clearly homologous, may have developed completely different or only partially overlapping spectra of molecular functions (for a recent comparison of HPV-16 and BPV-1 E7 proteins, see DeMasi et al., 2005).

6.8 Diagnosis, Treatment, and Prophylaxis

Papillomavirus infections differ from those of most other viruses by never becoming systemic and rather retain the form of a local and small epithelial neoplasia. It is obvious that skin or genital warts can be diagnosed by mere inspection, which can be complemented by detection of papillomavirus genomes in DNA preparations by hybridization or amplification. Research in the 1980s and 1990s confirmed that the traditional Papanicolaou (Pap) test, the diagnostic test of choice to detect precursors of cervical carcinomas since the 1950s, has been an HPV detection diagnosis, although this connection was not known for several decades. The Pap test attempts microscopically to detect in exfoliated cervical cells amorphous and intensely stained nuclei (now known as the result of aneuploidies due to E6 and E7 oncoprotein functions) and a light perinuclear halo (in hindsight, interpreted as a build-up of E4 protein). The Pap test has been of tremendous medical importance and resulted in a major reduction of the load of cervical cancer in all nations with satisfactory practice of gynecological check-ups (basically in all developed countries), while a lack of screens in most developing countries retained the role of cervical cancer as the leading cause of cancer-related death in women. When Pap tests identify dysplastic cells, suspected lesions are further examined by colposcopic

inspection. This procedure permits the sampling of biopsies for histological confirmation. Margin-free removal of visually apparent lesions is considered a treatment of choice of precancerous lesions.

These diagnostic and therapeutic treatments are not based on any information about the viral etiology of the disease process. More recently, diagnosis has become increasingly complemented by DNA tests of the same exfoliated cells that are targeted by the Pap test. The objective of these tests is to detect and type HPV infections. Repeated detection of specific high-risk HPV types in women older than 30 years attempts to identify chronically infected patients that are at risk to develop high-grade neoplasia.

Unfortunately, molecular biology has not yet led to rational design of drugs or therapeutic vaccination against HPV infections. Further, the ubiquitous spread of many high-risk HPV types and their high infectivity does not encourage to assume that traditional means of prophylaxis against sexually transmitted diseases are very useful. However, the expression of L1 protein in yeast and insect cells allows the mass production of non-infectious HPV particles, which have been developed by the companies Merck and GlaxoSmithKline into commercially available vaccines. Ongoing clinical studies of these vaccines established them as powerful prophylaxis against HPV infection. The presently available vaccines protect most of all against infection with HPV-16 and HPV-18, and development of vaccines against a broad spectrum of HPV types is underway. The worldwide application of such products to virgin girls and boys has the potential to eventually eliminate HPV-dependent carcinogenesis. However, past experience with the difficulties to distribute expensive vaccines worldwide (e.g., against hepatitis B), and the fact that all humans that presently are or have ever been sexually active are potential HPV carriers, places this goal many decades into the future.

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Chapter 7
Adenovirus Transformation

Patrick Hearing

7.1 Introduction

Adenoviruses (Ad) were first isolated by Rowe and colleagues in 1953 following the culture of primary cells from human adenoids (Rowe et al. 1953). Virus spread within these cultures was evident by a characteristic cytopathic effect of rounded and clumped cells detaching from the plate. The following year, Hilleman and Werner observed a similar type of cytopathic effect in cultured cells exposed to respiratory secretions from Army recruit with acute respiratory disease (Hilleman and Werner 1954). Common Ad infections are associated with pharyngitis, conjunctivitis and gastroenteritis. These infections are usually resolved quickly, resulting in lifelong immunity to the virus. Acute respiratory disease is a more severe pneumonia-like infection most often found in the military which results in significant morbidity. The adenovirus family is large and contains members that infect a wide range of animals, including monkeys, livestock, mice, birds, and humans. All these viruses consist of a naked icosahedral protein shell (70–100 nm in diameter) that encapsidates a linear, double-stranded DNA molecule.

A major interest in studying Ad followed the discovery that Ad serotype 12 (Ad12) caused malignant tumors in infected newborn hamsters (Trentin et al. 1962). This seminal finding by Trentin and colleagues coincided with the discoveries that SV40 and polyomavirus also induce tumor formation in animals in vivo and transformation in cultured cells in vitro and led to establishment of the field of DNA tumor viruses. Fortunately, to date, there has been no significant evidence to implicate Ad in the development of human cancer. The early work in the study of Ad oncogenesis in vivo and Ad transformation in vitro led to the conclusion that only members of Ad subgroup A (e.g., Ad12) caused tumors in animals, whereas Ad in subgroup A and other subgroups (e.g., subgroup C, Ad2 and Ad5) were able to transform rodent cells in culture with

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_7, © Springer Science+Business Media, LLC 2009

comparable efficiencies. More recent work has established that this reflects the specific experimental conditions used in the analyses and that other Ad (e.g., Ad9, subgroup D) are oncogenic in animal models.

The focus of early work in the field of Ad oncogenesis was the identification of viral proteins, mRNAs, and DNA sequences that are found in Ad-transformed cells and Ad-induced tumors (reviewed in Endter and Dobner 2004). The salient conclusions from these analyses include the observations that (1) Ad DNA sequences from the genomic left-end 3,000–5,000 bp are both necessary and sufficient to induce Ad transformation in vitro; (2) the same viral DNA sequences are found in Ad-induced tumors in vivo; (3) viral DNA sequences are integrated into host chromosomal DNA in a relatively non-specific manner; and (4) specific viral mRNAs and proteins from Ad early regions 1A (E1A) and 1B (E1B) are the major viral oncogenic determinants. More recent studies have shown the involvement of viral proteins encoded by early region 4 (E4) in Ad9-induced tumors in vivo and in enhancing Ad5 transformation in vitro. This review will focus on the roles of proteins encoded by E1A, E1B, and E4 in Ad transformation in vitro and oncogenesis in vivo, as well as distinctions between different Ad and their ability to cause cancer in animal models in vivo. Many excellent reviews on Ad transformation and oncogenesis have been published in the past, and the reader is directed to the following specific articles to supplement the material presented in this chapter (Berk 2005; Frisch and Mymryk 2002; Tauber and Dobner 2001; Turnell and Mymryk 2006; White 2006).

7.2 Early Region 1A (E1A)

Adenovirus encodes over 20 individual early gene products. The early genes are expressed in a temporal and coordinated manner. The first early region expressed after Ad infection is the immediate early transcription unit E1A since it requires only cellular proteins for its expression. The E1A gene products in turn activate transcription from the other early promoter regions. The E1A gene is comprised of two exons, and several E1A polypeptides are produced following alternative splicing of a primary RNA transcript (Fig. 7.1). The most abundant of the E1A proteins early after infection are referred to as the E1A 243 amino acid (243 aa) and 289 amino acid (289 aa) gene products (also referred to as the E1A 12S and 13S proteins, respectively, based on the size of the mRNA that encode each protein). The E1A 243 aa and 289 aa proteins act as major regulators of early viral transcription as well as important modulators of host cell gene expression and proliferation (reviewed in Berk 2005). The E1A 243 aa and 289 aa proteins share two conserved regions within the 5′ exon referred to as CR1 and CR2 as well as another conserved region (CR4) at the C-terminus (Fig. 7.1). The two proteins differ only in a 46-residue internal exon segment present in the 289 aa protein (Fig. 7.1). This region, referred to a conserved region 3 (CR3), is important for the transcriptional transactivation

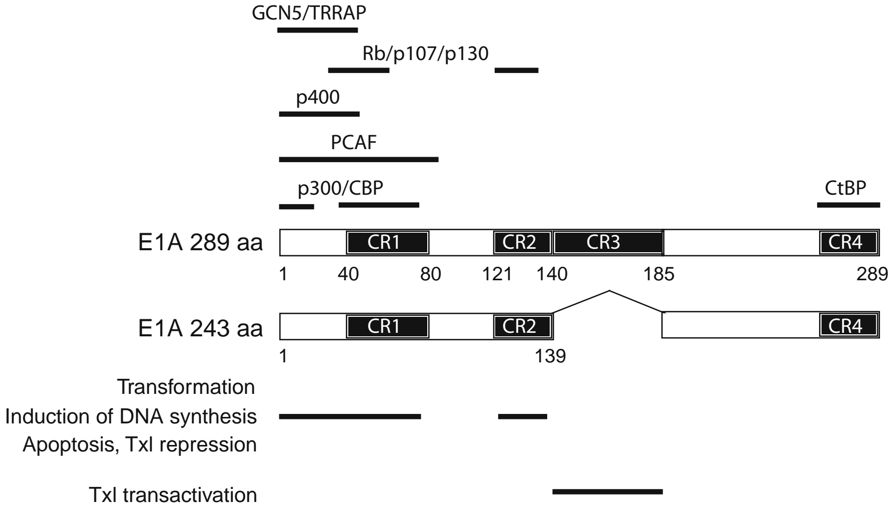

Fig. 7.1 Schematic diagram of the E1A proteins. The coding sequences of the 243 aa and 289 aa E1A proteins are shown with conserved regions depicted (CR1, CR2, CR3 and CR4). Binding sites for cellular proteins are indicated by bars along with E1A functional activities including transformation, induction of DNA synthesis, apoptosis, transcriptional (Txl) repression, and transcriptional activation

properties of the E1A 289 aa protein (reviewed in Berk 2005). Both proteins are localized to the nucleus due to a carboxy-terminal nuclear localization sequence (NLS).

The E1A gene products exert their effects by interactions with numerous cellular proteins, many of which are involved in transcriptional regulation (Fig. 7.1). The E1A products interact with a number of important cellular proteins including (1) the retinoblastoma tumor suppressor, pRb, and related family members p107 and p130 via CR1 and CR2; (2) transcriptional coactivators p300, CBP, PCAF, GCN5, TRRAP, and p400 amino terminal sequences and CR1; (3) a number of transcription factors such as TATA-binding protein (TBP), members of the ATF family (e.g., ATF-2, Sp1, and c-Jun), and the RNA polymerase II mediator complex via CR3; and (4) the transcriptional repressor CtBP via CR4. The regulation of these cellular proteins with respect to the transforming properties of E1A will be discussed below. The expression of E1A alone is sufficient to induce immortalization of primary rodent cells. E1A fully transforms such cells in conjunction with other oncogenes such as the Ad E1B proteins or activated Ras (Ruley 1983). The expression of E1A also is sufficient to induce S phase progression in quiescent cells. It appears as though the E1A to induce S phase is directly responsible for its ability to cause transformation (Frisch and Mymryk 2002).

E1A activation of gene expression via the E2F family of transcription factors has been well characterized. The E2F family of proteins was initially discovered

through studies of Ad E2 promoter regulation (Kovesdi et al. 1986). E2F transcription factors play a major role in the expression of cellular genes important for the regulation of cell cycle progression (reviewed in Dimova and Dyson 2005). E2Fs both positively and negatively regulate gene expression. E2Fs exist in the cell as heterodimers containing one of seven identified E2F proteins with one of two DP molecules. In general, E2F-1, -2, and -3 activate gene expression, while E2F-4, -5, and -6 repress gene expression. E2F-7 may function as dominant-negative effectors and, thus, may regulate both activating and repressing E2F complexes. Activating E2Fs recruit histone acetyl transferases (HATs) and other transcriptional activators to promoter regions (Lee et al. 1998; Trouche et al. 1996). In contrast, repressing E2Fs recruit histone deacetylases (HDACs) and other transcriptional repressors to promoter regions (Macaluso et al. 2006). These latter complexes are formed via the interaction of repressing E2Fs with members of the retinoblastoma gene family (pRb, p107, and p130). Specific members of the Rb family bind to different E2F complexes, determined by the member of the E2F present. E2F binding to Rb members involves the large binding pocket domain of Rb family members. Rb family binding to E2Fs is controlled through phosphorylation by cyclin-dependent kinases (cdks) (Dimova and Dyson 2005). The hyperphosphorylation of Rb family proteins by Cdks in G1 phase of the cell cycle results in dissociation of Rb from the E2F complexes, and derepression of E2F-responsive genes. The activation of E2F complexes results in the promotion of S phase of the cell cycle via the activation of cellular genes that promote cell cycle progression (e.g., cyclin E, cdk2, cdc25A, cyclin A, cdc2). E1A acts to subvert the tight control of E2F by binding directly to the pocket domains of Rb proteins via an LXCXE motif in E1A common to other DNA tumor virus transformating proteins. E1A sequesters Rb family members and frees E2F heterodimers to activate viral and cellular gene expression (Dimova and Dyson 2005). Both the E1A 243 aa and the 289 aa products direct the release of Rbs from E2Fs, and both E1A proteins promote cellular transformation, in part via this mechanism (Frisch and Mymryk 2002).

p300, CBP, PCAF, and GCN5 are all HATs, while TRRAP and p400 serve as scaffolding proteins to bridge interactions of HATs with other transcriptional regulators (reviewed in Roth et al. 2001). The ability of E1A to bind this class of transcriptional regulators makes sense with respect to the transcriptional activation properties of the E1A proteins. The binding of E1A to these proteins promotes cellular DNA synthesis, and E1A mutants that cannot interact with these effectors are defective for transformation (Jelsma et al. 1989; Wang et al. 1993). Furthermore, over-expression of p300 or CBP blocks E1A-mediated transformation. These results suggest that E1A and HATs act in the same pathway leading to cellular transformation. This may be via the direct activation by E1A of cellular genes that promote proliferation. Additionally, E1A represses p300 and CBP-mediated transcriptional activation most likely via sequestration of these HATs away from promoter regions and promoter-bound transcription factors (Arany et al. 1995; Lundblad et al. 1995). Direct

evidence for this model was obtained for E1A and its ability to inhibit GCN5/TRRAP-mediated transcriptional activation (Lang and Hearing 2003). Genetic evidence was provided in yeast that supports the binding of E1A with the GCN5/TRRAP complex (Gcn5/Tra1 in yeast) and showed that the E1A inhibition of cellular gene expression was mediated by the interaction of the N-terminal region of E1A with Gcn5/Tra1 (Kulesza et al. 2002). TRRAP was originally identified as a transcriptional coactivator of c-Myc via recruitment of GCN5 and this plays a role in c-Myc oncogenic function (McMahon et al. 1998, 2000). A c-Myc mutant that is defective for TRRAP binding is deficient in transformation properties. Replacement of the TRRAP-binding sequences in c-Myc with the E1A N-terminal domain restores c-Myc-transforming activity to a mutant that is otherwise transformation defective (Deleu et al. 2001). Collectively, these results indicate that E1A contributes to transformation by repression of the expression of cellular genes via the sequestration of transcriptional coactivator complexes.

Several recent reports have clarified the role of E1A binding to p300/CBP in transformation. To investigate the role of p300 in the regulation of cellular proliferation, cells were generated where p300 expression was inducible (Baluchamy et al. 2003). The induction of p300 protein levels significantly reduced S phase entry following serum stimulation. p300 induction was associated with down-regulation of c-Myc expression, but not that of c-Fos or c-Jun. Over-expression of c-Myc in this context reversed the inhibition of proliferation associated with p300 over-expression. These results are consistent with a previous report demonstrating that depletion of p300 in human cells results in premature G1 exit and up-regulation of c-MYC and they establish a role for p300 in the negative regulation of c-Myc protein levels (Kolli et al. 2001). E1A inhibition of p300 activity would be expected to reverse this process and, indeed, E1A induces c-Myc gene expression dependent on the binding of E1A to p300 (Baluchamy et al. 2007). This recent report also demonstrated that the binding of E1A to both p300 and pRb is required for c-Myc induction and the stimulation of S phase progression. These results are completely consistent with the earlier report showing the roles of cooperating oncogenes in cellular transformation and the ability of either E1A or c-Myc to collaborate with activated Ras to induce transformation (Ruley 1983).

E1A binds to p400 through the N-terminal domain (Fig. 7.1). p400 is related to the yeast chromatin-modifying proteins SWI2/SNF2 and forms a complex with TRRAP. An E1A mutant that is defective for p400 binding also is defective for transformation (Fuchs et al. 2001). Interestingly, E1A also causes the activation of $p19^{ARF}$ pathway which leads to the upregulation and stabilization of p53 and the induction of apoptosis (de Stanchina et al. 1998). The binding of E1A to p400 is involved in this process (Samuelson et al. 2005). p400 is part of a larger HAT complex, termed TIP60, that contains TRRAP, GCN5, PCAF, TIP48, and TIP49 (Frank et al. 2003). E1A induces $p19^{ARF}$ and p53 expression by binding to TIP60 and pRB, whereas p300 binding is dispensable for this process (Samuelson et al. 2005). A reduction in p400 expression levels reduces

p19^{ARF} and p53 protein levels and interferes with E1A induction of apoptosis (Samuelson et al. 2005). Finally, a recent report links the anaphase-promoting complex (APC) with E1A regulation of cell cycle progression (Turnell et al. 2005). The APC is an E3 ubiquitin ligase complex that regulates cell cycle progression via ubiquitin-mediated, proteasome-dependent degradation of specific substrate proteins. Several APC components interact with p300/CBP which stimulates p300/CBP HAT activity and potentiates p300/CBP-dependent transcription. APC5 and APC7 suppress E1A-mediated transformation in a p300/CBP-dependent manner suggesting that these activities also may be E1A targets during the transformation process (Turnell et al. 2005). All the aforementioned results demonstrate the complex regulatory circuit that governs the regulation of cellular proliferation and how disruption of this carefully coordinated system by the N-terminal domain of E1A leads to profound effects on cellular life and death decisions.

The C-terminal exon of the E1A proteins contains a nuclear localization signal and a binding site (CR4) for the transcriptional corepressor CtBP (reviewed in Chinnadurai 2002). E1A exon 2 exhibits transcriptional regulatory activities that antagonize exon 1 functions. E1A exon 2 negatively regulates E1A functions in transformation and tumorigenesis. In cooperation with activated Ras, E1A mutants containing deletions in exon 2 CR4 transform cells more efficiently that wild-type E1A (Subramanian et al. 1989). Tumors expressing these E1A C-terminal mutants are highly metastatic, whereas tumors expressing wild-type E1A are not. This activity correlates with binding of E1A to CtBP through the conserved motif PLDLS in CR4 of E1A (Boyd et al. 1993). CtBP functions as a transcriptional corepressor when tethered to a promoter region and binds to a number of HDACs including HDAC-1, -4, and -5 (Shi et al. 2003; Subramanian and Chinnadurai 2003). CtBP binds to another cellular protein, CtIP, as part of a corepressor complex that binds pRB and p130 (reviewed in Chinnadurai 2006). CtIP binds CtBP via a PLDLS motif and to pRb via an LXCXE motif (Meloni et al. 1999; Schaeper et al. 1998). The E1A proteins would be expected to disrupt these interactions and foster proliferation via inhibition of both pRb and CtBP activities. Yet the binding of CtBP to E1A inhibits its transforming properties. These contradictory ideas have not been resolved. The simplest model suggests that the binding of E1A to CtBP may antagonize the regulation of HAT complexes by the N-terminal region of E1A. The C-terminal region of E1A negatively regulates CR1-dependent transcriptional activation (Sollerbrant et al. 1996) and, thus, may antagonize the transforming activity of the N-terminal domain. The E1A 243 aa protein can be acetylated at residue Lys-239 in the C-terminal region by p300 and PCAF (Zhang et al. 2000). This residue is adjacent to the PXDLS motif in CR4 that mediates binding to CtBP. Mutation of Lys-239 in E1A blocks the interaction with CtBP in vitro and in vivo (Zhang et al. 2000). Thus, the binding of HATs to the N-terminal domain of E1A may reduce E1A binding to CtBP at the C-terminus.

E1A also plays a role in the induction of apoptosis in infected cells (reviewed in Chinnadurai 1998; White 1998). Sustained, unregulated E2F activity triggers a cellular checkpoint and causes an increase in the level of the tumor suppressor p53. Activated p53 induces gene expression by binding specific promoter sequences, which activates genes that are involved in a number of cellular processes. The presence of p53 can affect cells in primarily two ways (Chinnadurai 1998; White 1998). First, p53 can induce cell cycle arrest, thus inhibiting progression of cell division. This arrest can be facilitated by the transactivation of genes encoding inhibitors of cyclin-dependent kinases, e.g., p21$^{WAF\text{-}1/Cip\text{-}1}$, which prevents the phosphorylation of Rb family members. p53 also can induce cell death by the induction of apoptosis. It does so by inducing the activation of degradative enzymes, caspases, which generate the classic apoptotic pathway. This proteolytic cascade results in a characteristic apoptotic phenotype of shrinkage and rounding of the cell due to breakdown of the cytoskeleton, cleavage of cellular DNA and condensation of the chromatin, cytoplasmic vacuolization and membrane blebbing, and in the final stages, fragmentation of the cell membrane into vesicles or apoptotic bodies that can be taken up by neighboring cells. The activation of p53 and induction of cellular apoptosis would be deleterious to Ad replication. Therefore, Ad has evolved several proteins encoded by the E1B transcription unit that repress p53 activity and inhibit apoptosis. These proteins contribute in important ways to Ad-transforming properties (discussed below).

7.3 Early Region 1B (E1B)

The second Ad E1 gene expressed is early region 1B that leads to the production of two major species of mRNAs (Fig. 7.2). One mRNA codes for a 19 kDa polypeptide (E1B-19K) and the other codes for a 55 kDa protein (E1B-55K). The two proteins are encoded by alternative reading frames and share no sequence homology. The major roles of these proteins in Ad infection are to inhibit apoptosis and further modify the intracellular environment in order

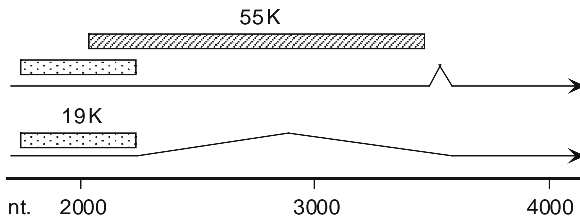

Fig. 7.2 The E1B transcription unit. Shown are nucleotide positions relative to the left end of the Ad5 genome, the two E1B mRNAs indicated by arrows, and reading frames for the E1B-19K and E1B-55K proteins indicated by rectangles

to make the cell more hospitable to viral protein production and viral DNA replication (Chinnadurai 1998; White 1998). Viruses with mutations in either or both E1B proteins are significantly reduced in virus yield due to cell death by apoptosis prior to the completion of the replicative cycle.

The E1B-55K protein is essential for a variety of important functions in the viral life cycle. One important function is inhibition of the p53 tumor suppressor and inhibition of the induction of p53-dependent apoptosis (Chinnadurai 1998; White 1998). The E1B-55K protein binds to the acidic, N-terminal transactivation domain of p53 and inhibits p53-induced transcription (Martin and Berk 1998). The binding of E1B-55K to p53 stabilizes p53 binding to DNA. However, the binding of E1B-55K to p53 alone is not sufficient to inhibit p53 functions. It is theorized that E1B-55K directs repression of promoters when held in a complex with p53 due to strong transcriptional repression by E1B-55K (Martin and Berk 1999). By doing so, E1B-55K inhibits the activation of p53-responsive promoter regions and blocks cycle arrest and apoptosis programs before they get underway. A cellular corepressor is required for the E1B-55K protein to exert its negative effects on promoter activity (Martin and Berk 1999). E1B-55K also disrupts the interaction of p53 with the HAT PCAF and interferes with p53 acetylation (Liu et al. 2000). Interestingly, it was recently shown that the E1B-55K protein promotes cell transformation independently of repression of p53 transcriptional activity (Sieber and Dobner 2007). This may relate to the ability of E1B-55K to inhibit other apoptotic activities in the cell including the pro-apototic cellular activity Daxx. E1B-55K coimmunoprecipitates with Daxx and co-localizes with Daxx in discreet nuclear structures that are distinct from PML nuclear domains (see below) (Sieber and Dobner 2007; Zhao et al. 2003a). Daxx augments p53-mediated transcription and E1B-55K interferes with this process (Zhao et al. 2003a). E1B-55K may also interfere with p53 function by cytoplasmic sequestration (Zhao and Liao 2003b). The E1B-55K protein is modified by the small, ubiquitin-like protein SUMO-1, and sumoylation of E1B-55K is required for its transformation activity (Endter et al. 2001). Finally, E1B-55K acts in a complex with another Ad early protein, E4-ORF6, which leads to the proteasome-dependent degradation of p53, further decreasing p53 effects on the infected cell (Steegenga et al. 1998). This function will be discussed below.

The E1B-19K protein is also involved in the inhibition of apoptosis. E1B-19K acts to block apoptotic pathways that do not rely on p53, such as the TNFα and Fas ligand cell death pathways (Chinnadurai 1998; White 1998). E1B-19K is a functional homologue of a cellular suppressor of apoptosis, Bcl-2 (reviewed in White 2006). Homodimers of a pro-apoptotic protein, such as Bax, result in the activation of caspases, leading to cell death. Bcl-2 heterodimerizes with Bax and inhibits its function, preventing the induction of apoptosis. The dimerization occurs through interaction of specific binding regions, Bcl-2 homology or BH domains. E1B-19K acts in the same manner as Bcl-2 and predominantly inhibits apoptosis by binding pro-apoptotic activities Bax and Bak (White 2006). E1B-19K shares sequence similarity with

Bcl-2 in two BH domains present in E1B-19K that are necessary to bind Bax. Similarly, the BH3 domain of Bax is sufficient for E1B-19K protein interaction. The binding of Bax and Bad by E1B-19K leads to inhibition of apoptosis (Chinnadurai 1998; White 1998). E1B-19K also plays a role in the inhibition of TNFa-induced apoptosis by blocking the oligomerization of death-inducing complexes involving FADD (Perez and White 1998). FADD is a protein that is activated by binding Fas via death domains, thus its name (Fas-associated death domain). The exact function of E1B 19K in FADD regulation is not well understood.

7.4 Early Region 4 (E4)

Whether they are early or late, a common theme among the Ad transcription units is that they encode multiple proteins of related functions. However, Ad early region 4 (E4) is the only transcription unit that produces proteins of relatively disparate functions. E4 encodes at least seven proteins according to analysis of open reading frames (ORF) and spliced mRNAs (Fig. 7.3). The gene products exhibit a wide range of activities. Proteins expressed from the E4 region have been shown to be important for transcriptional regulation, viral DNA replication, viral mRNA transport and splicing, shutoff of host cell protein synthesis, oncogenic transformation, and the regulation of apoptosis. Four proteins expressed by the E4 region are able to influence transformation, although none are essential to this process.

Subgroups D Ad9 is unique in its ability to induce estrogen-dependent mammary tumors in animals (reviewed in Tauber and Dobner 2001). The primary oncogenic determinant of these viruses is the E4-ORF1 protein, rather than the E1A and E1B proteins described above (Javier 1994). Based on sequence similarity, the E4-ORF1 protein appears to have evolved from a cellular dUTP pyrophosphatase gene, although E4-ORF1 does not possess this enzymatic activity (Weiss et al. 1996). Rather, E4-ORF1 appears to have utilized the structural aspects of dUTP pyrophosphatases in order to form homotrimers. The tumorogenic property of E4-ORF1 depends on a C-terminal

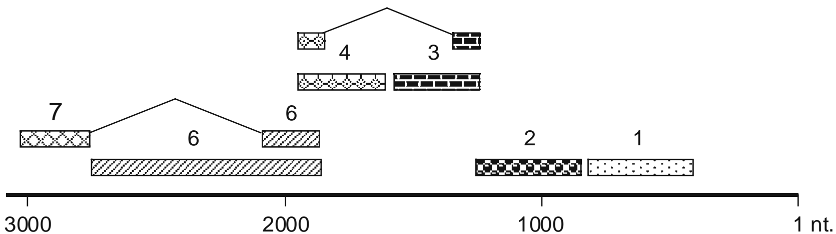

Fig. 7.3 The E4 transcription unit. Nucleotide positions are relative to the right end of the Ad5 genome. E4 open reading frames are indicated by rectangles

sequence motif referred to as a PDZ domain (Frese et al. 2003). Ad9 E4-ORF1 contains a class I PDZ-binding domain motif following the consensus sequence (S/T)-X-(V/I/L)-COOH (X is any amino acid) at the C-terminus of the protein. PDZ domains are involved in protein–protein interactions and in the case of Ad9 E4-ORF1, mediate the binding of E4-ORF1 to cellular proteins including Dlg1, MUPP1, PATJ, MAGI-1, and ZO-2 (Glaunsinger et al. 2001; Lee et al. 2000, 1997). PDZ proteins function as scaffolds to target signaling complexes to specific sites at the plasma membrane, and the PDZ proteins that bind to Ad9 E4-ORF1 have tumor suppressor activities. The binding of Ad9 E4-ORF1 to PDZ-containing proteins mediates the oncogenic function of this viral gene product (Glaunsinger et al. 2000, 2001; Lee et al. 2000, 1997). MUPP1, PATJ, MAGI-1, and ZO-2 localize to tight junctions at sites of cell–cell contact with epithelial cells. In epithelial cells, Ad9 E4-ORF1 prevents localization of PATJ and ZO-2 to tight junctions, and as such, disrupts tight junctions resulting in a loss of apicobasal polarity (Latorre et al. 2005). Tight junction disruption and the loss of apicobasal polarity are common features of epithelial cancers. An additional function of Ad9 E4-ORF1 is activation of PI-3 kinase and this activity also is involved in the oncogenic properties of this protein. Activation of PI-3 kinase is associated with many human cancers and the interaction of Ad9 E4-ORF1 with Dlg1 may mediate this process (Frese et al. 2006). Thus, Ad9 E4-ORF1 impacts on numerous cellular processes that are involved in the progression of cancer.

The E4-ORF3 protein is highly conserved among Ad and is multifunctional. The E4-ORF3 and E4-ORF6 proteins both bind to the E1B-55K product, although to different ends. E4-ORF6 enhances the inhibition of p53 by E1B-55K, whereas E4-ORF3 transiently relieves the repression of p53 by E1B-55K (Dobner et al. 1996; Konig et al. 1999; Steegenga et al. 1998). E4-ORF3 has been shown to localize with discrete nuclear structures alternatively known as PML nuclear bodies, PML oncogenic domains (PODs), or ND10 (Doucas et al. 1996). PML nuclear bodies exist as multiprotein complexes that exhibit a discreet, punctate appearance in the nucleus of a cell. E4-ORF3 is necessary and sufficient to cause redistribution of these protein complexes into long, track structures. PODs have been implicated in a number of cellular processes including transcriptional regulation, the regulation of apoptosis, DNA damage repair, protein modification, and an antiviral response (reviewed in Seeler and Dejean 1999). The exact function of PML is still unknown, but convincing evidence supports roles in all the aforementioned cellular processes. PODs have also been shown to react to stresses such as heat shock and heavy metals as well as interferon, suggesting a role in cellular defense mechanisms. The E4-ORF3 augments transformation of rodent cells in culture in concert with the E1A and E1B proteins (Nevels et al. 1999b). PML functions as a tumor suppressor and its functional loss is associated with the development of human acute promyelocytic leukemia. E4-ORF3 may augment transformation by Ad via the inhibition of PML activity, although this has not been demonstrated. Additionally, a second

important function of E4-ORF3 protein of the subgroup C Ad (e.g., Ad2 and Ad5) is the inhibition of cellular DNA damage response mechanisms. E4-ORF3 directs the reorganization of the Mre11-Rad50-Nbs1 complex (MRN complex) into PML-containing tracks (Evans and Hearing 2005; Stracker et al. 2002, 2005). The MRN complex serves as a sensor of DNA damage and is recruited to the ends of damaged DNA (D'Amours and Jackson 2002). This serves to trigger effector cascades that lead to cell cycle arrest and the repair of the DNA damage. In the context of Ad infection, this process if uninhibited results in the end-to-end ligation of viral genomes, effectively inhibiting viral DNA replication. E4-ORF3 interferes with this process by sequestering MRN proteins in the nucleus and blocking their function (Evans and Hearing 2005; Stracker et al. 2002, 2005). In the context of cellular transformation, inhibition of the DNA damage response by E4-ORF3 may contribute to oncogenesis by inhibiting the repair of damaged DNA and fostering the accumulation of mutations in cellular oncogenes and/or tumor suppressor genes. This intriguing possibility has not been formally demonstrated.

The E4-ORF4 protein is a multifunctional regulator. First, E4-ORF4 binds to the Bα subunit of the serine/threonine phosphatase PP2A (Kleinberger and Shenk 1993). By binding this subunit, the trimeric form of PP2A is activated which results in the dephosphorylation of target proteins such as mitogen-activated protein (MAP) kinases that are important in signal transduction pathways. Increased PP2A activity leads to decreased phosphorylation and inactivation of certain transcription factors, such as E4F, through direct interaction or through the inactivation of MAP kinases. E4-ORF4 expression also results in decreased E1A phosphorylation at MAP kinase consensus sites that are important for E4 transactivation (Whalen et al. 1997). Through decreasing the activity of E1A and E4F, E4-ORF4 regulates the expression of the E4 region itself and thus may suppress the oncogenic potential of the E1A proteins (Bondesson et al. 1996). Second, E4-ORF4 is able to induce p53-independent apoptosis in transformed cells (Lavoie et al. 1998; Marcellus et al. 1998; Shtrichman and Kleinberger 1998). Oncogenic transformation of cells sensitizes them to E4-ORF4-induced cell killing (Shtrichman et al. 1999). Depending on the cell type, E4-ORF4-induced apoptosis utilizes the classical pathway involving caspases or a non-classical pathway that is caspase independent (Livne et al. 2001). The binding to and regulation of PP2A by E4-ORF4 is essential for the induction of cell death (Marcellus et al. 2000; Shtrichman et al. 1999, 2000). E4-ORF4-dependent apoptosis also requires modulation of Src-family kinases (Lavoie et al. 2000). Thus, by an alternative mechanism, E4-ORF4 represses the oncogenic potential of the Ad E1 proteins. E4-ORF4 may be useful in the future as a therapeutic agent to target human cancers for apoptotic cell death.

The E4-ORF6 protein provides a number of functions that may contribute to Ad transformation. E4-ORF6 binds to and inhibits p53, providing Ad yet another defense for p53 effects within the cell (Dobner et al. 1996). E4-ORF6

augments the transformed phenotype of Ad E1-transformed cells through the down-regulation of p53 expression (Nevels et al. 1999a; Steegenga et al. 1998). These changes in transformation properties include morphological alterations, enhanced growth rates, higher saturation densities in culture, and accelerated tumor growth in animals. E4-ORF6 forms a direct protein complex with the E1B-55K protein. The E4-ORF6/E1B-55K complex recruits a CUL5-containing E3 ubiquitin ligase complex to target p53 and other cellular proteins, including proteins involved in DNA damage repair, for poly-ubiquitination and proteasome-dependent degradation (Baker et al. 2007; Harada et al. 2002; Querido et al. 2001, 1997; Stracker et al. 2002). By this mechanism, the E4-ORF6/E1B-55K complex counteracts the induction of p53 stability provided by E1A (Steegenga et al. 1998). Other targets of this virus-induced E3 ligase activity include Mre11, Rad50, and DNA ligase IV. Thus, E4-ORF6 enhances cellular transformation in conjunction with E1B-55K by two mechanisms: inhibition of p53 activity via degradation and the potential induction of DNA damage by inhibition of the MRN complex. These conclusions are consistent with previous results which demonstrated that E4-ORF6 contributes to oncogenic transformation by two distinct mechanisms: one that involves destabilization of p53 and another that is independent of this mechanism (Nevels et al. 2000). Further, the E1A and either E4-ORF3 or E4-ORF6 proteins can cooperate to transform primary rat cells, yet neither the E4-ORF3 nor the E4-ORF6 proteins are detected in transformed cells once established (Nevels et al. 2001). Such a "hit-and-run" type of transformation mechanism is consistent with the transient inhibition of DNA repair pathways by these viral gene products and the induction of mutations in oncogenes and/or tumor suppressor genes that foster oncogenic transformation.

The E4-ORF6/7 protein is produced from a spliced mRNA that encodes the amino terminus of E4-ORF6 linked to the unique E4-ORF7 sequence. E4-ORF6/7 molecules form stable homodimers that contribute to viral DNA synthesis by enhancing the production of E2 products. E4-ORF6/7 binds free E2F and induces cooperative and stable binding of E2F/DP heterodimers to inverted E2F-binding sites in the Ad E2 early promoter (Huang and Hearing 1989). E4-ORF6/7 induces expression from the cellular E2F-1 promoter and is able to functionally compensate for E1A in adenovirus infection by displacing Rb family members from E2Fs (O'Connor and Hearing 2000; Schaley et al. 2000). Further, the E4-6/7 protein alters the subcellular localization of E2F family members and directs E2F-4 from the cytoplasm to the nucleus (Schaley et al. 2005). Thus, E4-6/7 displays functional redundancy with the E1A proteins in terms of activating E2F family members and could contribute to transformation by this mechanism. Consistent with idea is the observation that E4-6/7 can complement an E1A mutant that is defective for pRb binding in the transformation of primary rat cells (Yamano et al. 1999). The results of mutational analysis of E4-6/7 linked the contribution of E4-6/7 to transformation with amino acid sequences that required for E2F interaction and regulation (Yamano et al. 1999).

7.5 Conclusions

The Ad life cycle represents a complex series of events that must occur in a temporally and stoichiometrically appropriate fashion in order for efficient production of progeny virus. The virus must usurp control of the cellular machinery while controlling the expression and functions of its own proteins. The manner with which Ad exerts these controls is through a myriad of protein–protein interactions. The use of Ad as a model system has led to a wealth of information regarding many aspects of viral as well as cellular processes, including oncogenic transformation. Ad evolved specific proteins that have transforming properties for different purposes: E1A to regulate viral and cellular gene expression and to promote cell cycle progression, Ad9 E4-ORF1 to promote cell proliferation, E4-ORF4 to regulate viral gene expression and cellular signaling pathways, and E4-6/7 to activate viral and cellular gene expression. Yet the unintended outcome of the function of these viral regulators is to induce cellular responses (e.g., cell cycle arrest, apoptosis) that would inhibit the viral replication program if left unabated. Thus, Ad evolved secondary regulators to inhibit these cellular responses: E1B-55K and E4-ORF6 to inhibit p53 and a cellular DNA damage response and E4-ORF3 to regulate many cellular processes including apoptosis and a cellular DNA damage response. Collectively, Ad inhibits two major tumor suppressor pathways that are deregulated in many human cancers (p53 and pRb) and blocks apoptotic responses that may otherwise promote cancer cell killing. The same tumor suppressor pathways and cellular signaling pathways are targeted by other DNA tumor viruses as described in other chapters in this book.

Acknowledgments I thank for colleagues for many helpful discussions. This work was supported by NIH grant CA122677.

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Chapter 8
Overview of the Large DNA Tumor Viruses

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8.1 Introduction: Historical Aspects

Approximately 20% of human tumors are associated with viruses which include RNA as well as DNA viruses. The RNA viruses linked to tumorigenesis are the human T-cell leukemia viruses 1 and 2 (HTLV-1/2) and human hepatitis virus C (HCV). The DNA viruses which primarily cause tumors in humans are Epstein–Barr virus (EBV), human papilloma virus 16 and 18 (HPV 16 and HPV 18), Kaposi's sarcoma-associated herpesvirus (KSHV) and human hepatitis virus (HBV). EBV was the first DNA virus detected in human tumor, Burkitt lymphoma of the African patients (Burkitt, 1958; Burkitt and O'Conor, 1961). The link between EBV and Burkitt lymphoma was established by the initial work of Epstein and further confirmed by the Henles working at the Children's Hospital of Philadelphia in 1964 (Epstein et al., 1964, 1965b). EBV was further identified as the causative agent of infectious mononucleosis (IM) by the Henles while analyzing the blood sample of one of their technical staff who had developed rubella-like rashes (Niederman et al., 1968; zur Hausen, 2005). Serologic tests of her blood showed antibody against EBV antigens which was undetected before, as she was a regular blood donor for experiments in the laboratory. Additionally, the isolated leukocytes of her blood grew in culture and produced permanent lymphoblastoid cell lines positive for EBV (Nilsson et al., 1971; Pearson et al., 1971; zur Hausen, 2005).

EBV was further linked to other diseases which include nasopharyngeal carcinoma (NPCs) and Hodgkin's lymphoma (Andersson-Anvret et al., 1977; Gunven et al., 1970; Henle et al., 1970, 1971; Johansson et al., 1970; Lanier et al., 1981; zur Hausen et al., 1970). This was demonstrated by the seroepidemiology of EBV which detected increased antibody titer against EBV in the

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_8, © Springer Science+Business Media, LLC 2009

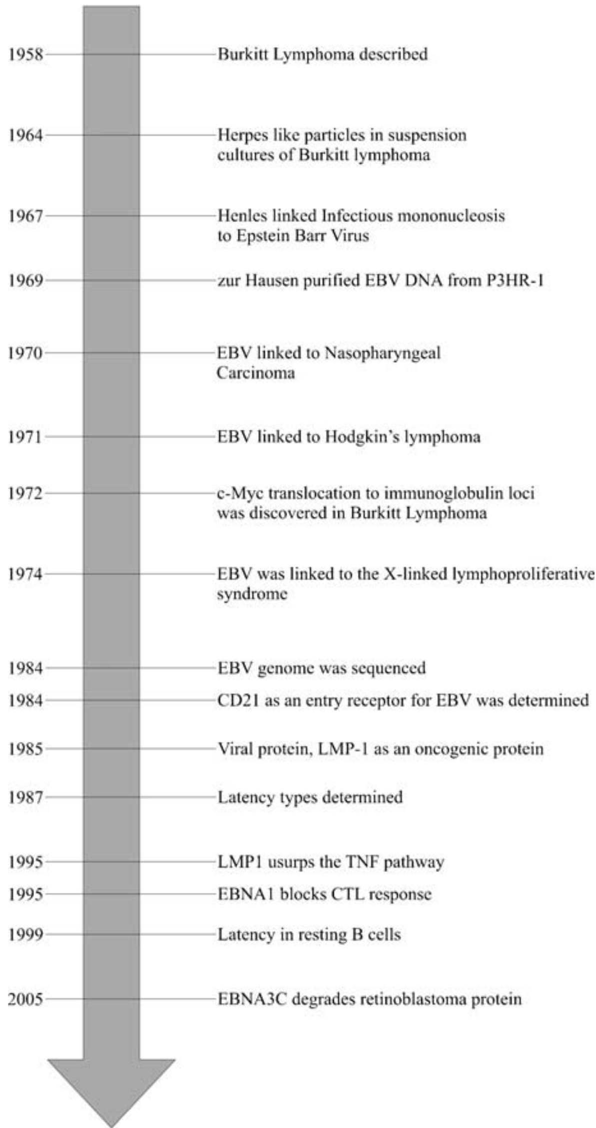

Fig. 8.1 Timeline of major developments in EBV-mediated tumors. Dennis Burkitt described Burkitt lymphoma in an African child with swellings in all the four angles of the jaws (Burkitt, 1958). Herpes-like viral particles were detected in the cultures from Burkitt lymphoma which was later characterized as Epstein–Barr virus (Epstein et al., 1964, 1965b). The genome of EBV was completely sequenced in approximately 170 kbp (Baer et al., 1984). Studies from Kieff group showed that LMP1 is able to engage signaling proteins of the tumor necrosis factor receptor family to usurp the TNF pathway (Mosialos et al., 1995). Internal Gly-Ala repetitive sequence of EBNA1 helps in viral escape from CTL surveillance, a critical requirement for EBV persistence and pathogenesis (Levitskaya et al., 1995). EBV establishes latency in resting memory B cells (Babcock et al., 1999). Epstein–Barr virus latent antigen 3C can mediate the degradation of the retinoblastoma protein through an SCF cellular ubiquitin ligase (Knight et al., 2005a)

later phase of the disease compared to the initial phase (Johansson et al., 1971). During the AIDS pandemic in the 1980s the Henles attempted to link the acquired immunodeficiency syndrome with a tumor in AIDS patients but, they were unable to link Kaposi's sarcoma lesions with EBV infection (Henle and Henle, 1981; Kaminsky et al., 1985). It is interesting to note that the etiologic agent associated with Kaposi's sarcoma was later identified to be other member of the γ-herpesvirus family (Chang et al., 1994; Verma and Robertson, 2003). This virus was named Kaposi's sarcoma-associated herpesvirus (KSHV) because of its origin from KS lesions and is also referred to as human herpesvirus 8 (HHV-8) as it is the eighth member of the human herpesvirus family (Moore and Chang, 2003; Verma and Robertson, 2003). KSHV was discovered from the Kaposi's sarcoma lesions using subtractive representational difference analysis (Chang et al., 1994). The genome of KSHV from two different sources, the Kaposi's sarcoma lesions and body cavity-based lymphomas, was sequenced and is a 165 kbp long *ds*DNA (Neipel et al., 1997; Russo et al., 1996). The coding region of the genome is flanked by high GC multiple terminal repeat units of 801 bp (Lagunoff and Ganem, 1997). The coding region which is also referred to as long unique region (LUR) encodes for approximately 90 genes (Neipel et al., 1997; Russo et al., 1996).

EBV was initially detected as a herpesvirus-like particle in Burkitt lymphoma tissues obtained from the African patients (Epstein et al., 1964). Due to the lack of proper identification methods these particles were referred to as herpes-like virus which was later classified as EBV when Harald zur Hausen purified EBV DNA from the EBV producer cells, P3HR-1 (Schulte-Holthausen and zur Hausen, 1970). EBV was further detected in the non-virus-producing cell line, Raji by DNA–DNA hybridization (Zur Hausen and Schulte-Holthausen, 1970). Additionally, studies by zur Hausen successfully showed that EBV not only infects the cells of lymphatic origin, B cells, but also establishes latent infection in epithelial cells of the nasopharynx and is suggested to be the major contributor to the development of nasopharyngeal carcinoma (Wolf, zur Hausen, and Becker, 1973). Detection of EBV DNA in Burkitt lymphoma and nasopharyngeal carcinoma was also confirmed by independent laboratories (Klein et al., 1974; Nonoyama et al., 1973). A historical timeline of the major discoveries in the EBV field is shown in Fig. 8.1.

8.2 Herpesviruses and Cancer

Herpesviruses are a family of double-stranded DNA viruses and are prevalent in most species throughout the animal kingdom. Herpesviruses are often co-carcinogens. They may have a "hit and run" mechanism of oncogenesis, perhaps by expressing proteins early in infection that lead to chromosomal breakage or other damage. So far, more than 130 herpesviruses have been identified. In humans, eight herpesviruses (HHV-1 to HHV-8) have been

identified: herpes simplex virus 1 and 2 (HSV-1 and HSV-2); varicella-zoster virus (VZV or HHV-3); Epstein–Barr virus (EBV or HHV-4); human cytomegalovirus (HCMV or HHV-5); human herpesvirus 6 and 7 (HHV-6 and HHV-7); and KSHV/HHV-8. There are considerable circumstantial evidences implicating these enveloped DNA viruses in human neoplasms. On the basis of their biological characteristics and genome sequences, they are classified into three subfamilies: α, β and γ. The γ-herpesviruses share more genes with each other than with members of either the α- or the β-subfamilies of herpesviruses (Fig. 8.2), and their genomes are organized in a co-linear fashion (Weiner et al., 1985).

The γ-subfamily of herpesviruses is lymphotropic and some are capable of undergoing lytic replication in epithelial and/or fibroblast cells. These viruses establish a lifelong period of latency in their host, with intermittent periods of lytic replication. The γ-*herpesvirinae* has been identified in many different animal species (Ensser and Fleckenstein, 2005). There are two subfamilies of γ-herpesviruses: the lymphocryptoviruses (γ-1) and the rhadinoviruses (γ-2). EBV is a lymphocryptovirus, whereas herpesvirus saimiri (HVS), which infects monkeys, and KSHV are rhadinoviruses (Neipel et al., 1998). Due in part to their recent identification and the generally benign nature of infection, information on herpesvirus-6 (HHV-6) and herpesvirus-7 (HHV-7) is still somewhat limited.

A striking characteristic of the members of the γ-herpesvirus family, including EBV and KSHV, is their ability to induce neoplasia in natural or experimental hosts. Both of these viruses (EBV and KSHV) are associated with different types of malignancies in their natural host and also related with lymphoproliferative diseases of B and/or T cells (Damania, 2004) (Table 8.1).

Additionally, EBV is associated with epithelial cancers such as nasopharyngeal carcinoma (NPC) and KSHV is linked to vascular endotheliosarcomas such as Kaposi's sarcoma (KS) (Damania, 2004) (Table 8.1).

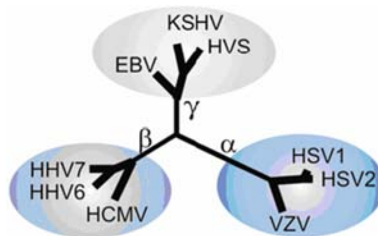

Fig. 8.2 A phylogenetic tree representing the family of herpesviruses. The tree was constructed by using full-length DNA polymerase gene sequences from the indicated herpesviruses. KSHV and HVS (rhadinoviruses) and EBV (lymphocryptovirus) are members of the γ-herpesvirus subfamily, whereas herpes simplex virus 1 (HSV-1) is an α-herpesvirus and cytomegalovirus (CMV) is a β-herpesvirus

Table 8.1 γ-Herpesviruses linked to human diseases

γ-Herpesvirus	Associated malignancies	Properties	Epidemic region
Epstein–Barr virus (EBV)	Burkitt lymphoma Hodgkin's lymphoma Post-transplant lymphoma X-linked lymphoproliferative syndrome T-cell lymphomas Nasopharyngeal carcinoma Gastric carcinoma	*c-myc* oncogene placed under the immunoglobulin heavy or light chain promoters causes BL (Dalla-Favera et al., 1987); HL is a B lymphoproliferative disease (Glaser et al., 1997); different genetic and environmental factors affect the occurrence of NPC (Pathmanathan et al., 1995)	Tropic regions of Africa, with common endemic malaria. NPC, China and SE Asia, where certain diets act as co-carcinogens
Kaposi's sarcoma-associated herpesvirus (KSHV)	Kaposi's sarcoma Primary effusion lymphomas Multicentric Castleman's disease	KS is a multifocal vascular tumor in cutaneous lesion (Antman and Chang, 2000); PELs are malignant B-cell lymphomas (Nador et al., 1996); MCD is characterized by vascular proliferation of the germinal centers of the lymph node (Moore and Chang, 2003)	Africa, the Mediterranean and East European descent

Herpesviruses have large genomes typically with over 90 open reading frames. Similar to other DNA tumor viruses, herpesviruses have a latent phase and a lytic phase to their life cycle (Fig. 8.3): In the latent stage, virus-infected cells are typically non-permissive for replication and viral DNA is integrated into the host cell chromosome (usually but not always) at specific sites. Only a subset (transformation-associated genes are expressed in all herpes-transformed cells) of the viral genes are expressed (Ackermann, 2006). These genes encode nuclear antigens or membrane proteins. Viral structural proteins are not expressed and no progeny virus is released. In the lytic stage, all ORFs of the viral genome are expressed (Ackermann, 2006). This leads to viral replication, cell lysis and cell death. Current evidence indicates that both the latent and the lytic genes of these herpesviruses contribute to viral oncogenesis through a transforming and/ or paracrine mechanism, causing normal cells to proliferate in an uncontrolled manner (Damania, 2007).

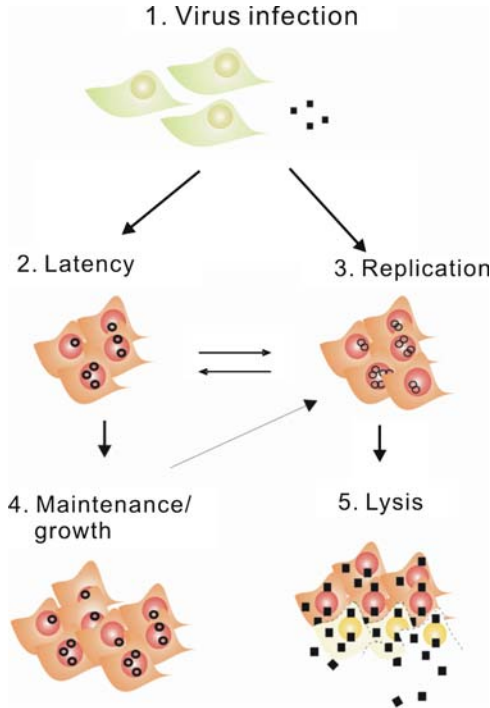

Fig. 8.3 Biphasic life cycle of γ-herpesvirus infection. (1) Dependent on the infected cell type, activation state and differentiation state, the viral DNA is delivered to the nucleus, where it circularizes and is subject to nuclear factors. Virus will establish latency either without (2) or following viral replication (3). During latency, viral genes will be expressed in a cell type-dependent manner and maintain virus latent infection to manipulate the immune response (4) or start to reactivate virus replication from latency, which will eventually lead to the lysis of infected cells and to the release of new virus (5)

8.3 Semi-permissive and Permissive Replication

B lymphocytes are only semi-permissive for replication of the virus and infection may either be latent or the host cell can be transformed by the virus. When lymphocytes are latently infected, the cell contains unintegrated copies (episomes) of the virus genome which are replicated in every cell division. In this case the immediate-early genes (including some nuclear antigens) are expressed (Ackermann, 2006).

In contrast, epithelial cells permit complete lytic replication of the virus. Epithelial cells allow the expression of the critical lytic genes (i.e., ZEBRA) which activates the early genes resulting in expression of the DNA polymerase followed by DNA replication. Subsequently, capsid proteins and the membrane glycoproteins are synthesized (Ackermann, 2006).

Kaposi's sarcoma-associated herpesvirus (KSHV) is also a member of the γ-herpesvirus family and establishes lifelong latency in B cells (Chang et al., 1994). The viral genome is ~160 kb in size and codes for 90 open reading frames (ORFs) (Russo et al., 1996; Taylor et al., 2005). KSHV encodes a diverse set of genes involved in transformation, signaling, prevention of apoptosis and immune evasion. It is associated with KS, PEL and MCD (Cesarman et al., 1995; Soulier et al., 1995). KSHV has also been shown to have the ability to immortalize primary bone marrow-derived endothelial cells and induce cell proliferation and survival of the infected cells (Flore et al., 1998).

8.4 Primary Infection

Detailed information on early events which correspond to infection of EBV and KSHV in humans is not available for obvious ethical concerns. Thus, the most detailed information on the early events following infection of live organisms with γ-herpesviruses is from MHV-68 infection in the murine model (Nash et al., 2001). The data showed that mice infected by MHV-68 virus via the respiratory routes result in lytic infection of lung epithelial cells followed by virus dissemination and latent infection of B lymphocytes as well as macrophages (Nash et al., 2001). The peak of virus latency in the spleen coincides with splenomegaly followed by an infectious mononucleosis-like syndrome and lymphoproliferative disease (Terry et al., 2000). However, for EBV, it has been demonstrated that EBV-infected resting memory B cells persist in vivo in a quiescent state (Thorley-Lawson, 2001) and later differentiate into plasma cells, but does not initiate any clear signs of viral replication (Laichalk and Thorley-Lawson, 2005). Nevertheless, it is still unclear as to whether this represents an active virus replication or proliferation of latently infected cells in humans. However, for KSHV, more recent models of de novo infection using cultured endothelial cells may address this issue at a similar level (Lagunoff et al., 2002; Moses et al., 1999).

8.5 Epidemiology of KSHV and EBV

Both KSHV and EBV belong to the γ-herpesvirus subgroup (Roizman, 1996). This subgroup was initially put together due to their common property of being able to establish latency in B lymphocytes and drive their proliferation (Roizman, 1996). Accordingly, the original name for the subgroup was lymphocryptoviruses. This same property of being able to drive B-cell proliferation can explain the association of these viruses with various lymphomas in immunosuppressed individuals. Both viruses express a completely distinct subset of latent genes, resulting in a phenotypically distinct set of malignancies, with

immunosuppression providing the common background theme (Cesarman and Mesri, 2007; Rickinson and Kieff, 1996).

Two common immunosuppression backgrounds in modern medicine are found in AIDS and transplant patients (Chang et al., 1994; Crawford et al., 1981). Accordingly, a number of AIDS-associated lymphomas are now being linked to herpesvirus infections. In such individuals, EBV is associated with a number of proliferative diseases (Epstein, 1971; Epstein et al., 1965a, 1967), including Burkitt lymphoma (Epstein, 1971; Epstein et al., 1965a, 1967), B-cell lymphoproliferative disease, infectious mononucleosis, nasopharyngeal carcinoma, natural killer cell lymphoma, primary effusion lymphoma, Hodgkin's disease, X-linked lymphoproliferative disease and post-transplant lymphoproliferative disease (Rickinson and Kieff, 1996). Although diseases associated with KSHV are not as numerous, they are much more common. Kaposi's sarcoma is the most common AIDS-associated malignancy and up until the advent of anti-retrovirals was the leading cause of mortality in AIDS patients (Chang et al., 1994).

Post-transplant lymphoproliferative disorder (PTLD) and Kaposi's sarcoma (KS) following solid organ transplantation are important post-transplant malignancies. PTLD is normally attributed to EBV infection and occurs due to the administration of immunosuppressive drugs to transplant patients to prevent organ rejection (Crawford et al., 1981). A direct result of the application of immunosuppression is depletion of EBV-specific cytotoxic T lymphocytes (CTLs), leaving the virus free to revert to latency III (Thomas et al., 1990). Some of the factors that are thought to contribute to the development of PTLD are an EBV mismatch between donor and recipient and genetic predisposition of recipients (Faye and Vilmer, 2005; Larson et al., 1996). The standard approach has been to titrate down the immunosuppression, but this is often insufficient to induce tumor regression. However, a complete withdrawal of immunosuppression often leads to not only tumor regression but also organ rejection at the same time (Starzl et al., 1984). Understanding the underlying causes of PTLD has resulted in adoption of preventive measures like improved EBV monitoring among solid organ transplant donors and recipients. There are at present no good treatment options for PTLD; however, recently adoptive immunotherapy using ex vivo-generated autologous EBV-specific CTLs or allogeneic CTLs (Khanna et al., 1999; Savoldo et al., 2001) and some dendritic cell-based therapies (Popescu et al., 2003; Subklewe et al., 2005) have shown promise.

The incidence of Kaposi's sarcoma (KS) after organ transplantation is two to three orders of magnitude higher than that in the general population, and its occurrence is also associated with immunosuppressive therapy (Moosa et al., 1998; Parravicini et al., 1997). The incidence rate of post-transplant KS is variable, depending on the donors/recipients country of origin and the type of organ received (Diociaiuti et al., 2000; Penn, 2000). There are several factors that are thought to contribute to post-transplant KS, including immunosuppressive therapy, KSHV mismatch between the organ donor and the recipient

and chronic stimulation by foreign antigens in allografts. Unlike EBV, there is lack of precise estimates of KSHV seroprevalence in different populations and preventative measures like closer monitoring of organ donor/recipient KSHV status have yet to be adopted.

Comparison of the disease states associated with these two γ-herpesviruses in the setting of the same immunosuppression (AIDS or immunosuppressive drugs) is a powerful tool in understanding the many pathways which compose the human immune system.

8.6 Genomic Organization of the DNA Tumor Virus

Following the discovery of EBV it took several years to completely sequence the genome, which was completely sequenced in 1984 and became the first herpesvirus to be completely sequenced (Baer et al., 1984). The EBV genome is approximately 170 kbp long and exists as a linear DNA in the virion particles, however, it exists as circular episomal copies in the nucleus of the infected cells (Farrell, 2005). EBV encodes for approximately 94 genes which were initially predicted based on the determined sequence but later found to be highly accurate with the expression profiling (Farrell, 2005). The nomenclature of the open reading frames was initially based on the size (A–X: largest to smallest) of the *Bam* HI fragments generated after entire genome digestion. The genes in these fragments were designated based on the sides of their initiation. For example, the left end genes are designated as LFs and similarly the right ends are RFs (Farrell, 2005).

KSHV and EBV belong to the γ-herpesvirus family of the herpesvirinae with a dsDNA genome which persists as a circular episome in the infected cells (Rickinson and Keiff, 2001). KSHV encodes for approximately 90 ORFs which are needed to complete the life cycle of the virus (Russo et al., 1996). Similarly, EBV encodes for 94 ORFs during its life cycle (Farrell, 2005). Amino acid sequence analysis of the proteins encoded by KSHV and EBV shows a high degree of conservation schematically shown as a collinear map (Fig. 8.4). There are five major regions of the genomes which show a high degree of conservation in the ORFs. These include region 1: ORFs 4–11 of KSHV with ORFs 6–11 of EBV; region 2: ORFs 17–50 of both KSHV and EBV; region 3: ORFs 52–57 in both viruses, region 4: ORFs 58–69 in both KSHV and EBV and region 5: ORF75 in both viruses (Farrell, 2005; Russo et al., 1996). Besides the above regions, both KSHV and EBV also encode unique ORFs. Some of these ORFs have orthologous functions and include the latency-associated nuclear antigen (LANA) of KSHV and the EBNA1 of EBV. They both contribute to the tethering of the viral episomal DNA to the host chromosomes (Ballestas et al., 1999; Barbera et al., 2006; Cotter and Robertson, 1999; Harris et al., 1985; Hung et al., 2001). The first open reading frame of KSHV termed as K1 also has functions similar to LMP1 of EBV (Lee et al., 1998; Wang,

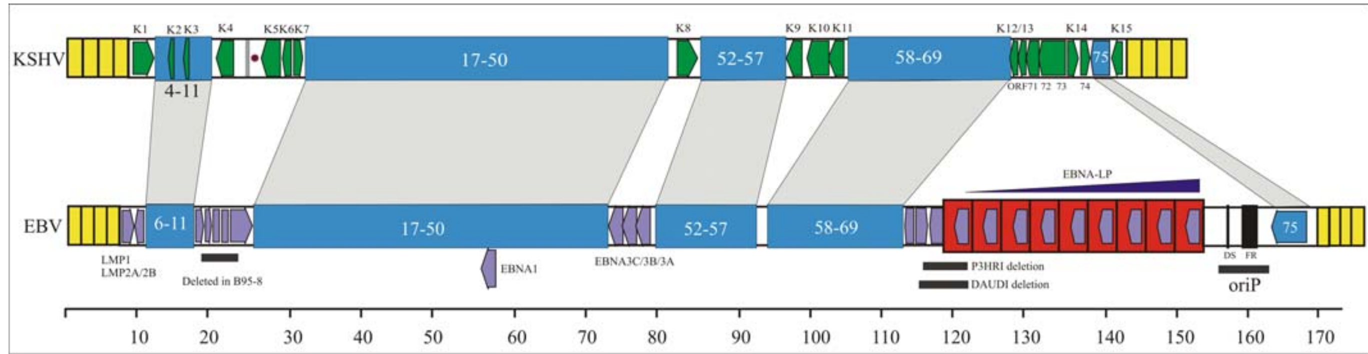

Fig. 8.4 Collinear homology of the KSHV and EBV genome. KSHV and EBV have a multiple terminal repeat which also acts as promoter for the immediate genes K1 in KSHV and LMP1 in EBV (DeWire, McVoy, and Damania, 2002; Farrell, 2005; Russo et al., 1996; Sadler and Raab-Traub, 1995; Verma et al., 2006b). Genes unique to KSHV are dark grey in color and these are designated as prefix K (1–15). The genes which are unique to the EBV are light grey in color. Majority of the genes have high degree of homology and are shown with colinear blocks

Liebowitz, and Kieff, 1985). Interestingly, these two proteins share similarity in terms of their location on the genome (Fig. 8.4). They are in close proximity to the terminal repeats of their genome which serve as the circularization region of the episomal DNA (Farrell, 2005; Russo et al., 1996).

LMP1, the EBV oncoprotein, is a transmembrane protein and is essential for immortalization of primary human B lymphocytes (Izumi et al., 1994; Kaye et al., 1993; Wang et al., 1985). The expression of LMP1 can be regulated directly or indirectly by elements within three promoters depending on the latency programs. In type III latency programs, LMP1 expression is regulated by EBNA2 and EBNALP which interact with the CSL bound to its *cis*-acting element within the 5′ promoter region of LMP1 and also activate expression of EBNA1, EBNA3A, 3B, 3C and LMP2 (Harada and Kieff, 1997; Kempkes et al., 1995; Wang et al., 1990). In type II latency program, LMP1 transcription is regulated by a promoter element located within the EBV direct terminal repeats (Sadler and Raab-Traub, 1995). During lytic replication, the first intron of LMP1 is activated and transcribes a 5′ truncated D1LMP1 mRNA (Fennewald et al., 1984). This truncated LMP1 RNA (D1LMP1) encodes for residues of the cytoplasmic domain, fifth and sixth transmembrane domains and the cytoplasmic domain (Fennewald et al., 1984). Immortalized fibroblasts expressing LMP1 showed growth in low serum and exhibited less contact inhibition, demonstrating the suggested oncogenic potential of the protein (Wang et al., 1985).

K1 of KSHV is the first open reading frame of the KSHV LUR and the promoter lies within the terminal repeat of the genome (Bowser et al., 2002, 2006; Verma et al., 2006b). K1 is expressed at very low levels during latency and cooperates in transformation of KSHV-infected cells (Lee et al., 1998, 2000; Wang et al., 2006). The expression of K1 is also regulated by LANA, one of the major latent antigens (Verma et al., 2006b).

8.7 Latency Programs

Although EBV has approximately 94 ORFs, only a limited number of genes are expressed in the latently infected cells (Thorley-Lawson, 2005). Depending on the expression profiles of the latent genes, EBV transcription during latency is categorized into four types (Rowe et al., 1987; Thorley-Lawson, 2005). Type 0 in which there is primarily expression of only small non-polyadenylated mRNA 1 and 2 (EBER1 and 2) (Lerner et al., 1981; Rickinson and Keiff, 2001). EBERs are the most abundant viral transcripts in the latently infected cells (Rymo, 1979). Type I latency present in Burkitt lymphoma leads to the expression of EBERs 1 and 2, BARTs as well as EBNA1 (reviewed in Rickinson and Keiff, 2001). EBNA1 is important for replication and tethering of the genome to the host chromosomes (Reedman and Klein, 1973; Yates et al., 1985). Type II latency is characterized by the expression of EBERs, BARTs, EBNA1 and two latent membrane proteins 1

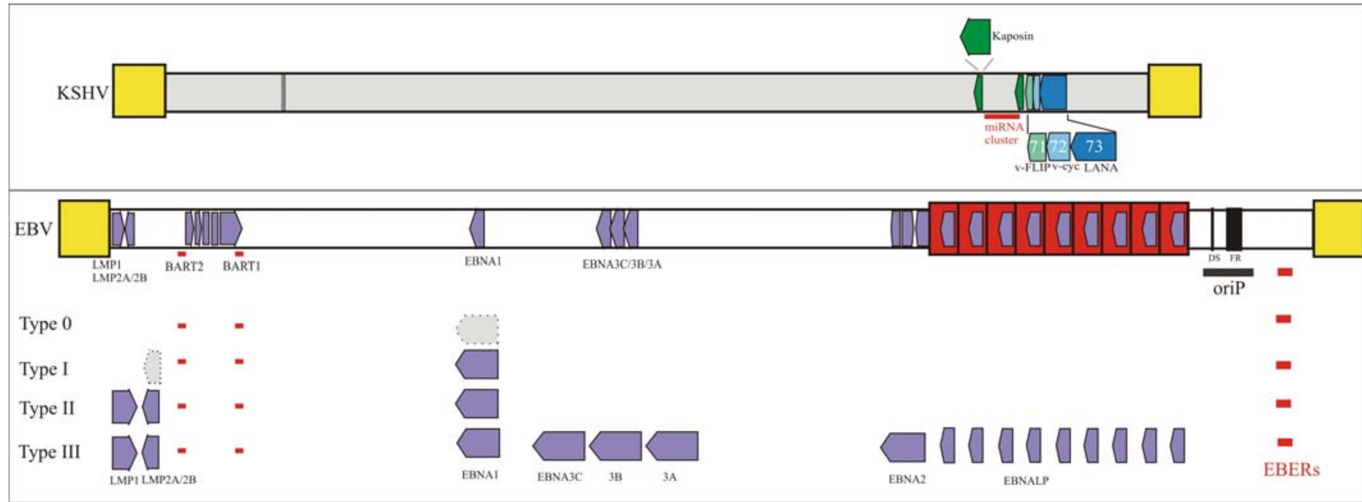

Fig. 8.5 Latent gene expression profile of KSHV and EBV. KSHV expresses cluster of genes ORF71, ORF72 and ORF73 with a polycistronic mRNA transcribed from a common promoter upstream of the ORF73 gene. Additionally, kaposins (A, B and C) are also detected during latent infection. EBV expresses a number of genes in latency types 0 to III. Latency type is characterized by the expression of EBERs, BARTs and very little expression of EBNA1 (shown as a dotted box). During latency type III all the latent transcripts are encoded and these proteins are able to drive primary B-cell proliferation leading to the development of lymphoblastoid cell line (LCL). Expression of LMP1 is also detected in latency types I and II which is essential for transformation of cell. EBNA3C, which has been shown to degrade tumor suppressor, Rb expresses only in latency type III

and 2 and is seen in nasopharyngeal carcinoma, gastric carcinoma, primary effusion lymphoma, AIDS-associated Burkitt lymphoma and Hodgkin's disease (Rickinson and Keiff, 2001). During latency type III almost all the latent proteins (LMP1 and 2 and EBNA1–6) as well as small RNAs, EBERs and BARTs are expressed (Katz et al., 1989; Rickinson and Keiff, 2001) (Fig. 8.5). Type III latency is detected in AIDS-associated immunoblastic lymphoma, infectious mononucleosis, X-linked lymphoproliferative disease and post-transplant lymphoproliferative diseases (Ho et al., 1988; Katz et al., 1989).

In contrast to EBV, KSHV does not seem to have different latency programs. KSHV establishes latent infection in endothelial as well as B lymphocytes with the expression of latent genes which includes LANA, a putative viral oncoprotein, viral cyclin (v-cyclin) encoded by ORF72 and viral Fas (vFLIP)-associated death domain (FADD) interleukin 1B converting enzyme (FLICE) inhibitory protein encoded by ORF71 (Dittmer et al., 1998). These genes lie adjacent to each other in the genome and are co-transcribed on polycistronic mRNAs (Dittmer et al., 1998; Grundhoff and Ganem, 2001). Other viral genes expressed during latency include viral interferon regulatory factor (vIRF) and kaposin encoded by open reading frame K10 and K12, respectively (Russo et al., 1996). Among these latent genes LANA is detected most consistently in all KSHV-infected cells of KS, PEL and MCD (Dupin et al., 1999).

8.8 Latency

During latency, the viral DNA is circularized in the nucleus of host cells. However, circularization of the genome may be inefficient. A similar manner usurped by the γ-herpesviruses will most often proceed with establishment and maintenance of latency. However, among γ-herpesviruses there is no common pattern of gene expression required for establishment and maintenance of latency. In EBV, there are nine latent viral genes expressed: six nuclear proteins (termed EBNAs) and three latency-associated integral membrane proteins (LMPs) along with two polyadenylated EBER transcripts and BARTs (Tsurumi et al., 2005). Among the nuclear antigens, EBNA1 plays a crucial role in the maintenance of EBV DNA in latently infected cells. It binds to the *cis*-acting element (*oriP*) on the viral genome to support efficient DNA replication and tethering it to chromosomes during mitosis and genome segregation. Based on the subsets of RNAs synthesized, at least four different states of latency by EBV are observed (Oehmig et al., 2004; Rickinson and Keiff, 2001). High copies of EBERs are detected during latency 0 (Lerner et al., 1981; Rickinson and Keiff, 2001). During latency I and II, a short EBNA1 mRNA is initiated from the Qp promoter, which lacks a recognizable TATAA element (Nonkwelo et al., 1997; Sadler and Raab-Traub, 1995). Type III latency transcription is mediated from far-upstream promoters, termed Cp and Wp. The corresponding RNAs are differentially spliced to encode EBNALP, EBNA2, EBNA3A, B, C and EBNA1 (Ho et al., 1988; Katz et al., 1989).

The latent membrane protein 1 (LMP1) has multiple transmembrane-spanning domains. The carboxyl terminus can interact with several tumor necrosis factor receptor-associated factors (TRAFs) (Mosialos et al., 1995) and is essential for the EBV's ability to transform variety of cell types. Additionally, LMP1 also upregulates several anti-apoptotic and adhesion genes expression, which includes *A20*, *bcl2*, *ICAM-1*, IRF-7, MMP-9 and FGF-2 (Mosialos et al., 1995). Another latent membrane protein, LMP2, located on the opposite end of the linear genome, has been shown to inhibit B-cell receptor (BCR) signaling (Miller et al., 1994). LMP2 sequesters the Src family members Fyn and Lyn and prevents them from translocating into lipid rafts with BCR, thereby inhibiting BCR activation (Dykstra et al., 2001).

Other viral proteins with transforming potential include EBV nuclear antigen 2 and 3 (EBNA2 and EBNA3) (Robertson and Kieff, 1995; Scala et al., 1993). EBNA2 is a promiscuous transcriptional activator and is essential for B lymphocyte transformation and upregulation of viral (EBNAs, LMP1 and LMP2) and cellular gene expression (CD23, CD21 and c-*myc*) (Cohen and Kieff, 1991; Grossman et al., 1994; Tong et al., 1995), while EBNALP has a critical role in upregulating cellular gene expression as a co-activator of EBNA2. EBNA3 proteins lie in a tandem array in the viral genome, and all either upregulate or downregulate specific subsets of cellular and viral genes. All three EBNA3 proteins can interfere with EBNA2 activation by disrupting its interaction with the DNA-binding protein RBP-Jk, thereby suppressing EBNA2-mediated transactivation (Robertson et al., 1995). EBNA3A and 3C are critical for transformation of primary B lymphocytes (Tomkinson and Kieff, 1992), whereas EBNA3B is dispensable (Tomkinson and Kieff, 1992). Importantly, EBNA3C can promote transformation, cellular proliferation and override the G1–S phase checkpoint by cooperating with the protooncogene Ras, Rb tumor suppressor as well as EBNA2 and EBNA3A in EBV-infected lymphocytes (Knight and Robertson, 2004; Knight et al., 2005a; Parker et al., 1996). These latency-associated transcripts and proteins of EBV thus create a complex regulatory network of activities important for cell proliferation as well as targeting of the latently infected cell from being recognized and eliminated.

Similar to the latent expression of genes required for EBV transformation of primary B lymphocytes, KSHV expresses seven latent genes with modulatory effects on host cell growth: ORF73 (LANA), ORF72 (v-cyclinD), K13 (vFLICE), K12 (kaposin A), K11.5 (vIRF-2), K10.5 (LANA-2) and K15 (LAMP) (Dourmishev et al., 2003). LANA, v-cyclinD and vFLICE are all expressed from the same locus by polycistronic, differentially spliced mRNAs (Dittmer et al., 1998; Sarid et al., 1999). LANA has been demonstrated to be a multiple function protein. It associates with transcriptional activators/repressors (An et al., 2004; Cai et al., 2007; Groves et al., 2001; Hyun et al., 2001; Jeong et al.,, 2001; Knight et al., 2001; Muromoto et al., 2006; Renne et al., 2001; Verma et al., 2004), subverting tumor suppressors, blocking apoptosis and stimulating cellular transformation (Cai et al., 2006b; Friborg et al., 1999;

Fujimuro et al., 2003; Radkov et al., 2000; Si and Robertson, 2006) as well as maintaining viral episomes (Kedes et al., 1997; Kellam et al., 1997; Rainbow et al., 1997). Another critical latent protein in KSHV is viral cyclin D (vCyc or kCyc). It diverges from its cellular counterpart with a wider range of substrates as targets when associated with cdk6 complexes (Swanton et al., 1997). The phosphorylation of RB1, histone H1, p27, Bcl-2 and ORC-1 stimulated by vCyc/cdk6 is required for efficient stimulation of cell cycling, apoptosis and replication by the vCyc (Chang et al., 1996; Ellis et al., 1999; Godden-Kent et al., 1997; Swanton et al., 1997).

8.9 Maintenance of Latency

Once latency is established by the expression of viral latent genes, the resident virus needs to further maintain the infection during latency. So far, little is known about the required factors for maintenance of latency in the γ-herpesviruses. But cytokine signaling of γ-herpesvirus membrane proteins seems to play a major role in the maintenance of latency as well as in developing malignancies (Damania et al., 2000). For example, several latent membrane proteins (i.e., HHV-8 K1 and K15; EBV LMP1 and LMP2A) of the γ-herpesviruses have been shown to provide constant chemokine signaling, which results in growth and activation of the cells, without disturbing the latent state of the virus (Ackermann, 2006). Also, the host-derived genes like vIL-10, vBcl-2, vFLIP and vFGAM-S may provide signals that favor the survival of latently infected cells within the organism (Ackermann, 2006). Therefore, failure of the immune system to maintain the delicate balance between restricted growth of latent cells for maintenance of latency and uncoordinated multiplication of infected cells can contribute to the development of diseases associated with γ-herpesvirus infections.

8.10 Sites of Latency

The specific sites of in vivo latency for different γ-herpesviruses are not yet clear. However, for EBV, the memory B cells have clearly been identified as the site of latent EBV infection (Ackermann, 2006). Additionally, the latent KSHV and MHV-68 are considered to reside in the B-cell compartment.

8.11 Lytic Cycle

In the life cycle of herpesvirus, the full lytic replication of herpes simplex virus type 1 (HSV-1) or cytomegalovirus (CMV) can be accomplished by infection of numerous cell types. However, such an efficient lytic replication system does

not exist for EBV and KSHV. The lytic replication process of EBV and KSHV is quite distinct. During lytic stage, virus expresses the lytic ORF proteins by an ordered cascade. Based on the order of gene expression, the virus-encoded ORFs expressed can be further divided into immediate early (IE), early (E) and late (L) (Ackermann, 2006). For immediate-early genes, there are three key transactivating proteins encoded which are critical to initiate lytic stage: Two of these are encoded by the highly conserved open reading frames (ORF50/Rta and ORF57/Mta) (Byun et al., 2002; Malik et al., 2004) and the third by a non-conserved gene (K8 of HHV-8; ZEBRA of EBV) (Sinclair, 2003; Zhu et al., 1999). The immediate-early proteins are critical for viral reactivation and their direct targets of transactivation will also be essential for lytic reactivation of EBV and KSHV (Hammerschmidt and Sugden, 1988; Rickabaugh et al., 2005; Wang and Yuan, 2007; Yu et al., 2007). The promoter activation mediated by these proteins has a strong effect on DNA synthesis from the origins of lytic DNA replication due to expression of the DNA polymerase, helicases and other ancillary proteins. As a result, virions are generated and released from the productively infected cells.

Lytic replication differs from the latent replication in which multiple rounds of replication are initiated within the viral lytic origin (i.e., EBV *oriLyt*) (Hammerschmidt and Sugden, 1988) and the replication process is (for the most part) dependent on virus-encoded proteins. Once the immediate-early lytic genes are expressed, these transactivators will activate viral and certain cellular promoters, leading to an ordered cascade of viral gene expression: activation of early gene expression followed by the lytic cascade of viral genome replication and late gene expression (Renne et al., 1996a, b). In the viral productive cycle, the virus genome is amplified more than 100-fold (at least for EBV) (Renne et al., 1996a, b). The head-to-tail concatemeric viral DNA caused by DNA replication is subsequently cleaved into unit length genomes and packaged into virions in the nucleus (Renne et al., 1996a, b).

Although a low level of spontaneous lytic gene expression can be detected in most latent EBV- or KSHV-infected cell lines (Fakhari and Dittmer, 2002; Jenner et al., 2001; Paulose-Murphy et al., 2001), the lytic cycle of the γ-herpesviruses is initiated only on rare occasions (Oehmig et al., 2004). Therefore, the expression of Zta or Rta not necessarily known to be important for pathogenicity is now shown to be required for initiation of the replication cycle. So far, studies on the expression of EBV or KSHV in lytic phase predominantly benefited from the ability to culture latently infected LCL or PEL cells with the induction of the lytic cycle with common laboratory chemicals (such as TPA or sodium butyrate) (Yu et al., 1999) or hypoxic treatment (Cai et al., 2006a; Davis et al., 2001).

Additional studies investigating lytic gene expression following reactivation of latent virus have demonstrated that in EBV, *oriLyt*-mediated DNA replication is likely to be biphasic suggesting a two-stage model (Pfuller and Hammerschmidt, 1996). After induction of the lytic cycle, viral DNA is amplified to yield monomeric progeny DNA which is dependent on a functional

oriLyt in *cis* (Tsurumi et al., 2005). The BZLF1 protein then binds to *oriLyt* and the viral DNA is preferentially nicked by DNase I to provide an ideal template for rolling-circle replication. In the late phase, the EBV genome is amplified. To accomplish this *oriLyt*-dependent DNA replication, many lytic proteins that are required have been identified (Tsurumi et al., 2005). One of these proteins, the BZLF1 is assumed to be a key protein controlling the switching of EBV from latent to lytic infection (Adamson et al., 2005; LaJeunesse et al., 2005; Wen et al., 2007). In KSHV, many lytic proteins with a range of different functions have been identified (see Table 8.2). There are six immediate-early

Table 8.2 The lytic proteins associated with KSHV pathogenesis

Proteins	Characteristic	Function	Associated diseases
K2/vIL-6	Homology to cellular IL-6	Associated with gp130 but not gp80, regulate IFN-α, VEGF	Cell proliferation; hematopoiesis, tumorigenesis and angiogenesis
ORF74/ vGPCR	Seven-transmembrane, IL-8 receptor homolog; encoded by a major bicistronic transcript that also encodes the K14/viral OX2	Protein kinase C, protein kinase B, Akt, NF-αB and mitogen-activated protein kinases, IL-1β, TNF-α, IL-6, IL-8, granulocyte macrophage colony-stimulating factor, VEGF, bFGF and MCP-1	Increased transcriptional activity of their nuclear targets, stimulation of cellular proliferation, promotion of cell survival and transformation
K6/vMIP, K4/ vMIP-II and K4.1/ vMIP-III	Homologs of human MIP-Iα, a βCC chemokine; vMIP-II and vMIP-III are encoded together on an IE mRNA	vMIP-I and vMIP-II both engage the chemokine receptor CCR-8; vMIP-III engages the CCR-4 chemokine	Immune evasion and proinflammation
K9/vIRF-1; K10.5/ K10.6/ vIRF-3	CBP co-activates c-myc with vIRF-1	Inhibiting the transcriptional programs; vIRF-3 blocks IFN signaling	Transformation; blocks programmed cell death
ORF16/ vBCl-2	Similarity to human bcl-2	Interaction with the proapoptotic cellular protein Diva	Anti-apoptotic
K7/vIAP	Homolog of the cellular protein survivin-Ex3	Targets two critical arms of the early and late cellular apoptotic response	Protect cells from mitochondrial damage and apoptosis
K3/MIR1, K5/MIR2	A bicistronic transcript for IE gene expression	Stimulates the ubiquitylation; K3 targets all four HLA allotypes, K5 specifically targets HLA-A and HLA-B	Rapid endocytosis of mature major histocompatibility complex MHC class I

Table 8.2 (continued)

Proteins	Characteristic	Function	Associated diseases
K14/viral OX2	A homolog of the cellular OX2 protein	Activates inflammatory cytokine production (IL-1β, TNF-α and IL-6)	Paracrine induction
K1	Oncogenic protein	Activates the NF-κB and PI3K pathways; upregulates VEGF and MMP-9	Transformation; cell proliferation; blocking Fas-induced apoptosis
vGPCR	Oncogenic protein	Activates the phospholipase C (PLC) and PI3K pathways; upregulation of many cytokines and paracrine factors	Transformation; cell proliferation

(IE) loci with multiple transcripts identified: (1) ORF50 (replication and transcriptional activator, Rta), K8 and K8.2 (K-basic-leucine zipper, Bzip; replication-associated protein, RAP); (2) ORF45; (3) K4.2, K4.1 and K4 (vMIPs); (4) ORF48 and ORF29b; (5) K3 and ORF70 and (6) a transcript with no apparent coding potential. Sequentially, for both EBV and KSHV (Liang and Ganem, 2003), the IE-encoded transactivator activates early gene expression which involves DNA replication and metabolism, followed by expression of late genes which produces the viral structural protein (Dourmishev et al., 2003). Studies of transcript architecture from individual loci have also demonstrated that numerous virus transcripts are spliced and many are polycistronic in the *Herpesviridae*.

8.12 Tethering of Viral Genomes

KSHV-encoded LANA and EBNA1 of EBV tether the viral genome to the host chromosomes (Rickinson and Keiff, 2001; Verma et al., 2007). Although these proteins do not share any amino acid sequence similarity they perform similar functions in terms of tethering and replication of the viral genome (Harris et al., 1985; Verma et al., 2007; Yates et al., 1984). EBNA1 is the most promiscuously expressed antigen which helps in harbouring the viral genome in actively dividing as well as resting memory B cells, the likely host for lifelong virus persistence in vivo (Hochberg et al., 2004; Miyashita et al., 1997; Reedman and Klein, 1973; Rowe et al., 1987). EBNA1 is a 641aa long protein with distinct amino and carboxyl terminus. The amino terminal region has the chromosome-binding domain which includes linking regions 1 and 2 (LR1 and LR2) (Mackey et al., 1995; Mackey and Sugden, 1997). The linking region mediates association of

EBNA1 to the mitotic chromosomes. Linking region 2 has been shown to interact with the host cellular protein EBP2 (EBV-binding protein 2) and this interaction is necessary for bridging EBNA1 to the mitotic chromosome (Kapoor and Frappier, 2003; Kapoor et al., 2001; Shire et al., 1999; Wu et al., 2000). However, it has been experimentally shown that replacing LR2 with multiple copies of LR1 in EBNA1 was sufficient to support replication and maintenance of the *oriP*-containing plasmids (Sears et al., 2004). Amino acid sequence analysis of LR1 and LR2 revealed that both LRs are rich in glycine–arginine repeats (Sears et al., 2004). Glycine–arginine repeat motifs are generally referred to as AT hooks which bind to AT-rich DNA and so the LR1 and LR2 of EBNA1 can function as an AT hook protein (Sears et al., 2004). Additionally, replacement of both the linking regions of EBNA1 with the cellular protein HMGI/Y which has AT hook activity supports replication and maintenance of the *oriP* plasmids (Hung et al., 2001; Reeves and Beckerbauer, 2001; Sears et al., 2003, 2004).

KSHV-encoded latency-associated nuclear antigen (LANA) is a 1162aa long protein which migrates at a molecular size of 220–230 kDa on denaturing SDS-PAGE (Schwam et al., 2000). LANA also has a distinct amino, carboxyl terminus and a large middle glutamic acid and glycine repeat region (Verma et al., 2007). The amino terminus of LANA has a nuclear localization signal and a chromosome-binding sequence but lacks any distinct AT hooks seen in EBNA1 (Verma et al., 2007). The nuclear localization signal (NLS) sequence was mapped to amino acids 24–30 of LANA (Piolot et al., 2001; Schwam et al., 2000; Szekely et al., 1999). The LANA NLS is highly homologous to the NLS of EBNA1 (Schwam et al., 2000). In particular, these sequences have phosphorylation sites, and phosphorylation of the residues close to or within the NLS may modify the nuclear import of LANA similar to numerous cellular proteins during the course of the cell cycle (Boulikas, 1993). Besides the amino terminus, the carboxyl terminus of LANA also has a NLS and studies have demonstrated that both the amino and the carboxyl termini of LANA can localize to the nucleus (Schwam et al., 2000). The amino terminus of LANA exhibits a diffuse pattern seen in interphase nuclei typical of many transcription factors, however, the carboxyl terminus accumulates as discrete nuclear speckles reminiscent of the punctate pattern shown by full-length LANA (Friborg et al., 1999; Glenn et al., 1999; Schalling et al., 1995).

LANA tethers the viral genome to the host chromosome through interaction with a number of host cellular proteins (Verma et al., 2007). The cellular proteins which are shown to bind with LANA are histone H1 (Cotter and Robertson, 1999; Verma et al., 2007), a chimeric protein in which the chromosome-binding sequence (CBS; 5–32aa) replaced with histone H1 tethered the chimeric LANA and genome to the host chromosome and supported replication of TR-containing plasmid(Shinohara et al., 2002). Other cellular proteins which are shown to interact with LANA are methyl CpG-binding protein MeCP2 and the 43 kDa DEK protein (Krithivas et al., 2002). The MeCP2-binding domain of LANA was mapped to the amino terminal chromosome-

binding domain of LANA and the DEK binding region to the carboxyl terminus of LANA (Krithivas et al., 2002). Additionally, LANA was also shown to interact with the host chromatin-binding protein Brd2/RING3 (Viejo-Borbolla et al., 2005). A recent report has suggested that LANA can associate and attach to the surface of the core nucleoproteins, H2A and H2B (Barbera et al., 2006). However, this study was primarily done with the extreme amino terminus of the LANA (1–32aa) which contains a high ratio of charged residues that may have influenced the high affinity to extremely charged core histone proteins and needs to be further analyzed in terms of function. Recent data based on the pull-down assay of the host nuclear proteins with the LANA amino terminal domain followed by sequencing of the bands did not reveal any core nucleosomal proteins (Kaul et al., 2007). However, a large number of cellular proteins were detected which are currently under investigation. Based on the data so far LANA can recruit a number of cellular proteins and is involved in tethering and efficient segregation of the viral episomal DNA in dividing tumor cells (S. Verma, H. Si and E. S. Robertson, unpublished data).

Based on the information so far it is clear that both EBNA1 and LANA can utilize a complex set of proteins to tether to the host chromosome through their amino terminal domain (Rickinson and Keiff, 2002; Verma et al., 2007). The carboxyl terminal domain binds to the respective viral DNA by recognizing a unique nucleotide sequence (cognate sequences) (Ballestas and Kaye, 2001; Cotter, Subramanian, and Robertson, 2001; Garber et al., 2002; Yates et al., 1984). The crystal structure of the DNA-binding domain of EBNA1 has been solved (Bochkarev et al., 1996). This crystal structure revealed the presence of a core domain which is required for dimerization and a flanking domain which contains the DNA-contacting residues (Bochkarev et al., 1996; Summers et al., 1996). The DNA-binding domain of LANA has not been crystallized but the secondary structure of the carboxy terminal domain of the protein shows a highly similar organization of amino acid residues important for binding to the DNA (Grundhoff and Ganem, 2003).

8.13 Replication of Viral Genomes

EBV genome has multiple binding sites for EBNA1 and these regions are referred to as the family of repeats (FR) and the dyad symmetry (DS) (Yates et al., 1984). The family of repeats has approximately 20 binding sites for EBNA1 and is required for efficient persistence of the viral genome, whereas the DS element contains 4 EBNA1-binding sites and is considered as the replication initiation site (Gahn and Schildkraut, 1989). Each binding site is comprised of an 18 bp sequence (5′-CGGGAAGCATATGCTACCCG-3′). EBNA1 binds to a pair of EBNA1-binding sites which are spaced 21 bp apart center to center (Reisman et al., 1985). Similar to EBV, KSHV has a replication initiation site located within the terminal repeat region which is present in

approximately 30–40 copies in each genome (Russo et al., 1996). Each terminal repeat unit is an 801 bp long, high GC-containing DNA element capable of supporting replication (Lagunoff and Ganem, 1997; Verma et al., 2006a, 2006c). LANA binding sites have been identified in the terminal repeats which comprise a 13 bp sequence separated by a 22 bp center to center and these binding sites are termed as LBS1 and LBS2 (LBS1/2) (Ballestas and Kaye, 2001; Cotter et al., 2001; Hu and Renne, 2005). Both these sites are required for efficient replication of the plasmid (Hu and Renne, 2005; Verma et al., 2007). However, LBS1/2 alone cannot support replication of a plasmid but requires a 32 bp region upstream of the LBS1, suggesting that replication initiation starts within the upstream region called RE (Hu and Renne, 2005). Binding of EBNA1 and LANA at their cognate sites leads to the bending of DNA which in turn provides the site for binding of other cellular replication proteins (Bashaw and Yates, 2001; Wong and Wilson, 2005).

Chromatin immunoprecipitation assays performed on the EBV and KSHV genome suggest that origin recognition complexes are recruited to these sites in an EBNA1- and LANA-dependent manner (Chaudhuri et al., 2001; Schepers et al., 2001; Stedman et al., 2004; Verma et al., 2006a). EBNA1 and LANA have been shown to interact with human origin recognition complexes in vitro as well as in vivo (Verma et al., 2006a). Binding of ORCs to the DNA in close proximity to the cognate sequence of EBNA1 and LANA assembles the replicative helicase minichromosome maintenance proteins (MCMs) required for firing of cellular DNA origins (Cook et al., 2004; Dhar et al., 2001; Verma et al., 2006).

In the recent years while analyzing the genome of EBV for initiation of replication, Norio and Schildkraut reported the presence of a number of replication initiation sites in the EBV genome (Norio and Schildkraut, 2001, 2004). Although the DS element serves as the primary site for replication initiation other sites within the genome are capable of initiating replication as detected by single molecule analysis of the replicated DNA (Norio and Schildkraut, 2004). Similar to EBV, we recently reported the presence of another replication initiation site within the long unique region of the KSHV genome (Verma et al., 2007). This replication origin site is capable of replication even in the absence of LANA expression (Verma et al., 2007). Thus, EBV and KSHV to a large extent share antigens of similar structure and functional characteristics, important for persistence of the virus in the infected cells.

8.14 Cellular Signaling Pathways Usurped by the Large DNA Tumor Viruses

All viruses, however distinct, have rules of survival, which, if not followed, can lead to extinction. They have to escape or overcome immune surveillance and ensure proliferation and survival of the infected cells. These viruses also strive

toward becoming non-pathogenic in an effort to persist indefinitely in the infected host. KSHV and EBV follow these basic rules, almost to the letter. A common set of rules means that similar cellular pathways are targeted by both viruses. Interestingly, a non-homologous set of latent genes results in both viruses targeting the same pathways in somewhat distinct ways but achieving the same end result.

8.14.1 Immune Evasion

Viruses use various means to avoid detection by the host immune system. One obvious mechanism is to maintain as low a viral antigen profile as possible (Banks and Rouse, 1992). Herpesviruses are unique in that they have two distinct infection profiles (Roizman, 1996). The number of viral antigens expressed during de novo infection and the latent life cycle are significantly smaller than the number expressed during the lytic phase of all herpesviruses. Accordingly, EBV and KSHV express eight and six antigens, respectively, during the latent phase of their life cycles (Cesarman and Mesri, 2007; Rickinson and Kieff, 1996). Another trick that viruses can employ to escape immune recognition is mimicry of host epitopes. In herpesviruses, the latent and immediate-early epitopes are also more similar to host epitopes than are the lytic epitopes (Stevenson, 2004).

KSHV latency-associated nuclear antigen (LANA) is functionally similar to the EBV nuclear antigen-1 (EBNA1) protein expressed during viral latency, although they have little or no amino acid similarities (Grundhoff and Ganem, 2003). As outlined earlier, both proteins are instrumental in maintenance of their respective viral episomes. Since expression of both antigens is critical, the viruses have devised a clever way to escape immune recognition of these proteins. EBNA1 escapes antigen processing by inhibiting its own proteasomal degradation and slowing down its own synthesis to reduce defective ribosomal product processing (Levitskaya et al., 1995; Yewdell, 2003; Yin et al., 2003). EBNA1 includes a unique glycine–alanine repeat domain that renders the protein stable by blocking its proteasome-dependent degradation. This inhibits EBNA1 antigen presentation through the class I pathway (Blake et al., 1999; Tellam et al., 2001). Similarly, the QED-rich central repeat region of LANA also retards LANA synthesis and markedly enhances LANA stability in vitro and in vivo (Kwun et al., 2007). Although there is little to no homology in the sequences of LANA and EBNA1, the sequences of both proteins have evolved to minimize provoking CTL-mediated immune responses.

Other mechanisms of immune evasion target innate immunity rather than adaptive immunity. KSHV has developed a unique mechanism for antagonizing cellular IFN-mediated antiviral activity by incorporating viral homologs of the cellular IRFs (Moore et al., 1996). KSHV-encoded vIRFs specifically interact with either the DNA-binding domain or the central IRF association domain of cellular IRFs, and this interaction leads to the inhibition of IRF-

stimulated IFN-mediated immunity (Burysek et al., 1999; Gao et al., 1997; Lubyova and Pitha, 2000; Moore et al., 1996). EBV on the other hand seems to recruit IRF7 to regulate its latency (Zhang and Pagano, 1999).

8.14.2 Cell Cycle Deregulation

KSHV and EBV have evolved to promote proliferation of infected cells, thereby expanding the pool of infected cells without progeny virus production and so minimizing a strong CTL response (Banks and Rouse, 1992). Since most cells in disease states associated with both viruses are latently infected, it falls upon latent genes to drive infected cell proliferation. Accordingly, some latent genes have the ability to modulate the mammalian cell cycle control mechanisms. KSHV-encoded v-cyclin (Li et al., 1997) mimics its cellular homolog, cyclin-D2, in binding to CDK6 (Li et al., 1997). This functional heterodimer can phosphorylate and inactivate the retinoblastoma protein (Li et al., 1997), a major cell cycle regulator. However, the v-cyclin/CDK6 complex surpasses its cellular homolog in displaying broader substrate specificity, enhanced stability and constitutively active state (Swanton et al., 1997). In addition to CDK6 substrates, this complex can also phosphorylate CDK2 substrates (Li et al., 1997) and it does not require activation by the upstream kinase, CAK (Kaldis et al., 2001). In addition, viral CDK complexes are also resistant to cellular CDK inhibitors, $p27^{Kip1}$ and $p21^{Cip1}$. Resistance to inhibition by $p27^{Kip1}$ is thought to be conferred by v-cyclin/CDK6 mediating its phosphorylation on Ser10 and consequent mislocalization (Jarviluoma and Ojala, 2006). Similarly, v-cyclin/CDK6 also inactivates $p21^{Cip1}$ by phosphorylating it on serine130 (Jarviluoma and Ojala, 2006). Another KSHV protein that plays an important role in cell cycle regulation is its nuclear antigen, LANA (Fujimuro and Hayward, 2004; Fujimuro et al., 2003; Ottinger et al., 2006; Verma, Lan, and Robertson, 2007). LANA has been reported to inactivate Rb (Radkov, Kellam, and Boshoff, 2000), p53 (Friborg et al., 1999) and to enhance E2F activity (Radkov, Kellam, and Boshoff, 2000) for S-phase entry. These LANA functions during latency can modulate the host cell growth machinery in multiple ways and so contribute to B-cell hyperplasia (Fig. 8.6A).

EBV also targets similar pathways to deregulate the cell cycle; however, due to its distinct set of latent genes, its ability to regulate these pathways is somewhat different than that of KSHV. EBV nuclear antigen 3C recruits the SCF^{Skp2} ubiquitin ligase complex (Knight et al., 2005b). This complex is an integral player in cell cycle regulation (Bashir and Pagano, 2004). By recruiting and modulating the activity of this cellular complex, EBNA3C can bypass the G1/S cell cycle checkpoint (Knight et al., 2005a, b). Along with the ubiquitin ligase complex, EBNA3C also appears to facilitate the ubiquitylation and subsequent proteasomal degradation of some of its substrates. In this fashion, EBNA3C is able to inactivate Rb (Knight et al., 2005a) and p27 (Knight et al.,

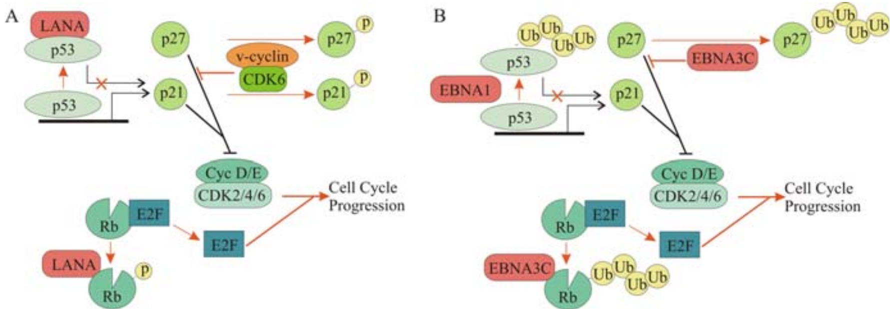

Fig. 8.6 Common cell cycle regulators targeted by KSHV and EBV. Viral molecules are shown in shades of red and cellular molecules in shades of green. Signals/pathways usurped by the viruses are shown in orange. (**A**) Mechanism of inactivation of important cell cycle inhibitors, p21, p27, Rb and p53 by KSHV. (**B**) Mechanism of inactivation of the same inhibitors by EBV

2005b), both major cell cycle regulators. Evidence also suggests that EBV infection also results in modulation of p53 activity (Holowaty et al., 2003a), but exactly how this is achieved remains to be elucidated (Fig. 8.6B).

8.14.3 Anti-apoptosis

Escape of infected cells from apoptosis is advantageous for both viral expansion and its survival. In the latent stage of infection, latent genes of EBV and KSHV usurp cellular apoptotic pathways to prevent infected cell death. p53 is another important apoptosis-related gene targeted by both KSHV and EBV. KSHV LANA interacts with p53, leading to abrogation of p53-induced cell death during latency (Friborg et al., 1999). LANA's functional EBV homolog, EBNA1, plays a similar role in inactivation of p53-induced apoptosis in EBV-infected cells (Holowaty et al., 2003b). USP7/HAUSP is a de-ubiquitylation enzyme that has been implicated in regulating the p53–Mdm2 pathway by stabilizing p53. Competitive binding of EBNA1 to USP7/HAUSP disrupts p53 stabilization and thus protects cells from p53-induced apoptosis (Holowaty et al., 2003b). Recently, one of the cellular E3 ubiquitin complexes, EC_5S, was found to be specifically recruited by LANA for degradation of the tumor suppressors von Hippel-Lindau (VHL) and p53 (Cai et al., 2006b).

For infected cells in the lytic phase of infection, KSHV and EBV, both encode homologs of cellular anti-apoptotic genes to protect the host cell from itself. The Bcl-2 homolog expressed by both viruses seems to protect infected cells in the lytic cycle from apoptosis. KSHV-encoded vBcl-2 has both sequence and functional homology to cellular bcl-2 (Cheng et al., 1997; Sarid et al., 1997). vBcl-2 is expressed in spindle-shaped cells at the late stages of the Kaposi's sarcoma lesions, and the anti-apoptotic effect of KSHV vBcl-2 seems to contribute to Kaposi's sarcoma progression (Widmer et al., 2002). Similarly, EBV

also encodes a Bcl-2 homolog, BHRF1, an early lytic cycle protein (Becker et al., 1991; Marchini et al., 1991). The function of BHRF1 resembles anti-apoptotic Bcl-2 in some cell types and contributes to both the initial evasion of apoptosis during early infection and the establishment of latency for cellular transformation (Becker et al., 1991; Marchini et al., 1991).

One of the most interesting features of EBV and KSHV biology is the complex relationship between infection and host immunity. This point is illustrated by the increased incidence of specific types of EBV- and KSHV-infected lymphoproliferative diseases/malignancies in immunocompromised individuals (Boulanger et al., 2001; Theate et al., 2003; Verma et al., 2005; Webster-Cyriaque et al., 2006). EBV infects asymptomatically most adults worldwide and establishes latency in a small proportion of B lymphocytes. However, in severely T-cell-immunodeficient organ transplant recipients, these latently infected cells can grow unbridled, much like primary cultures of EBV-infected B cells in vitro (Waller et al., 1993). Reducing immunosuppressive medications can effectively control the EBV-infected proliferating B cells (Bunnapradist et al., 2002; Cao et al., 1999; Raghavachar et al., 1996). Kaposi's sarcoma, a multifocal angioproliferative disease composed of KSHV-infected spindle cells, is a very rare malignancy in the general population, yet it is the most common cancer in immunocompromised acquired immunodeficiency syndrome (AIDS) patients (Silvestris, 1999; Swinnen, 2001).

Molecular virologic studies support the hypothesis that herpesvirus reactivation not only enhances dissemination but also potentially contributes directly to lymphoma through expression of viral lytic genes, many of which encode growth deregulatory and immunomodulatory proteins (Ackermann, 2006) (Table 8.2). Numerous lytic proteins also have the potential to enable infected cells to avoid or inhibit the host immune system (Ackermann, 2006). Collectively, these viral proteins counteract multiple levels of the immunological response to viral infection and may play dual roles in growth modulation and immune evasion; for example, anti-apoptotic proteins could enable infected cell proliferation while inhibiting destruction by host immunocytolytic activities.

Despite having developed numerous strategies for spread in humans and evasion of immune recognition, the most common outcome of infection with EBV and KSHV in humans is the establishment of a symbiotic relationship with the host. After primary infection, these viruses establish long-term latent infection in humans with only intermittent reactivation and shedding for horizontal transmission (Ackermann, 2006). All of these stages of virus infection commonly occur without apparent disease. Over the last several years, we have gained considerable insight into the biochemical mechanisms of cell–virus interactions and the diseases associated with herpesvirus infection, and progress toward the development of therapeutic strategies is based on this knowledge.

Cancers are the result of a disruption of the normal restraints of gene expression on cellular proliferation. There are two kinds of genes in which altered expression can lead to loss of growth control (Damania, 2007): (1) Oncogenes: The encoded proteins are stimulatory for growth and cause cancer when

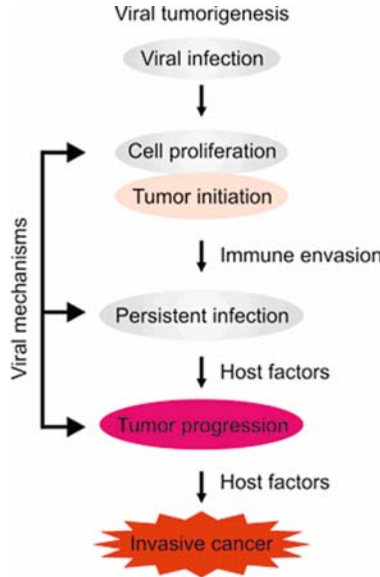

Fig. 8.7 The invasive process of γ-herpesvirus-mediated tumorigenesis. Cancers are usually the result of a disruption of the normal restraints on cellular proliferation. Once viruses infect host cells, the virus which has oncogene or inhibitor of tumor suppressor initiates normal cells aberrant proliferation and induces primary tumor formation. Then, based on the viral infection mechanism, the virus further blocks the host immune response system to establish persistent infection, followed by usurped host co-factor to promote tumor progression and cause the cancer invasion

hyperactive. (2) Tumor suppressor genes: The proteins can inhibit cell growth and cause cancer when inactivated (Damania, 2007). Normally, cellular oncogenes are not expressed in quiescent cells since they are involved in growth and development or they are expressed at low levels (Damania, 2007). Therefore, it is apparent that tumor viruses can obtain a cellular gene during its evolution and this gene can subsequently become altered or aberrantly expressed even though the cells do not themselves carry a viral oncogene. The involvement of viral and cellular oncogenes in tumors has led to the elucidation of the mode of immune invasion of DNA tumor virus (Fig. 8.7).

8.15 Applications

8.15.1 Large DNA Viruses as Vectors for Gene Therapy

The efficient gene transfer of bioengineered genetic material into target cells is a prerequisite for any gene therapeutic approach to alleviate disease states (Kohn et al., 1989). The efficiency of gene delivery to B cells is one of the limiting

factors in cell-based therapeutic strategies as B cells are commonly refractory to transducing gene vectors (Hellebrand et al., 2006). Due to its natural tropism for human B cells, EBV might be a viable option for efficient delivery of genetic material to these cells (Hellebrand et al., 2006). EBV also has many other properties that make it extremely attractive as a potential gene therapy vector. They are essentially episomal self-replicating systems (Conese et al., 2004), with the capacity for carrying large amounts of foreign DNA material. Episomal maintenance mitigates the safety issues associated with inadvertent activation of protooncogenes associated with integration gene of the therapy vectors into the host genome (Kurth, 1995). Self-replication ensures long-term transgene expression, a problem often associated with non-viral gene therapy approaches. However, it has an order of magnitude higher transgene-carrying capacity that truly sets large DNA virus-based vectors apart from their RNA and small DNA virus-based vectors. Truly successful therapy, it has long been felt, requires the delivery of an extra minichromosome. For it is often desirable to deliver not only the therapeutic gene but also the control genes accompanying the therapeutic gene for the therapy to be successful. Also, it has been demonstrated that persistent gene expression can best be obtained by using the genomic locus of a transgene (White et al., 2002). This includes endogenous tissue-specific promoters and potential *cis*-acting elements in upstream/downstream and intronic DNA (White et al., 2002). However, the size of most genomic loci precludes their use in RNA and small DNA virus-based gene therapy vectors. EBV-based vectors have been shown to carry as much as 120 kb of transgenetic material (White et al., 2002).

Nonetheless, EBV is an oncogenic virus and any vector based on EBV has to overcome the associated safety concerns. Accordingly, EBV vectors missing critical viral genes have been made that are consequently non-replicating and thus far non-oncogenic (Banerjee et al., 1995). A number of disease states are excellent candidates for such a therapeutic intervention. Over 120 primary inherited immunodeficiency diseases have been identified and the involved genes identified. A large fraction of these diseases involve antibody deficiencies and are therefore B cell specific.

A better understanding of γ-herpesvirus biology would undoubtedly lead to design of vector systems that safely eliminate the associated safety concerns and yet retain the efficiency of the wild-type virus. One important feature of these vectors is to have a stable replicating element which can persist in the delivered human cells for an extended period of time. Herpesvirus genomes inherently possess such replication element which supports replication in the presence of *trans*-acting viral proteins (Verma et al., 2007; Yates et al., 1985). These *trans*-acting proteins are mostly immunogenic and therefore are not ideal to use in the gene delivery vectors. A recently published report has identified a region in the genome which can replicate even in the absence of any *trans*-acting viral proteins (Verma et al., 2007). This replication element can potentially be used as a replicon origin for gene therapy-based vectors as this element can support autonomous replication in human cells and therefore the DNA can persist long term, a critical function for gene therapy vectors.

Acknowledgments This work was supported by Public Health service grant from NCI CA072510, CA091792 NIDCR DE014136, DE017338 and NIAID AI067037 (E.S.R.). E.S.R. is a scholar of Leukemia and Lymphoma Society of America. S.C.V. is supported by the NIH pathways to independence award (K99CA126182).

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Chapter 9
Epidemiology of EBV Infection

Rosemary Rochford

9.1 Introduction

Following the discovery of EBV, studies by Gertrude and Werner Henle and others were done to determine the distribution of EBV in the general population. Because EBV was initially isolated from a Burkitt's lymphoma cell line, there was some thought that the virus would not be very prevalent and would be geographically restricted like the tumor from which it was derived. As it turned out, EBV is ubiquitous in the world's population with an estimated greater than 90% of adults worldwide infected. Even remote populations such as those living in the Melansian islands or the jungles of the Amazon are infected with EBV (Black et al. 1970; Lang et al. 1977). For most, primary infection is asymptomatic and the virus remains as a benign latent infection for the lifetime of the host. But primary infection in young adulthood can cause infectious mononucleosis, a benign self-limiting disease. In addition, EBV has been associated with a number of different malignancies in both immunocompetent and immunocompromised hosts and the classification of the virus as a group 1 carcinogen highlights the impact of this virus on human health.

How then to link infection with disease? Numerous studies—both epidemiologic and molecular—have etiologically linked EBV with the endemic form of Burkitt lymphoma, nasopharyngeal carcinoma, a subset of Hodgkin disease and gastric carcinomas (see Chapters 10). Associations of the virus with other malignancies including breast cancer still need further analysis to determine a causal link. The ubiquity of EBV in the population points to the requirement for other co-factors in the etiology of EBV-associated cancers. For endemic Burkitt lymphoma, malaria is a clear co-factor (Morrow 1985) but the cellular processes leading to malignancy still need to be determined. For nasopharyngeal carcinoma, familial history, certain HLA class I genotypes and consumption of salt-preserved fish are considered risk factors (reviewed in (Chang and Adami

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_9, © Springer Science+Business Media, LLC 2009

2006)) but the decades long period between EBV infection and onset of disease remain challenges to discovering the pathways to malignancy.

Although the elegant studies done in the 1960 s and 1970 s by the Henle's, De The and others clearly mapped out many aspects of EBV epidemiology, research on EBV epidemiology is, perhaps, not yet complete. New technologies have allowed detection of viral DNA as a marker of infection rather than relying on serological markers of infection. And the increased sensitivity of the assays is changing some of what we know of the transmission of EBV. The ability to rapidly amplify and sequence the viral genome has led to renewed questions regarding the complexity of EBV infection and the link of different EBV sub-types to disease. This chapter will focus on the natural biology of EBV infection in the population. For comprehensive reviews of the early studies on EBV epidemiology, readers are referred to the papers of De The (de-The 1980; de The 1982).

9.2 Modes of Transmission

EBV is a strict human pathogen although some species of monkeys (e.g., cotton top marmosets) can be experimentally infected with EBV. EBV is transmitted primarily through contact with saliva. Virus in saliva was initially measured using a transformation assay that detects infectious virus by the ability of saliva extracts to transform lymphocytes in culture. Transforming virus can be detected for years after infectious mononucleosis (Miller et al. 1973). Studies using PCR for direct amplification of viral DNA indicate that viral DNA can be readily detected in saliva in 100% tested infected adults (Ling et al. 2003) and children (Mbulaiteye et al. 2006).

Oral transmission through direct contact with infectious saliva is considered to be the primary route of transmission in young adults. Infectious mononucleosis was originally called the "kissing disease" to indicate its spread through direct contact with saliva. The virus can also be spread through indirect contact with contaminated eating utensils, toys or fingers. Indirect contact with infected saliva is thought to be the primary route for infection of infants and young children (Henle and Henle 1970). In developing countries, premastication of food by mothers suggest a possible reason for higher rates of transmission to infants.

EBV DNA, cell-associated virus and infectious virus can be detected in cervical secretions of women and men (Sixbey et al. 1986; Israele et al. 1991; Andersson-Ellstrom et al. 1997; Enbom et al. 2001) suggesting that sexual transmission of the virus is possible. Two extensive cross-sectional studies examined whether sexual activity was a risk factor for EBV positivity in heterosexual transmission and increased risk of infectious mononucleosis (Woodman et al. 2005; Higgins et al. 2007) and a third, smaller study examined the possibility of sexual transmission in homosexual males (van Baarle et al.

2000). Although it is difficult in these studies to segregate sexual activity that precludes oral transmission, there was a greater risk for EBV seropositivity with increased number of sexual partners (Crawford et al. 2002; Woodman et al. 2005). Questions remain as to whether levels of virus in cervical secretions are high enough to transmit virus and whether the route of infection (oral versus sexual) predispose to infectious mononucleosis.

If EBV is shed in the cervix, this also suggests the potential for perinatal transmission of mother to child at birth. Although there is no direct evidence for perinatal transmission, de The had hypothesized this to be a risk factor for endemic BL (de-The 1977). Maternal antibodies are likely to be a barrier in most cases of transmission to occur from mother to child, but in some circumstances perhaps maternal antibodies would be at a level insufficient to protect the infant from infection. Transmission of EBV through organ transplantations can also occur mostly likely via infected B lymphocytes in the transplanted organ (Cen et al. 1991). Infection via this route is a significant risk factor for development of post-transplant lymphoproliferative disorder in EBV-seronegative donors (Haque et al. 1996).

9.3 Seroepidemiology of EBV Infection

The pioneering studies of the Henle's used acetone-fixed smears of the Burkitt's lymphoma cell lines EB1 and EB2, followed by indirect immunofluorescence staining to identify the elevated levels of what we now know is anti-viral capsid antigen (VCA) antibodies in serum from patients with endemic Burkitt's lymphoma ((Henle and Henle 1966; Henle et al. 1969). These studies were then extended to defining unique patterns of EBV antibodies in serum from patients with nasopharyngeal carcinoma and infectious mononucleosis (reviewed in (Henle and Henle 1980)) and helped to define the etiologic association of EBV with these diseases. Newer methods have emerged to characterize EBV serology including ELISA-based assays using well-characterized EBNA-1 and VCA peptides (Fachiroh et al. 2006). These methods are used primarily for diagnosis of EBV-associated diseases but their utility in well-defined seroepidemiologic studies to identify populations at risk before the emergence of malignancy remains an important area of study.

9.4 Age of Infection

There are two peaks of EBV infection as measured by seroconversion, age 2–4 years and 15 years (Henle and Henle 1970). What is striking is the profound difference of EBV seropositivity in children depending on geography and socioeconomic status (Henle et al. 1969; Biggar et al. 1978a). In developing countries, infection occurs early in life for most children while in developed countries,

many are not infected until later in life. For example, in a recent study of Kenya children, 94% of children are EBV seropositive by 3 years of age (Moormann et al. 2005). This result is in concordance with earlier studies on EBV seropositivity in other countries of Africa (Biggar et al. 1978a) as well as more recent studies based in Nigeria (Martro et al. 2004) as well as Ethiopia (Tsega et al. 1987). Children in Hong Kong were reported to be 61% EBV seropositive by 2 years (Chan et al. 2001), while children in Germany and the USA were between 45 and 47% EBV seropositive (Martro et al. 2004). The latter observation is consistent with earlier studies done in the 1970 s that found US children of 1–2 years of age to be 49% EBV seropositive (Fleisher et al. 1979).

The greater rate of infection of children with EBV early in life has been linked to socioeconomic status (de The 1982) and hypothesized to be due to differences in hygiene (Biggar et al. 1978b). Other possible, but not exclusive, explanations could be a higher rate of viral shedding in family members (Biggar et al. 1978b) or having to do with a lack of protection from maternal antibodies. Maternal antibodies can be detected in infants under 6 months of age and in a comprehensive longitudinal study, Biggar found that evidence of maternal antibodies was protective against EBV infection (Biggar et al. 1978b). However, a separate study showed that some mothers had lower levels of EBV-specific antibodies leaving infants especially vulnerable to infection before 6 months of age (Geser et al. 1982). The converse was true as well, e.g. if children had high titers of maternal antibody, they were protected from EBV infection in the first 2 years of life (Chan et al. 2001). Whether early infection (e.g. <6 months) alters the establishment and/or maintenance of EBV persistence is unknown. The age at infection is important as it impacts on outcome of disease and risk for cancer. Early age at infection is asymptomatic (Biggar et al. 1978a) but increases the risk for endemic Burkitt's lymphoma (de-The 1977). Later age of infection leads to infectious mononucleosis in 20–50% of cases and increased risk for Hodgkin's disease (Glaser et al. 1997, 2005).

9.5 EBV Viral Load in Populations

EBV exists as a latent infection in memory B cells. In healthy, EBV-seropositive adults, there is a stable, low frequency of latently infected cells estimated at 1–200 EBV-positive B cells in 10^5 total B cells (Miyashita et al. 1997). Without enrichment for B cells from peripheral blood, it is difficult to detect EBV-infected cells in healthy EBV seropositive adults by PCR amplification using real-time PCR (Kimura et al. 1999) or quantitative competitive PCR (Rowe et al. 1997). This feature has been exploited to monitor EBV infection by the measurement of EBV DNA in blood, that is the EBV viral load, in individuals thought to be at risk for development of post-transplant lymphoproliferative disorder (PTLD) (Rowe et al. 2001). PTLD occurs in organ transplant recipients following iatrogenic immune suppression and is caused by EBV. Increases

in EBV viral load precede development of the lymphoproliferation and as such, measurement of EBV viral load has become an important diagnostic tool.

A number of methodologies have been used to measure EBV viral load including competitive quantitative PCR and more recently, real-time quantitative (RTQ)-PCR (Rowe et al. 1997; Kimura et al. 1999; Stevens et al. 1999). An important issue is whether to analyze DNA extracted from whole blood, from purified lymphocytes or from plasma (Stevens et al. 2001). Viral DNA in plasma is indicative of both virions as well as viral DNA from lysed cells. Isolation of DNA from lymphocytes would only be primarily indicative of cell-associated infection. However, isolation of lymphocytes is not feasible for large-scale studies and routine clinical monitoring. DNA extracted from whole blood captures both the cell-associated and the cell-free viral DNA and has been used most commonly for purposes of standardization (Stevens et al. 2001). In all cases, it is important to note that measurements of EBV viral load cannot distinguish latent from lytic virus but can measure the burden of infection.

An advantage of PCR-based assays is the applicability to large-scale population-based studies to identify individuals at risk for EBV-associated malignancies. Several studies have used RTQ-PCR to examine EBV viral load in children at risk for endemic Burkitt's lymphoma. EBV viral load was found to be significantly elevated in young children living in a region of Kenya that experiences holoendemic malaria in comparison to children living where there is little exposure to malaria (Moormann et al. 2005) supporting a role for malaria in dysregulating EBV persistence. Two separate studies reported elevated EBV viral load in children living in Ghana (Rasti et al. 2005) and Gabon (Yone et al. 2006) experiencing an episode of acute malaria. Elevated viral loads detected in plasma (Rasti et al. 2005) suggested that malaria induced reactivation of EBV although it cannot be ruled out that malaria induced apoptosis of EBV-infected cells. An interesting point taken from all these studies is the relatively high proportion of African children with a readily detectable EBV viral load suggesting a different pattern of EBV persistence in developing countries. Whether this is due to early age of infection or other factors is unknown.

9.6 Molecular Epidemiology of EBV Genetic Variants

The EBV genome was the first large DNA virus genome to be completely sequenced (Baer et al. 1984). The prototypic virus used was isolated from the B95-8 cell line that was originally derived from a patient with infectious mononucleosis in the USA. Subsequently, a second strain of EBV was described that had significant differences in the sequences of the EBV-transforming genes, EBNA-2, -3A, -3B and -3C (Dambaugh et al. 1984; Rowe et al. 1989; Sculley et al. 1989; Sample et al. 1990; Dolan et al. 2006). The strain based on the B958 sequence is referred to as Type 1, and the second strain is referred to as Type 2. These two strains have also been called Type A and B, respectively. The

complete sequence of an EBV Type 2 strain, the AG876 strain, derived originally from a patient with endemic Burkitt's lymphoma living in Ghana (Pizzo et al. 1978), was completed in 2006 (Dolan et al. 2006). These two strains not only differ in their genotype but also have functional differences in their transforming capacity. Type 1 readily transforms cells in culture, while Type 2 is poorly transforming (Rowe et al. 1989; Sample et al. 1990). In addition, Type 1-infected cell lines poorly reactivate from latency readily, while Type 2 readily reactivates from latency (Buck et al. 1999).

Early assays to assess the prevalence of these two strains in the population relied on amplifying viral DNA first by outgrowth of lymphoblastoid cell lines derived from peripheral blood lymphocytes (Yao et al. 1985). Some argued that this approach resulted in an under reporting of the prevalence of the Type 2 strain of EBV because of its relatively poor transforming capacity. With the advent of more sensitive PCR methods, direct amplification of EBV DNA from peripheral blood has confirmed that Type 1 is the dominant strain in Western countries, a higher prevalence of Type 2 in South America and relatively equal distribution of Type 1 and 2 as measured in a select number of African countries (Zimber et al. 1986; Yao et al. 1991b; Gratama et al. 1994; Falk et al. 1997: Young, 1987 #453). Most studies have reported that co-infection with Type 1 and 2 is detected in less than 10% of adults tested in US and European studies with the exception of HIV-infected homosexual males and transplant recipients where co-infection rates are high (Katz et al. 1988; Sixbey et al. 1989; Gratama et al. 1994; Yao et al. 1996).

Repeats found in the EBNA proteins have also been used to detect minor heterogeneity in virus types and to track EBV infection between transplant recipients and within families (Gratama et al. 1990, 1994). Ebnotyping, as this was called, found that a dominant transforming strain of EBV was found in both oropharynx and blood in a healthy individual and this type was relatively stable over time (Gratama et al. 1994) consistent with the work of others (Yao et al. 1991a). However, more recent studies, as described below, raise questions whether studies relying on the transforming capacity of EBV isolates to assess infection under-represents the diversity of EBV strains in the healthy hosts.

In addition to the differences observed in EBNA 2, -3, 3b and 3c, sequence variations have also been detected in the latent membrane protein (LMP)-1. LMP-1 is an oncogenic protein shown to be essential for EBV transformation (Kaye et al. 1993) and able to independently induce transformation (reviewed in (Rickinson and Kieff 2001)). The description of sequence variation in LMP-1 opened the door to numerous studies investigating the possibility of a link between LMP-1 variants and increased risk for malignant transformation. Common patterns observed include addition of Xho I restriction site in the amino terminus, deletion of 30 bp in the carboxy terminus, variable number of 33 bp repeat and amino acid changes in the amino and carboxy terminus of the protein. At least seven different strains have been identified based on these changes (Miller et al. 1994; Walling et al. 2003). The genetic changes observed in

LMP-1 do not co-segregate with the different EBV types (Khanim et al. 1996; Tierney et al. 2006).

The high frequency of co-infection with EBV Type 1 and 2 in the immunocompromised population raises the question of whether co-infection with EBV types is more frequent due to the immunocompromised state of host or if the immunocompromised state amplifies an already existing diversity of EBV infection. Since the EBV types are immunologically distinguishable based on antibody responses to the EBNA proteins, the detection of co-infection in healthy hosts could suggest that protective immunity is not achieved and superinfection is possible. Multiple studies have examined this question by PCR amplification of EBV DNA from blood or saliva in a number of different populations (reviewed in Walling et al. 2003). Some studies have focused on detection of EBV Type 1 and 2, while others have focused on detection of LMP-1 variants based on heteroduplex tracking assays or PCR amplification followed by sequencing. The percentage of co-infection with Type 1 and 2 has ranged from 0 to 53% (reviewed in Walling et al. 2003) but the consensus has been that co-infection with these two types is rare in healthy adults (Yao et al. 1991a; Gratama et al. 1994). In contrast, studies focusing on detection of LMP-1 variants have concluded that co-infection in healthy adults is relatively common. For example, in a study of nine healthy adults, co-infection with up to five genotypes—based on variants of LMP-1 sequence—was observed in two individuals and differed both in compartment the virus was isolated from (e.g. saliva and blood) and over time (Walling et al. 2003). A separate study using heteroduplex tracking assay to distinguish LMP-1 variants in blood and saliva from 20 healthy EBV seropositive donors found that all individuals tested harbored multiple LMP-1 variants ranging from two to six different variants (Sitki-Green et al. 2003).

A more recent study has extended these studies by identifying allelic variants to the EBNA-2 gene (Tierney et al. 2006). Five different EBNA-2 variants were found in Type 1 EBV strains. Fourteen patients with infectious mononucleosis were followed, and transforming virus was isolated as well as amplification by PCR and heteroduplex tracking assay to identify both EBNA-2 and LMP-1 variants. Thirteen patients had evidence of infection with multiple EBV EBNA-2 and LMP-1 variants confirming other studies that focused on only detection of LMP-1 variants (Sitki-Green et al. 2004; Fafi-Kremer et al. 2005). While the weight of these studies argues that co-infection with multiple EBV variants can occur in immunocompetent hosts, the question remains as to transmission of multiple variants occurs during primary infection or whether it reflects the ability to be re-infected with EBV throughout life. The answer to this question has important implications for the development of an EBV vaccine.

With all the differences in EBV genetics and evidence of phenotypic differences between variants, a key question is whether there are genotypic variants of EBV that correlate with increased disease risk. While there have been numerous studies, a consensus has not yet been reached.

Many questions remain regarding the genetic diversity of EBV. Is the sequence polymorphism found in EBV latent proteins reflective of random genetic variation over time (e.g. is there quasispeciation occurring) or is it evidence of selective pressure? Importantly, do infection with particular EBV strains pre-dispose an individual to risk for a particular cancer? Finally, if that risk exists, how do the sequence changes correlate with functional changes in the protein or pathogenesis of the virus to increase risk? Comprehensive molecular epidemiologic studies with well-defined populations are still needed to answer these questions.

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Chapter 10
EBV Diseases

Joseph S. Pagano

Abstract Epstein-Barr Virus, the first human tumor virus, also causes infectious mononucleosis and hairy leukoplakia. How the same virus can cause both malignant and benign conditions is best understood through the pathogenesis of EBV infection. The virus was discovered in 1962 in African Burkitt's lymphoma; however, the basic molecular lesions in all BL, EBV-negative or positive are chromosomal translocations that activate *c-myc*. EBV causes immunoblastic B-cell lymphomas and is associated with Hodgkin's lymphomas. Monoclonal EBV episomes are universally present in the distinctive epithelial malignancy, nasopharyngeal carcinoma, and in parotid tumors, but only a subset of gastric tumors. Other associations, NK/T-cell lymphomas, some breast cancers, and leiomyosarcoma, are inconsistent and obscure pathobiologically. Vaccines to prevent infection and regimens to treat IM remain under development. However, exploiting virus-specific features of the EBV malignancies therapeutically is showing progress while at the same time illuminating the biology of cancer cells and their genesis.

10.1 Introduction

That EBV is so closely associated with both benign and malignant conditions has been puzzling but can now be rationalized in most cases. The framework for such an understanding includes considerations of virus–cell relations that are etiologic, others that alter cell phenotype during tumorigenesis and immunologic competence and genetics of the host. In some diseases the virus association is inconsistent. In all cases the foundations are in the pathogenesis of EBV infection, given in perspective here and presented in detail in other chapters.

In only three diseases can EBV be taken as the etiologic agent: infectious mononucleosis (IM), EBV lymphoblastic lymphomas and hairy leukoplakia of

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_10, © Springer Science+Business Media, LLC 2009

Table 10.1 Epstein-Barr Virus-Associated Diseases

Benign	
Infectious Mononucleosis	
Hairy Leukoplakia	
Malignancies	
Lymphomas	
Burkitt's	Endemic
B-cell, immunoblastic	Innate and acquired immunodeficiency
Hodgkin's	~40–50%
NK/T-cell*	Japan, Korea
Carcinomas	
Nasopharyngeal	100%
Parotid	Alaskan Inuits
Gastric	~15%
Breast	Association?
Others	
Leiomyosarcoma	AIDS (children)

* See also Chronic Active EBV infection

the tongue (HLP) (see Table 10.1; for discussion of what constitutes an etiologic relation, see [1]). Post-transplantation lymphomas, solitary CNS lymphomas and leiomyosarcoma occur exclusively in hosts with immunodeficient states, mostly acquired. The only EBV disease caused by a heritable genetic disorder is X-linked lymphoproliferative syndrome; others such as EBV-associated hemophagocytic syndrome and chronic active EBV infection may have genetic components. EBV lymphomas also occur frequently in certain rare congenital immunodeficiency diseases.

Burkitt's lymphoma (BL) is of course the original source of the virus and was thought to be its cause in endemic regions where viral genomes were originally detected in most, but not all, of the tumors but in less than a fifth of sporadic cases elsewhere. In contrast common to all BL, endemic or sporadic, whether EBV infected or not, are characteristic chromosomal translocations that activate expression of the *c-myc* proto-oncogene. These molecular lesions are basic to the pathogenesis of BL and are causative for this distinctive lymphoma. EBV can be considered a potent contributory co-factor.

The direct association of Hodgkin's lymphoma (HL) with EBV infection is more recent, based on detection of EBV episomes which, because they are monoclonal, are considered significant—but only in subsets of HL that approximate 50% of HL overall.

Of the carcinomas associated with EBV, NPC is by far the most consistent and longstanding in its connection to the virus, whereas a minor but intriguing subset (~15%) of gastric carcinoma is infected with EBV. Quite distinct are the rare B-cell lymphomas of the stomach that seem to arise as a result of intense immunostimulation by EBV antigens. Uniting all the EBV malignancies is the presence of monoclonal EBV episomes in each tumor cell, which implies that

infection is nontrivial because it must have been present in a single cell that expanded from the outset of the malignancy, not acquired later. Breast cancer is a special case with EBV detected inconsistently and only in some tumor cells of more aggressive forms of the carcinoma.

Presented here is a consideration of this diverse assemblage of diseases, not so much from a descriptive clinical point of view as from a pathogenetic perspective, which supplies threads of connection among them. For complete clinical and diagnostic descriptions the reader must consult reviews cited later. Concluding the chapter is a perspective on this complex panoply of diseases.

10.2 Pathogenesis of EBV infection

EBV is transmitted primarily by virus excreted in saliva, but it can also be transmitted sexually [2, 3]. Like all other human herpesviruses EBV infects both primary and secondary cell types that are different. Initially the virus infects and replicates in differentiated oropharyngeal epithelial cells, but it also infects B lymphocytes in the tonsillar region. Whether the virus infects epithelial cells first or lymphocytes first moving then by cell fusion to epithelial cells (like other herpesviruses, EBV can produce syncytia) is debated; but generally it is the latently infected cell that is the secondary cell type. For the receptors used in these cells, see Chapter 15 (Epstein–Barr Virus Entry) [4]. The virus prefers to replicate in differentiated epithelial cells, an interesting parallel with HPV (see Chapter 4: The Life Cycle of Human Papillomaviruses).

The outcome of infection of the two cell types is quite different. In epithelial cells there is abundant viral replication and cytolysis. Oropharyngeal mucosal cells and perhaps parotid ductal cells are the source of virus released into saliva and in similar fashion virus from cervical epithelial cells [5–7]. In contrast in lymphocytes the infection is predominantly latent, although there is probably an early phase of infection in tonsillar lymphocytes in which the virus does replicate; B cells that differentiate into plasmablasts also support viral replication [9]. Virus regularly gains entry to circulating B lymphocytes, but causes them to proliferate rather than to lyse. This ability to immortalize B cells is the hallmark of EBV infection (see Chapter 12: Epstein–Barr Virus-Transforming Proteins: Biologic Properties and Contribution to Oncogenesis). In normal hosts the wave of proliferation of these cells triggered by infection is checked by intense T-cytolytic responses to EBV antigens expressed by them. Consequently the number of infected B cells subsides, but a few circulating memory B cells survive, and they or their progeny persist for life. The EBV episomes in such cells are maintained by the long-lived EBNA-1 protein, transcripts for which are re-expressed from time to time when resting memory B cells are stimulated to divide [8, 9]. Episodically there is reactivation of viral replication in occasional infected lymphocytes, which can later infect additional epithelial cells and renew virus excretion. Shedding of virus fluctuates usually at low levels for many years. Thus EBV like all other herpesviruses infects a primary cell type

where it freely replicates, producing a virus which can immortalize cells with high efficiency, whereby it moves concurrently to a secondary cell type where it persists in a latent state.

10.3 Infectious Mononucleosis

The classic syndrome occurs typically in post-pubescent young people [10]. Earlier in life silent infection or mild nondescript symptoms are the rule beginning, depending on socioeconomic status, as early as 4–6 months after birth, for example, in BL-endemic regions as maternal antibodies wane. The disease has a long incubation period—4–6 weeks; before that there may be an insidious onset with fever and malaise. IM classically presents with pharyngitis, often severe, that may have its origin in sloughing of infected epithelial cells. The findings include lymphadenopathy, prominent in head-and-neck sites but generalized, and splenomegaly. There is a distinctive atypical lymphocytosis (up to 40%), a manifestation of the characteristic T-cell response; it is not confined to circulating cells, but causes lymphadenopathy and splenomegaly as well as mild hepatic dysfunction, often subclinical, that combine in the familiar syndrome. The Monospot test which detects heterophil antibodies is positive in >90% of cases, and almost all cases of IM can be diagnosed clinically together with these two laboratory findings.

The many complications of IM that affect several organ systems are thought to be caused by the onslaught of T-cell responses and are not infrequent. The general idea is that cytolysis caused directly by viral replication is restricted largely to mucosal surface epithelial cells, whereas abnormalities affecting parenchymal and other organs are due to exuberant T-cell responses to infiltrating infected B lymphocytes and not to replication in and direct damage to these cell types including hepatic, hematologic or neural. A positive consequence of this scenario is that malfunctions in these systems tend not to persist even if severe. Mild hepatitis detected by elevated serum transaminase is a regular feature of IM, but jaundice is rare and transient. The complications of IM include airway obstruction in the posterior oropharynx. Mild hematologic abnormalities such as neutropenia are common; hemolytic anemia, agranulocytosis, aplastic anemia and severe thrombocytopenia are serious complications that require hospitalization. Neurologic complications are diverse and include Guillan–Barré syndrome, aseptic meningitis, encephalitis and transverse myelitis, most of which are rare. Spontaneous splenic rupture is strongly associated with IM but rare.

Since IM represents the results of the first encounter with the virus in normal hosts, almost all the other EBV diseases can be viewed against the backdrop of the responses to infection in this basic pathobiologic scheme.

Symptoms of IM ordinarily last for several weeks but some, especially fatigue, may persist for months. *Chronic active EBV infection* is an extremely rare disorder, more often reported in East Asians than in Caucasians, in which typical IM

becomes protracted into a severe illness lasting more than 6 months that may include chronic fever, hypoplastic bone marrow, uveitis, persistent hepatosplenomegaly and various hematologic abnormalities together with extremely high EBV antibody titers and EBV DNA and antigens detected in affected tissues. This condition is distinctive in that EBV-infected cells include CD_4^+ T cells and NK cells [11]. It is not to be confused with *chronic fatigue syndrome*, a distressing but ill-defined illness consisting of heterogeneous conditions, often somatizations, in most cases not associated with EBV infection [12, 13].

X-linked lymphoproliferative syndrome is triggered by primary EBV infection in rare kindred. The infected boys develop severe unrelenting IM that is ultimately fatal usually because of liver necrosis and failure caused by lymphocytic infiltration, which is widespread also in other organs. Hypogammaglobulinemia is a feature of the disease and may become apparent before EBV infection. Another outcome is development of B-cell lymphomas [14] (for a review, see [15]). XLP is essentially a disorder of regulation of levels of EBV-infected B cells relative to reactive T cells. Hypersensitivity to other viruses has not been recognized. XLP arises through mutation of a gene that encodes an SH2 domain-containing protein called SAP [16] which is involved in lymphocyte signaling pathways and is expressed in T and NK cells.

10.3.1 Burkitt's Lymphoma (BL)

Noted by Dennis Burkitt in the 1950s as a fast-growing tumor of the jaw in children in Uganda, but not reported until 1962 [17], he saw that the disease occurred in geographically distinct areas and lower elevations overlapping the malaria belt and suspected a mosquito-borne virus might be the cause. At this point although many viruses were known to cause cancer in animals, none was mosquito borne, and no human tumor virus was recognized. Initial efforts by Achong and Barr in Epstein's laboratory to detect a virus in BL tissue by EM were unsuccessful until they succeeded in establishing cell lines from BL, in which herpesvirus-like particles were then visualized, although not in all BL lines [18]. Since none of the then-known human herpesviruses was lymphotropic, a new virus was suspected and confirmed to exist by W. Henle and G. Henle because serum from BL patients that recognized an antigen in the cultured BL cells did not react with HSV, CMV or VZV [19]. EBV was named, and H. zur Hausen working with the Henles detected EBV DNA in a BL cell line [20].

Surprisingly EBV antibodies were detected not only in all African BL patients but also in a cross-section of the normal healthy population in the USA. The association with IM was discovered serendipitously when an EBV-negative technician in the Henle laboratory contracted IM and had an antibody response to the virus [21]. Her B lymphocytes reacted with antibodies from BL patients and could grow as an immortalized cell line. That EBV was the causative agent for IM was proven in a seroepidemiologic study of Yale students [22] and by molecular hybridization analyses that detected EBV in saliva [23].

Similar to BL, not all the tumor samples or cells revealed EBV by EM, and it was not until analyses by molecular hybridization of a set of African BL samples collected by G. Klein could it be said that virtually every African BL contained EBV genomes [24–26]. BL remains the most common childhood cancer in the endemic regions of sub-Saharan Africa with an incidence of 10 per 100,000 in children between 2 and 15 years of age.

Endemic BL presents as a localized maxillary or mandibular tumor that is also detected intra-abdominally, especially in the ileocecal region, but it does not usually involve the bone marrow in contrast to the sporadic form of the disease [27]. The histopathology is distinguished by the so-called "starry sky" appearance produced by brightly staining macrophages that stud the otherwise homogeneous field of atypical neoplastic, often mitotic B-lymphoblastoid cells [28]. The rapid growth of the tumor and the equally remarkable response to even as little as a single injection of cyclophosphamide are features usually not shared by sporadic BL. A major difference is the presence of EBV genomes in only about 20% of sporadic cases in contrast to more than 98% of endemic BL [29]. The incidence of BL in the USA and European countries as well as in Africa now exceeds that of endemic regions because of its frequency in patients with AIDS. The presence of EBV in such tumors (20–30%) roughly reflects the presence of the virus in BL in nonimmunocompromised persons, which is remarkable since virtually 100% of persons with AIDS are EBV infected. Classically BL occurs in children without overt immunodeficiency.

However, all BL whether in Africa, the USA, Europe or in other endemic areas in Brazil and New Guinea have in common one of a set of three specific reciprocal chromosomal translocations that result in the reactivation of the *c-myc* proto-oncogene at chromosome 8q24 by placing it adjacent to immunoglobulin regulatory elements on chromosomes 14, 22 or 2 [30]. This then is the essential feature of BL that serves as its molecular pathogenetic basis, and indeed the principal EBV oncoprotein LMP1 is not expressed in BL. EBV antibodies when detected in high titers in children in endemic areas seem to anticipate a greater risk of development of BL [31], with EBV probably contributing as a co-factor by stimulating B-cell proliferation which allows favoring selection of cells with c-myc translocations. Holoendemic malaria, also a likely co-factor, suppresses T-cell responses [32]. However, BL is endemic in Northeastern Brazil, which is not a holoendemic region [33]. BL can be distinguished from diffuse large B-cell lymphomas, some of which have the (8;14) translocation, by gene expression profiling [34].

10.3.2 EBV Immunoblastic Lymphomas

EBV immunoblastic lymphomas are exclusively B cell in type and occur only in severe immunodeficiency, either acquired or inborn. These lymphomas arise from the proliferation of B cells that occurs in the normal course of primary

EBV infection. However, in immunocompromised persons this lymphoproliferative phase is unchecked and escalates because of lack of T cells to recognize and destroy the proliferating infected B cells. More usually such lymphoproliferation has its onset long after primary EBV infection, having its origin in circulating infected memory B cells.

There is better understanding of the genesis of post-transplantation lymphomatous disease (PTLD) since its onset tends to be recognized earlier and progression can be followed. The main factors that predispose to such lymphomas are not only the intensity but the kind of immunosuppressive regimen used. In general, the more intensive the regimen the more likely are lymphomatous complications, but suppression of specific components of the cell-mediated immune response may have disproportionate effects. Lymphoproliferation may be diffuse or localized and is usually not detected in peripheral blood, but it may infiltrate the bone marrow. The proliferation is at first polyclonal in type (as is B-cell proliferation in normal hosts during IM), then evolves to a biclonal state, finally becoming monoclonal [35]. This evolution has implications for treatment. Lifting or altering the immunosuppressive regimen may produce reversal of the proliferation in the polyclonal but not later phases. Such a maneuver is practicable in renal transplants (although it may cause rejection of the kidney), but generally not in heart, liver and bone marrow transplants, where highly intensive therapy must be sustained. Elaboration of a virally encoded cytokine, BCRF1, a homolog of IL-10, is a feature of PTLD that may serve as an autocrine growth factor for the infected B cells.

Somewhat surprisingly since infection in the proliferating cells is latent, there is some anecdotal evidence that antiviral therapy during this phase of the disease may also contribute to its reversal at least transiently [36]. It is far from clear why such a therapy should work, but if it does the effects may depend on whether it is a primary infection and on catching disease early, perhaps by curbing secondary infection of new populations of B cells and by dampening cytokine production in the viral cytolytic cycle [37]. When lymphoproliferation becomes monoclonal it is essentially an autonomous neoplasm no longer responsive to manipulation of immunosuppression, although such cells may still respond to specific virally directed immunotherapy as they continue to express EBV antigens (see Chapter 16: Epstein–Barr Virus Immunotherapy). Monoclonal lymphoblastic disease is notoriously refractory to treatment by conventional means and tends to worsen rapidly and be fatal.

Although continuously proliferating EBV-infected B lymphocytes may acquire various translocations and other genetic abnormalities, there is no stereotypic mutation, and the translocations characteristic of BL do not arise (nor have they been reproduced by prolonged serial passage of EBV-infected B cells in tissue culture [38]). Recent reports have suggested a scenario whereby *c-myc* expression can also be activated by stabilization of β-catenin which acts as a transcriptional co-activator for expression of *c-myc*. Activation of *c-myc* by this route obviates the need for *c-myc*-activating translocations, which are not detected in immunoblastic lymphomas. This signaling pathway is called into

play by deubiquitination and stabilization of β-catenin, which ordinarily turns over continuously in most cells, but not in Type III EBV-infected cells. In contrast in Type I (BL) cells β-catenin turns over rapidly and is transcriptionally inactive [39] (for review, see *Essays in Biochemistry* [40]).

The cells are B-lymphoblastoid in type and express all the Type III EBV latency proteins. They are veritable in vivo counterparts of Type III cells that are cultivated in vitro from normal peripheral blood of EBV-infected persons. EBV alone is able to drive proliferation of such cells in vitro, and it is safe to assume that EBV is the sole cause of such lymphomas in the human culture vessel presented by a patient with profound immunosuppression.

The EBV B lymphomas that arise in AIDS are similar in cellular origin and basic character, with every tumor cell containing EBV episomes and expressing Type III latency proteins, but clinically they are unique, presenting as solitary CNS lymphomas, often without manifestations elsewhere. This distinctive localization perhaps results from the combination of the immune incompetence and the immunologically sheltered site presented by the brain, to which EBV-infected B cells do have access. These CNS lymphomas although invasive and lethal do not metastasize outside the brain. Interestingly not only do the latently infected lymphoma cells express LMP1, the principal EBV oncoprotein, but they also express and activate interferon regulatory factor 7 (IRF7), which itself has oncogenic properties [41] as well as being, somewhat paradoxically, the master regulator of Type I interferon responses induced during viral cytolytic replication [42].

Before immunoblastic lymphomas became a common occurrence in these acquired immunodeficient states, B-cell lymphomas in patients with inborn immunodeficiencies such as Wiskott–Aldrich syndrome, ataxia telangiectasia and severe combined immunodeficiency syndrome were found to be EBV positive, generally intractable to treatment and fatal [43].

EBV is detected frequently but not always in primary effusion lymphomas, which are primarily KSHV-infected B-cell tumors. EBV is not etiologic in these tumors, but whether it is in some way contributory or perhaps alters tumor progression is unknown.

10.3.3 Hodgkin's and Other Lymphomas

Speculation that HL might have an infectious etiology because of the pattern of its age-related incidence and other epidemiologic features antedated its association with EBV infection. That persons with a history of IM have an increased risk for HL has been substantiated in epidemiologic studies, but only with the detection of EBV genomes in the malignant cell population of subsets of HL has this association been placed on a firm basis [44, 45]. While evidence implicating EBV has been gathering, the role of the virus in this malignancy is quite uncertain, yet probably nontrivial and compatible with aspects of the

pathogenesis of EBV infection. The variety of clinical and pathological types of HL argues for multiple etiologic and modifying factors, and whether EBV is etiologic or a modifier of tumor progression remain open questions.

Clinically HL comes to attention with painless swelling of lymph nodes, especially in young adults, but the age-related incidence is biphasic with the second peak above 55 years and has poorer prognosis. In the younger patients cure is achieved in 80%. HL may also present in a disseminated form with involvement of spleen, lung, liver and bone marrow. Systemic symptoms, which occur in about a third of patients, include fevers and night sweats as well as pruritus without its usual causes. Key to prognosis is whether the enlarged lymph nodes are detected in a single nodal region, usually the mediastinum (Stage I), on the same (Stage II) or both sides (Stage III) of the diaphragm or with widespread involvement in extranodal sites (Stage IV) [46]. Diagnosis is based on finding a scattering of Reed–Sternberg (RS) cells in biopsied tissue, the clonal malignant cell, in a background of abundant reactive lymphocytes, eosinophils and histiocytes characteristic of HL. Of special interest for EBV investigations is the recent recognition that RS cells derive from B-cell lineage, which has in part led to the reclassification of HL as *classical* (2–3% RS cells) and *nodular lymphocyte predominant* (variant RS cells with polypoid nuclear contour, but also of B-cell origin) [47]. These comprise distinct disease entities which include the previously classified histologic subtypes, each with their own set of clinical features. Classical HL, the most common, embraces the nodular sclerotic subtype, affects younger people and presents with localized cervical and mediastinal disease; mixed cellularity HL is usually in pediatric and older age groups with more advanced disease; and lymphocyte-depleted HL, which is infrequent, is found in older patients with extensive disease and is also associated with AIDS.

Because of its variegated histopathology the malignant component of HL remained obscure for many years. In particular the origin of the RS cell and indeed whether it was malignant were unsettled. It is now accepted that the RS cells scattered in the abundant cellular response usually found in the lymphoma are its malignant element and that this pathologic cell is germinal B cell in origin. Identification was elusive because RS cells do not express detectable B-cell markers such as immunoglobulin despite containing appropriate Ig gene arrangements. This histopathology sets apart the nodular lymphocyte-predominant form of HL with its atypical RS cells that are described as lymphocytic and histiocytic and resemble “popped corn”; some classical RS cells are also present [48].

Nodular lymphocyte-predominant HL is unique clinically and pathologically with limited nodal disease in the neck, not the mediastinum, but without systemic symptoms, usually in men and indolent in course. The malignant cells are CD22 positive and CD30 and CD15 negative. There may be late recurrences (>10 years) after treatment. The variety and intensity of cytotoxic radiation treatment is based initially on stage of disease guided by histologic subtypes [49].

Type and intensity of therapy depends on stage of disease and includes combination chemotherapy and radiotherapy [50, 51]. In general, cure rates for HL overall are high, but disease is refractory to treatment in 5–10% of patients, and despite complete responses relapses occur in 10–30% of patients.

10.3.4 EBV and HL

A history of IM has been identified as a risk factor for HL in reports dating back to 1981 [52]; even earlier elevated EBV antibody patterns were noted in HL [53]. Direct evidence came with detection of monoclonal EBV episomes in RS cells of some HL. Strikingly, LMP2a antigen was visualized by immunoperoxidase staining on the outer membrane of RS cells, and it is postulated that the anti-apoptotic effect of LMP2a allows cells of B-cell lineage that do not express immunoglobulin to escape apoptosis [54–57]. Hence EBV may rescue such cells and thus contribute directly to HL oncogenesis. LMP1 is also expressed in RS cells of HL and may contribute by activating NF-κB, which is a key feature of RS cells [58, 59]. However, EBV is detected in only about a third of HL cases overall. Unexpectedly it is rarely found in young adults. Rather it is more prevalent with increasing age and in classic HL of the mixed cellularity than the nodular sclerosis subtype. Importantly, the most recent large study shows that IM is associated only with EBV-positive HL [60]. This finding emphasizes the link between symptomatic EBV infection, but it makes no statement about EBV infection generally. The interval between diagnosis of EBV-positive HL and a recalled episode of IM was 4.1 years (median time; range 1.8–8.3 years) [61]. These findings may reflect a causal association but cannot be interpreted as a histologic subtype-specific risk factor. Finally there is some evidence of increased mortality in patients with EBV-positive HL who are more than 45–50 years old [62, 63].

10.3.5 Other Lymphomas

EBV can depart from its B-cell tropism and rarely infect not only T cells but NK cells and produce natural killer/T-cell lymphoma (NKTL). In Korea these tumors account for ~9% of non-Hodgkin's lymphoma (NHL). The T cells affected are NK cell-like and are in the cytotoxic lineage. Clinically the tumors present in extranodal sites, some in the skin or GI tract. Not all these tumors are EBV positive. In general these are aggressive malignancies that do not respond well to either chemotherapy or irradiation. Histopathologically there are distinctive angiogenic and angiocentric features along with fibrinoid necrosis. NKTL is considerably more common in Korea, Japan and China and also in Peru and Mexico, where the association with EBV is stronger than in Europe and the USA [64, 65]. A second group consists of mature T-cell lymphomas that

are peripherally located (PTCL). A clinically distinctive type of NKTL involves the nasopharynx (see 10.5.1, *Hemophagocytic Syndromes*).

10.4 Epithelial Diseases

The early association of EBV with an epithelial malignancy, nasopharyngeal carcinoma (NPC), was at first baffling since EBV was viewed as a lymphotropic virus. The paradox was resolved by finding that the virus could gain entry to and replicate in epithelial cells in the normal course of infection, but presented another paradox: EBV did not normally persist in oropharyngeal epithelial cells, rather it destroyed them (see Sections 10.2–10.4). In Section 10.4 a non-malignant and several malignant conditions arising in epithelial cells are considered.

10.4.1 Hairy Leukoplakia (HLP)

One of the two herald lesions of AIDS, HLP of the tongue, was not recognized until the disease became endemic. It does occur in organ transplant recipients, but less commonly and so must have predated AIDS. HLP lesions are distinctive: they appear as gray-white corrugations on the lateral edges of the tongue and their histopathology is also distinctive, perhaps driven by expression of EBV genes and is characterized by acanthosis (hypertrophy) of the stratum spinosum, hyperkeratosis and koilocytosis but a scanty inflammatory response [66]. Uniquely among EBV diseases HLP is the manifestation of cytolytic and not latent infection. The most remarkable feature of the lesion is the massive number of EBV particles it harbors [67]. The lesion presents a striking instance in vivo of EBV replication in the squamous epithelium and led to the discovery that latency genes could be expressed in cytolytic infection along with expected lytic cycle RNAs [68, 69]. Replication in these epithelial cells is not obviously cytolytic perhaps because of the expression of latency genes that are anti-apoptotic. The lesions are not considered to be premalignant in contrast to oral leukoplakias in smokers, which differ both in location and in histopathology. HLP lesions resolve upon EBV-specific antiviral therapy but tend to recur depending on fluctuations in host immune status. Pseudo-hairy leukoplakia lesions are detected rarely; they do not contain EBV, and their histopathology while similar is not identical. HLP lesions usually harbor several strains of EBV and because of the density of infection may be a preferred site for recombination of viral strains [69]. Perhaps most puzzling is the localization of the infection in tongue rather than buccal or parotid epithelial cells where EBV replicates normally. The virus is not detected in the tongue in normal hosts, but subclinical infection there can be detected in patients with AIDS. The possible

contribution of other viruses and microorganisms to the pathogenesis of HLP continues to be investigated.

10.4.2 Nasopharyngeal Carcinoma (NPC) and Parotid Tumors

Viewed worldwide, NPC is the EBV-associated malignancy with the highest incidence. While not proven to be the etiologic agent of this distinctive tumor, EBV by reason of its universal presence in the form of monoclonal genomes, coupled with unique EBV serologic responses that precede or accompany its onset, make it almost certain that the virus triggers or is at least an indispensable contributor to NPC. Clinically the tumor occurs on average at an earlier age than other head-and-neck malignancies and predominantly in men. It arises in a specific site in the posterior nasopharynx, the fossa of Rosenmuller in Waldenstrom's ring. However, more often than not NPC presents as an enlarged lymph node in the neck, the result of local metastasis, which points to the notoriously invasive character of this tumor.

NPC's histopathology is also distinctive among head-and-neck tumors because it is almost always that of undifferentiated carcinoma. There are, however, three subtypes classified by WHO: Type I, keratinizing carcinoma, which is rare, and Type II nonkeratinizing carcinoma and Type III, the undifferentiated and by far the most common form. Type II and III tumors are made up of single-file rows of tumor cells that have lost typical epithelial tissue structure and histologically show diffusely infiltrative growth. NPC is characterized by lymphocytic infiltration which consists of T cells that are reactive to viral antigens expressed by the tumor. LMP1 is usually expressed in Type II and III NPC, but not often detected in Type I because the EBV episome is absent or its copy number is low [70]. However, the first EBV episome cloned from NPC tissue was from a Type I tumor [71]. NPC tumor cells exhibit phenotypes similar to cells that have undergone epithelial-mesenchymal transition (EMT) found in various carcinomas—strikingly so in Type III NPC—and marked by loss of expression of epithelial genes such as E-, α- and β-catenins and expression of fibronectin, vimentin, N-cadherin and sm-actin, hallmarks of EMT. LMP1 can produce such histologic changes in cell culture models via induction of the metastasis factors, Twist and Snail. These transcriptional factors are indispensable in embryogenesis and are also overexpressed in metastatic but present at lower levels in less-invasive stages of NPC [72]. In fact LMP1 can induce a whole spectrum of invasion, metastasis and angiogenic factors that probably determine the metastatic character of NPC (for a review of this topic, see [73]).

Treatment with surgery, radiotherapy and chemotherapy can improve survival significantly for 5 years and beyond, but cure is rare because of the likelihood of early metastasis. Experimentally, because some of the same Type III antigens are expressed in NPC as in immunoblastic lymphomas, immunotherapeutic approaches are beginning to be entertained. Phosphonated

nucleoside analogs used as antiviral drugs have been shown to cause apoptosis and inhibit growth of NPC grown in nude mice, which may offer another kind of therapeutic approach [74]. Currently the best means to monitor the effects of treatment of NPC is by determining amounts of EBV DNA in the plasma [75]. Various biomarkers that may be applicable for monitoring progression of NPC are under study but not generally available [76].

In deciding whether to assign etiologic status to a tumor virus, the epidemiology of a malignancy may provide the first clues to a possible infectious cause [77]. In the case of NPC the epidemiology is complex and fascinating. As with other cancers caused by viruses NPC exhibits regions of striking endemicity, chiefly in Southern China. This high incidence is retained in Chinese who reside in Hong Kong, Taiwan, Singapore and Kuala Lumpur as well as California, where, however, the incidence wanes after one to two generations, which has been thought to suggest a dietary co-factor retained in Chinese émigrés in the Far East but lost in the USA. North Africa also has endemic foci for NPC, but in Caucasians, although the incidence is lower than in China. Similarly emigrants from Africa to France at first have a relatively high incidence of NPC which wanes in succeeding generations, perhaps again associated with changes in diet. NPC also has relatively high incidence in Alaskan Inuit people. Although there is some evidence for genetic predisposition to NPC the heterogeneity of the Chinese population has defied clear-cut genetic patterns. Finally NPC occurs sporadically in Western countries at much lower incidence (~1/100), and the peak age distribution shifts from ages 40 to 50 or 60 years in Chinese to younger ages; indeed sporadic NPC while rare is the most common carcinoma in children. Even more baffling is the bimodal age distribution of endemic NPC in North Africa, with peaks in incidence in the teens and young adults as well as the middle-aged. Etiologically complex, NPC has been linked to dietary, environmental and genetic co-factors. Exposure to several EBV-inducing tumor-promoting agents including nitrosamines has been implicated in high incidence areas, and polymorphism in a nitrosamine-metabolizing gene may affect susceptibility to NPC [78]. EBV isolates can be classified as Type I or Type II based on DNA sequence divergence in the EBNA-2 and -3 genes. Type I EBV is more prevalent in China, but none of the type-discriminating EBNA genes is expressed in NPC. No one EBV type or strain correlates with NPC incidence nor do LMP1 gene polymorphisms [79].

In endemic regions of China programs for early detection of NPC are practiced by screening for the distinctive circulating IgA responses to EBV-replicative antigens such as VCA and EA, which prompts biopsy in the posterior nasopharynx. These antibody responses arise early in the genesis of NPC and may even herald its onset. The profile of antibody responses points to active viral replication and so suggests that a wave of viral reactivation precedes onset of NPC. Since EBV causes cytolysis when it replicates in epithelial cells it has been hypothesized that entry of EBV in the premalignant cells must be a late event [80].

Cytogenetic analysis indicates that members of the populations at high risk for NPC may share genetic susceptibility to the effects of EBV and other environmental co-factors. Family studies have linked one NPC susceptibility locus to the human leukocyte antigen (HLA) region [81], and candidate oncogenes and tumor suppressor genes have been identified (see [82] for review). Mutations in the p53 tumor suppressor gene are rarely found in NPC except in metastases. How does EBV work, in concert with such genetic and environmental conditions, to contribute to the initiation or progression of this malignancy? One hypothetical scenario that may account for many features of NPC is as follows: the elevated immunoglobulin A (IgA) antibody titers to EBV that precede the development of NPC may be a result of viral reactivation from latency. The presence of the virions in mucosa would induce IgA synthesis, which could in turn facilitate entry of EBV into new epithelial cells of the nasopharynx, possibly via a "secretory component" IgA receptor on epithelial cells [83]. The program of cellular gene expression in a subset of these cells, altered by years of exposure to environmental carcinogens especially in endemic regions, may increase their susceptibility to EBV infection which then leads to viral latency and transformation, triggering a clonal proliferation leading rapidly to dysplasia and NPC. A model of rapid rather than stepwise progression to malignancy after virus infection of target cells is consistent with the observation that premalignant lesions and carcinoma in situ are rare in NPC and are usually only detected concomitantly with carcinoma. This scenario leaves unexplained what causes the surge of viral reactivation as well as the specific reason why some of the newly infected epithelial cells would proliferate rather than be destroyed by the virus (for review, see [84]).

Among *salivary gland tumors* only those of the *parotid* gland are linked to EBV. They, like NPC, are undifferentiated carcinomas with high incidence both in North American Inuits and Chinese in endemic regions and regularly contain clonal EBV episomes localized to the epithelial and not the lymphocytic component of the tumor. It is intriguing that no other tumors of the nasooropharynx are infected by EBV [84].

10.4.3 Gastric Carcinoma and Breast Cancer

Gastric cancer is unique in being associated with two infectious agents: *Helicobacter pylori* and EBV. *H. pylori* is the major causative agent for gastric cancer that is located anatomically beyond the cardia. The pathogenetic sequence is chronic infection and inflammation leading to chronic atrophic gastritis and dysplasia culminating in gastric carcinoma of the intestinal type. The *cag*+ strain of this bacterium is able to inject *H. pylori* CagA protein into epithelial cells where it is tyrosine phosphorylated and changes intracellular signaling along with another *H. pylori* protein VacA, high-risk genotypes of which suppress T-cell responses. These strain-specific exposures coupled with persons

with specific alleles in the IL-1β or TNFα genes have 20- to 80-fold increased rates of gastric adenocarcinoma [85]. *H. pylori* also causes gastric lymphomas of the so-called "MALT" type which are B-cell lymphomas thought to be driven by intense antigenic stimulation by the bacterium. Such lymphomas also occur in chronic hepatitis C infections [86, 87].

The association of EBV with gastric cancer is more recent than the other EBV malignancies (for review see [88]) probably because the ability of the virus to replicate in gastric epithelium had not been recognized and because EBV is detected in only about 10% (range 2–16%, depending on geographic region) of gastric carcinomas, but in 35% of gastric stump carcinomas that occur in remnants of the stomach removed after gastrectomy for peptic ulcer in the Billroth II procedure—now much less common as the incidence of gastric ulcers has fallen. Histopathologically the tumors are poorly differentiated and marked by lymphocytic infiltration in and around tumor cell nests [89].

In a study of patients in the Netherlands (1989–1993) almost all the EBV-positive cases were in males. In contrast to *H. pylori* case findings, most of the cancers were in the upper part of the stomach including the cardia [90]. This and other (but not all) studies report that EBV-positive gastric cancers tend to be detected at an earlier stage and have longer disease-free survival, although mortality is generally as high ultimately as for EBV-negative tumors [91]. At the molecular level there are distinct chromosomal aberrations in the tumor cells but p53 mutations are rare as in NPC. EBV monoclonal episomes are present in all tumor cells, and EBNA1 and BARF0 as well as EBER RNAs are always expressed; LMP2B is detected in about half the cases [92]. These features point to EBV-positive gastric cancer as a distinct clinicopathologic entity and suggest that the virus may contribute etiologically.

The association of some *breast cancers* with EBV has been contentious mostly because the significance of detection of the virus has been viewed only from an etiologic perspective [77, 93]. An etiologic role should require consistent detection of viral genomes in the tumors, in every cell, generally in a latent form, none of which is the case in breast cancer.

EBV can be detected in breast milk of healthy women with high frequency (46% up to 74% post-natally) without known accompanying pathology [94]. Breast cancer is usually detected as a painless localized mass before it has metastasized. Early sites for metastasis are axillary and supraclavicular nodes, the finding of which does not preclude long-term survival with appropriate therapy. Patients with extranodal metastases usually respond to therapy for a time, but at this stage the disease is ultimately fatal. A subset of breast cancers occurs relatively early in life—in the twenties and thirties—and in these cases tumor growth is often aggressive especially in African-American women. Mutations in the BRCA1 and BRCA2 genes also predispose to or if homozygous cause aggressive breast cancer with early onset. *HER2* and *HER3* are amplified and overexpressed in up to 30% of breast cancer and correlate with poor prognosis [95].

EBV has been detected more frequently in tumors with poor prognosis, such as less differentiated, estrogen receptor (ER) and progesterone receptor

(PR)-negative tumors with high mitotic index [96] as well as in those with metastatic lymph nodes (for review, see [93]). The EBV copy number in the tumors as a whole is very low, well under a single copy per cell. However, laser microdissection of tumors changes the picture with considerably higher numbers of genomes detected by QPCR in pure tumor cell populations [97]. Nevertheless EBV genomes are not detected in every tumor cell in a given specimen. Moreover in some tumors higher genome copy numbers can be attributed to scattered virus-producing cells [98]. The viral gene expression profile in at least some EBV-positive tumors includes EBNA1, LMP1 and BARF0 but does not include the other Type III latency genes and so distinguishes the viral expression pattern from that of EBV-infected lymphocytes that may infiltrate the tumor [99]. Breast cancer tissue itself is heterogeneous with unequal distribution of ER, PR, EGFR and other cellular markers that play a role in its biology [100]. Finally LMP1 expressed in epithelial cells including breast cancer cell lines can induce a wide range of invasion, metastasis (MUC1, MMP9 and MMP1) and angiogenic factors (IL-8, FGF-2, COX2, HIF1α and VEGF) that together participate in every stage of tumor progression. LMP2A and EBNA3C may also contribute by affecting cell motility and counteracting a metastasis suppressor protein.

Consequences of expression of these factors are numerous and well studied (for review, see [73, 74]). One example in breast cancer is the cell anti-adhesive effect of MUC1 binding to β-catenin. All these findings together indicate that EBV is not etiologic in breast cancer but suggest that the virus might contribute to tumor progression. EBV infection is also reported to alter responses to tamoxifen and induce multiple drug resistance and to dysregulate HER2/HER3 signaling in EBV-infected breast cancer cells [101]. HER1 and HER3 are members of the EGFR family, and EGFR is constitutively upregulated in LMP1-expressing cells and could thus contribute in another way to the association of the virus with aggressive tumors with poor prognosis. The significance of these findings will need to be verified by studies directly in breast cancer tissues. Additionally the finding of occasional virus-producing cells in tumor tissue does not exclude an oncogenic role, early or late, for the virus as the evidence is mounting that EBV lytic cycle products such as cytokines may contribute to tumor development [37]. Moreover LMP1, EBNA2 and other oncogenic EBV products conventionally considered to be latency genes are in fact expressed and functional during the cytolytic cycle [68]. Thus the relation between EBV and breast cancer even though inconsistent could be significant when viewed from a perspective other than one of primary etiology.

10.5 Other Associations

10.5.1 Hemophagocytic Syndromes

EBV is associated with hemophagocytic lymphohistiocytosis (EBV-HLH), a rare fulminant disease of T and NK cells, which is related to chronic active EBV

infection (CAEBV). In HLH EBV is found in $CD8^+$ T cells, whereas in CAEBV the virus is detected in $CD4^+$ T cells or NK cells, where the infection is regarded as ectopic in contrast to IM where the virus is found only in B cells [11]. Clinically EBV-HLH is distinguished from severe IM mainly by histiocytic phagocytosis in bone marrow and secondary lymphoid organs and is characterized by damage to the vascular system which involves cytokines including IFN-γ and TFNα. HLH is an unremitting disease with high mortality resulting from multiple organ failure and coagulopathy [64, 65]. For CAEBV, see Section 10.3.

10.5.2 Leiomyosarcoma

In immunologically intact children leiomyosarcomas are not infected by EBV despite the fact that the C2R receptor is expressed in smooth muscle cells (for review, see Jensen [102]). In children with AIDS leiomyosarcomas are regularly EBV positive and contain an average of about 4.5 genomes per cell, present as episomes. The restricted range of copy numbers (~1.7–6.6 per cell) suggests that infection is latent, and it is generally monoclonal. The viral gene expression pattern is not well documented but does include EBNA1. Detection of EBV-replicative cycle antigens has been reported in cultured leiomyosarcoma cells, which may be the result of viral reactivation under culture conditions. EBV genomes have been detected in nonsarcomatous muscle cells in AIDS patients, which suggest that infection is present at an early stage in oncogenesis. EBV-infected leiomyosarcomas occur less frequently under other immunosuppressive conditions such as in recipients of organ transplants but are not reported in adults, which suggests that the tumor begins with primary EBV infection.

10.6 Integration and Conclusions

EBV, like most of the known tumor viruses, is a ubiquitous agent that infects untold numbers of people without, for the most part, untoward consequences. It is certainly the etiologic agent for some malignancies, but more usually causes benign conditions if any disease at all. The virus is clearly the sole etiologic agent in immunoblastic lymphomas in the immunocompromised. Except for the rare leiomyosarcomas none of the other EBV-associated malignancies requires overt immunodeficiency.

For NPC worldwide the intimate and consistent association with the virus argues strongly that it contributes to the genesis of the tumors. This conclusion is fortified by the presence of monoclonal EBV episomes in each tumor cell, which must then either be present at the beginning of malignant clonal expansion or confer a selective advantage on proliferation of the tumor cells. Detection of EBV episomes in nasopharyngeal cells with earliest changes of NPC suggests that the virus may even be indispensable. At the same time the inferential evidence is that

infection of these cells occurs decades after primary infection of the host and that malignant progression is rapid, probably in cells made susceptible by acquired mutations and perhaps genetic predisposition as well. Clearly a wave of viral reactivation accompanies and likely precedes onset of NPC, but the tissue source of the virus and what provokes its replication in middle life, mainly in men, in the most endemic regions and how it enters the nasopharyngeal cells without causing their cytolysis remain continuing mysteries.

Next in the causal hierarchy is BL. Historically the virus was accepted at first as the etiologic agent, and EBV DNA was detected in almost all BL specimens from the African endemic region with sensitive, quantitative techniques. However, even then EBV was not detected in the odd BL specimen (less than 2%). Subsequently the dominant oncogenic role of the chromosomal translocations that reactivate expression of *c-myc*, even in BL in nonendemic parts of the world, came to be appreciated. The absence of EBV genomes in more than 80% of American BL may relate to its different pathobiology, but there are no recognized histopathologic categories for EBV-positive and EBV-negative BL. Now as the incidence of BL has soared worldwide because of its frequency in AIDS, the tumors are often EBV negative, remarkably, even in infected persons, but the hallmark translocations are found consistently. In the end the role of EBV and, especially, other possible contributory factors remains suppositious. Recent discovery of the high incidence of BL in Southern Brazil, a region populated with descendants from the African endemic region, adds to this intriguing, still unfolding chapter in the history of tumor viruses.

For the Western world Hodgkin's lymphoma is the most important EBV malignancy. The association of the virus with HL rests on both solid molecular and suggestive epidemiologic evidence. Recognition that the Reed–Sternberg is the malignant cell of HL and that it is of B-cell lineage were landmark findings, and detection of monoclonal EBV episomes in RS cells fortifies the significance of EBV in the pathogenesis, but because of the low numbers of RS cells in HL few analyses have actually detected the episomes specifically in these cells. Of course detection of EBV is quite variable, with the EBV-positive and EBV-negative subsets that are currently recognized, based mainly on age, geography and to some extent histopathologic differences, being approximate and ill-defined. Clarification may only be provided when subsets of HL can be distinguished not by differences in histopathology but by genomic and proteomic profiling.

What about the other associations? Even though only about 10% of gastric carcinomas are EBV infected, in these cases each tumor cell harbors EBV episomes and has like the other EBV malignancies a stereotypic pattern of viral gene expression. Also there is at least superficial similarity of NPC, parotid tumors and gastric cancers in that all three have lymphoepithelial features which might hint at a common mechanism. In contrast the association of EBV with breast cancer is inconsistent both among tumors and within them, and if EBV is contributing to these tumors it is probably not etiologically but perhaps by infecting existing tumor cells and affecting their oncogenic progress. The observations that EBV is detected more consistently in T and NK cells in hemophagocytic syndromes more

frequently in Southeast Asian than in Western countries raise the question whether here too the virus modifies progression of disease rather than causes it. New chapters in the odyssey of EBV are sure to come. How to prevent infection with EBV and how to treat infectious mononucleosis are questions that are yet to be answered 42 years after its discovery [103–106].

Finally although most viral malignancies have virus-specific features, the goal of targeting tumor cells therapeutically through bioengineered agents has until recently remained elusive, yet it is a rational enterprise that should one day lead to a final chapter.

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Chapter 11
The Epstein–Barr Virus Genome

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11.1 Introduction

In 1984, the B95-8 isolate of Epstein–Barr virus (EBV), a commonly used laboratory strain whose DNA genome had been partially or completely cloned by several groups, was the first herpesvirus to have its genome completely sequenced (Baer et al. 1984). The information gained from this first genomic sequence (accession number V01555) provided a wealth of new information on the coding potential of this DNA tumor virus, and consequently was the basis for the rapid advancement of the EBV field that soon followed. The complete sequence data were particularly critical, for example, to the characterization of the complex latency-associated genes of EBV, whose highly spliced mRNAs span upward of 85 kilobase pairs (kbp) of the genome. Sequence information missing from the B95-8 genome – the result of a 12-kbp deletion – was subsequently provided by analysis of the corresponding genomic region of the Raji EBV isolate (Parker et al. 1990). An updated and fully annotated wild-type EBV genome sequence, reconstituted from the Raji and B95-8 sequences, is now available (accession number AJ507799) (de Jesus et al. 2003). Moreover, the complete sequence of two additional EBV genomes has recently been reported, one of which is that of the prototype type 2 strain of EBV, AG876 (Dolan et al. 2006; Zeng et al. 2005). Though it has been 25 years since publication of the B95-8 EBV sequence, this information is still invaluable today as it continues to provide insight into the biology and evolution of EBV as a highly successful human pathogen.

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_11, © Springer Science+Business Media, LLC 2009

11.2 Basic Properties and Organization

The EBV genome is approximately 172,000 bp, and exists in two configurations: the linear form present within the virion that is produced during virus replication (i.e., the lytic cycle of infection) and the circular form that is maintained as an episome within the nuclei of latently infected cells. As described later in this chapter, replication of the EBV genome during lytic and latent infections is mediated through distinct mechanisms and origins of DNA replication. In its linear configuration, the dsDNA genome is bounded on each end by several copies of a direct 538-bp terminal repeat (TR); the episomal form is generated via covalent fusion of the left and right TR domains upon infection of a cell that supports latent EBV infection. The genome is divided into five predominantly unique sequence domains (U1–U5) by the four major direct internal repeat domains (IR1–IR4), each of which encodes viral polypeptide (Fig. 11.1). The nucleotide sequence of the genome is numbered from the first base before the leftmost *Eco*RI restriction site, which is located 50 bp into the U1 domain. The sizes of the IR elements themselves are 3,072 bp (IR1), 123 bp (IR2), 708 (IR3) and 103 bp (IR4). The actual number of repeats in each repeat domain varies between virus isolates. The GC content of the EBV genome is approximately 60%, though these two nucleotides are generally enriched within repeat domains.

11.2.1 Coding Content and Gene Nomenclature

Analysis of the genomic nucleotide sequence of the B95-8 isolate of EBV revealed 84 likely translational open reading frames (ORFs) (Baer et al. 1984). From this first completely sequenced genome, an ORF nomenclature was adopted that is based on relative position and orientation within the *Bam*HI restriction fragment linkage map of the B95-8 genome, e.g., ORF BARF1 for *Bam*HI-*A* fragment *r*ightward *f*rame 1 (Fig. 11.2). If an ORF spans two or more *Bam*HI fragments, it

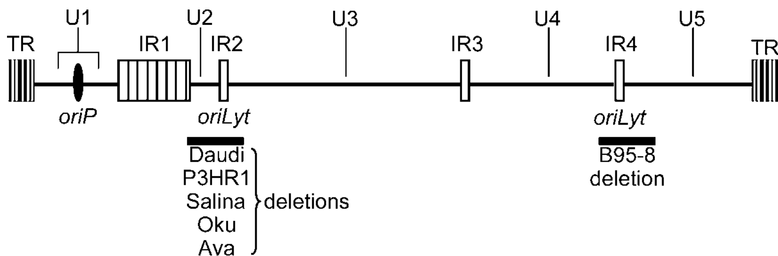

Fig. 11.1 Structure of the EBV genome. The EBV genome is flanked by reiterating terminal direct repeats (TR) and is divided into five largely unique-sequence domains (U1-U5) by the four major internal direct repeat domains (IR1-IR4). Also shown are the positions of the latent (*oriP*) and lytic (*oriLyt*) infection origins of DNA replication, the postions of the 6.6-8.5-kbp deletion present within the EBV genomes within approximately 20% of EBV-positive BLs, and the 12-kbp deletion present in the B95-8 EBV genome

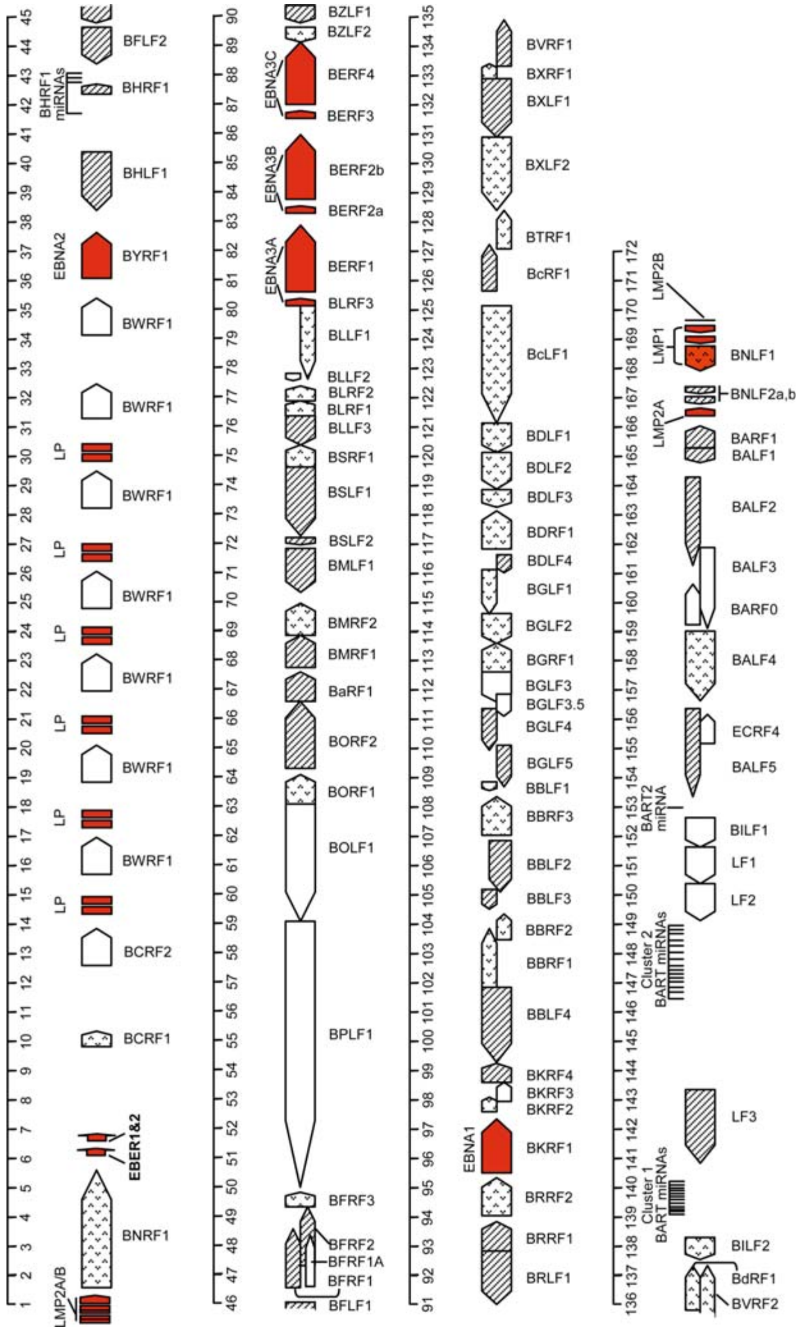

Fig. 11.2 Coding content of the EBV genome. Translational ORFs (block arrows) are shown, as are the positions of the noncoding RNAs *EBER-1* and *EBER-2*, and the *BHRFI* and *BART* miRNAs. The latency-associated ORFs or the exons that comprise these ORFs are colored

is assigned to the fragment that contains its 5′ end. Genes absent from the B95-8 genome, as a consequence of a ~12-kbp deletion (Fig. 11.1) (Raab-Traub et al. 1980), were identified by sequence analysis of the corresponding segment of the Raji EBV genome and revealed the presence of an additional three leftward ORFs that are designated LF1, LF2 and LF3 (Parker et al. 1990). A number of the smaller ORFs were subsequently discovered to be present within short exons and spliced in-frame with adjacent longer ORFs during pre-mRNA processing, reducing the likely repertoire of viral proteins. However, several of the latency-associated genes were not apparent from genomic sequence due to the highly spliced nature of their mRNAs (and ORFs), the primary transcripts of which can span considerable distances of the genome. In addition to the B95-8 genome, the nucleotide sequences of the EBV type 1 isolate GD1 (accession number AY961628) and type 2 isolate AG876 (see below; accession number DQ279927) have recently been reported (Dolan et al. 2006; Zeng et al. 2005). Collectively, the protein coding potential of EBV now stands at approximately 84 polypeptides (the originally predicted repertoire), though to date not all predicted genes have been confirmed or characterized.

In addition to protein, the EBV genome encodes for at least two classes of noncoding RNAs. The best studied of these are the latency-associated EBV-encoded small RNAs, EBER-1 and EBER-2 (Arrand and Rymo 1982; Glickman et al. 1988). The second class of EBV noncoding RNAs contains approximately 30 microRNAs (miRNAs) believed to be derived either from primary transcripts of mRNAs containing the BHRF1 ORF or those that give rise to the *Bam*HI-*A* *r*ightward *t*ranscripts (*BARTs*), a family of alternatively spliced polyadenylated RNAs whose protein-coding potential is unclear (Cai et al. 2006; Pfeffer et al. 2004).

In addition to the organization of ORFs and other information provided in Fig. 11.2, a comprehensive transcriptional map of the EBV genome can be viewed at http://www.med.ic.ac.uk/ludwig/ebv.htm (de Jesus et al. 2003).

11.2.2 Lytic-Cycle Genes

Ninety percent of EBV genes contribute to the production of progeny virus. Whereas a very few of these lytic-cycle genes may be expressed at some point

Fig. 11.2 (continued) red; early lytic-gene ORFs are shaded with diagonal lines and late lytic-gene ORFs with chevrons. Open arrows depict ORFs of unknown gene class or that are hypothetical (see Table 11.1). ORFs are named, e.g., BCRF1, according to convention as described in the text (Section 11.2.1); common names, eg., EBNA2, are presented above the appropriate ORF or coding exon(s) (LP of EBNA-LP). Note that the *LMP 2A* and *2B* mRNAs originate at the right end of the genome and transverse the fused TRs of the EBV episome, and thus the majority of the LMP2A and all the LMP2B ORF are located at the left end of the genome in its linear configuration as shown here. The scale bar above each row is in kbp. (*See* Color Insert)

during latent infection, e.g., *BHRF1* (Altmann and Hammerschmidt 2005; Austin et al. 1988; Pearson et al. 1987), this is the exception, as the overwhelming majority are restricted to expression during the virus replication cycle. Herpesvirus lytic-cycle genes are primarily classified according to their temporal pattern of expression: immediate early, early and late. The former is expressed immediately upon infection and, by definition, do not require prior protein synthesis for their transcription, whereas the latter require the onset or completion of viral DNA replication. Because of the lack of cell lines that will actively support EBV replication upon infection, the lytic cycle has historically been studied within latently infected B-cell lines following their treatment with chemical inducers of EBV replication, such as the phorbol ester TPA, or cross-linking of surface immunoglobulin. However, because lytic-cycle gene expression is absent in these virus-positive cells prior to induction, it has been questioned whether any of the EBV lytic-cycle genes can be classified as true immediate-early genes. Thus, these EBV genes are typically classified as either early or late, depending on whether their expression can occur in the presence of an inhibitor of EBV lytic-cycle DNA replication following induction of virus replication.

The organization of ORFs within the EBV genome, illustrated in Fig. 11.2, reveals considerable conservation of genetic material, with generally little or no gap between ORFs. Also, there are multiple instances of overlapping ORFs encoded on the same or opposing DNA strands and sharing of mRNA 3′-processing signals. There is a lack of strict clustering of lytic-cycle genes according to temporal class, and lytic-cycle genes are also interspersed with those encoding the latency-associated proteins. While a complete discussion of the lytic-cycle genes is beyond the scope of this chapter, a brief listing of known properties is presented in Table 11.1.

11.2.3 Latency-Associated Genes

The latency-associated gene products promote the establishment and maintenance of latent EBV infection, primarily within B lymphocytes. Whereas expression of the lytic-cycle genes is regulated temporally upon initiation of the virus replication cycle, the latency genes are expressed according to specific EBV transcriptional programs maintained by the latently infected cell. These range from expression of the full complement of latency genes in the growth or Latency III program, to the more restricted Latency I and 0 programs in which little or no viral protein is expressed, respectively (see Chapter 14, Epstein–Barr Virus Latent Infection Nuclear Proteins, Genome Maintenance and Regulation of Lymphocyte Cell Growth and Survival). The majority of the latency-associated genes, in stark contrast to their lytic-cycle counterparts, encode extensively spliced mRNAs with as many as 21 exons and which are derived from primary transcripts believed to span up to 85 kbp of the EBV genome (Fig. 11.3). The few

Table 11. 1 EBV ORFs and gene function

ORF	Alternative designation	Genome coordinates	Gene class	Function/properties
LMP-2A[1]	TP-1	166103–1680	Latent	Signal transduction
LMP-2B[1]	TP-2	59–1680	Latent	Negative regulator of LMP-2A
EBNA-LP[1]	EBNA-5	14409–35694	Latent	EBNA-2 coactivator
BYRF1	EBNA-2	36216–37679	Latent	Transcriptional activator
BLRF3, BERF1[2]	EBNA-3A, EBNA-3	79955–80293, 80382–82877	Latent	Transcriptional regulator
BERF2a,b[2]	EBNA-3B, EBNA-4	83065–83421, 83500–85959	Latent	Transcriptional regulator
BERF3, BERF4[2]	EBNA-3C, EBNA-6	86083–83421, 83500–85959	Latent	Transcriptional regulator
BKRF1	EBNA-1	95662–97587	Latent	Viral genome maintenance
RPMS1	BART, CST	150323–155552	Latent	Unknown
A73	BART, CST	156652–159865	Latent	Unknown
BARF0	BART, CST	159121–160536	Latent	Unknown
BNLF1	LMP-1	169016–167702	Latent	Mimics constitutively active CD40
BNRF1		1736–5692	Late	Major tegument protein
BCRF1	vIL-10	9675–10187	Late	IL-10 homolog
BCRF2		12541–13692	Unknown	Hypothetical protein
BWRF1[3]		15613–16764	Unknown	Hypothetical protein
BHLF1		40269–38287	Early	Unknown
BHRF1		42088–42663	Early	Bcl-2 homolog
BFLF2		44647–43691	Early	Nuclear membrane protein
BFLF1		46237–44660	Early	DNA packaging
BFRF1A		46236–46643	Unknown	DNA packaging
BFRF1		46603–47613	Early	Nuclear membrane protein
BFRF2		47520–49295	Early	Virion egress
BFRF3		49219–49749	Late	Capsid protein
BPLF1		59239–49790	Unknown	Tegument protein
BOLF1		62951–59232	Unknown	Tegument protein-binding protein, capsid assembly
BORF1		62950–64044	Late	Minor capsid binding protein
BORF2		64119–66599	Early	Ribonucleoside reductase, large subunit
BaRF1		66612–67520	Early	Ribonucleoside reductase, small subunit
BMRF1		67611–68825	Early	Processivity factor, DNA replication
BMRF2		68830–69903	Early	Receptor for cellular integrins
BMLF1, BSLF2	SM, EB2, Mta	71833–70455, 72000–71940	Early	mRNA export factor, regulator of splicing
BSLF1		74593–71969	Early	Primase, DNA replication
BSRF1		74636–75292	Late	Tegument protein
BLLF3		76186–75350	Early	dUTPase

Table 11. 1 (continued)

ORF	Alternative designation	Genome coordinates	Gene class	Function/properties
BLRF1		76259–76567	Late	Glycoprotein
BLRF2		76637–77125	Late	Tegument protein
BLLF1	gp350/220	79865–77142	Late	Envelope glycoprotein
BLLF2		77725–77279	Early	Unknown
BZLF2	gp42	89828–89157	Early	MHC class II binding protein, fusion
BZLF1	ZEBRA, Zta, EB1	90867–89922	Early	Transcriptional activator of lytic cycle
BRLF1	Rta	92895–91078	Early	Transcriptional activator of lytic cycle
BRRF1		92894–93826	Early	Enhancement of lytic-cycle induction
BRRF2		94014–95627	Late	Tegument protein
BKRF2	gp25, gL	97670–98083	Late	gH chaperone
BKRF3		98065–98832	Unknown	Uracil DNA glycosylase
BKRF4		98846–99499	Early	Tegument phosphoprotein
BBLF4		101971–99542	Early	Helicase, DNA replication
BBRF1		101916–103757	Late	Capsid protein
BBRF2		103660–104496	Late	Unknown
BBLF3, BBLF2		105098–104493, 106792–105227	Early	Primase-associated factor, DNA replication
BBRF3	gM	106849–108066	Late	Glycoprotein M
BBLF1		108686–108459	Late	Tegument protein
BGLF5		110053–108641	Early	Alkaline exonuclease
BGLF4		111326–110037	Early	Protein kinase
BGLF3.5		111666–111205	Unknown	Tegument protein
BGLF3		112651–111653	Unknown	Unknown
BGRF1		112650–113585	Late	Packaging protein
BGRF1, BDRF1		112650–113585, 116927–118063	Unknown	Putative DNA packaging protein
BGLF2		114585–113575	Late	Tegument protein
BGLF1		116086–114563	Late	Tegument protein
BDLF4	gp115	11673–-116056	Early	Glycoprotein
BDRF1		116927–118063	Late	Packaging protein
BDLF3	gp150	118778–118074	Late	Envelope glycoprotein
BDLF2		120101–118839	Late	Tegument protein, Cyclin B1 homolog?
BDLF1		121017–120112	Late	Minor capsid protein
BcLF1		125178–121033	Late	Major capsid protein
BcRF1		125177–127429	Early	Unknown
BTRF1		127353–128627	Late	Capsid maturation
BXLF2	gH, gp85	130747–128627	Late	Fusion
BXLF1		132572–130749	Early	Thymidine kinase
BXRF1		132571–133317	Late	Basic core protein
BVRF1		133127–134839	Early	Tegument protein
BVRF2	P40	135638–137455	Late	Capsid protein, protease

Table 11. 1 (continued)

ORF	Alternative designation	Genome coordinates	Gene class	Function/properties
BdRF1		136418–137455	Late	Tegument protein, capsid scaffolding protein
BILF2	gp55/80, gp78	138236–137490	Late	Glycoprotein
LF3		143344–140570	Early	Unknown
LF2		150324–149035	Unknown	Unknown
LF1		151694–150285	Unknown	Unknown
BILF1	gp64	152641–151703	Unknown	Constitutively active G protein coupled receptor
BALF5		156288–153241	Early	DNA polymerase
BALF4	gB, gp110	158864–156291	Late	Fusion
BALF3		161220–158851	Unknown	Terminase large subunit/ ATPase
BALF2		164312–160926	Early	ssDNA binding
BALF1		165059–164397	Early	Bcl-2 homolog
BARF1		165046–165711	Early	CD80 homolog, binds CSF-1
BNLF2b		166845–166540	Early	Potential gp141
BNLF2a		167028–166846	Early	Potential membrane protein

[1]ORF with highly spliced mRNA; coordinates represent distance between 5′ and 3′ ends of ORF; LMP-2A/B mRNAs span fused termini of episome.
[2]ORF generated by mRNA splicing.
[3]Present in each copy of IR1.

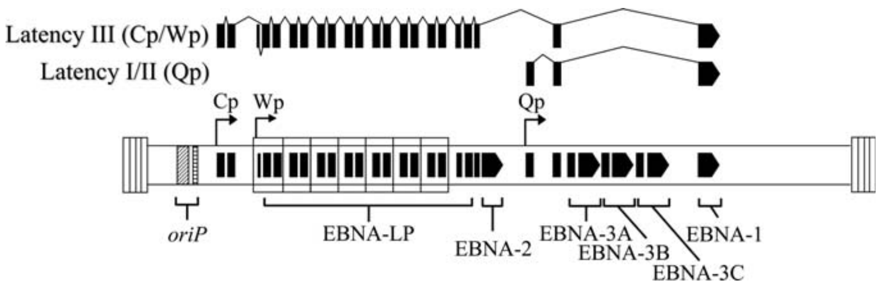

Fig.11.3 *EBNA* promoter usage. Shown relative to the linear EBV genome are the structures of the *EBNA-1* mRNAs expressed from Cp or Wp during the Latency III program of EBV latency, or from Qp during the Latency I and II programs. Whereas all *EBNA* mRNAs can be expressed from Wp or Cp, Qp is used exclusively for EBNA-1. Exons (black boxes) that make up the individual EBNA ORFs are bracketed; the IR1 repeat elements that contribute to the majority of the EBNA-LP ORF are boxed. Non-*EBNA-1* mRNAs originating from Wp or Cp have the same 5′-leader structure derived from IR1 as the *EBNA-1* mRNAs shown here, but contain the EBNA 2, 3A, 3B or 3C coding exons in their 3′ ends. The 172-base exon from the *Bam*HI-U fragment (see noncoding exon common to Cp/Wp and Qp *EBNA-1* mRNAs) is also present within the *EBNA-3* mRNAs. Illustration is not to scale

lytic-cycle mRNAs that are spliced are modest by comparison, containing only 2–5 exons and relatively short introns, most of which are under 500 bp. The EBV proteins known to be expressed during latent infection are divided into two classes: the EBV-encoded nuclear antigens (EBNAs) and the latent membrane proteins (LMPs).

11.2.3.1 The *EBNA* Gene Family

There are six members of the EBNA family of nuclear proteins – EBNAs 1, 2, 3A, 3B, 3C and LP – that upon infection of B lymphocytes are expressed from a multicistronic transcription unit spanning approximately one half of the EBV genome. Shortly after infection, *EBNA* transcription is mediated by the RNA polymerase II promoter Wp (within the *Bam*HI-W fragment) that is present in each copy of the large internal repeat, IR1, though the promoter within the 5′ -most repeat is believed to become dominant (Fig. 11.3) (Yoo et al. 1997). Within hours after infection and the initiation of transcription from Wp, there is a switch in *EBNA*-gene transcription from Wp to the promoter Cp (within *Bam*HI-C) approximately 3 kbp upstream of the leftmost Wp (Woisetschlaeger et al. 1990). This switch is mediated primarily by EBNA-2, and thus is believed to afford the virus control over EBNA expression and its consequences (Rooney et al. 1992; Woisetschlaeger et al. 1991). The individual *EBNA* mRNAs are generated by alternative splicing and 3′ processing of the *EBNA* primary transcripts originating from these two common *EBNA*-gene promoters. Furthermore, the common leader sequence of the *EBNA* mRNAs that is composed primarily of a two-exon repeat element (W_1-W_2), each derived from a copy of IR1, encodes the sixth EBNA, hence its designation leader protein or EBNA-LP (Rogers et al. 1990; Sample et al. 1986; Speck et al. 1986). All other EBNA ORFs are either contained within a single exon (EBNAs 1 and 2) or generated by an in-frame splice between a short and a long exon (EBNAs 3A, 3B and 3C) at the 3′ end of their respective mRNA (Fig. 11.3). All EBNA mRNAs, therefore, are potentially bicistronic, though the 5′ splice required to generate the EBNA-LP ORF has only been observed in cDNAs also encoding EBNA-2.

The above scenario of EBNA expression occurs only during the growth or Latency III program (full latency-gene expression), and only within B lymphocytes. During the more restricted Latency I and II programs common to EBV-associated tumors, Cp and Wp are epigenetically silenced through mechanisms that are not fully understood, and EBNA expression is limited to EBNA-1 (the genome-maintenance protein) from the promoter Qp (within *Bam*HI-Q) approximately 40 kbp downstream of Cp (Fig. 11.3) (Nonkwelo et al. 1996; Schaefer et al. 1995). Although the coding exons for each of the EBNA-3 proteins lie between Qp and the 3′ exon encoding EBNA-1, transcription from Qp does not give rise to EBNA-3 mRNAs,

presumably due to strict regulation of pre-mRNA processing. The inability of epithelial cells to support a Latency III pattern of EBNA expression is apparently due to a requirement for the B-cell-specific transcription factor BSAP/Pax5 for activation of Wp (Tierney et al. 2007). Consequently, epithelial cells are unable to initiate the expression of EBNA-2 needed to sustain *EBNA* transcription via Cp, and express only EBNA-1 by apparent default through Qp.

The *EBNA-3* genes are of particular note from the standpoint of the genetic evolution of EBV. The EBNA 3A, 3B and 3C coding regions are organized in tandem within the genome and have a similar gene structure, with each ORF composed of a short and long exon of approximately 0.35 and 2.5 kbp, respectively, separated by an intron of less than 100 bp. The EBNA-3 ORFs encode proteins of similar size (938–992 amino acids) that bear distant sequence similarity and contain one highly conserved motif that binds to the cellular transcription factor CBF-1, through which the EBNA-3 proteins modulate transcription. In addition, each EBNA-3 protein has a repetitive motif in its C-terminus, though the sequence is unique to each protein. The apparent grouping, structural similarities and distant sequence homology of the EBNA-3 proteins suggest that their genes may have arisen by gene duplication. As a member of the genus *lymphocryptovirus* (LCV) of the *Gammaherpesvirinae* subfamily of *Herpesviridae*, EBV is highly related genetically and biologically to LCVs that infect Old World non-human primates. A homolog of each of the EBV EBNA-3s is encoded by these LCVs, and though their respective homologies to the EBV EBNA-3 proteins are only 35–40%, protein function is largely conserved (Dalbies-Tran et al. 2001; Jiang et al. 2000; Rivailler et al. 2002; Zhao et al. 2003). The recent discovery and sequence analysis of the genome of an apparent primitive LCV isolated from a New World non-human primate revealed that, while some of the genes of EBV and this LCV share homologies as high as 75%, no obvious sequence homologs exist for EBV latency-associated genes other than for EBNA-1. However, there does appear to be positional homologs of the other EBV *EBNA* and *LMP* genes, and that at least the LCV LMP-1 is functionally equivalent to its EBV counterpart (Rivailler et al. 2005). Interestingly, the equivalent *EBNA-3* locus of this LCV contains only a single gene (*C3*) with a size and gene structure similar to those of the EBV EBNA-3s, suggesting that this may represent a predecessor to the *EBNA 3A*, *3B* and *3C* genes of EBV and other Old World primate LCVs.

11.2.3.2 The *LMP* Genes

The *LMP* genes of EBV encode three integral membrane proteins: LMP 1, 2A and 2B. The LMPs are expressed during the EBV Latency II and III programs and are present within the plasma membrane with their N- and C-terminus orientated toward the cell cytoplasm. LMP-1 contains 6 transmembrane domains, whereas both LMP-2 proteins have 12. Unlike the other latency-

associated genes, *LMP-1* is transcribed in a leftward direction and encodes a modestly spliced mRNA containing only three exons that span approximately 2.5 kbp of the genome (Fig. 11.2) (Fennewald, Van Santen, and Kieff 1984). By contrast, the *LMP-2A* and *LMP-2B* mRNAs each contain nine exons, of which exons 2–9 are identical between the *2A* and the *2B* mRNAs. The unique 5′ exons of the *LMP-2* mRNAs reflect the fact that they are transcribed from separate promoters (Laux et al. 1989; Sample et al. 1989). The LMP-2A ORF begins in the 5′ exon, whereas translation of the *LMP-2B* mRNA begins in its second exon, in-frame with LMP-2A translation. Thus, although *LMP-2A* and *LMP-2B* are separate genes, they encode highly related proteins that differ only by the additional 119 amino acids encoded by the *LMP-2A* mRNA. Another unique feature of the *LMP-2* genes is that they span the fused termini of the viral genome, and therefore can only be expressed from its episomal form; the promoters and 5′ exons are located to the left of the TR domain in U5, whereas exons 2–9 are in U1 (Laux et al. 1988, 1989; Sample et al. 1989). Thus, *LMP-2A* and *LMP-2B* genes span at least 11.5 and 8.5 kbp of the viral genome, respectively, assuming at least four copies of TR are present within the episome.

11.2.3.3 The *BARTs*

These alternatively and highly spliced transcripts appear to arise from the same promoter, span approximately 22.5 kbp of the EBV genome and contain poly(A) tails on their mostly common 3′ termini (de Jesus et al. 2003; Sadler and Raab-Traub 1995; Smith et al. 2000). The presence of several ORFs (BARF0, RK-BARF0, RPMS1 and A73) among members of this family of RNAs suggests that they do indeed function as mRNAs. However, as yet there has not been a definitive demonstration of *BART*-encoded protein expression within latently infected cells. The *BARTs* are widely expressed within latently infected cells (both B and epithelial) *in vitro* and are detectable by PCR in the peripheral blood of healthy EBV carriers (Chen et al. 1999; Chen et al. 1992).

11.2.3.4 Noncoding RNAs

The two classes of small noncoding RNAs encoded by EBV and which are associated with latent EBV infection are the *EBER* RNAs (*EBER-1* and *EBER-2*) and approximately 30 miRNAs. *EBER-1* and *EBER-2* are highly structured RNAs of 167 and 172 nucleotides, respectively (Glickman et al., 1988). Their genes lie in tandem immediately upstream of the latent infection origin of DNA replication, *oriP*, at the left end of the genome (Fig. 11.2) and are transcribed by cellular RNA polymerase III (Howe and Shu, 1989). *EBER* RNAs are highly expressed in all programs of EBV latency and are restricted to the cell nucleus, where they are present at approximately 10^7 copies per cell (levels of *EBER-1* are approximately ten-fold higher than *EBER-2*). Although

they are known to enhance the tumorigenic potential of Burkitt lymphoma (BL) cells and the transformation of primary B cells by EBV in vitro, their precise mechanisms of action in these regards are unclear (Komano et al. 1999; Ruf et al. 2000; Wu et al. 2007; Yajima et al. 2005).

The EBV miRNAs are derived from four loci within the viral genome: the *BHRF1* locus encoding for a pro-survival Bcl-2 homolog, and two clusters and one single miRNA (BART2) within introns of the *BART* locus (Fig. 11.2) (Cai et al., 2006; Pfeffer et al., 2004). Analysis of miRNA expression in various EBV-infected cells has indicated that the *BART* miRNAs are expressed in latently infected epithelial cells (Latency II program), and at much lower levels in B-cell lines. By contrast, the BHRF1 miRNAs are present in high levels within B cells maintaining the Latency III program, but are very low or undetectable during the restricted latency programs in B (Latency I) and epithelial (Latency II) cell lines. Further, three of the *BART* and one of the *BHRF1* miRNAs are induced to higher levels upon induction of the lytic cycle (Cai et al., 2006). For a thorough discussion of the EBV miRNAs see Chapter 25.

11.3 Genome Subtypes

There are two true genetic strains of EBV: type 1 strains (formerly type A) of which the prototype is the B95-8 isolate, and type 2 strains (formerly type B) represented by the AG876 and Jijoye isolates. Type 1 EBV is more common in Europe and the United States, whereas type 2 isolates are relatively common (along with type 1 isolates) in New Guinea and Africa, as well as within the HIV-positive male homosexual population (Yao et al. 1998; Young et al. 1987; Zimber et al. 1986). The two EBV strains are distinguished by differences between their respective EBNA-2 and EBNA-3 proteins, which bear amino acid sequence homologies of 53, 84, 80 and 72% between the EBNA-2, EBNA-3A, EBNA-3B and EBNA-3C of B95-8 and AG876, respectively (Dambaugh et al. 1984; Sample et al. 1990). These differences are sufficient to prevent many sera from individuals infected with a type 1 isolate from effectively recognizing their counterparts encoded by a type 2 isolate, and vice versa (Rowe et al. 1989). Apparent type-specific nucleotide differences have also been noted for the *EBER* RNAs (Arrand et al., 1989). Recent determination of the complete genomic sequence of the AG876 isolate and its comparison to the fully sequenced genomes of the B95-8 and GD1 type 1 isolates has confirmed that strain-specific differences are limited to these loci (Dolan et al. 2006). The true biological significance of this diversity is currently unclear. In vitro, type 1 isolates are more efficient than their type 2 counterparts in B-cell immortalization, a property primarily attributed to differences at the *EBNA-2* locus (Cohen et al. 1989; Rickinson et al. 1987). However, the relevance of this to the pathobiology of EBV within the human population is unknown.

11.4 Naturally Occurring Deletion Mutants

There is at least one naturally occurring deletion within EBV genomes that is of particular interest, as it has and continues to reveal significant insight into EBV biology. This is a deletion of 6.6–8.5 kbp, present in the EBV genomes within a number of BL cell lines (Fig. 11.1), that removes the 3′ -coding exons of EBNA-LP and the entire EBNA-2 (BYRF1) and BHLF1 ORFs (the latter encodes an early lytic-cycle protein of unknown function). Viruses with this deletion are unable to transform B cells in vitro due to the loss of EBNA-2 and a fully functional EBNA-LP (Cohen et al. 1989; Hammerschmidt and Sugden 1989; Skare et al. 1985). Although originally thought to have arisen during long-term cell culture, it is now evident that this deletion may occur naturally within approximately 20% of EBV-positive BLs (Kelly et al. 2002). These tumors are unusual in that they do not maintain a Latency I program (expression of only EBNA-1, from Qp), but express EBNA-1 and the EBNA-3 s from Wp. The EBNA promoter Cp is inactive in these cells due to the absence of EBNA-2 (Kelly et al. 2005). Of considerable interest is the observation that in BL cells that contain both wild-type and deletant genomes, the wild-type genome is transcriptionally silent (Kelly et al. 2002). This suggests that the locus targeted by this deletion, in addition to coding for EBNA-2, may provide a function *in cis* necessary for the epigenetic silencing of Wp and Cp in the normal progression from Latency III to the restricted latency programs. This transition to restricted latency is critical in both the EBV-infected tumor cells and the normal B-cell reservoir of EBV to promote immune evasion of latently infected cells. Thus, this particular deletion has provided critical insight into the roles that EBNA-LP and EBNA-2 play in EBV latency and their importance in B-cell transformation, and suggests that this locus may also play a pivotal and previously unanticipated role in the epigenetic regulation of EBV latency.

11.5 Genome Replication

EBV employs independent strategies to replicate its genome during lytic and latent infections. DNA replication during lytic infection is mediated by viral DNA polymerase and accessory proteins, whereas EBV primarily relies on the cellular DNA replication machinery to duplicate its genome during latency. Lytic and latency-associated DNA replication also occurs through distinct origins of DNA replication. The EBV proteins involved in lytic-cycle replication are encoded by ORFs BALF2 (ssDNA-binding protein), BALF5 (DNA polymerase), BMRF1 (processivity factor), BBLF4 (helicase), BSLF1 (primase), BBLF2/3 (primase-associated factor) and BZLF1 (*oriLyt*-binding protein, transcriptional activator) (reviewed in Tsurumi, Fujita, & Kudoh 2005). There are two origins of lytic-cycle DNA replication, $oriLyt_{\text{left}}$ and $oriLyt_{\text{right}}$ (Fig. 11.1). However, a single *oriLyt* appears sufficient, as the B95-8 B-cell line

containing the B95-8 isolate, which has lost *oriLyt*$_{\text{right}}$ as a consequence of its genomic deletion, is a common laboratory source of virus. Replication is believed to occur through a rolling circle mechanism, resulting in concatemers of the viral genome that are cleaved within the TR domains during packaging (Zimmermann and Hammerschmidt 1995).

Replication of the EBV genome during latent infection occurs simultaneously with replication of the host DNA during S phase of the cell cycle, and is mediated by the cellular DNA replication machinery. Latency-associated EBV DNA replication initiates at the plasmid origin of replication, *oriP*, located at the left end of the genome (Fig. 11.1), and requires a single viral gene product: the genome-maintenance protein, EBNA-1. EBNA-1 is a sequence-specific DNA-binding protein that binds to two functionally distinct elements of *oriP*. These are the family of repeats (FR), consisting of approximately twenty 30-bp repeats, each containing an EBNA-1-binding site, and following ~800 bp of intervening DNA, a region of dyad symmetry (DS) that has four additional EBNA-1-binding sites. EBNA-1 bound to DS orchestrates the assembly of cellular replication proteins at *oriP* and the initiation of DNA synthesis (Chaudhuri et al. 2001; Deng et al. 2002; Dhar et al. 2001; Schepers et al. 2001). EBNA-1 bound to FR serves to partition the EBV genome to daughter cells by tethering its DNA cargo to host metaphase chromosomes via its interaction with a cellular chromosome-associated protein (Kapoor et al. 2005; Shire et al. 1999) or interaction with host DNA through an AT hook mechanism (Sears et al. 2004).

Acknowledgments Much of the material presented in Fig. 11.2 and Table 11.1 was adopted from information available at http://www.med.ic.ac.uk/ludwig/ebv.htm that is maintained by Dr. Paul Farrell. We apologize to our many colleagues whose work could not be fully cited due to space constraints. We gratefully acknowledge our research support from U.S. Public Health Service grants CA056639 and CA073544 (to J.T.S.), CA056645 and CA117827 (to C.E.S.), and the Penn State Hershey Cancer Institute.

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Chapter 12
Epstein–Barr Virus Transforming Proteins: Biologic Properties and Contribution to Oncogenesis

Nancy Raab-Traub

12.1 Introduction

The Epstein–Barr virus (EBV) is perhaps the most successful human herpesvirus in that greater than 95% of the world's population are infected (Kieff and Rickinson 2001). The virus establishes a persistent usually benign infection that is marked by the presence of EBV-infected B-lymphocytes in the peripheral blood and continued secretion of low levels of virus in saliva (Miller et al. 1973). EBV is the prototype of the gamma herpesviruses. The gamma herpesviruses are distinguished by their ability to remain dormant in lymphoid cells and the ability to expand the infected cell population to induce cell growth. Pathogenesis induced by these viruses usually results from uncontrolled cellular replication rather than cell death caused by viral replication. EBV is linked to the development of several malignancies, primarily of lymphoid and epithelial cell origin (Raab-Traub 1996). These cancers include African Burkitt's lymphoma, post-transplant lymphoma (PTL), AIDS-associated lymphoma, Hodgkin's disease (HD), T-cell lymphoma, nasopharyngeal carcinoma (NPC), parotid gland carcinoma, and gastric carcinoma.

In vitro, infection of B-lymphocyte induces permanent growth transformation (Pope et al. 1973). During growth transformation, the virus does not replicate and produce progeny virions but rather is replicated by the host DNA polymerase as an extrachromosomal episome (Adams and Lindahl 1975). This "latent" transforming infection is not silent and is dependent on the carefully regulated expression of multiple viral genes. These genes include three integral membrane proteins, latent membrane proteins 1, 2A, and 2B (LMP), six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, and EBNA-LP), and two small, non-coding nuclear RNAs (EBERs) (Kieff and Rickinson 2001). Differences in viral expression comparing EBV expression in transformed lymphocytes to NPC were identified in early studies (Raab-Traub et al. 1983).

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_12, © Springer Science+Business Media, LLC 2009

These studies showed that the sequences later shown to encode EBNA2 and EBNA3 were not transcribed in NPC but there was abundant transcription of sequences that encode LMP1, LMP2, and the BamHI A restriction fragment. These findings led to the identification of three distinct types of latent infection with differing patterns of viral expression (Rowe et al. 1987). Transformed B-lymphocytes represent Type 3 latency which is also characteristic of post-transplant lymphoma. Many of the cancers associated with EBV represent Type 2 latency with expression of LMP1, LMP2, and the EBER and BART RNAs (Raab-Traub 1996). EBNA2, 3A, 3B, and C are not expressed. These proteins are the major transcriptional regulators during latent infection and are also the primary targets of cytotoxic lymphocytes (Murray et al. 1990). Thus one event that likely contributes to the development of cancer is the expression of the transforming proteins, LMP1 and LMP2, in the absence of the proteins recognized by the immune system.

The underlying molecular reasons for some of the differences in expression have been identified. One factor is different promoter usage for EBNA expression. In latency 1 and 2, a promoter in BamHI F/Q is used for EBNA1 transcription while in latency 3 expression of EBNA2 apparently activates a promoter within BamHI C that regulates a primary transcript that is differentially spliced to produce all the EBNAs (Kieff and Rickinson 2001; Sample et al. 1991). In lymphoid cells, LMP1 expression is dependent on transactivation of its promoter by EBNA2 and EBNA-LP (Wang et al. 1990). The factors that regulate the usual LMP1 promoter in epithelial cells are not known; however in NPC and in an unusual epithelial lesion that develops in AIDs patients, hairy leukoplakia, LMP1 can be expressed from a larger mRNA (Gilligan et al. 1990a, b). This mRNA initiates within the first terminal repeat (TR) from a promoter that is regulated by SP1 and STAT3 (Chen et al. 2003; Sadler and Raab-Traub 1995). This TR promoter is considerably more active in epithelial cells. STAT3 is activated by IL6 and LMP1 which also induces IL6. This may represent a positive regulatory loop that regulates LMP1 in Type 2 latency and NPC.

As latent infection is essential for lymphocyte transformation, it is difficult to separate the two processes. Genetic studies have identified the viral genes that are required for transformation and establishment and maintenance of latent infection (Marchini et al. 1992; Marchini et al. 1993). These genetic studies, the regulation of viral gene expression during latent infection, and the properties of the EBV nuclear antigens during B-lymphocyte transformation will be reviewed in another chapter.

Most of the EBV latent genes have been studied in cell culture systems for effects on growth regulation. Several EBV genes including LMP1, LMP2, BARTs, and the EBERs have been shown to affect cell growth properties and induce transformation in vitro. This chapter will review the properties of these genes in transformation models and transgenic mice and discuss the relationship of these biologic properties to characteristics of EBV-associated malignancies.

12.2 Latent Membrane Protein 1

Expression of LMP1 has been detected in most EBV-associated cancers including NPC, early pre-malignant dysplastic nasopharynx, HD, and PTL (Herbst et al. 1992; Pathmanathan et al. 1995; Young et al. 1989). Genetic studies have revealed that it is absolutely required for B-lymphocyte transformation (Kaye et al. 1993). Expression of LMP1 alone induces many of the properties of EBV-transformed B-cells and induces expression of many cellular genes including B-cell activation antigens, adhesion molecules, transferrin receptor, interleukins 6 and 10, and sensitivity to TGF-beta (Wang et al. 1990). LMP1 expression in epithelial cells affects their biologic properties and induces characteristics of malignant cells including migration and cell cycle properties (Dawson et al. 1990; Dawson and Young 2001; Everly et al. 2004; Mainou et al. 2005).

12.2.1 LMP1 Structure and Activation of Signaling Pathways

LMP1 is an integral membrane protein with a complex molecular structure containing a cytoplasmic amino terminus, six transmembrane domains, and a long cytoplasmic carboxy terminal portion (Fig. 12.1). The hydrophobic transmembrane domains oligomerize without ligand binding; thus, LMP1 apparently functions as a constitutively active growth factor receptor. Motifs have been identified within the transmembrane domains that promote specific interactions between the transmembrane regions and enhance signaling (Soni et al. 2006).

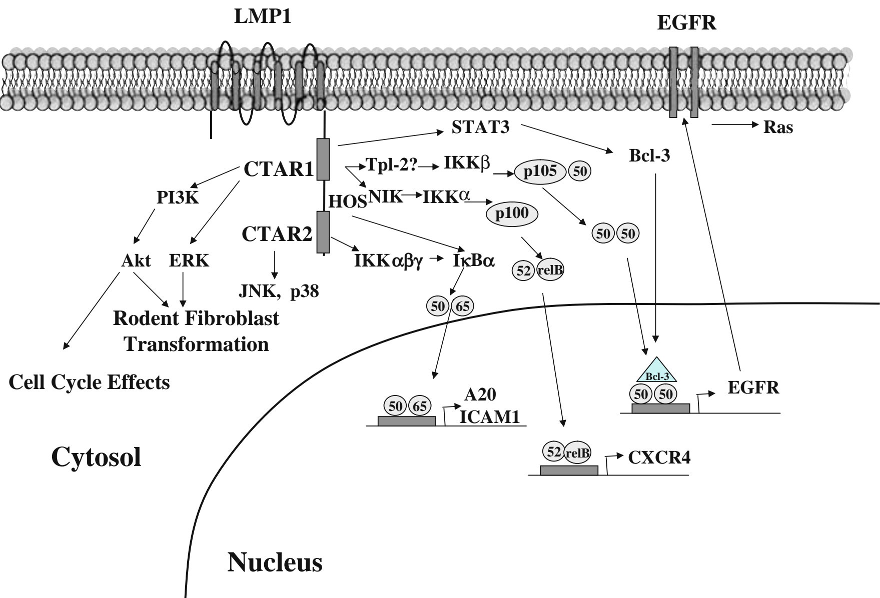

Fig. 12.1 LMP1 effects on signaling pathways

Two hybrid analyses identified cellular proteins that complex with LMP1 (Mosialos et al. 1995). These molecules were shown to also be signaling adaptors for the tumor necrosis factor receptor family and are entitled TRAFs. The TRAFs form heteromeric complexes that transduce signals that depending on the receptor may activate NFκB, induce cellular growth, or induce apoptosis. Two domains have been identified within the cytoplasmic carboxy terminus of LMP1; both can activate the NFκB transcription factor (Fig. 12.1) (Huen et al. 1995). These carboxy terminal activation domains (CTAR1 and CTAR2) bind distinct TRAFs and activate distinct pathways. The TRAFs that associate with LMP1 are basically the same as those that bind to CD40, and LMP1 has been shown to partially substitute for CD40 using CD40 knockout and LMP1 transgenic mice (Uchida et al. 1999). Genetic studies with virus containing LMP1 deleted for CTAR1 or CTAR2 have shown that CTAR1 is necessary for B-lymphocyte transformation while CTAR2 is dispensable if the cells are provided with fibroblast feeder layers (Kaye et al. 1999). The membrane proximal domain, CTAR1, interacts with TRAF1/2 or TRAF3/5 heterodimers through a consensus TRAF binding domain (PQQAT) whereas CTAR2 recruits TRAFs 2 and 6 through adapter molecules, TRADD, RIP, or BS69 (Izumi et al. 1999; Soni et al. 2007; Wan et al. 2006). Studies using knockout mouse embryo fibroblasts (MEFs) have shown that TRAF6 is required for CTAR2-induced signaling (Luftig et al. 2003; Schultheiss et al. 2001).

LMP1, like many proteins that transduce signals, localizes to cholesterol-rich membrane domains called rafts. LMP1 affects the cellular localization of TRAFs resulting in partitioning of TRAF3 into raft domains (Ardila-Osorio et al. 1999). In contrast, CD40 had much less pronounced effects on TRAF3 relocalization (Xie and Bishop 2004). The role of TRAF3 in LMP1 signaling is controversial and several studies using dominant negative forms, KO cells, or TRAF domain mutants have indicated that it is required for some signaling events and inhibitory for others (Miller et al. 1998; Xie and Bishop 2004).

LMP1 has many biologic properties and can inhibit differentiation, block apoptosis, affect cell cycle regulation, induce migration, and reduce growth factor requirements (Lo et al. 2003). Many of these properties result from LMP1 induction of the cellular proteins that regulate these processes. For example, the inhibition of apoptosis by LMP1 is attributed to its induction of the expression of the anti-apoptotic molecules, A20, bcl2, mcl1, and bfl1. The effects of LMP1 on cell expression differ between cell types such that induction of bcl2 and mcl1 occurs only in lymphocytes while effects on the EGFR are detected in epithelial cells.

12.2.2 NFκB Activation

NF-κB is a family of transcription factors that regulate a broad range of biological processes including inflammation, angiogenesis, cell cycle regulation,

apoptosis, and oncogenesis (Basseres and Baldwin 2006). There are five mammalian NF-κB family members including p50, p52, p65 (RelA), c-Rel, and RelB. The NF-κB family members dimerize and bind NF-κB consensus sequences in cellular and viral promoters through their rel homology domain (RHD) (Karin 2006; Rothwarf and Karin 1999). The p65, c-Rel, and RelB family members have transactivation domains that recruit transcriptional machinery to promoters. These proteins interact with either p52 or p50 homo or heterodimers to bind DNA and activate transcription. This activation is tightly regulated through interactions with inhibitors of NFκB (IκB), which sequester NFκB members in the cytosol through binding of the NFκB to the ankyrin repeats within the IκB proteins. Extracellular stimuli, such as binding of tumor necrosis factor to its receptor, induce a kinase cascade that ultimately results in phosphorylation, ubiquitination, and degradation of an IκB, leading to the release and nuclear translocation of bound NFκB. The mammalian IκBs include p105 (NFκB1, the p50 precursor), p100 (NFκB2, the p52 precursor), IkBα, IkBβ, IkBγ, IkBε, and Bcl-3. The first NFκB pathway identified is regulated by a trimeric IκB kinase (IKK) complex which is composed of catalytic IKKα and IKKβ subunits and the regulatory IKKγ (NEMO) subunit. Activation of the trimeric complex leads to phosphorylation and proteasome-dependent degradation of IκBα and release of bound p50/p65 NF-κB subunits. This pathway is considered the "canonical pathway" (Ghosh and Karin 2002). A second pathway has also been identified. This "non-canonical" pathway is mediated by IKKα independently of the trimeric complex. The NFκB activating kinase (NIK) phosphorylates and activates IKKα which then phosphorylates p100 and mediates processing of p100 to p52 (Eliopoulos et al. 2003). Homodimeric forms of p52/p52 or p50/p50 are also formed (Fig. 12.1). These complexes lack transactivation domains and have been shown in some cases to inhibit transcription. However, in some cases, these complexes can bind to bcl3 and activate transcription (Heissmeyer et al. 1999). Although bcl3 has ankyrin repeats and is considered an IκB, it also has a transactivation domain which can activate transcription (Dechend et al. 1999). The multiple genes that are regulated by NFκB are likely uniquely activated by distinct complexes.

CTAR1 and CTAR2 were originally defined by their ability to activate NFκB (Huen et al. 1995). CTAR2 is considered the major NFκB activator as it has the most pronounced activation of NFκB in reporter assays. However, CTAR1 activates multiple forms of NFκB and is now known to activate both the canonical and the non-canonical pathways (Soni et al. 2007). Early studies had determined that in epithelial cells CTAR1 activates multiple distinct forms of NFκB including p50/p50 homodimers, p50/p52, and p52/p65 heterodimers while CTAR2 only activates the canonical pathway forming p50/p65 and p52/p65 heterodimers (Paine et al. 1995). CTAR1 also retains the unique ability to upregulate the epidermal growth factor receptor (EGFR) and TRAF1 through its interactions with TRAFs (Miller et al. 1997, 1998). Studies with knockout MEFs deleted for IKKα, IKKβ, IKKγ, or NIK have shown that LMP1 activates NFκB-regulated genes through at least three distinct pathways (Luftig

et al. 2004). The IKKα-dependent pathways represent the non-canonical pathway while NFκB activation that requires IKKβ and IKKγ would represent the canonical pathways. A third pathway was IKKβ dependent but IKKγ independent and was considered an atypical, canonical. The actual forms of NFκB induced by these pathways were not identified although regulation of some genes was assigned to canonical, non-canonical, and atypical. Recent work specifically analyzing LMP1 induction of EGFR revealed that this gene was regulated by p50/p50 homodimers that formed a complex with bcl3 (Thornberg and Raab-Traub 2007). This complex has also been shown to be bound to the EGFR promoter in vivo in samples of NPC by chromatin immunoprecipitation (Chip) indicating that the LMP1-mediated effects identified in vitro mirror the processes affected during oncogenesis (Thornburg et al. 2003). These findings indicate that LMP1 activates NFκB through different mechanisms that activate specific forms of NFκB which regulate specific genes.

12.2.3 Phosphatidylinositol-3-kinase (PI3K/Akt)

PI3kinase contributes to the regulation of many cellular processes including proliferation and motility. It has multiple targets including protein kinase C and GTPases. One critical target is the Akt kinase (PKB) (Brazil et al. 2002). This serine/threonine kinase has multiple targets including the apoptotic protein, bad, the forkhead transcription family, and glycogen synthase kinase β. (GSK3β). GSK3β has multiple targets and when localized in the axin complex phosphorylates β-catenin leading to its degradation (Morin 1999). When GSK3β is inactivated, β-catenin accumulates and can translocate to the nucleus and interact with the lef/tcf transcription factors. LMP1 has been shown to affect the levels of cytoplasmic β-catenin, but it does not apparently induce its translocation to the nucleus (Shackelford et al. 2003) (Everly et al. 2004). LMP1' effects on β-catenin may be due to LMP1-mediated induction or suppression of expression of ubiquitin ligases and deubiquitinating enzymes. One candidate ubiquitin ligase is the SIAH ubiquitin ligase that targets β-catenin. LMP1 has been shown to reduce levels of SIAH mRNA and protein (Jang et al. 2005). LMP1 has also been shown to interact directly with the E3 ubiquitin ligase component homologue of slimb (HOS), a member of the β-TrCP/Fbw1 subfamily of F box proteins, which regulates the ubiquitin-dependent destruction of IκBα and β-catenin (Tang et al. 2003).

LMP1 was first shown to induce Akt phosphorylation in a PI3kinase-dependent fashion in epithelial cell lines where inhibition of Akt activation resulted in apoptosis (Dawson et al. 2003). Subsequent studies have shown that PI3kinase activation is mediated through CTAR1 and that this property is required for rodent fibroblast transformation (Mainou et al. 2005). Surprisingly, activation of NFκB was not required for rodent fibroblast transformation although it is absolutely critical for transformation and growth of B-lymphocytes.

Using a series of LMP1 mutants in the carboxyl terminal tail and the TRAF-binding domain indicated that CTAR1 mediates the majority of PI3K-Akt signaling and this was associated with deregulation of cellular markers associated with G_1/S transition (Mainou et al. 2007). The cyclin-dependent kinase, cdk2, and its target, Rb, were elevated in CTAR1-transformed fibroblasts while the cdk inhibitor, p27, and the desmosome-associated protein plakoglobin were decreased. These effects strictly required CTAR1 in truncation mutants. However, mutants of LMP1 that had inactivated CTAR1 and CTAR2 within the full-length molecule could still affect these markers indicating that sequences between these two domains could provide this property (Mainou et al. 2007). These forms of LMP1 were not transforming, indicating that activation of PI3kinase is required but is not sufficient for transformation.

12.2.4 Mitogen-Activated Kinase (MAPK) – ERK, p38, and JNK

MAPK regulates many cellular responses to growth factors and stress through its activation of the downstream kinases ERK, p38, and JNK. Activation of JNK is primarily mediated by CTAR2 while both domains can activate p38 (Eliopoulos et al. 1999a,b; ; Eliopoulos and Young 1998). CTAR2 mediates signals through TRADD and RIP (Izumi and Kieff 1997). In TNFR signaling, TRADD induces pro-apoptotic signals through its death domain (DD). However, in context with LMP1, it activates NFκB and JNK (Soni et al. 2007). The inability of CTAR1 to activate JNK in epithelial cells has been suggested to be due to low levels of TRAF1 expression which is required for JNK activation (Eliopoulos et al. 2003). Initial studies indicated that CTAR2 mediates signals through TRADD and TRAF2 since a dominant negative TRAF2 could impede NFκB signaling from both CTAR1 and CTAR2 (Devergne et al. 1996). However, in recent studies using knockout mouse embryo fibroblasts, it has been shown that TRAF6 is responsible for CTAR2 signaling (Wu et al. 2006). The interactions between the adaptor protein, BSM2, that was shown to bind CTAR2 and TRAF6 may specifically activate JNK (Wan, et al. 2006).

LMP1 has also been shown to induce activation of MAPK and ERK1/2 in Rat-1 fibroblasts and in T-cells (Roberts and Cooper 1998). In rodent fibroblasts, activation of ERK strictly required CTAR1 and only LMP1 mutants that could activate ERK were transforming (Mainou et al. 2007). Inhibitors of ERK activation blocked transformation. These studies indicate that PI3kinase and ERK activation are required for rodent fibroblast transformation and reveal that transformation of fibroblasts differs in requirements compared to B-lymphocytes transformation. It is likely that a subset of the factors required for lymphocyte transformation is sufficient for rodent fibroblast transformation.

12.2.5 Effects of LMP1 Strain Variation on Transformation

Several types of sequence variation have been detected in LMP-1. Within the carboxy terminus, the number of an 11-amino acid (aa) repeat element is variable (Miller et al. 1994). In the prototype Type 1 B95-8 sequence and in the Type 1 RAJI and Type 2 HR-1 strains, the third repeat element contains an insertion of 5 aa which are also the first five unique aa after the last partial repeat element. Additionally, a deletion of amino acids 343–352 of the B95-8 LMP-1 has been described (Miller et al. 1994). This deletion has been the focus of many studies that indicated that LMP1 with the deletion had enhanced transforming potential in vitro and may be present in more aggressive disease forms (Knecht et al. 1993). Beyond these differences, identification of distinct, consistent signature amino acid changes led to the identification of seven variants of LMP1 (Edwards et al. 1999). The CTAR1 and CTAR2 motifs are highly conserved and unchanged in all variants. Comparison of the biologic and signaling properties of the variants indicated that all of the variants were capable of transforming Rat-1 fibroblasts as measured by blockage of contact-inhibited and anchorage-independent growth and induced homotypic adhesion in EBV-negative B-cells (Mainou and Raab-Traub 2006). All variants activated the PI3K-Akt signaling cascade and induced changes in cellular markers that are frequently associated with cell cycle deregulation. However, three of the variants had decreased binding to the ubiquitin ligase, HOS resulting in increased NFκB reporter activity (Mainou and Raab-Traub 2006).

Most of the conserved sequence changes in the variants were shown to be within known and predicted potential human leukocyte antigen (HLA)-restricted epitopes, suggesting a potential immune-modulated selection mechanism (Edwards et al. 2004). Although all variants have been detected in various malignancies, the CAO/China 1 LMP1 variant is significantly more prevalent in NPC tumors from the southern endemic region of China. This variant is not presented by individuals who are A2 or A24, the most common HLA types found in patients with NPC.

12.2.6 Effects in Transgenic Mice

Transgenic mice expressing LMP1 under an immunoglobulin heavy chain promoter and enhancer have increased incidence of B-cell lymphomas by 12 months of age (Kulwichit et al. 1998). Analysis of the activated-signaling pathways indicated that the transgenic tumors were similar to tumors that developed in LMP1-negative littermates (Thornburg et al. 2006). Subsequent studies have determined that lymphoma specifically develops in the B-1a subset of B-lymphocytes, a population that can be clonally expanded in older mice and is associated with the development of murine B-cell malignancies (Shair et al. 2007). The lymphoma cells have deregulated cell cycle markers including

phosphorylated Rb, elevated cdk2, and decreased p27. The malignant cells have constitutively activated STAT3 and also secrete IL10. These same targets are also deregulated in wild-type B-1a lymphomas that arise spontaneously through age-predisposition. Inhibitors of PI3K, Akt, NFκB, and STAT3 signaling inhibit the enhanced viability and growth of healthy LMP1 transgenic lymphocytes and lymphoma cells in vitro. These results indicate that LMP1-induced activation of the IL10/STAT3, Akt, and NFκB-signaling pathways underlies the ability of LMP1 to promote malignant transformation in this model system.

Transgenic mice that express LMP1 in epithelial cells using either the polyoma promoter or a keratin 14 promoter have also been produced (Wilson et al. 1990). The polyoma promoter transgenic mice develop epithelial hyperplasia and have abnormal patterns of hair growth. In other studies where the transgenic mice were treated with carcinogens and phorbol esters, the mice developed papillomas and carcinomas at levels higher than control mice (Curran et al. 2001). These studies suggest that LMP1 effects on proliferation and differentiation contribute to the development of carcinomas.

12.3 Latent Membrane Protein 2

The LMP2 proteins are integral membrane proteins with 12 transmembrane domains and a 119 bp amino terminal cytoplasmic domain (Longnecker et al. 1991). The LMP2 mRNAs are unique in that they are highly spliced mRNAs and contain exons located at both ends of the linear EBV genome (Sample et al. 1989). Thus, they could only be expressed from the episomal form of EBV during latent infection. LMP2B lacks the first exon which encodes the N-terminal cytoplasmic domain. Similarly to LMP1, LMP2 oligomerizes in the plasma membrane (Longnecker and Kieff 1990). It has been shown that LMP2B can downregulate LMP2 effects possibly due to interactions with LMP2A through the transmembrane domains.

Elegant genetic studies determined that LMP2 is not required for transformation (Longnecker et al. 1992, 1993a, b;). However, the importance of LMP2 to pathogenesis is indicated by its consistent expression in most of the malignancies associated with EBV including NPC and HD, where it is abundantly detected within the malignant Reed–Sternberg cells (Busson et al. 1992). Antibodies to LMP2 are not usually found; however, patients with NPC do have readily detectable antibodies to LMP2 (Lennette et al. 1993). In addition, several CTL epitopes have been identified within LMP2 and novel immunotherapies are currently being developed to target LMP2 expression in NPC and HD (Bollard et al. 2006).

The N-terminal cytoplasmic domain contains eight tyrosines in potential-signaling motifs (Fig. 12.2). Two of the tyrosines form an immunoreceptor-tyrosine-based activation motif (ITAM). LMP2 was initially shown to have a

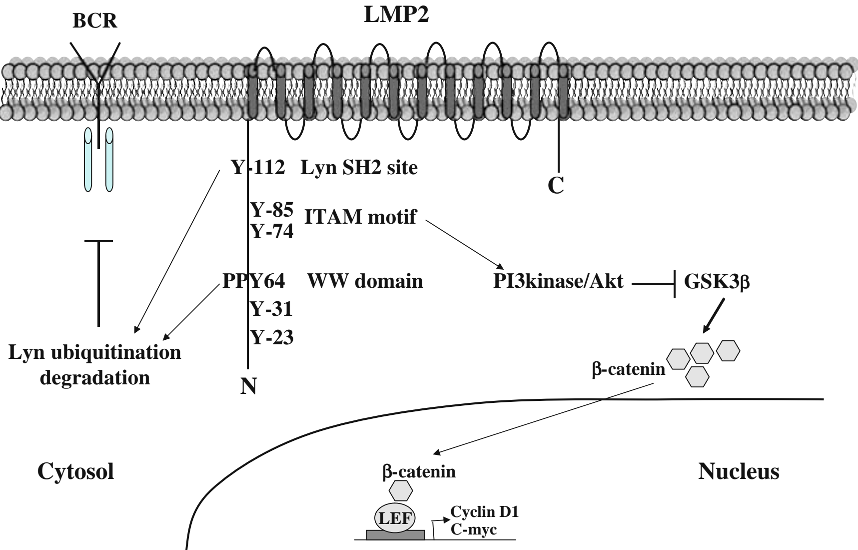

Fig. 12.2 LMP2 effects on cell signaling

potent effect on B-cell signaling (Miller et al. 1993). The B (BCR) and T-cell receptors contain ITAMs that are phosphorylated by src family kinases and bind syk or ZAP70 leading to activation of multiple signal transduction cascades. LMP2 blocks signaling from the BCR and all of the subsequent phosphorylation events (Miller et al. 1994). LMP2A apparently sequesters lyn and syk kinases within lipid-rich rafts and induces their degradation. This property is linked to a PY motif within LMP2 that has been shown to bind members of the HECT domain-containing Nedd4 ubiquitin ligases (Ikeda et al. 2001). Association of these ligases and the lyn tyrosine kinase induces lyn degradation. One important consequence of this inhibition is that it prevents the activation and differentiation of lymphocytes, a process that can induce activation of the EBV replicative cascade. Thus, expression of LMP2A helps maintain a latent infection in B-lymphocytes.

In epithelial cells, LMP2A becomes phosphorylated on tyrosines upon adhesion to extracellular matrix, ECM, proteins that are ligands for integrin receptors (Scholle et al. 1999). Integrins that are expressed in the proliferating, basal layer of the epithelium are in contact with the basement membrane and integrin ligands. As cells move from the basal layer to form the upper epithelium, integrin signaling is affected and the cells begin to differentiate. LMP2 inhibits differentiation of the HaCat epithelial cell line in organotypic raft cultures (Scholle et al. 2000). The LMP2A expressing HaCat cells also formed colonies in soft agar indicating that they continued to proliferate without attachment to matrix and these cells formed aggressive tumors in nude mice. The cells had activated Akt kinase that indicated activation of PI3 kinase and

inhibition of PI3 kinase blocked their ability to grow in soft agar. Thus in some epithelial cell lines, LMP2A may be transforming, possibly due to activation of Akt. A new study revealed that not all HaCat cells can be transformed; however, in this study LMP2 was able to induce transformation of a second epithelial cell line (Fukuda and Longnecker 2007). One important difference was the activation of ras in the transformed epithelial cells. LMP2 may only be transforming or oncogenic in cells with activated ras or where ras can be activated by LMP2. Subsequent studies have also shown that LMP2 induces epithelial cell motility and increases expression of the a6 integrin (Pegtel et al. 2005). Blocking of a6 integrin inhibited migration indicating that this signaling is required for increased motility.

In normal epithelial cells immortalized with the catalytic subunit of telomerase, LMP2 expression induced the activation of Akt via PI3K signaling and the Akt targets, FKHR and GSK3β, were phosphorylated (Morrison et al. 2003). LMP2 expression resulted in dramatically increased levels of β-catenin in the cytoplasm and increased translocation of β-catenin to the nucleus. The translocated β-catenin was functional and in reporter assays activated a TCF-sensitive reporter. The translocation of β-catenin to the nucleus was dependent on the ITAM motif and PI3kinase activation (Morrison and Raab-Traub 2005). Although LMP2 activated this critical pathway, the telomerase-immortalized epithelial cells that expressed LMP2 were not transformed and could not form tumors in nude mice.

Activation of Akt is likely to be an important property of LMP2 that may be responsible for its ability to enhance B-cell survival and contribute to oncogenesis. The constitutive nuclear β-catenin and activation of TCF/LEF target genes are hallmarks of the vast majority of colon cancers that have inappropriate activation of the Wnt pathway. GSK3β, β-catenin, APC, and axin are in a complex that is part of the Wnt pathway (Segditsas and Tomlinson 2006). APC and axin are considered tumor suppressor genes due to their inhibitory effects on this pathway while the wnt ligand protein and β-catenin have been shown to have oncogenic capacity. Mutations in the phosphorylation sites on β-catenin and mutations in APC, which also affect β-catenin turnover, have frequently been identified in colon, prostate, and skin cancers and in medulloblastomas and hepatocellular carcinomas. In carcinomas that lack mutations in APC or β-catenin, mutations in axin have frequently been identified, indicating that constitutive activation of this pathway can occur through multiple genetic events. The activation of this pathway by LMP2 may preclude the genetic activating mutations detected in other carcinomas.

Phosphorylated, activated Akt, phosphorylated GSK3β, and nuclear β-catenin were also detected in the xenografted NPC tumor, C15, and in primary NPC samples (Morrison et al. 2004). These findings indicate that the β-catenin/Wnt pathway is activated in NPC. Expression of LMP2 and activation of β-catenin regulated expression are likely important components of EBV effects on epithelial cell growth. Interestingly, activated Akt was also detected in HD; however, effects on β-catenin were not detected (Morrison et al. 2004).

This finding suggests that similar to LMP1, LMP2 also has cell type specific effects.

A recent study also revealed potentially powerful effects of LMP2 on epithelial cell growth (Moody et al. 2003). In primary infected epithelial cultures, a few EBV-infected clones rapidly overtook the culture. The clones that had this growth advantage had fewer numbers of TR and it was shown that this property resulted in increased expression of LMP2. Thus cells that expressed more LMP2 as a result of fewer terminal repeats grew significantly faster in culture.

12.3.1 Effects in Transgenic Mice

Transgenic mice that express LMP2 using the heavy chain enhancer and promoter have revealed LMP2 has intriguing effects on B-cell development and differentiation. In one line with high levels of LMP2 expression, B-cells are produced that do not express the BCR and lack heavy chain rearrangement (Caldwell et al. 1998, 2000). Such cells are usually eliminated through apoptosis; however, elevated levels of LMP2 apparently provide a viability signal that bypasses normal regulation. This ability required the ITAM and its association with syk. These transgenic mice have been further characterized. All of the lymphocytes were shown to be B1-a lymphocytes, a subset of B-cells that are primarily found in the peritoneal cavity and may be activated independently of T-cell help (Ikeda et al. 2004). Apparently expression of LMP2 during development substitutes for those signals that usually come from the BCR and this signaling skews development to the B-1 subset. Interestingly, transgenic mice that express LMP1 under the same regulatory sequences have normal B-cell development but develop lymphomas in the B-1a subset (Shair et al. 2007).

Transgenic mice that express LMP2 in squamous epithelial cells have also been produced using the K14 promoter (Longan and Longnecker 2000). These mice do not have any apparent defects in skin development and without detectable effects on differentiation. These studies suggest that LMP2 lacks transforming properties in normal epithelia and may require activation of other pathways or genetic events for transformation.

12.4 EBNA1

EBNA1 enables replication of the EBV episome by binding to a series of 30 bp repeats that comprise the EBV origin of plasmid replication (Orip) (Yates et al. 1984). A transcriptional enhancer within orip is activated by EBNA1 and is suggested to influence the activity of the Wp/Cp promoters that regulate transcription of the primary EBNA1 transcript in Type 3 latency, and also the promoter for LMP1 (Altmann et al. 2006). As EBNA1 is required for maintenance of latent infection, it is expressed in all forms of latency and in

all of EBV-associated malignancies. It also has the unique property of escaping proteasomal degradation and presentation by HLA class I (Levitskaya et al. 1995).

Because of the strict requirement for EBNA1 in latent infection, it has been difficult to genetically separate its latency maintenance properties for any contribution to growth transformation. Several studies indicate that it is not transforming. In a study of a cell line containing integrated EBV that would not require the EBNA episomal maintenance function, expression of a dominant negative form of EBNA1 had no effect on cell growth (Kang et al. 2001). In addition, EBV deleted for EBNA1 was still transforming in the rare cell lines that developed integrated EBV (Humme et al. 2003).

A role for EBNA1 in malignancy has been reported using a line of transgenic mice that expressed EBNA1 using the heavy chain promoter and a polyoma enhancer and developed B-cell lymphoma with 100% penetrance (Wilson et al. 1996). This study has been controversial as the protein was inconsistently detected in this lineage but was readily detectable in a second lineage that did not develop lymphoma. This inconsistency suggested that the oncogenic properties of the first lineage may reflect insertional mutagenesis. To further evaluate this, three additional EBNA1 transgenics were produced using the heavy chain promoter and enhancer (Kang et al. 2005). Although the mice were maintained until late age (18–26 months), lymphomas were not detected in any organs. The transgenic mice were identical to control littermates with normal weight and spleen size and splenocyte differentiation and antibody production. These studies strongly suggested that EBNA1 did not possess oncogenic properties.

However, EBNA1 like many EBV proteins has interesting properties that could have contributing transforming effects. Protein profiling revealed that EBNA1 interacted with a ubiquitin ligase called HAUSP that has been implicated in stabilizing p53 (Saridakis et al. 2005). EBNA1 was shown to bind with higher affinity to the region of HAUSP that binds p53 and MDM2. A functional consequence of this interaction was demonstrated in the ability of EBNA1 to protect from p53-induced apoptosis.

Additional intriguing properties were identified using transcriptional profiling of a carcinoma epithelial cell line that stably expressed EBNA1. One important gene that was upregulated was STAT1 (Wood et al. 2007). Upregulation by EBNA1 was confirmed at the protein level and this increase resulted in elevated, activated phosphorylated STAT1 after treatment with IFNα and IFNγ. Analyses of EBV-related tumors have identified elevated STAT1 in NPC tissues suggesting that this property occurs during oncogenesis in vivo (Chen et al. 2001). A second gene that was identified in this profiling study was βig-h3, protein that is induced by TGFβ treatment and is regulated by SMAD2 (Wood et al. 2007). EBNA1 was shown to decrease SMAD2 levels with subsequent impairment in TGFβ response.

The powerful molecular properties of EBNA1 with regard to chromatin and DNA binding and transcriptional regulation are likely to have the ability to also

affect cellular growth and gene regulation. The unique interactions of EBNA1 that could affect p53 function and its ability to enhance STAT signaling and impair TGFβ signaling could well be contributing factors in EBV transformation and oncogenesis in vivo.

12.5 BARF1

The BARF1 gene encodes a 31 kD protein that is expressed early after lytic infection. This protein is secreted and can function as a decoy receptor for colony stimulating factor 1 (Strockbine et al. 1998). BARF1 can inhibit secretion of αIFN from mononuclear cells (Cohen and Lekstrom 1999). Because BARF1 is not expressed during latency, it has not been considered likely to contribute to tumor growth. However, several studies have identified expression of BARF1 in most latently infected NPC cells (Seto et al. 2005).

The potential growth-transforming properties of BARF1 were first demonstrated using BALBc3T3 cells and the EBV-negative BL Louckes cell line (Ooka 2001). The BARF1 expressing 3T3 cells formed aggressive tumors in nude mice while the Louckes cell line had elevated expression of c-myc and activation antigens.

The transforming properties have also been demonstrated using the Akata Burkitt lymphoma cell line (Sheng et al. 2003). Clones of Akata cells can be isolated that have lost the EBV genome. These EBV-negative Akata cells do not grow in soft agar and cannot form tumors in nude mice and reinfection with EBV restores these properties. Thus this has been a useful system to identify the viral protein(s) that confer these properties. In this system, BARF1 induced expression of bcl2 and induced tumor formation in SCID mice. These studies suggest that BARF1 may also contribute to EBV-induced tumorigenesis. This could occur if there was consistent expression in some situations such as NPC or as a result of paracrine effects on neighboring cells if expressed in some tumor cells during lytic reactivation.

12.6 The EBV-Encoded Noncoding RNAs – EBERs

The most abundant RNAs in EBV-infected cells are small nuclear EBER RNAs (Arrand and Rymo 1982). They are present at approximately 10^5 copies per cells but are not necessary for lymphocyte transformation (Howe and Steitz 1986). The EBERs are expressed in many of the malignancies linked to EBV and contribute in some way to the maintenance of latency in vivo. Examples of NPC that have differing degrees of differentiation lack EBER expression in differentiated areas and the EBER RNAs are also not detected in the permissive EBV infection, hairy leukoplakia (Gilligan, et al. 1990a; Pathmanathan et al. 1995). In vitro studies have also shown that they are downregulated during viral

replication. These findings suggest that expression of the EBER RNAs is downregulated during differentiation and replicative infection and most likely contributes to latency or transformation.

EBER1 and EBER2 are 167 and 172 base pairs long with complex predicted secondary structure. They are present in ribonucleoprotein complexes that contain the LA protein. Antibodies to this protein are produced in patients with systemic lupus erthematosus (Lerner et al. 1981). LA binds the 3' termini of all PolIII transcripts. The EBERs also bind the ribosomal protein L22 which binds to the EBER1 stem loop structure (Toczyski et al. 1994). L22 has been implicated in tumorigenesis as it is the target of a chromosomal translocation detected in leukemia.

Functionally, the EBERs were shown to inhibit IFN-induced PKR activation and block phosphorylation of eIF2a which controls protein synthesis and blocks it after IFN treatment (Nanbo et al. 2005; Sharp et al. 1993). This ability to block IFN could be a major factor during in vivo infection. In addition, some studies have suggested that PKR can function as tumor suppressor so it might be important to block this property.

The contribution of the EBERs to transformation has primarily been demonstrated in the EBV-negative Akata cells where expression of the EBER RNAs partially restores growth transformation and induces the ability to form tumors in SCID mice (Yajima et al. 2005). These tumors have elevated bcl2. Subsequent studies of Akata cells have shown that EBV infection or EBER expression induced IL10 through EBER interactions with RIG1 (Samanta et al. 2006). IL10 can function as a B-cell growth factor and antibodies to IL10 inhibited EBER-induced growth stimulation indicating that its induction was required for the EBER-induced growth transformation.

12.7 BamHI A Rightward Transcripts (BARTs)

Studies of EBV expression in NPC identified a family of rightward transcripts from the *Bam* HIA region that were abundantly expressed in NPC but were barely detectable in lymphoid cell lines (Gilligan et al. 1991). The BamHIA transcripts (also called BARTs or CSTs) are differentially spliced and give rise to several different family members that are 3' co-terminal (Sadler and Raab-Traub 1995). Cloning and sequencing cDNAs from the C15 tumor revealed patterns of alternate splicing of at least seven exons that form several ORFs (Fig. 12.3). It is unclear if the BamHIA mRNAs actually encode protein as none of the proteins have been identified as yet (Thornburg et al. 2004). However, several interesting properties have been identified for the putative proteins. All of the *Bam* HIA transcripts contain the BARF0 ORF at their 3' ends, which potentially encodes a 174-amino acid protein. The BARF0 ORF is unusual in that the stop codon is embedded in the polyadenylation codon and it is unclear how efficiently this mRNA would produce protein (Sadler and Raab-Traub

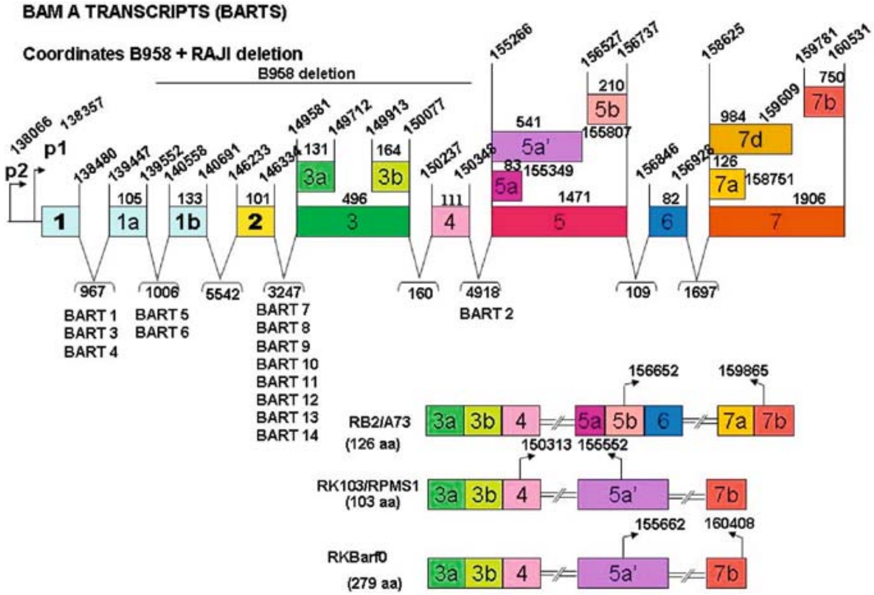

Fig. 12.3 Structure, open reading frames, and miRNAs

1995). One cDNA, termed RK-BARF0, contained the BARF0 ORF extended at its 5' end. RK-BARF0 could potentially encode a 279-amino acid protein with a highly hydrophobic N-terminal region that resembles an endoplasmic reticulum (ER) targeting sequence (Sadler and Raab-Traub 1995).

A yeast two-hybrid analysis of the RK-BARF0 ORF revealed that it binds unprocessed Notch1, scramblase, and the human homologue of I-mfa (HIC) (Thornburg, et al. 2004). The interaction of Notch and RK-BARF0 induces proteasome-mediated degradation of both endogenously and exogenously expressed Notch. This reduction of overall Notch protein could inhibit Notch signaling.

HIC was initially identified as a cellular protein that interacted with HIV tat and affected tat-regulated promoter function. HIC is the human homolog of I-mfa, which has been shown to inhibit myogenin and myo-D-induced transcription. HIC can also bind and inhibit LEF-induced transcription (Ghogomu et al. 2006). Thus by inhibiting HIC function, BARF0 could enhance B-catenin-induced transcription and possibly enhance the properties of LMP2.

Potential interesting properties of other BART ORFs have also been shown (Fig. 12.3). The A73 or RB2 ORF interacts with the receptor for activated src (RACK1) with as yet unknown consequences on RACK1 or src function (Smith 2001). A fragment of the RPMS1 or RK103 ORF contains a WW motif that interacts with RBPJk. This is the same cellular transcription factor that interacts with EBNAs 2, 3A, 3B, and 3C. Thus, this protein could possibly

modulate the interaction of EBNA2 with RBPJk or substitute for EBNA2 in epithelial cells. This interaction could also affect Notch signaling; thus, co-expression of BARF0 and RPMS1 could effectively reduce Notch signaling. The Notch pathway regulates development and cell fate decisions through control of diverse mechanisms such as proliferation, cell cycle arrest, differentiation, cell survival, and apoptosis (Kato et al. 1996; Osborne and Miele 1999). Due to its diverse functions, disruption of controlled Notch activation, either through constitutive activation or through constitutive repression, could promote transformation and oncogenesis.

Virally encoded miRNAs were first described in EBV (Pfeffer et al. 2004). These RNAs are noncoding and can post-transcriptionally downregulate the expression of mRNAs with complementary sequences (Bartel 2004). Using the EBV B95 laboratory strain, three miRNAs were cloned from the BamHI H fragment and two from the BART region (Pfeffer et al. 2004). However, B95 has a major 12 kb deletion that encompasses most of the BART region (Raab-Traub et al. 1980). Subsequent cloning using the NPC C15 tumor and the BC1 KSH/EBV lymphoma cell line identified 14 additional miRNAs produced from the introns of the BARTs (Fig. 12.3) (Cai et al. 2006). This study also revealed that the three BamHI H miRNAs were actually produced from a large intron in the primary EBNA transcript that is only expressed in B-lymphocytes. One of these miRNAs has subsequently been shown to also be processed from the BamHI H replicative transcript (Xing and Kieff 2007). In keeping with the first description of the BART transcripts, the BART miRNAs are abundant in NPC samples but are only detected at low levels in some latently infected lymphocytes. Nine of the BART miRNA are highly conserved with miRNAs cloned from the rhesus homologue of EBV (Cai et al. 2006). This conservation is considerably greater than the sequence homology in the rest of the genomes and suggests that these miRNAs target cellular mRNAs. It will be important to identify the targets of these miRNAs to determine how they contribute to regulation of cellular growth.

Additional roles for some of these transforming genes in viral latency are described in Chapter 14 (Epstein–Barr Virus Latent Infection Nuclear Proteins; Genome maintenance and regulation of lymphocyte cell growth and survival).

12.8 Summary

This review of the properties of EBV proteins that affect cell growth indicates that they alter a myriad of regulatory pathways that intersect and cross-regulate. Some pathways are affected directly while others are modulated by cellular genes that are induced by EBV. It is also apparent that some pathways are targeted by multiple viral genes.

The Notch pathway is used and abused by multiple EBV proteins. Four of the EBNAs interact with the Notch targeted DNA-binding protein, RBPJk, to

regulate viral and cellular transcription. RBPJk also binds the putative RPMS1 protein encoded by the BART transcript. Another BART protein, BARF0, also interacts with Notch and induces its degradation. Thus it seems that EBV blocks cellular Notch signaling and also uses the Notch interacting DNA-binding protein for its transcription factors.

The Wnt pathway regulates expression through effects on β-catenin and its activated transcription factors, Tcf/lef. EBV apparently targets β-catenin through multiple mechanisms including LMP1-mediated effects on ubiquitin ligases and LMP2 inactivation of GSK3β. Tcf/lef transcription is also regulated by interactions with HIC which is targeted by BARF0.

LMP1 affects multiple mechanisms that regulate NFκB and activates both the canonical and the non-canonical pathways. These activations are mediated through effects on different kinases mediated by distinct molecular interactions of LMP1 and adaptors. LMP1 effects on ubiquitin ligases also contribute to levels of components of the pathway. It is also likely that LMP2 effects on ubiquitin ligases also affect NFκB regulation.

The further study of the multiple ways that EBV mediates transformation will possibly identify additional pathways that are critical for normal growth regulation. The pathways that are targeted by EBV are likely to be affected by other mechanisms in other transformation models and in oncogenesis. In addition, the targeting of these pathways by multiple viral proteins through distinct mechanisms will aid in identifying critical regulatory points. The new finding that EBV also encodes multiple miRNAs will also likely lead to new understanding of pathways and genes that are regulated by these new macromolecules. The study of EBV continues to illuminate mechanism by which viruses usurp cellular growth regulation and contribute to oncogenesis.

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Chapter 13
The Epstein–Barr Virus Lytic Life Cycle

Sankar Swaminathan and Shannon Kenney

13.1 Introduction

The lytic phase of Epstein–Barr virus (EBV; Zalani et al. 1996) infection is an essential part of the virus life cycle, since only this form of viral replication results in the production of infectious viral particles, and allows the virus to be transmitted from cell to cell and host to host. Although development of EBV-associated malignancy is primarily associated with growth of latently EBV-infected cells, immunosuppressed patients often have abnormally high levels of infectious viral particles in their plasma, and enhanced intercellular transmission of virus may play a role in increasing the likelihood that immunosuppressed patients eventually develop EBV-induced lymphoproliferative disease (LPD) (Cohen 2000; Feng et al. 2004a) Anti-viral drugs (acyclovir and ganciclovir) which inhibit the lytic form of EBV replication decrease the risk of post-transplant lymphoproliferative disease in renal transplant recipients (Funch et al. 2005). In addition, early-passage B cells immortalized with lytic-defective EBV (missing either viral immediate-early gene) have a reduced ability to form LPD-like lesions in SCID mice relative to B cells immortalized with wild-type EBV (Hong et al. 2005). Lytically infected B cells secrete paracrine factors (including cellular IL-6 and VEGF) that may promote the growth of the latently infected B cells in LPD lesions (Hong et al. 2005; Hong et al. 2005; Jones et al. 2007). Finally, although a small number of lytically infected cells within tumors may promote their growth through the above mechanisms, it is extremely unlikely that tumors can continue to grow if the majority of tumor cells become lytically infected. Therefore, there has been substantial interest in defining agents which can efficiently induce the lytic form of EBV infection in tumor cells, thereby using

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_13, © Springer Science+Business Media, LLC 2009

the virus itself to kill tumors (Feng and Kenney 2006; Fu et al. 2007). The results of the first phase 1/2 study using intentional induction of lytic EBV infection as a method for treating EBV-positive lymphomas in patients were very promising (Perrine et al. 2007). Somewhat surprisingly, however, the only human disease currently known to be caused by the lytic form of EBV infection is oral hairy leukoplakia (OHL). OHL, which predominantly occurs in immunosuppressed patients, is a relatively innocuous white lesion on the side of the tongue that results from lytic EBV infection of differentiated tongue epithelial cells (reviewed in Greenspan et al. 1985). Thus, although EBV-associated cancers are largely composed of cells harboring latent EBV, understanding how EBV converts from the latent to the lytic form of infection and produces infectious viral particles is relevant to the pathogenesis and treatment of EBV-associated malignancies.

13.2 Lytic EBV Infection in Humans

In immunocompetent humans, it has been difficult to identify EBV-positive cells with the lytic form of infection, although such cells must surely exist given that EBV is commonly present in the saliva of normal hosts. The extreme rarity of lytically infected cells in normal hosts likely reflects the robust cytotoxic T-cell response directed against lytic viral proteins (Steven et al. 1997), such that most lytically infected cells are rapidly eliminated. To date, lytic EBV infection in normal hosts has only been found in oropharyngeal epithelial cells (Steven et al. 1997; Pegtel 2004 #1388) and in tonsillar plasma cells (Laichalk and Thorley-Lawson 2005). In completely lytic OHL lesions, EBV infection only occurs in the more differentiated epithelial layers (Niedobitek et al. 1991); there is no evidence suggesting that EBV normally infects undifferentiated epithelial cells (Feederle et al. 2007; Niedobitek et al. 1991).

13.3 Lytic Viral Gene Cascade: Overview

The lytic genes of herpesviruses, including EBV, are expressed in a temporally regulated manner. The current model of the progression of lytic herpesvirus infection is largely derived from studies performed with herpes simplex virus. In this model, the first viral genes to be transcribed are the viral immediate-early (IE) genes. Since the transcription of viral IE genes is activated by viral proteins present in the tegument of the incoming virion particle and by host cell proteins, these genes are transcribed even in the presence of protein synthesis inhibitors and DNA replication inhibitors. Next, the viral IE proteins, which include one or more transcription factors, activate expression of the viral early genes. Viral early proteins include proteins which mediate replication of the viral DNA. Transcription of early genes is inhibited by protein synthesis inhibitors, but not by replication inhibitors. Following replication, transcription of late viral genes

is activated. Late viral genes encode structural proteins which allow the virus to be encapsidated and produce infectious virion particles. Transcription of late genes is reduced by both protein synthesis inhibitors and replication inhibitors.

In the case of EBV, due to the difficulty of studying lytic infection in vitro, it has not been possible to confirm some aspects of the above model, and there is evidence to suggest that certain aspects of the model may not be accurate during lytic EBV infection. Instead of defining IE genes as the first viral genes transcribed during primary infection of cells, EBV immediate-early genes have been previously defined as the first viral genes expressed (in the presence of protein synthesis inhibitors) when latently infected cells are treated with stimuli that induce lytic gene transcription. By this definition, BZLF1 and possibly BRLF1 are EBV IE genes (Biggin et al. 1987; Flemington et al. 1991; Shimizu et al. 1989). Only recently has it been confirmed that BZLF1 is indeed an IE gene during primary EBV infection of B cells (Wen et al. 2007); the status of BRLF1 as an IE gene during primary infection of B cells or epithelial cells has not yet been reported. Whether the EBV tegument protein, BPLF1, which has homology to the HSV VP16 protein (Schmaus et al. 2004), plays a similar role in inducing transcription of EBV IE genes during primary infection remains unknown (but likely). The growing evidence that a subset of “late” EBV genes encoding structural proteins is expressed even in the absence of viral replication, and that this subset is transcriptionally activated by the BRLF1 protein (Chua et al. 2007; Feederle et al. 2000; Francis et al. 1999; Han et al 2007; Lu et al. 2006), suggests that late gene transcription in EBV may be regulated in a different manner than late gene transcription in herpes simplex virus.

13.4 Reactivation of Lytic EBV Infection In Vitro

Because a cell culture system which supports efficient primary lytic EBV infection does not currently exist, it has been technically challenging to study many aspects of lytic EBV infection in vitro. In contrast, many latently infected EBV-positive cell lines are available. The discovery that high-level expression of a single viral protein, BZLF1, in latently infected cell lines is sufficient to reactivate the lytic form of viral infection (Countryman et al. 1987; Countryman and Miller 1985; Takada et al. 1986) was a major advance in the field and has allowed investigators to define the mechanisms by which BZLF1 induces lytic EBV replication. Furthermore, this discovery indicated that regulation of BZLF1 gene expression by cellular transcription factors must play a key role in determining if EBV infection is latent or lytic in host cells. Another major advancement was the discovery that a particular EBV-positive Burkitt line (Akata) can be efficiently switched to the lytic form of infection in a near-synchronous manner by activating (cross-linking) the B-cell receptor with anti-immunoglobulin (Takada and Ono 1989). This finding has allowed investigators to examine cellular regulation of viral IE promoters in the context of the intact viral genome in a physiologically relevant cell type. Our knowledge

regarding the key aspects of lytic EBV infection, discussed below, was largely derived from transfecting latently infected cells with BZLF1 expression vectors and/or treating Akata cells with anti-IgG. Other agents which can be used to induce the lytic form of EBV infection in latently infected cells include the phorbol ester, 12-0-tetradecanoyl phorbol-13-acetate (TPA), sodium butyrate (an HDAC inhibitor) and calcium ionophores (Faggioni et al. 1986; zur Hausen et al. 1978). Ultimately, all of these inducing stimuli share the ability to activate transcription of BZLF1 from the latent viral genome.

13.5 Initial Steps in Viral Reactivation

The promoters driving BZLF1 and BRLF1 transcription, Zp and Rp, are inactive in B cells containing the latent form of EBV infection (Biggin et al. 1987; Flemington et al. 1991; Shimizu et al. 1989). Epigenetic modifications of viral DNA, such as DNA methylation and histone deacetylation, probably inhibit IE gene transcription in latently infected cells (Ambinder et al. 1999; Batisse et al. 2005; Bhende et al. 2004; Falk and Ernberg 1999; Jenkins et al. 2000; Nonkwelo and Long 1993; Paulson et al. 2002; Paulson and Speck 1999; Szyf et al. 1985). However, even “naked” DNA reporter gene constructs driven by the Zp and Rp promoters are essentially inactive in many EBV-negative B-cell lines (Bhende et al. 2004; Feng et al. 2004b; Kenney et al. 1989; Sinclair et al. 1991; Zalani et al. 1995), but can be activated by various types of lytic-inducing stimuli such as B-cell receptor stimulation. Thus, the inactivity of the Zp and Rp promoters in unstimulated B cells is due both to the lack of *trans*-acting transcriptional activators as well as the presence of cellular repressors which regulate the two IE promoters.

13.6 Induction of Lytic EBV Gene Expression During Plasma Cell Differentiation

The human protein XBP-1, a basic-region leucine zipper transcriptional activator protein of the CREB/ATF family, is activated early during plasma cell differentiation, where it plays an essential role (Liou et al. 1990; Reimold et al. 2001). XBP-1 reactivates lytic EBV infection when combined with activated protein kinase D (PKD) (Bhende et al. 2007; Matthews et al. 1994), a type II histone deacetylase inhibitor which is activated following antigen receptor engagement in B cells. XBP-1 alone efficiently activates Rp and in concert with PKD activates Zp. The mechanism by which XBP-1 activates Zp and Rp is not yet defined (but does not seem to involve direct binding to either promoter). Thus XBP-1, in conjunction with activated PKD, promotes the switch from latent to lytic EBV infection as B cells become activated by antigen and differentiate into plasma cells.

13.7 Activation of Zp by B-Cell Receptor Engagement and Other Lytic-Inducing Stimuli

Two types of *cis*-acting motifs, the “ZI” and “ZII” motifs (Fig. 13.1), are critical for activation of Zp by a variety of different lytic-inducing stimuli, including B-cell receptor engagement. The four ZI motifs are AT-rich sequences (Chatila et al. 1997) that have dual roles as negative and positive regulators of Zp transcription (Binne et al. 2002; Borras et al. 1996; Daibata 1994 #163; Flemington 1990 #148; Binne 2002 #111; Daibata et al. 1994; Flemington and Speck 1990d). While the ZI motifs inhibit Zp activity in the absence of lytic-inducing agents, they are also essential for Zp activation by a variety of different inducing stimuli, including TPA, anti-Ig, calcium ionophores and chemotherapy (Binne et al. 2002; Feng et al. 2004a). During viral latency, ZI binds to the cellular protein MEF2D, and MEF2D is thought to interact with type II histone deacetylating complexes (HDACs), thereby repressing Zp transcription (Gruffat et al. 2002). Alterations in the phosphorylation status of MEF2D that occur during B-cell activation may convert MEF2D to a positive regulator (Bryant and Farrell 2002). In addition, B-cell receptor engagement results in phosphorylation of type II HDACs, which induces relocalization of type II HDACs to the cytoplasm, preventing the interaction between MEF2D and HDACs (reviewed in Wang et al. 2006).

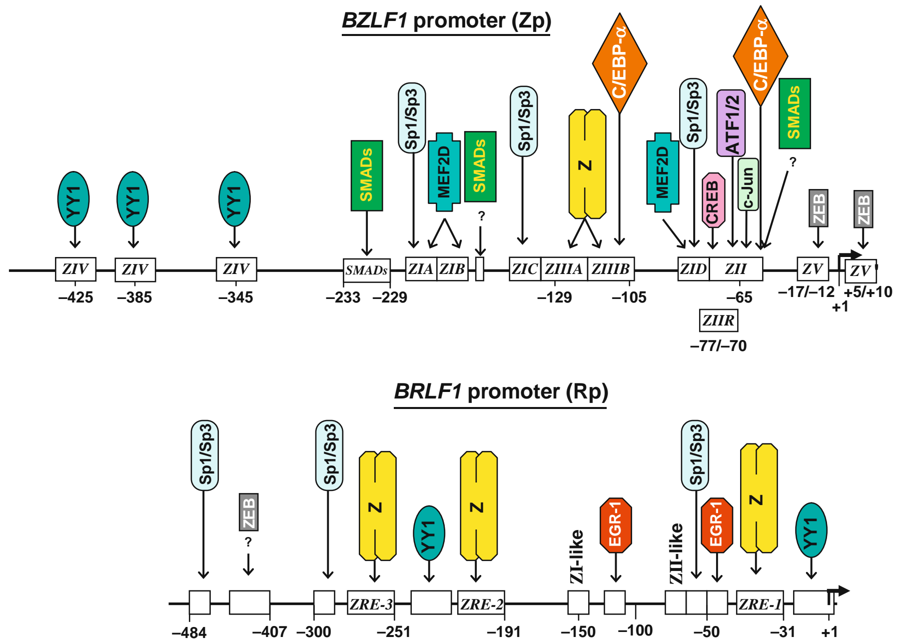

Fig. 13.1 The BZLF1 and the BRLF1 promoters

The "ZII" motif , a slightly atypical CREB-responsive element (CRE) that binds CREB, ATF-1, the ATF-2/c-jun heterodimer and possibly CCAAT/enhancer-binding proteins and the c-jun/c-fos ("AP1") heterodimer (Adamson et al. 2000; Fitz et al. 1997; Flamand and Menezes 1996; Flemington and Speck 1990; Liu et al. 1998), is also essential for induction of BZLF1 transcription by most stimuli (Binne et al. 2002; Chatila et al. 1997; Daibata et al. 1994; Feng et al. 2004a; Flamand and Menezes 1996; Flemington and Speck 1990d). Activation of the cellular transcription factors which bind to CRE motifs is generally mediated through phosphorylation by cellular kinases, including the c-jun N-terminal kinase (JNK) and stress Map kinase p38. Many of the stimuli that induce lytic EBV infection in vitro are known to activate the p38 and JNK kinases, and conversely, inhibitors of p38 and JNK kinases reduce the effectiveness of a number of lytic-inducing stimuli (Adamson et al. 2000; Feng et al. 2004a; Feng et al. 2002). Lytic induction may also be mediated through the activated (phosphorylated) forms of ATF-1 or CREB binding to the ZII motif,, particularly during epithelial cell differentiation (Fitz et al. 1997; Bryant and Farrell 2002; Karimi et al. 1995; MacCallum et al. 1999). TGF-beta activation of Zp, which occurs in certain BL lines (Fahmi et al. 2000; Liang et al. 2002), is also at least partially mediated through the ZII motif (Liang et al. 2002).

Negative regulation of BZLF1 transcription also plays a critical role in promoting viral latency in B cells. In particular, there is a "ZV" motif located near the TATA box that binds to the zinc finger protein, ZEB (Kraus et al. 2001; Kraus et al. 2003), and strongly inhibits BZLF1 transcription (Binne et al. 2002). YY-1-binding sites ("ZIV" elements) and a *cis*-acting sequence, ZIIR (which binds to an as-yet-unknown cellular factor), also function as negative regulators of BZLF1 transcription (Liu et al. 1998; Montalvo et al. 1995; Montalvo et al. 1991).

Once BZLF1 transcription has been activated by cellular factors, the BZLF1 gene product may autoactivate its own transcription through ZRE sites in Zp (Binne et al. 2002; Flemington and Speck 1990a). Nevertheless, whether BZLF1 actually activates the Zp promoter in the context of the intact genome during viral reactivation remains controversial (Binne et al. 2002; Le Roux et al. 1996; Zalani et al. 1996). In contrast, the BRLF1 protein clearly activates BZLF1 transcription in the context of the intact viral genome (Zalani et al. 1996) through its effects on c-jun, ATF-2 and PI3 kinase (Adamson et al. 2000; Darr et al. 2001).

13.8 Cellular and Viral Regulation of Rp Activity

Less is known about the regulation of the BRLF1 promoter (Rp). The Rp promoter, like Zp, is activated by B-cell receptor stimulation (Feng et al. 2004a; Sinclair et al. 1991; Zalani et al. 1995), and two EGR-1 (Zif-268)-binding motifs in Rp are required for this activation (Feng et al. 2004a; Zalani et al. 1995). As

mentioned previously, Rp is also highly activated by the plasma cell differentiation protein, XBP-1 (Bhende et al. 2007). Rp also contains several Sp1 sites (Zalani et al. 1992), which are required for constitutive promoter activity as well as efficient autoactivation of Rp by the BRLF1 protein (Ling et al. 2003; Ragoczy and Miller 2001). Like Zp, the Rp promoter contains binding sites for YY-1 which function as negative regulators (Zalani et al. 1997) (Fig. 13.1).

There are also at least three BZLF1-binding sites (ZREs) in Rp, and BZLF1 binds to, and activates, Rp through these ZRE sites (Bhende et al. 2004; Liu and Speck 2003; Sinclair et al. 1991). Of note, two of the ZRE sites in Rp contain CpG motifs which are methylated in latently infected cells (Bhende et al. 2005). Surprisingly, BZLF1 actually binds to, and activates, the methylated forms of these Rp ZRE sites more efficiently than the unmethylated forms (Bhende et al. 2004). This ability of BZLF1 to preferentially activate the methylated form of the BRLF1 promoter likely helps to ensure that under appropriate stimuli, the virus can rapidly reactivate to the lytic form of infection even when its viral genome is methylated in latently infected cells.

13.8.1 BZLF1 Protein

High-level expression of the BZLF1 protein (Z, ZEBRA, Zta, EB1) is sufficient to induce the switch from the latent to the lytic form of EBV infection (Chevallier-Greco et al. 1989; Countryman and Miller 1985; Ragoczy et al. 1998; Rooney et al. 1989; Takada et al. 1986; Westphal et al. 1999; Zalani et al. 1996). Conversely, a BZLF1-deleted EBV mutant cannot undergo the lytic form of viral replication unless the BZLF1 gene product is expressed in *trans* (Feederle et al. 2000). BZLF1, a homolog of c-jun and c-fos, is a member of the bZip family and binds as a homodimer to AP-1-like motifs (including the consensus AP-1 site) known as Z-responsive elements (ZREs) (Chang et al. 1990; Farrell et al. 1989; Flemington and Speck 1990c; Lieberman and Berk 1990; Lieberman et al. 1990; Packham et al. 1990). BZLF1 activates expression of immediate-early, and early, lytic EBV genes in conjunction with the other major lytic viral transcription factor, BRLF1 (Holley-Guthrie et al. 1990; Kenney et al. 1989; Lieberman et al. 1989; Rooney et al. 1989; Urier et al. 1989; Zetterberg et al. 2002). BZLF1 contains an amino terminus transactivator domain (Deng et al. 2001; Flemington et al. 1992), a basic DNA-binding domain (homologous to the basic DNA-binding domains of c-jun and c-fos (Farrell et al. 1989; Flemington et al. 1994)) and a bZip homodimerization domain in the carboxy-terminal portion of the protein (Flemington and Speck 1990c; Kouzarides et al. 1991). BZLF1 does not heterodimerize with c-fos or c-jun but interacts with another cellular bZIP protein, C/EBP-alpha (Wu et al. 2003), and can activate at least some promoters through C/EBP-alpha-binding sites.

BZLF1 binds directly to ZRE motifs in IE and early lytic EBV promoters, and activation of these promoters requires ZRE sites (Flemington and Speck

1990a; Urier et al. 1989). During viral reactivation, all evidence to date suggests that BZLF1 initially activates the BRLF1 IE promoter (Rp) (Adamson and Kenney 1998; Francis et al. 1999), and both the BZLF1 and the BRLF1 gene products are required for activation of many (if not most) early lytic genes (Adamson and Kenney 1998; Bhende et al. 2005; Feederle et al. 2000; Francis et al. 1997). Early viral promoters often contain binding sites for both BZLF1 and BRLF1. The interesting ability of BZLF1 to preferentially bind to the methylated form of the ZRE sites in Rp requires serine residue 186 in the basic DNA-binding domain (Francis et al. 1999; Heston et al. 2006; Petosa et al. 2006). BZLF1 may also activate certain cellular promoters through a non-DNA-binding mechanism (Flemington et al. 1994).

The crystal structure of the carboxy-terminal portion of BZLF1 bound to a consensus AP-1 site was recently published (Petosa et al. 2006). The basic DNA-binding domain and homodimerization domain of BZLF1 form a long alpha helix. Residues N182, A185, S186 and R190 in the basic DNA-binding domain directly contact bases in the AP-1 site, while a series of arginine/lysine residues stabilizes the interaction by contacting the phosphate backbone of DNA. Some but not all of the predictions of the BZLF1 crystal structure have been confirmed by mutagenesis studies of BZLF1 (Heston et al. 2006); interestingly, mutation of the N182 residue (which directly contacts AP-1 bases in the crystal structure) does not impair the ability of BZFL1 to disrupt viral latency. The BZLF1 residues which are essential for binding to ZRE sites clearly differ depending on the target promoter (Bhende et al. 2005; Heston et al. 2006). Residues of BZLF1 outside the canonical bZip basic DNA-binding domain (including C171) are required for binding to degenerate ZRE sites, including C/EBP motifs (Wang et al. 2006). Furthermore, mutation of certain residues in the BZLF1 basic DNA-binding domain has resulted in proteins which are competent to induce BRLF1 transcription but not early lytic gene transcription as well as proteins which are competent to activate BRLF1 and early gene transcription but cannot undergo viral replication or late gene expression (Heston et al. 2006).

Once bound to DNA, the ability of BZLF1 to interact directly with histone acetylating complexes (including CBP and p300) presumably helps to acetylate chromatin, converting it to a conformation favorable for transcription (Adamson and Kenney 1999; Chen et al. 2001; Deng et al. 2003; Zerby et al. 1999). BZLF1 also interacts directly with basic transcription factors, including TFIID and TFIIA (Chi and Carey 1993; Chi et al. 1995; Lieberman and Berk 1994; Lieberman and Berk 1991; Lieberman et al. 1997; Manet et al. 1993). BZLF1 transcriptional activity is regulated by post-translational modifications, including phosphorylation of serine 173 by casein kinase 2, which modulates DNA binding of BZLF1, the ability of BZLF1 to repress BRLF1-mediated activation of late viral genes and viral replication (Baumann et al. 1998; Daibata et al. 1992; El-Guindy et al. 2002; Gradoville et al. 2002). BZLF1 is also sumo-modified at lysine residue 12, although the effect of this modification on BZLF1 function has not yet been well defined (Adamson and Kenney 2001),

since mutation of this residue by itself does not prevent viral replication (unpublished observations).

BZLF1 also plays a direct role in lytic EBV DNA replication, binding directly to a series of essential ZRE sites in the lytic origin of replication, oriLyt (Fixman et al. 1995; Fixman et al. 1992; Hammerschmidt and Sugden 1988; Schepers et al. 1993; Schepers et al. 1993). Mutation of residues 12/13 in BZLF1 results in a protein which is transcriptionally competent but replication incompetent (Sarisky et al. 1996). Mutation of several other BZLF1 residues (including S173, C189, Y180 and K188) likewise predominantly produces a defect in viral replication rather than lytic gene transcription (El-Guindy et al. 2007; Heston et al. 2006). BZLF1 interacts directly with some of the core viral replication proteins, which may promote formation of the initial replication complex (Gao et al. 1998; Takagi et al. 1991; Zhang et al. 1996). Thus, BZLF1 plays roles in mediating lytic replication that are distinct from its transcriptional functions.

BZLF1 also regulates the activity of a number of cellular genes. For example, BZLF1 activates transcription of c-fos (Flemington and Speck 1990b), the tyrosine kinase TKT (Lu et al. 2000), matrix metalloproteinases 1 and 9 (Lu et al. 2003; Yoshizaki et al. 1999), cellular IL-10 (Mahot et al. 2003) and cellular IL-6 (Jones et al. 2007). Conversely, BZLF1 inhibits expression of the interferon gamma receptor (Morrison et al. 2001) and the major TNF-alpha receptor (TNFR1) (Morrison et al. 2004). BZLF1 activation of the immunosuppressive cytokines, TGF-beta and IL-10, and repression of the receptors for gamma-interferon and TNF-alpha could contribute to viral pathogenesis by protecting the virus from the innate host immune response. Likewise, activation of the B-cell growth factor, IL-6, may promote viability of infected B cells and possibly contribute to EBV-associated B-cell malignancies.

BZLF1 alters the host cell environment in numerous ways that may enhance the efficiency of lytic viral replication. For example, BZLF1 expression disperses promyelocytic leukemia (PML) bodies, nuclear structures which contain several cellular proteins (Adamson and Kenney 2001; Bell et al. 2000). PML bodies have been proposed to play an important role in cellular anti-viral defenses. Consistent with this model, several DNA viruses, including CMV, adenovirus and HSV, disperse PML bodies during replication (for review, see Maul 1998). BZLF1 also inhibits p53 transcriptional function (Mauser et al. 2002; Zhang et al. 1994), thereby ensuring that the host cell cannot undergo p53-mediated apoptosis during lytic viral infection. In addition, BZLF1 interacts with and inhibits the function of the p65 component of NF-KB (Keating et al. 2002; Morrison and Kenney 2004) and likewise interacts with and inhibits IRF-7 (Hahn et al. 2005). The ability of BZLF1 to inhibit NF-KB and IRF-7 function presumably provides the virus with even further protection against the host immune response.

BZLF1 also regulates cell cycle progression, although the exact effect is very cell-type dependent. In primary fibroblasts, BZLF1 produces a profound G1/S block (Cayrol and Flemington 1996; Cayrol and Flemington 1995; Cayrol and

Flemington 1996; Mauser et al. 2002; Rodriguez et al. 1999; Rodriguez et al. 2001; Wu et al. 2003) by decreasing expression of cyclin A and c-myc (Mauser et al. 2002; Rodriguez et al. 2001) and increasing p21 expression (Cayrol and Flemington 1996; Wu et al. 2003). In HeLa cells, BZLF1 induces both a G2 and a mitotic block (Cayrol and Flemington 1996; Mauser et al. 2002) associated with decreased cyclin B expression and a defect in chromosome condensation (Mauser et al. 2002). In sharp contrast, in primary keratinocytes and the EBV-immortalized marmoset B-cell line, B95-8, BZLF1 enhances expression of a number of S-phase-dependent cellular proteins and increases the activity of cyclin-dependent kinases (Mauser et al. 2002), although cellular DNA replication is blocked (Kudoh et al. 2003). Furthermore, agents that inhibit the activity of cyclin-dependent kinases also inhibit lytic EBV gene expression (Kudoh et al. 2004), although the exact mechanism for this effect is not clear. These results suggest that a "pseudo late G1/S-phase" environment, in which certain late G1/S-phase-restricted cellular proteins such as E2F-1 are expressed but cellular DNA cannot replicate, may be the ideal host cell environment for lytic EBV replication.

13.8.2 BRLF1 Protein

In a subset of latently infected cell lines, particularly EBV-infected epithelial cells, expression of the BRLF1 protein induces lytic EBV infection (Feederle et al. 2000; Ragoczy et al. 1998; Westphal et al. 1999; Zalani et al. 1996). Even in cell lines that can only be induced to the lytic form of infection by BZLF1 (such as Raji cells), BRLF1 is required in concert with BZLF1 to activate many early lytic genes (Feederle et al. 2000; Ragoczy and Miller 1999). A mutant EBV recombinant in which the BRLF1 gene is deleted is unable to enter the lytic form of infection, or efficiently express BZLF1, unless the BRLF1 gene product is supplied in *trans*. These results confirm that the BRLF1 gene product is an important and essential activator of the Zp IE promoter and early lytic promoters (Feederle et al. 2000). Like BZLF1, BRLF1 binds directly to the EBV oriLyt (Gruffat and Sergeant 1994; Hammerschmidt and Sugden 1988), although BRLF1-binding sites in oriLyt are not absolutely essential for oriLyt replication in plasmid-based replication assays (Fixman et al. 1992).

BRLF1 contains an amino-terminus DNA-binding domain and homodimerization domain (Manet et al. 1991) and a carboxy-terminal transcriptional activation domain (Hardwick et al. 1988; Hardwick et al. 1992) which interacts directly with TBP and TFIIB (Manet et al. 1993). In some cases, BRLF1 activates lytic EBV gene promoters by binding directly to a GC-rich motif (consensus $GGCCN_7GTGGTG$) present in the promoters of a number of different lytic genes (Gruffat et al. 1992; Gruffat et al. 1990; Gruffat and Sergeant 1994; Kenney et al. 1989; Quinlivan et al. 1993). The BRLF1-binding motifs in the BHRF1 and SM early promoters function as powerful enhancer elements in the presence of the BRLF1 protein (Chevallier-Greco et al. 1989; Cox et al. 1990; Kenney et al.

1989). In contrast, BRLF1 activates its own promoter (Rp) and the BZLF1 promoter (Zp) through indirect mechanisms. BRLF1 stimulates its own promoter through Rp Sp1 motifs (Liu and Speck 2003; Ragoczy and Miller 2001); this effect is mediated by a complex containing BRLF1, Sp1 and the cellular protein, MCAF1, binding to Sp1-binding sites (Chang 2005). BRLF1 stimulates the Zp promoter through its effect on the c-jun and ATF-2 transcription factors, which bind to and activate Zp through the ZII motif (Adamson et al. 2000). BRLF1 activates the stress Map kinases (p38 and c-jun N-terminal kinase) (Adamson et al. 2000) as well as PI3 kinase (Darr et al. 2001), and inhibiting any of these three kinases abolishes the ability of BRLF1 to activate BZLF1 transcription or disrupt viral latency (Adamson et al. 2000; Darr et al. 2001). The early viral protein, BRRF1, which activates c-jun phosphorylation, cooperates with BRLF1 to induce transcription of BZLF1 in the context of the intact viral genome (Hong et al. 2004). Like BZLF1, BRLF1 also interacts with the histone acetylase, CREB-binding protein (CBP) (Swenson et al. 2001).

There is growing recognition that a subset of early genes and late lytic cycle genes can be activated by BRLF1 alone without concomitant BZLF1 expression. These BRLF1-responsive genes (which can be identified in Raji cells where BRLF1 does not induce BZLF1 expression) include the early gene SM (Ragoczy and Miller 1999). Furthermore, BRLF1 by itself can activate transcription of a subset of "late" viral genes even in the absence of viral replication (Chua et al. 2007; Feederle et al. 2000; Lu et al. 2006; Ragoczy and Miller 1999). The direct interaction between BRLF1 and the cellular protein, TSG101, was recently shown to be required for the ability of BRLF1 to activate some viral late genes, although the exact mechanism for this effect has not been fully defined (Chua et al. 2007).

Like BZLF1, BRLF1 also activates some cellular genes. BRLF1 activation of the cellular gene, fatty acid synthase (FAS), may be an essential component in BRLF1-mediated induction of lytic EBV infection (Li et al. 2004) since agents which inhibit FAS also inhibit the ability of transfected BRLF1 to induce the lytic form of EBV gene expression (Li et al. 2004). BRLF1 activation of a decoy receptor for TNF-alpha (Ho et al.2007) probably helps protect the virus from the effects of TNF-alpha. BRLF1 also affects the regulation of the host cell cycle, increasing the number of cells in S-phase in both primary human fibroblasts and HeLa cells through its effect on E2F-1 expression (Swenson et al. 1999). BRLF1 also interacts directly with, and inhibits the function of, the tumor suppressor protein, Rb (Zacny et al. 1998).

13.9 Post-Transcriptional Gene Regulation

The SM protein is an early gene product with multiple post-transcriptional gene regulatory functions that are essential for lytic EBV replication (Gruffat et al. 2002; Han et al. 2007; Swaminathan 2005). In addition to enhancing expression

of EBV lytic genes, SM has both positive and negative effects on cellular gene expression (Batisse et al. 2005; Buisson et al. 1989; Nicewonger et al. 2004; Ruvolo et al. 2003; Ruvolo et al. 1998; Semmes et al. 1998). SM, also known as EB2, Mta and BMLF1, is a member of a highly conserved family of proteins represented in most human and primate herpesviruses as well as several other mammalian herpesviruses, including bovine, ovine, murine, equine and alcelaphine species (Albrecht et al. 1992; Bello et al. 1999; Chapman et al. 1992; Chee and Barrell 1990; Gupta et al. 2000; Winkler et al. 1994). The homologs of SM in human herpesviruses include herpes simplex virus (HSV) ICP27, human cytomegalovirus (hCMV) UL69, varicella-zoster virus (VZV) ORF4 and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8) ORF57.

SM is an RNA-binding protein that interacts with RNA both in vitro and in vivo, although no stringent sequence specificity has been demonstrated (Buisson et al. 1999; Gao et al. 1998; Hiriart et al. 2003; Ruvolo et al. 2001; Ruvolo et al. 1998). SM increases mRNA accumulation of its target genes by post-transcriptional mechanisms. There is evidence that SM interacts with cellular export proteins and may thus serve as a bridge between viral mRNAs and the export machinery of the host cell (Boyle et al. 1999; Chen et al. 2001; Hiriart et al. 2003). Such an effect may be particularly important for the many intronless mRNA transcripts expressed during the lytic cycle of EBV replication. The majority of herpesvirus genes expressed during the lytic cycle of virus replication does not contain introns. This lack of introns may pose an intrinsic obstacle to gene expression as splicing is intimately linked to nuclear export of cellular mRNAs (Cheng et al. 2006; Moore 2005). Proteins comprising the mRNA export machinery (TREX complex) are deposited on mRNA molecules consequent to splicing and facilitate nuclear export by interaction with TAP/NXF1, a central mediator of export, via the nuclear pore (for review, see Reed and Cheng 2005). Intronless cellular and viral mRNAs are nevertheless exported by a variety of mechanisms and it has been hypothesized that SM compensates for the lack of access of EBV mRNAs to splicing-coupled export pathways. Evidence also exists that SM may increase the nuclear accumulation of transcripts post-transcriptionally, possibly by stabilizing mRNAs and enhancing polyadenylation (Key et al. 1998; Ruvolo et al. 1998).

The action of SM depends partially on whether the target gene contains introns. The majority of spliced cellular genes and reporter genes with introns is not activated by SM and several are downregulated (Ruvolo et al. 2003). Although the HSV homolog ICP27 inhibits splicing (Sciabica et al. 2003), a direct effect of SM on splicing has not yet been demonstrated. On the other hand, the mRNAs of many intronless target genes accumulate to much higher levels in the presence of SM. Examination of SM effects on all EBV transcripts using a microarray approach showed that while the majority of EBV genes which are upregulated by SM are late lytic phase genes, several early genes are upregulated by SM (Han et al. 2007). Two of these genes are essential components of the core DNA replication complex, BSLF1, the EBV DNA primase and BALF5, the EBV DNA polymerase (Fixman et al. 1992). Lytic EBV

DNA replication is thus dependent on SM. In addition, while the efficient accumulation of many late EBV lytic transcripts is indirectly dependent on SM due to its effect on DNA replication, several late transcripts also appear to be directly dependent on SM for enhanced expression (Batisse et al. 2005; Han et al. 2007).

SM also has effects on cellular gene expression and growth. Inducible expression of SM in EBV-negative B-cell lymphoma cells leads to inhibition of cell growth and a global decrease in mRNA accumulation (Ruvolo et al. 2003). However, several cellular genes are upregulated, including several targets of STAT1. In addition to increasing overall levels of STAT1 mRNA, SM increases the relative levels of the beta isoform of STAT1, which may act as a dominant-negative modulator of gamma-interferon signaling (Baran-Marszak et al. 2004). Whether SM has immune modulatory effects on EBV-infected cells during the course of lytic replication remains to be demonstrated. Interestingly, one gene whose expression is activated by SM and is STAT1 responsive encodes a PML body protein designated Sp110b, which interacts with SM and synergizes with SM in enhancing mRNA accumulation (Nicewonger et al. 2004). Recently, yeast interaction screens have indicated that SM also interacts with Sp100, another PML body component, and with several cellular RNA-binding proteins (Hiriart et al. 2005; Johannsen et al. 2004). The physiological relevance of these interactions remains to be determined.

13.10 EBV Proteins Involved in Regulating Apoptosis, Host Cell Proliferation and Immune Evasion

13.10.1 EBV Anti-Apoptotic Proteins

The BHRF1 gene of EBV, expressed during early lytic replication, encodes a 17 kD protein homologous to the anti-apoptotic cellular protein bcl-2. BHRF1 can perform a bcl-2-like function in preventing cell death in the face of various types of apoptotic stimuli (Henderson et al. 1993; Tarodi et al. 1994). BHRF1 protein expression has only been convincingly demonstrated during lytic replication, and EBV mutants deleted for BHRF1 are fully capable of transforming primary B lymphocytes and maintaining latent infection (Lee and Yates 1992; Marchini et al. 1991). Furthermore, such mutants are unimpaired in their ability to reactivate from latency and replicate lytically. Since BHRF1 genes are highly conserved among EBV homologs in other species, it is likely that they serve an important function in vivo. BHRF1 protein may be important for maintaining cell viability in the face of a cellular apoptotic response to viral infection, thereby prolonging the time available for EBV replication and production of virus proteins prior to cell lysis. Another EBV lytic protein, expressed during early lytic replication, and homologous to bcl-2, is encoded

by the BALF1 open reading frame. One report suggested that BALF1 has a function similar to that of bcl-2, inhibiting apoptosis (Marshall et al. 1999). However, another study was unable to demonstrate an anti-apoptotic function and suggested that BALF1 may play a role in counteracting BHRF1 (Bellows et al. 2002). The exact role of BALF1 in EBV infection remains to be conclusively demonstrated. However, experiments with recombinant EBV deleted for either BALF1 or BHRF1, or both, indicate that BALF1 and BHRF1 are expressed transiently upon primary infection in vitro and are important in the establishment, but not maintenance, of the transformed state in B lymphocytes (Altmann and Hammerschmidt 2005). These studies found that BALF1 and BHRF1 were anti-apoptotic and were functionally redundant as viruses mutated in either BALF1 or BHRF1 were still transformation competent.

13.10.2 EBV vIL-10, a Viral Cytokine

EBV expresses a gene that is highly homologous to human IL-10 (hIL-10), which is an important mediator of the balance between the Th1 and Th2 immune responses (Hsu et al. 1990; Vieira et al. 1991). The EBV vIL-10 is encoded in the BCRF1 open reading frame as an intronless gene, whereas the human counterpart is extensively spliced. EBV vIL-10 inhibits IFN-gamma release and enhances B lymphocyte proliferation, similar to hIL-10. EBV recombinants deleted for BCRF1 transform B lymphocytes in vitro and produce lymphomas in SCID mice, demonstrating that vIL-10 is dispensable for latent infection and latently infected cell growth (Swaminathan et al. 1993). vIL-10 null EBV elicits higher amounts of IFN-gamma release from cocultured lymphocytes, indicating that vIL-10 may play a role in suppressing the local immune response during lytic reactivation (Swaminathan et al. 1993). EBV vIL-10 may also be released from lytically infected cells in vivo and enhance growth of nearby latently infected lymphocytes.

13.10.3 BARF1, a Decoy Cytokine Receptor

EBV expresses the BARF1 protein early after induction of lytic replication in latently infected B lymphocytes (Zhang et al. 1988). The BARF1 gene contains an N-terminal signal sequence and BARF1 protein is secreted from infected cells (Strockbine et al. 1998). Expression cloning identified the cytokine CSF-1 as a BARF1 ligand, suggesting that BARF1 might act to inhibit CSF-1 function (Cohen and Lekstrom 1999). BARF1 competitively inhibits CSF-1 stimulation of bone marrow macrophage proliferation and IFN-alpha release from mononuclear cells. Furthermore, a recombinant EBV in which BARF1 was mutated was reduced in its ability to inhibit IFN-alpha secretion by mononuclear cells compared to wild-type EBV. BARF1 was dispensable for transformation of

primary B lymphocytes in vitro (Cohen and Lekstrom 1999). However, the BARF1 transcript has also been detected in latently infected epithelial cells and has been implicated as a possible oncogene in epithelial carcinomas (Seto et al. 2005; Sheng et al. 2003).

13.10.4 Lytic DNA Replication

Initiation of lytic DNA replication begins at the oriLyt origin, which is present in two copies in the EBV genome. However, they are apparently functionally redundant, as the genome of the B95-8 strain of EBV carries a deletion which removes one of the two oriLyts but is fully functional in lytic replication. In addition to BZLF1 (see above), six EBV proteins that comprise the core DNA replication complex are essential for mediating replication of plasmids containing oriLyt in transfection assays (Fixman et al. 1992, 1995). These are BALF5, the DNA polymerase; BALF2, the single-stranded DNA-binding protein; BMRF1, the DNA polymerase processivity factor; BSLF1 and BBLF4, the primase and helicase homologs; and BBLF2/3, a third component of the helicase–primase complex. Each of these proteins appears to interact directly with the other two and with BALF5, the DNA polymerase (Fujii et al. 2000; Yokoyama et al. 1999). The BBLF2/3 protein also interacts with a DNA-binding human zinc finger protein ZBRK1 and the ZBRK1 corepressor, KAP-1 (Liao et al. 2005). It has been suggested that these cellular proteins may bind to oriLyt DNA and via interactions with BBLF2/3 may help to tether the replication complex to oriLyt. A similar role has been postulated for the cellular transcription factors Sp1 and ZBP-89, which bind to oriLyt and to the processivity factor BMRF1, and to EBV DNA polymerase (Baumann et al. 1999). Lytic DNA replication is thought to occur via a rolling circle mechanism with the formation of complex intermediate plasmid forms (Pfuller and Hammerschmidt 1996).

13.11 Viral Proteins Involved in Nucleocapsid Formation and Egress

13.11.1 The BFRF1/BFLF2 Complex

During the process of virion formation, the herpesviral nucleocapsid is thought to undergo envelopment as it traverses the nuclear membrane, followed by de-envelopment, acquisition of tegument proteins and final envelopment as it traverses the *trans*-Golgi or plasma membrane prior to virion release (Granzow et al. 2001). Two conserved herpesvirus genes, designated as UL31 and UL34 in alphaherpesviruses, are required for nuclear egress of the nascent virion. Two proteins expressed early in the lytic phase of EBV replication, BFRF1 and

BFLF2, are the EBV homologs of UL31 and UL34 and are involved in the process of initial nuclear envelopment (Farina et al. 2005; Gonnella et al. 2005). Recombinant EBV with deletions of BFRF1 are able to replicate their DNA and do not appear to be deficient in late gene expression. However, they are unable to efficiently produce and release extracellular EBV. Mutant BFRF1-deleted EBV capsids can be observed to accumulate in the nucleus and are not as prevalent in the cytoplasm as BFRF1-expressing EBV. The deficiency can be rescued by complementation with BFRF1, indicating that BFRF1 is necessary for nuclear egress of nucleocapsids. BFLF2 colocalizes with BFRF1 at the nuclear membrane, but does not localize normally in the absence of BFRF1. Both BFRF1 and BFLF2 cooperate to induce morphological changes in the nuclear membrane, similar to those observed during virus replication, and interact with lamin B, suggesting that they cooperate in enhancing EBV nuclear egress. Expression of HSV pUL31 and pUL34 has recently been shown to induce formation of nuclear membrane-derived vesicles, indicating that these two proteins alone are sufficient to initiate primary envelopment and vesicle formation at the nuclear membrane (Klupp et al. 2007).

13.11.2 BGLF4, a Protein Kinase

EBV expresses a serine–threonine kinase (EBV-PK) encoded by the BGLF4 ORF that is expressed during early lytic replication (Chen et al. 2000; Gershburg et al. 2004). EBV-PK is a nuclear protein that has several potential cell and EBV substrates. EBV-PK phosphorylates the BMRF1, BZLF1 and EBNA-LP proteins although the biological effect of these modifications is not known (Asai et al. 2006; Chen 2000; Gershburg and Pagano 2002; Kato et al. 2003). EBV-PK also phosphorylates the cellular translation elongation factor EF-1 δ and activates topoisomerase II (Lee et al. 2007). These activities may be correlated with the chromosome condensation induced by EBV-PK expression which is observed during EBV lytic replication (Lee et al. 2007). Recently, knockdown of EBV-PK in recombinant EBV-infected 293 cells demonstrated that infectious virus production was dependent on EBV-PK (Gershburg et al. 2007). Electron microscopic examination of cells induced to permit lytic replication showed that the ratio of intracytoplasmic to intranuclear capsids was reduced by EBV-PK knockdown, suggesting that BGLF4 is important for nuclear egress. Although previous studies had not shown a relationship between EBV-PK and BFRF1/BFLF2, two proteins required for nuclear egress, the more recent report observed a reduction in the levels of BFLF2 as a result of knocking down EBV-PK expression (Gershburg et al. 2007). Whether EBV-PK plays a role in regulating BFRF1/BFLF2 function by phosphorylation has not been experimentally confirmed but has been proposed to do so by analogy to the mechanism of the HSV US3 kinase, which phosphorylates both UL34 and UL31, the homologs of BFRF1/BFLF2. EBV-PK has been identified as a

tegument protein, and it has been suggested that it may phosphorylate Z or EBNA-LP proteins upon primary infection, although the actual substrates of BGLF4 during infection have not been identified (Asai et al. 2006).

13.11.3 Virion Proteins

Many of the important structural proteins with unique roles in EBV infectivity are discussed in the chapter on EBV binding and infection. The function of many of the structural proteins of EBV is inferred from their homology to HSV proteins whose function has been established. After DNA replication is complete, linear EBV genomes are packaged into icosahedral capsids. A portal protein (BBRF1) is a component of the capsid which serves as a tunnel through which the DNA is thought to be propelled and packed into the capsid (Lebedev et al. 2007), with the addition of BVRF1, a homolog of HSV UL25, which is thought to be the “cork” that prevents genomic DNA from escaping through the portal (Sheaffer et al. 2001).

A direct analysis of the protein content of EBV virions by mass spectrometry that was made possible by the highly efficient induction of lytic replication in EBV-infected cells has recently been reported (Johannsen et al. 2004). EBV BcLF1, BDLF1 and BORF1, the major capsid protein, minor capsid protein (mCP) and mCP-binding protein were identified in the virion. Portal protein (BBRF1) and the “cork” protein BVRF1 were also detected in the virion preparation. As expected, the major glycoproteins were identified in relative abundance, and both large tegument proteins, LTP (BPLF1) and LTP-binding protein BOLF1, and the middle tegument protein BNRF1 were also measurable.

BNRF1, one of the tegument proteins unique to gammaherpesviruses, has been shown to be important for EBV transport to the nucleus after infection (Feederle et al. 2006). BNRF1-deleted recombinant EBV when transfected into epithelial cells are capable of replicating DNA and producing virus particles. The released BNRF1-negative EBV are able to bind and enter uninfected B lymphocytes, but are impaired in the ability to travel from endosomes to the nucleus.

13.11.4 MicroRNAs

EBV has been shown to express 17 microRNAs (miRNAs) (Cai et al. 2006; Grundhoff 2006; Pfeffer 2004). These are encoded in two clusters, one located in the BHRF1 gene and the other in the BART gene (Cai et al. 2006). The majority of the BART miRNAs lie in the region of the EBV genome deleted in the prototype B95-8 strain and were therefore not identified in initial studies. The B95-8 strain is fully competent for lytic replication, indicating that the majority of EBV BART miRNAs are dispensable for both lytic replication and transformation. Although recombinant EBV from which various portions of the BHRF1

gene has been deleted have been described, and are replication competent, BHRF1 miRNA expression from these viruses has not been characterized (Lee and Yates 1992; Marchini et al. 1991). Both BART and BHRF1 miRNA clusters are expressed during various types of latent EBV replication. The BHRF1 cluster, consisting of three miRNAs, appears to be expressed primarily in type III latency, whereas BART miRNAs are expressed at high levels in type II latency (Cai et al. 2006). The location of all but one of these miRNAs suggests that their transcription might increase during lytic replication. Two of the BHRF1 miRNAs are encoded in the 3· UTR of the BHRF1 gene, whereas one is encoded upstream of the lytic promoter from which BHRF1 mRNA is transcribed during lytic replication. Transcription of the BART gene, which is detected at highest levels in NPC cells, in other types of cells also increases during lytic replication (Yuan et al. 2006). Increases in levels of one of the BHRF1 miRNAs and several of the BART miRNAs have been demonstrated after lytic replication was induced in EBV-infected cell lines in vitro (Cai et al. 2006).

One of the BART miRNAs is antisense to the 3· UTR of the EBV DNA polymerase, and it has been suggested that this miRNA may play a role in regulating DNA polymerase activity (Pfeffer et al. 2004). Seven of the EBV miRNAs were found to have highly conserved homologs in the rhesus lymphocryptovirus (rLCV), suggesting that they play an important biological role in vivo (Cai et al. 2006). In addition to possibly regulating viral gene expression, EBV miRNAs are likely to modulate cellular gene expression as has recently been shown for KSHV miRNAs. Such effects on cell gene expression may be important to facilitate EBV replication or infected cell survival during lytic replication.

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Chapter 14
Epstein–Barr Virus Latent Infection Nuclear Proteins: Genome Maintenance and Regulation of Lymphocyte Cell Growth and Survival

Eric Johannsen, Michael Calderwood, Myung-Soo Kang, Bo Zhao, Daniel Portal, and Elliott Kieff

Epstein–Barr Virus (EBV) was discovered through its presence in African Burkitt lymphoma. Denis Burkitt, a British surgeon working in Kampala, Uganda in the late 1950 s, encountered children with massive jaw or abdominal lymphomas that he had not seen in the United Kingdom. He corresponded with other physicians and traveled through Sub-Saharan Africa to gather information about these lymphomas. He discovered that lymphomas were prevalent in children of migratory tribes that had settled in regions with hyper-endemic malaria. Burkitt wrote and lectured about the unusual clinical and epidemiologic features of African lymphoma and suggested that there might be an infectious etiology [1]. After attending Burkitt's lecture in London, Anthony Epstein obtained samples of live tumor tissue and succeeded in growing lymphoma cells in continuous culture. Epstein identified a Herpes virus in electron micrographs of a very small fraction of cultured lymphoma cells [2]. The Burkitt lymphoma (BL) associated virus differed from known human herpes viruses in being non-infectious for cultured cell lines and non-reactive with antibodies to other human Herpes viruses. Epstein had discovered the first human tumor virus; the prototype for the "oncogenic" Gamma Herpesviruse subfamily.

We now recognize that the human Gamma Herpesviruses, EBV and the Kaposi's Sarcoma Herpesvirus (KSHV), which was discovered three decades later by Chang and Moore [3], replicate in epithelial cells and establish long-term latency in B-lymphocytes (for review see [4–6]). Latently infected lymphocytes periodically become permissive for virus replication near epithelium, nucleate a focus of virus replication, and thereby enable the virus to complete its life cycle by spreading to the epithelium of a nonimmune host. EBV and KSHV are distinctive among human Herpesviruses in causing lymphomas, carcinomas, or sarcomas, particularly in humans with immune deficiencies.

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_14, © Springer Science+Business Media, LLC 2009

EBV is a gamma 1 Herpesvirus or lymphocryptovirus (LCV), and KSHV is a gamma 2 or rhadinovirus (RDV) [7–11]. Many old and new world primate species have an endemic LCV. LCV genomes are nearly collinear and highly homologous to EBV [9, 12–18]. In contrast, RDV are endemic not only in primate species but also in many other mammalian species [19–24]. Overall, RDV genomes are more diverse, but have collinear and largely homologous blocks of genes in common with LCV genomes; the common genes are mostly those that are important for virus replication. Fifty-nine of the eighty-one KSHV predicted open reading frames are collinearly homologous to EBV open reading frames [11]. Most are more distantly homologous to alpha and beta herpesvirus genes.

The principal differences between LCV and RDV genomes are that LCVs have six genes encoding nuclear antigen proteins (EBNAs) and two encoding intrinsic membrane proteins (LMPs) that are expressed in a form of latent infection, termed latency III. These genes encode the proteins that enable the virus genome to cause infected lymphocyte proliferation and persist as an episome in dividing cells. KSHV has genes, which are similar to the two EBV LMP genes in exon intron structure and in encoded protein domains. K1 is the positional homolog of LMP1 and also activates NF-kB signaling, but only has two transmembrane domains, whereas K15 is the positional homolog LMP2A/B. LMP2A/B has 12 transmembrane domains and interacts with non-receptor protein tyrosine kinases. Further, KSHV nuclear antigen gene, LANA is similar to EBNA1 in being essential for KSHV genome persistence as an episome [25–27]. KSHV also has a gene encoding a cyclin homologue and several IRF homologues, which have some similarities to EBNAs [11, 23, 24]. However, KSHV does not efficiently convert cells to perpetual replication, whereas EBV efficiently causes lymphocytes to replicate continuously in culture as lymphoblasoid cell lines (LCLs) [28, 29] and can cause polyclonal lymphopro-liferative diseases in severely immune compromised people and non-human primates [4, 30–34]. The neoplastic potential of latency III EBV infection in the absence of an effective T-cell response is most evident in EBV infection in congenital SH2D1A deficient or SCID humans, in T-cell depleted, iatrogenically immune suppressed, or EBV-negative transplant recipients, and in CD4 T-cell-depleted HIV-infected humans, where EBV infected lymphoblasts can cause malignant lymphoproliferative proliferative diseases (LPD), including polyclonal LPD, Burkitt-like or other lymphomas or even Hodgkin disease (HD). EBV-associated HD is usually latency II infected with EBNA1, LMP1, LMP2 expression and EBERs [4, 35–48], whereas latency III lymphocyte infections are characterized by the expression of EBNA2, EBNALP, EBNA3A, EBNA3B, EBNA3C, and EBNA1, integral membrane proteins, LMP1, LMP2A, and LMP2B, EBERS, Bam A rightward transcripts and microRNAs. In contrast, most EBV-associated Burkitt lymphomas are latency I and express only EBNA1, EBERs, Bam A rightward transcripts and micro RNAs, whereas most nasopharyngeal carcinomas are latency II infected and express latency I-associated gene products along with LMP1 and LMP2 (for review see [5, 49]).

Recombinant EBV reverse genetic and biochemical analyses establish that EBNA2, EBNALP, EBNA3A, EBNA3C, and LMP1 have essential roles in effecting B-cell conversion to LCLs [50–60] and that EBNA1 is critical for efficient EBV episome persistence and transcription [61–70]. Coupled recombinant EBV-based reverse molecular genetic and downstream biochemical analyses link EBNALP [51, 71–77], EBNA2 [50, 55, 57, 78–85], EBNA3A [56, 58, 86–92], EBNA3C [93–95], and LMP1 domains [96–99] to critical cell gene transcription, growth, and survival pathways. EBNA2 and EBNALP are the principal initial regulators of virus and cell gene transcription in latency III conversion of B-lymphocytes to proliferating lymphoblasts [100]. EBNA2 alters transcription through RBP/CSL, the key DNA-binding protein in Notch signaling, through PU.1/SpiB, important B-cell *ets* family transcription factors, and through AUF1; EBNALP potentiates EBNA2 activity [50, 57, 71, 72, 78–80, 101].

Substantial evidence favors a working hypothesis in which EBNA3A and EBNA3C participate in EBV latency III conversion of B-lymphocytes to LCLs by coordinately regulating virus and cell gene transcription with EBNA2 through their common associations with RBP/CSL. In contrast to EBNA2 and EBNALP which activate and co-activate transcription through RBP/CSL, EBNA3A and EBNA3C have repressive effects when tethered at artificial promoters or when transfected into cells with EBNA2 and the EBNA2 responsive Cp promoter. EBNA3C can however also activate the LMP1 promoter (LMP1p) with EBNA2 [48, 87, 90, 91, 94, 95, 102–107]. Other evidence indicates that EBNA3A and EBNA3C can affect changes in cell growth or survival through interaction with Rb, SCF^{SKP2}, CtBP, Nm23-H1, cyclinA, or $p27^{KIP1}$ [86, 106, 108–119], although these interactions have not as yet been linked to physiologic levels of EBNA3A or EBNA3C expression or residues that are essential for EBNA3A or EBNA3C affects on cell proliferation.

To enable EBV episome persistence through multiple rounds of infected cell division, EBV expresses the EBV-encoded nuclear antigen 1 (EBNA1). EBNA1 binds to multiple cognate sites in the EBV episome oriP DNA element and tethers EBV episomes to chromosomes for partitioning to progeny cell nuclei, wherein EBNA1 also enhances episome replication and transcription [25, 62, 65, 67, 69, 120, 121]. EBNA1 is essential for the efficient persistence of EBV episome DNA in all dividing and malignant cells. Considerable evidence with dominant negative EBNA1, EBNA1 antisense oligonucleotides or EBNA1 RNAi indicate that 70% inhibition of EBNA1 results in EBV genome loss and decreased tumor cell growth and survival, validating EBNA1 as a potential target protein for therapeutics against EBV-associated diseases [122–124]. EBNA1 has three essential domains for EBV genome persistence and increased transcription; argine–glycine (RG) domain 1 (RG1) aa33-89, RG2 aa327-386, and the oriP DNA-binding (DBD)/homodimerization domain (DD) aa459-607. Targeting any of these domains would interrupt viral gene expression or terminate EBNA1-mediated episome persistence.

Estimates of the EBV efficiency in conversion of primary human B-lymphocytes to LCLs range as high as 10%. EBV infects human B-lymphocytes through EBV's principal outer membrane glycoprotein gp350/220 adsorption to CD21, a complement receptor [125, 126]. EBV gp42 interaction, part of the gH/gL complex, then interacts with MHC class II, a co-receptor, which likely initiates gH/gL and gB interaction with the lymphocyte membrane [127–130]. Within 8 h, the genome is circularized in the nucleus [100, 131]. Transcription initiates clockwise from the Wp promoter in the IR1 repeats and most initial RNAs terminate at the polyA site downstream of the EBNA2 exon (Fig. 14.1) [100, 132–134]. The transcript encodes EBNALP from N-terminal exons and EBNA2 from a C terminal exon. A 5′ alternative splice determines whether the RNA lacks an upstream ATG for EBNALP translation and translates only EBNA2 from the downstream open reading frame (ORF) or have an ATG at the beginning of the EBNALP ORF, and translate EBNALP, rarely reinitiating translation of EBNA2 [133].

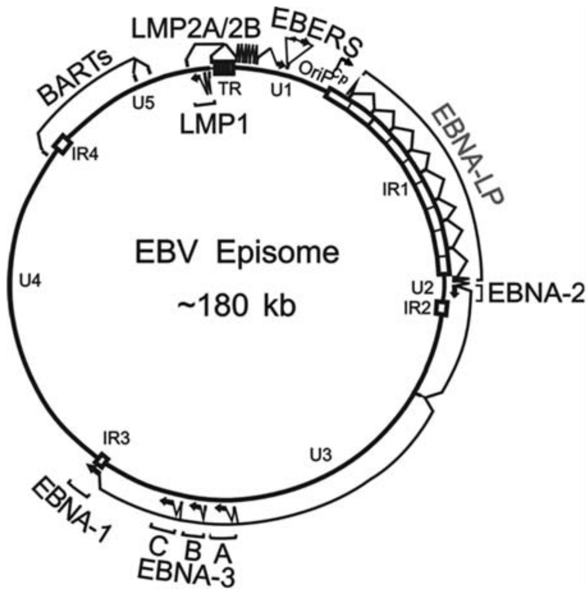


Fig. 14.1 Diagram of the EBV episome, which is formed through covalent linkage of the terminal repeat DNA within the first 8 hours of latent lymphocyte infection. In latency III, the Wp promoter in the long internal repeat (IR1) nearest the first unique DNA (U1) initially transcribes RNA, which is differentially spliced to encode EBNALP and EBNA2. EBNALP and EBNA2 up regulate the EBV U1 Cp and U5 LMP1p and LMP2p promoters as well as the cell c-myc promoter. Increased transcription through EBNA2 results in a longer transcript that terminates after EBNA1 and is differentially spliced to encode EBNALP, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA1. LMP1, LMP2A, and LMP2B are also expressed. Cells enter S phase and EBER RNAs are expressed. EBNA1 up regulates episome transcription, replication, and maintenance. In response to latent EBV infection, cells continuously proliferate

EBNA2 activates and EBNALP co-activates Cp (in U1 in Fig. 14.1) and EBV LMP1p [135, 136]. EBNA2 also activates the LMP2 and cell *c-myc* promoter. EBNA2 targets most promoters through the EBNA2 $PWWP_{325}$ sequence, which is a ligand for RBP/CSL, a Notch transcription factor that binds the cognate DNA sequence "5′-CG**TGGGAA-3′**"[78, 80, 137]. The EBNA2 WWP mimics the Notch WFP sequence in the "RBP/CSL association motif (RAM)" domain, which binds to a hydrophobic pocket in the beta trefoil domain of RBP/CSL, and cannot be replaced by EBNA2 with FFP [54, 78, 80, 138–142]. Adjacent EBNA2 residues (aa292-310) are also critical for EBNA2 association with RBP/CSL, for EBNA2-mediated transcription through RBP/CSL, and for EBNA2-mediated lymphocyte growth transformation [54, 55]. These residues bind to SKIP and to SMRT, which also binds to RBP/CSL [143]. These data support a model in which EBNA2 interacts with RBP/CSL and SKIP and displaces the SMRT-associated repressor complex from RBP/CSL to activate promoter-specific transcription [143, 144]. EBNA2 also enhances transcription of EBV latent promoters through interaction with PU.1/SpiB/Elf-1 at the LMP1p [145] or AUF1 at Cp [146]. The EBNA2 C-terminal acidic activation domain recruits TAF40, TFIIB, TFIIH, p100 with associated TFIIE, and p300/CBP with associated P/CAF to activate transcription from specific promoters and thereby convert B-lymphocytes to LCLs [81, 147–149].

EBNALP can also interact, but not stably associate with the EBNA2 acidic domain [73] and co-activates transcription from the Cp and LMP1 promoters and may have similar activity at other EBNA2 responsive cell promoters [71, 72]. Not all EBNA2 responsive promoters are EBNALP co-activated in their native state. The LMP2A gene and cell CD21 and Hes-1 promoters are EBNA2 responsive, but not EBNALP co-activated [150]. EBNALP is unusual among the EBNAs in localizing not only to the nucleus but also to the cytoplasm [77, 151]. Recent data are consistent with a model that EBNALP co-activation is due to EBNALP removal of Sp100 and Hp1α repressors from EBNA2-targeted promoters to other nuclear sites [76] and of HDAC4/5 to the cytoplasm [77]. Hsp72 interaction with EBNALP likely facilitates these "load-on and off-load" protein transport actions of EBNALP [152].

At 24–36 h after B-lymphocyte infection, EBNA2 and EBNALP-enhanced Cp (or Wp) transcripts extend through the EBNA2 polyA site and through the EBNA3A, EBNA3B, EBNA3C, and EBNA1 polyA sites (Fig. 14.1). EBNA3A, EBNA3B, EBNA3C, EBNA1, and LMP1 are then also expressed [100, 153]. By 36–48 h EBNA3A, EBNA3B, EBNA3C, EBNA1, and LMP1 are at full levels and infected cells enter S phase [100]. After the first S phase, EBERs are expressed and latency III protein expression remains stable. Most cells continue to proliferate and double every 36–48 h. Some cells proliferate better than others and dominate. Depending on growth conditions, infected cells gradually increase replication rate over the first 6 months in culture and then double every 18–24 h. Some cells go through a telomere-related crises and emerge with increased telomerase expression, more rapid doubling, and more frequent chromosomal abnormalities [154].

14.1 EBNA2

Given the similarities among old and even new-world LCVs in biochemical and biological properties in latency III infection, amino acid sequence conservation is useful in identifying sequences that are important for tertiary structure and protein–protein interactions. For EBNA2 and EBNA3A, 3B, and 3C there are also two different alleles that are the major genome sequence differences between the two human EBV types [132, 155]. The type 2 (T2) EBNA2 is deficient in primary B-lymphocyte conversion to LCLs, whereas type 1 (T1) EBNA2 and T1 and T2 EBNA3A, EBNA3B and EBNA3C are fully wild type for LCL outgrowth [57, 60, 101, 156]. Comparison of the T1 and T2 EBNA2 with Papio LCV (pLCV) EBNA2 identified nine conserved regions (CR), depicted in the upper line of Fig. 14.2 (numbered 1–9) [157]. Subsequent sequencing of the Rhesus LCV (rLCV) EBNA2 reveals six broader and less densely conserved domains, including the polyproline (PP) domain as depicted in the line below CR1-9.

Combined recombinant EBV reverse molecular genetic and biochemical analyses of the role of EBNA2 in primary B-lymphocyte growth transformation indicate that at least four EBNA2 domains (CR1/2, CR4, CR5/6, and CR8) are very important or essential for transcription from virus and cell promoters and for conversion of human B-lymphocytes into LCLs [50, 53–55, 57, 158–163]. Two of these, aa1-58 and aa102-148, are intrinsically complex, potentially partially overlapping in function, and have a role in EBNA2 promoter regulation and affects in cell transformation. The two N-terminal domains are largely independent self association domains (SAD 1 and 2 in Fig. 14.2), which mediate and/or affect EBNA2 transcriptional regulation through cell transcription factors other than RBP/CSL. The PP domain is critical for the coordinate

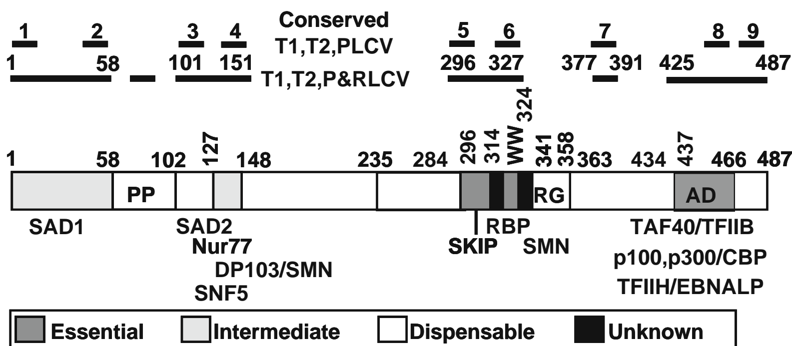


Fig. 14.2 Schematic of EBNA2 amino acids 1-487 that are essential, intermediately important, or dispensable for lymphocyte transformation. Residues that have not been tested are in black. Protein interactions are shown below the interactive EBNA2 amino acids. Conserved domains among EBV types 1 and 2 and the closely related Pan and Rhesus Lymphocryptiviruses are shown in the top two lines

positioning of SAD 1 and 2 [158, 164]. Deletion of SAD1 and the entire poly proline domain impairs SAD2 function [158]. While some mutations in aa1-58 or the PP domain have major effects on transformation and on LMP1p, but not Cp activation, EBV expressing EBNA2d2-88 has 10% of WT efficiency in initial primary B-lymphocyte transformation to LCLs. The EBNA2d2-88 LCLs grow out and expanded as rapidly as WT LCLs and once isolated free of defective interfering virus, EBV EBNA2d2-88-infected primary B-lymphocytes convert to LCLs and expand with only moderately reduced efficiency. EBNA2d2-88 is hypofunctional in LMP1p activation and is substantially over-expressed in EBV EBNA2d2-88-converted LCLs. LMP1 levels are WT at the high EBNA2d2-88 level [159]. Despite the only moderately reduced overall effect on transformation of EBNA2d2-88, insertion of GRSS after EBNA2 aa18 or deletion of aa19-33 substantially weakens LMP1p activation, delays LCL outgrowth, and substantially reduces transformation efficiency indicating that mutations in the N-terminal 58 aa can substantially inactivate EBNA2 [50]. EBNA2d3-30 is also defective in maintaining growth of EBNA2-HT converted LCLs in the absence of 4HT, activates Cp, but not LMP1p, and does not associate with WT EBNA2, indicating a broad effect on both SAD1 and SAD2 [164]. EBNA2 1-58 are also important for activation and co-activation by EBNALP [71, 158, 165]. Deletions of most, but not all, of the PP domain are well tolerated in LCL outgrowth assays and EBNA2 deleted for the entire PP domain can support LCL growth in a conditional EBNA2 complementation test [159, 166]. Interestingly, PP-deleted EBNA2 is three- to fourfold hyper-functional in LMP1p activation, twofold hyper-functional in LMP2Ap activation, and similar to EBNA2 in Cp activation. EBNA2d3-19, d18-60, or d117-199 are hypofunctional in LMP1p activation and WT in Cp activation [167]. EBNA2 is in large complexes in lymphoblasts, sediments as dimers to octamers when expressed in lymphoblasts, and even baculovirus-expressed recombinant EBNA2 is an octomer, whereas EBNA2d2-88 is a dimer [158, 168, 169]. Thus, considerable evidence supports the hypothesis that EBNA2 N-terminal residues 1–58 and 101–199 are self-association domains linked by a poly proline domain to enable EBNA2 activation of promoters such as the LMP1p, but are substantially less important in Cp activation. EBV with EBNA2d102-127 is similar in phenotype to EBNA2d2-88 in being significantly reduced in initial efficiency of B-lymphocyte conversion to LCLs, WT in initial time for LCL outgrowth, WT in subsequent LCL expansion, and near WT in immortalization of B-lymphocytes with defective-free EBV EBNA2d102-127 [159]. EBNA2d102-127 differs from EBNA2d2-88 in that EBNA2d102-127 is WT for LMP1p activation [50]. However, aa102-127 are important for SAD2 homodimerization, consistent with the notion that aa1-58 can be sufficient for multimer formation [158]. EBV with EBNA2d117-146 has 5% of WT EBNA2 efficiency in lymphocyte immortalization, and LCL outgrowth requires 4–8 weeks instead of the WT 3 weeks [50]. EBNA2d123-147 complementation of a conditionally inactivated EBNA2-HT fusion in an LCL gives only slightly reduced LCL growth compared to WT EBNA2, and EBNA2d123-147 is WT

in LMP1p and Cp activation [170]. Overall, EBV EBNA2aa102-127 is less impaired in conversion of primary B-lymphocytes to LCLs than EBV EBNA2d117-146. The significant growth defect with EBNA2d117-146 is likely to be due to the role of these residues in binding Nur77. Nur77 is a nuclear hormone receptor transcription factor that can translocate to mitochondria in the cytoplasm to induce apoptosis [170]. Nur77 binding to Notch can retain Nur77 in the nucleus and prevent Nur77-mediated apoptosis in T cells [171]. EBNA2aa128-154 can also bind Nur77 and EBNA2 deleted for aa123-147 fails to bind to Nur77 [172]. Furthermore, EBNA2 binding of Nur77 in the nucleus can protect cells against Sindbis virus infection or etoposide-mediated cell death [170, 172]. Moreover, EBV conditional EBNA2 LCLs that are trans-complemented by EBNA2d123-147 are sensitive to etoposide-induced apoptosis relative to WT EBNA2 trans-complemented cells, whereas both cell types are equally resistant to TNF-induced apoptosis [170]. EBNA2 association with Nur77 could be important in preventing apoptosis following the stress of EBNA2-induced c-myc expression in the conversion primary B-lymphocytes to LCLs or for EBNA2-mediated transcriptional activation. Also, EBNA2aa117-146 could be important for DP103 or SNF5/INI1 recruitment to promoters. DP103 binding to EBNA2 is dependent on EBNA2 aa121-216, and INI1 binding to EBNA2 is dependent on EBNA2 aa112-170 [173, 174]. DP103 can co-activate the LMP1p with EBNA2 [174] and SNF5/INI1 is important in activation of transcription from previously silenced sites and in DNA repair processes.

Two EBNA2 domains are absolutely essential for EBV conversion of lymphocytes to LCLs. CR5 and CR6 coordinately mediate association with RBP/CSL, the DNA-binding protein that targets Notch and EBNA2 to promoters, and CR8 is an acidic-activating domain similar to VP16 [50, 81, 101, 147–149, 175, 176]. The CR6 PWWPPIC domain mimics Notch1 LWFPEGF in WW or WF binding to a hydrophobic pocket in RBP/CSL and EBNA2 WW_{324} mutation to FF or SS are inactive in RBP/CSL binding, Cp activation, and primary B-lymphocyte conversion to LCLs [54, 78, 80, 177]. Furthermore, a CR6 peptide blocks EBNA2 binding to RBP/CSL and reverses EBNA2 mediate promoter activation in cells, indicating that inhibition of EBNA2 binding to this site might halt LCL growth [177, 178], as is also evident with EBNA3A overexpression and dissociation of EBNA2 from RBP/CSL [179]. EBNA2 association with RBP/CSL is an important or nearly determinative feature of promoters that have been investigated, including the Cp, LMP1p, LMP2Ap, CD21p, and CD23p [54, 78, 136, 160, 163, 177, 180–186]. However, some EBNA2 activations are RBP/CSL independent; the LMP1p is 50% activated by EBNA2 mutants defective in RBP/CSL binding and the minimal LMP1p responsive element does not bind RBP/CSL [145]. Moreover, IL18 and RAP-GEF2 are EBNA2 activated in an RBP/CSL independent fashion [187, 188]. Even in RBP/CSL-dependent promoters, isolated RBP/CSL-binding sties are not necessarily sufficient to convey EBNA2 responsiveness. EBNA2 activation

of Cp depends on AUF1 [146] and of LMP1p is dependent on PU.1, SpiB, or Elf-1[145].

CR5 is 10aa N-terminal to CR6, which binds to RBP/CSL, and is highly associated with SKIP, an RBP/CSL-binding protein. EBNA2 directly interacts at a high level with SKIP in yeast 2-hybrid tests and EBNA2 activates transcription through Gal4DBD-SKIP in mammalian 2-hybrid assays; interaction with SKIP is dependent on CR5, while mutation of CR6 has no affect on SKIP interaction [143, 189]. However, EBNA2 d296-314 is deficient in RBP/CSL association and in Cp activation, although LMP1p activation is unaffected [55]. These data are consistent with a model in which EBNA2 interacts with RBP/CSL directly through CR6 and engages SKIP on RBP/CSL through CR5 to stabilize RBP/CSL association. Both are essential for LCL outgrowth and for Cp-type promoter activation, but CR5 is significantly less important and CR6 not fully required for LMP1p activation [55, 143].

CR8 is part of the conserved and essential aa437-466, an acidic-activating domain that is similar to VP16 [50, 101, 175]. The acidic domain is essential for EBNA2 transcriptional activation and for LCL outgrowth [50, 101, 175]. The acidic domain recruits TFIIB, TFIIH, TBP, TAF40, p100, TFIIE, p300/CBP and can interact with EBNALP [71, 81, 147–149].

In contrast to the importance of most of the conserved domains for transcription and primary B-lymphocyte conversion to LCLs, EBNA2 d148-234 has >30% EBNA2 efficiency in conversion of primary B-lymphocytes to LCLs, with only slight delay in time to outgrowth [50]. EBV EBNA2d200-234 has >60% WT EBV efficiency in LCL outgrowth and is identical to EBNA2 in time to outgrowth [50]. Further EBV EBNA2 d235-284 has ~50% WT EBV efficiency in conversion of primary B-lymphocytes to LCLs and is only slightly delayed in outgrowth. EBNA2 aa341-358 is an RG repeat, which mediates interaction with histone H1 and chromatin, but is poorly conserved. EBV EBNA2 d341-358 is efficient in infected LCL outgrowth, although outgrowth is slower than with EBNA2-infected LCLs [53]. Deletion of the EBNA2 RG domain results in hyper activation of LMP1p in transient assays, but not of Cp [53]. Furthermore, EBNA2 is methylated and methylated EBNA2 can bind SMN, which may be important in splicing or transport of RNA from EBNA2-activated promoters [190]. Thus, the EBNA2 RG domain has potentially significant biochemical activities, but a weak overall role in LCL outgrowth. Recombinant EBV with EBNA2 d363-387 or EBNA2 d389-434 are similar to EBV with WT EBNA2 in transformation efficiency, indicating that EBNA2 aa363-424 do not have a critical role in LCL outgrowth, despite inclusion of CR7 [53]. Moreover, CR9 includes an NLS, but EBV with EBNA2d467-487 is WT for primary B-lymphocyte conversion to LCLs and for LMP1p activation [50]. Consequently, EBNA2 aa148-284, aa341-434, and aa467-487 are of less specific interest for further reverse genetic or biochemical analysis.

A range of EBNA2 responsive cellular promoters have recently been identified by transcription profiling of LCLs that are conditional for EBNA2

expression [82, 187, 191]. From these lists of EBNA2 upregulated genes *c-myc* is obviously important as a central mediator of cell growth effect. Notch also targets c-myc through RBP/CSL in the causation of human childhood T-cell ALL, and Notch1 activation of c-myc is through an RBP/CSL site upstream of the c-myc promoter [142, 192]; EBNA2 is likely to interact with this site. The crystal structures of RBP/CSL bound to DNA with and without Notch or Mastermind (MAML) reveal aspects of Notch promoter targeting and activation that are likely to be relevant to EBNA2 interaction with RBP/CSL and upregulation of transcription [193].

14.2 EBNALP

EBNALP is encoded in the leader of EBNA2 mRNAs and potentially co-activates transcription with EBNA2 from the start of EBV latency III infection [71, 72]. EBNALP is composed of N-terminal 66 aa repeats, encoded by repeating 66nt W1 and 132nt W2 exons followed by an 11 aa encoding Y1 exon and a 34 aa encoding Y2 exon (Fig. 14.3). The W1 and W2 exons are derived from successive copies of the EBV 3 kbp internal repeat [133, 194]. Most EBV strains encode EBNALP with 3–6 W1W2 repeats. These EBNALP N-terminal 66 amino acid repeats have most of the EBNALP effect in EBNA2 co-activation assays. Two W1W2 repeats are sufficient for some activity and 4–5 confer maximal co-activation [71, 72]. Core residues of the 66aa W1W2 repeats that are critical for transient co-activation include components of the NLS and a critical serine residue within W2 (S34) which is a p34cdc2 substrate; S34A renders EBNALP inactive, whereas S34E renders EBNALP constitutively active [74, 75, 195–198]. EBNALP requires EBNA2 for previously described promoter-specific effects and can effect 50–100 fold transcriptional co-activation with EBNA2 over EBNA2 alone [71, 72]. EBNALP repeats provide EBNALP dimerization, substantial co-activation, and transient binding to EBNA2 through the W2 repeat C-termini [71, 73, 199]. The EBNALP C-terminal unique 45aa regulates activity and EBNA2 association. Deletion of the EBNALP C-terminus 10 aa results in abrogation of co-activation and stable

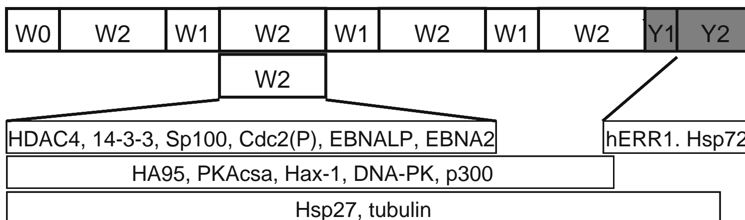


Fig. 14.3 Schematic of EBNALP repeat W1 and W2 (22 and 44 amino acids, respectively) repeats and Y1 and Y2, unique amino acids. Below are shown interactive cell proteins

association with EBNA2 [71, 73]. The EBNA2 acidic domain can bind EBNALP and is critical for EBNALP co-activation, although EBNA2 aa1-58, 360-420 and the RG domain also affect EBNA2 co-activation by EBNALP [73, 165]. The C-terminal 45 amino acids are critical for primary B-lymphocyte conversion to LCLs [51, 79]. The importance of the 45 aa C-terminus could be due to associations with cell proteins and/or to their role in regulating EBNALP W1W2 associations and co-activation. Because of the intrinsic difficulty in constructing EBV recombinants with mutations in the EBNALP W1W2 repeats, their role in LCL outgrowth has not been directly assessed by reverse genetics.

EBNALP-associated proteins have been identified in EBNALP immune precipitates [76, 200, 201], limited yeast 2-hybrid screens [196, 202], and EBNALP affinity chromatography [203]. EBNALP immune precipitates, microsequencing, and Western blot confirmations identify specific associations with alpha and beta tubulins, Hsp27, Hsp72, DNA-PK, and HA95 [204], a gene duplication of AKAP 95 [200, 201, 203]. EBNALP W1W2 repeats also bind to the DNA-PK C-terminus near the kinase domain and are phosphorylated by DNA-PK, in vitro [201]. Hsp72 and HA95 associate with EBNALP at moderately high and very high levels, respectively [201]. Surprisingly, Hsp72 has a positive role in EBNALP co-activation with EBNA2 [152]. HA95 association enables PKA to interact with EBNALP and to down modulate EBNALP co-activation [205]. The role of HA95 may be more complex since HA95 is also associated with RNA helicase A, a major nucleo-cytoplasmic-shuttling factor, and a substantial fraction of EBNALP is in LCL cytoplasm [151, 201, 205, 206]. An EBNALP yeast two hybrid bait retrieved a mitochondria-associated survival factor, Hax-1, which associates with EBNALP W1W2 in the cytoplasm, and hERR1, which associates with leucine residues in EBNALP Y2 and increases EBNALP activation of a Gal4 promoter 50% [196, 202]. Interactions with p53, Rb, and p14/ARF have also been described [207–210], but not confirmed. EBNALP associates with PML bodies [133, 211] and co-localizes with CBP in LCL PML bodies; EBNA2 co-localizes with p300 in other nuclear areas [212].

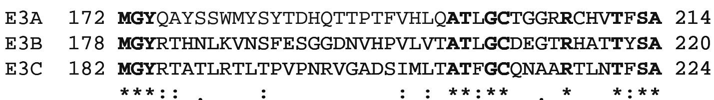
EBNALP interaction with PML body components implicate Sp100 in EBNALP co-activation. EBNALP can relocalize Sp100 and HP1α, which are associated with repressive activity and activate transcriptional. An Sp100 mutant that is deficient in PML body interaction partially co-activates with EBNA2, consistent with a model in which localization of Sp100 outside of PML bodies results mimics EBNALP effects [76]. Further, an Sp100 mutant that does not associate with Hp1α does not cause this effect, consistent with Sp100 movement of Hp1α being a major factor in EBNALP affects through Sp100. Also, PML deficient Sp100 co-activates EBNA2 effects on the EBNALP responsive Cp and LMP1ps and not on the LMP2A promoter. Thus, EBNALP displacement of Sp100 and HP1α from PML bodies likely contributes to EBNALP co-activation, although in LCLs EBNALP and Sp100 localize to PML bodies. EBNALP movement of HDAC4 to the cytoplasm also removes repressors from EBNA2 transcription sites [77]. Surprisingly, Hsp72 is also a

factor in EBNALP co-activation; Hsp72 may affect EBNALP folding and interaction with repressors.

14.3 EBNA3 Proteins

The EBNA3 proteins are each ~1000aa and are encoded by low copy mRNAs. However, the proteins are very stable and highly abundant in LCLs [151, 194, 213–215]. EBNA3A, EBNA3B, and EBNA3C appear to have evolved from tandem triplication of a single ancestral gene (for review see [17, 216]). The highest degree of homology among the EBNA3 proteins is within the N-terminal third of the proteins which mediate binding to RBP/CSL (discussed further below). The C-terminal third of the EBNA3 proteins are most divergent and comprised largely of residues encoded by expanded oligonucleotide repeats [213, 214]. The fact that EBNA3A, EBNA3B, EBNA3C, differ between the EBV types I and II has been useful for comparative purposes [23, 155]. The type I and II EBNA3A, EBNA3B, or EBNA3C genes are collinearly homologous [155]. Type II EBNA3A, EBNA3B, or EBNA3C can replace type I EBNA3A, EBNA3B, or EBNA3C without an effect on the conversion of primary B-lymphocytes to LCLs [60]. Domains that are important for the conversion of primary B-lymphocytes to LCLs are therefore likely to be particularly conserved between type I and type II EBNA3A, EBNA3B, or EBNA3C. Since the non-human old world primate lymphocryptoviruses (LCVs) transform B-lymphocytes from their endemic species, have collinearly homologous genomes with EBNA3A, EBNA3B, and EBNA3C genes, convert their autologous primary B-lymphocytes to LCLs, and cause LPD when the host species is immune suppressed, EBNA3A and EBNA3C residues required for LCL outgrowth are likely to be conserved in primate LCVs as well [91, 217]. Importantly, the conservation among the EBNA3As, among the EBNA3Bs and among the EBNA3Cs of different LCVs is significantly greater at every level of overall conservation than the conservation among EBNA3A, EBNA3B and EBNA3C of the same LCV, consistent with a working hypothesis that these proteins have similar functions, with divergent specificities.

The conservation among EBNA3 proteins is most evident in the amino terminal third. Within aa110–340 are 49 residues that are highly conserved among all EBNA3 proteins of all sequenced old world primate lymphocryptoviruses (Fig. 14.4). Twenty conserved and 11 identical residues are within



```
E3A  172 MGYQAYSSWMYSYTDHQTTPTFVHLQATLGCTGGRRCHVTFSA 214
E3B  178 MGYRTHNLKVNSFESGGDNVHPVLVTATLGCDEGTRHATTYSA 220
E3C  182 MGYRTATLRTLTPVPNRVGADSIMLTATFGCQNAARTLNTFSA 224
         ***:: .    :           : : **:**  . *   *:**
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Fig. 14.4 Schematic of EBNA3A, EBNA3B and EBNA3C conserved amino acids (Homology Domain) that mediate interaction with RBP/CSL

aa172–244, termed the EBNA3 core homology domain [91, 103, 218]. A substantial body of evidence indicates the core homology domain mediates EBNA3 binding to RBP/CSL [88–91, 102, 103]. Deletion of EBNA3A aa170–240 disrupts RBP/CSL association and prevents EBNA3A from blocking EBNA2 activation of the Cp promoter in transfection assays [58]. Mutation of EBNA3A $TLGC_{202}$ to $AAGA_{202}$ prevents the interaction of EBNA3A aa125-222 with RBP/CSL by mammalian two-hybrid [103], prevents overexpressed EBNA3A repression of EBNA2 Cp promoter activation [58, 102], prevents overexpressed EBNA3A from blocking EBNA2 activation of *c-myc* in B lymphoblasts [179], and is null for EBNA3A maintenance of LCL growth [91]. Similarly, EBNA3B aa1-311 is sufficient for RBP/CSL binding, and mutation of $TLGC_{208}$ to $AAAA_{208}$ is disruptive. Within the EBNA3C homology domain, mutation of $TFGC_{212}$ to $AAAA_{212}$ disrupts EBNA3C's ability to repress EBNA2 activation of promoters with RBP/CSL sites and abrogates EBNA3C inhibition of an RBP/CSL gel shift [91, 218]. Surprisingly, EBNA3C $AAAA_{212}$ coactivates the LMP1p with EBNA2 to the same extent as EBNA3C, indicating that strong stable interaction with RBP/CSL is not required for EBNA3C coactivation of the LMP1p [91]. More recent data indicate that EBNA3A, EBNA3B, and EBNA3C each have unique and substantially exclusive transcriptional and lymphocyte-immortalizing effects [219]. While at least some essential EBNA3A and EBNA3C transcriptional effects are through RBP/CSL, other important functions may be RBP/CSL independent.

EBNA3A and EBNA3C are essential for lymphocyte conversion to LCL out growth, while EBNA3B is not. EBV reverse genetic analyses placing a nonsense codon after EBNA3B codon 109 or deletion of the entire EBNA3B ORF results in minimal effect on LCL outgrowth, indicating that EBNA3B is not critical for primary B-lymphocyte conversion to LCLs [60, 220, 221]. EBNA3B may in part have evolved to add to the EBNA3A and EBNA3C effects in enabling EBV-infected cell recognition by immune T cell (for review see [4]). EBV conversion of primary B cells to immortal proliferating lymphoblasts could not have evolved as a strategy for EBV persistence without engendering strong T-cell containment of lymphoblast proliferation [222–224]. Indeed, a bone marrow transplant recipient with latency III LPD, who was treated with T cells that had an EBNA3B dominant CD8 T-cell response, initially responded, only to later die from latency III LPD with cells that had lost EBNA3B expression as a consequence of an out of frame immune escape mutation [225].

In contrast to EBNA3B, insertion of nonsense codons after EBNA3A codon 302 or EBNA3C codon 365 results in EBV recombinants that can only transform resting human B-lymphocytes when the cells are co-infected with the EBNA2 null transformation defective P3HR1 EBV to provide EBNA3A or EBNA3C in trans [52, 226]. In other experiments, a mini-EBV genome with a nonsense mutation in EBNA3A codon 304 was used to transform LCLs and P3HR1 co-infection was required, consistent with EBNA3A encoded by P3HR1 being essential for LCL outgrowth [227].

More recently, LCLs transformed by reverse genetically engineered EBV recombinants that express a conditional EBNA3A [56] or EBNA3C [93]. Hydroxy-tamoxifen (HT) mutant estrogen receptor fusion have been used to further evaluate the role of EBNA3A or EBNA3C in the maintenance of LCL growth. As a consequence of the HT fusion, the EBNA3A-HT and EBNA3C-HT proteins are expressed at lower than wild-type EBNA3A or EBNA3C levels in the presence of HT. Nevertheless, EBNA3A-HT and EBNA3C-HT, in media supplemented with HT, double every 2 days and expand 1,000 fold over 3 weeks. In each instance, shift of the infected LCLs to non-permissive conditions in media without HT results in EBNA3A-HT or EBNA3C-HT movement to the cytoplasm within 24 hr and gradual fall in EBNA3A-HT or EBNA3C-HT to undetectable levels over the next several days. LCL growth ceases over 5–7 days after shift to medium without HT and can be slowly restored by addition of HT to culture medium. Without HT, EBNA3A-HT or EBNA3C-HT-infected LCL cells slowly die over days 7–21. Expression of the other EBNAs, LMP1, CD21, CD23, and *c-myc* are unaffected by EBNA3A or EBNA3C inactivation, indicative of a non-essential or non-unique role for EBNA3A or EBNA3C in regulating these genes. LCLs can be transfected with an efficiency of at least 20% and an oriP-based episome can be used to provide WT or mutant EBNA3A, EBNA3B, or EBNA3C expression in trans, following shift to non-permissive conditions. Importantly, growth of EBNA3A-HT LCLs can be rescued by expression of EBNA3A, but not by expression of EBNA3B or EBNA3C [56]. This complementation system has also been used to define EBNA3A domains essential for LCL growth (see below) [58]. Using an oriP-based system allows cells with various levels of transfected episomes and resultant EBNA3 protein levels to self select for appropriate levels by their ability to proliferate after EBNA3A-HT or EBNA3C-HT inactivation [91]. After transfection, EBNA3A-HT or EBNA3C-HT LCL cells kept in medium with HT express low levels of transfected EBNA3A or EBNA3C as they grow out at 21–30 days, whereas cells kept in medium without HT express full wild-type levels of transfected EBNA3A or EBNA3C as they grow out at 21–30 days [93]. Thus, this system dramatically shows the dependence of LCL cells on normal level wild-type EBNA3A and EBNA3C protein expression.

14.4 EBNA3A

Conditional EBNA3A inactivation in LCLs and complementation with EBNA3A or EBNA3A mutants identify EBNA3A aa170-240, $TLGC_{202}$, aa300-386, and aa386-410 to be essential for LCL growth and aa240-300 and aa827-944 to be important for robust LCL growth (see Fig. 14.5, [52]). In contrast, aa2–124, 410–439, 440–470, 470–500, 500–523, 523–612, and 620–820 can be deleted without negative effect on LCL growth [52]. EBNA3A mutants impaired or null for RBP/CSL binding ($TLGC_{202}$-

Domain	Mutant	LCL Growth	EBNA2 Cp Repression	RBP/CSL Association
1	d170-240	–	–	–
1	TLGC-AAGA	–	–	+/–
2	d240-300	+/–	+	+
3	d300-386	–	–	+
4	d386-410	–	+	+
5	d827-944	+/–	+	+

Fig. 14.5 Table of the EBNA3A amino acids essential (LCL Growth -) or important (LCL growth +/–) for continuous lymphoblast cell growth and the effect of deletion on LCL growth, EBNA3A repression of EBNA2 activation of the EBV Cp promoter, or EBNA3A association with RBP/CSL

$AAGA_{202}$ or d170-240) were unable to support growth of EBNA3A-HT LCLs upon HT withdrawal. This indicates that RBP/CSL association is essential for EBNA3A-mediated LCL growth [52]. EBNA3A d240–300, d300–386, and d386–410 were partially (d240–300) or fully (d300–386 and d386–410) defective for LCL growth maintenance, but associated normally with RBP/CSL (Fig. 14.5) [91]. The failure of EBNA3A d300–386 to repress EBNA2 Cp promoter activation may due to a slight decrease in RBP/CSL-binding affinity that impairs its ability to compete with EBNA2, but is not detectable in the coimmunoprecipitation assay. These EBNA3A residues are at the C-terminus of the broader EBNA3A homology domain and may important for interactions with RBP/CSL-associated proteins or make direct secondary contacts with RBP/CSL. Alternatively, EBNA3A repression of EBNA2 Cp promoter activation may require inhibition of RBP/CSL binding to DNA in addition to competition with EBNA2. EBNA3A aa827–944 was also required for efficient LCL outgrowth in the EBNA3A-HT complementation assay. These residues include binding motifs that mediate CtBP association (Fig. 14.6)[111].

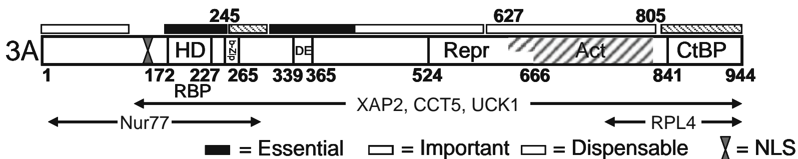

Fig. 14.6 Schematic of EBNA3A amino acids that are essential (Black overlay), important (Hatched overlay) or dispensable (clear overlay) for continuous lymphoblast proliferation. Repr and Act indicate domains that have repressive or activating effects when localized to promoters by fusion to the Gal4 DNA binding domain. Interactive cell proteins are indicated below except for CtBP binding, which is shown within amino acids 841–944

When fused in-frame to the Gal-4DBD and assessed in cells with a reporter containing multiple upstream Gal-4DBDs, EBNA3A aa524–666 [103, 228] and EBNA3C aa363–545 [104] are strong repressive domains, whereas EBNA3A aa627–805 [102] and EBNA3C aa724–826 [87, 91] are strong activating domains, consistent with both up and downregulating potential (Fig. 14.6). Overall, EBNA3A or EBNA3C fused in-frame to the Gal-4DBD strongly repress transcription [87, 102, 104]. Further, EBNA3A and EBNA3C associate with cell proteins that up or downregulate transcription [86, 106, 110, 111, 114, 116, 229]. Moreover, the highly associated RBP/CSL is itself associated with repressors such as SKIP, NCoR, SMRT, and SHARP, and EBNA3s may interact with RBP/CSL or with EBNA2 or Notch1 to stabilize, displace, or recruit repressors or activators [116, 189, 230, 231]. EBNA3A also has an $LXXLL_{105}$ complex nuclear hormone receptor interaction motif [232, 233]. RBP/CSL, the epsilon subunit of the chaperonin T-complex protein 1, the p38 subunit of the aryl hydrocarbon receptor complex, uridine kinase 1, Nur77, and RPL4 associate with EBNA3A in yeast two-hybrid (Y2H) screens [234–237] (Fig. 14.6).

14.5 EBNA3C

EBNA3C-HT LCLs have also been derived and used to investigate the role of EBNA3C in LCL growth. EBNA3C-HT LCLs are similar in that their growth can be rescued by EBNA3C expression in trans, but not by EBNA3A or EBNA3B expression [93]. Within 7 days after HT withdrawal from EBNA3C-HT LCLs $p16^{INK4A}$ is expressed, phosphorylated Rb decreases and cyclin A levels decrease slightly, whereas $p21^{CIP1}$, $p27^{KIP1}$, Cdk4, Cdk6, and cycD2 levels do not change [93]. EBNA3C expressed in trans-prevented $p16^{INK4A}$ mRNA induction and protein expression. These results indicate that EBNA3C has an essential transcriptional regulatory role in repressing $p16^{INK4A}$ and permitting cell cycle progression and LCL growth. Candidate EBNA3C-regulated genes have also been identified by expression profiling of LCLs impaired for EBNA3C expression by insertion of an antibiotic resistance marker upstream of the EBNA3C gene; TCL1 was found to be downregulated in EBNA3C low relative to cells with WT EBNA3C levels and could be an important mediator of EBNA3C affects on LCL outgrowth [91].

EBNA3C also has a repression domain (aa346-543), encompassing aa363–398, which are similar to the EBNA3A acidic (DE) region, a polyproline repeat (aa551–610), a transcriptional activation domain (aa724–826), and multiple NLSs [87, 104] (Fig. 14.7). Residues within the EBNA3C aa365–545 repression domain are also important for LMP1p co-activation [48]. A Y2H study identified 19 potential EBNA3C-associating proteins [48]. EBNA3C residues that bind these 19 proteins are represented Fig. 14.7. Binding of TFIID, ProTalpha, SMN, DP103, SUMO, CtBP, CycA, Rb, HDAC1, Nm23, p300, Skp2, C8/alpha7,

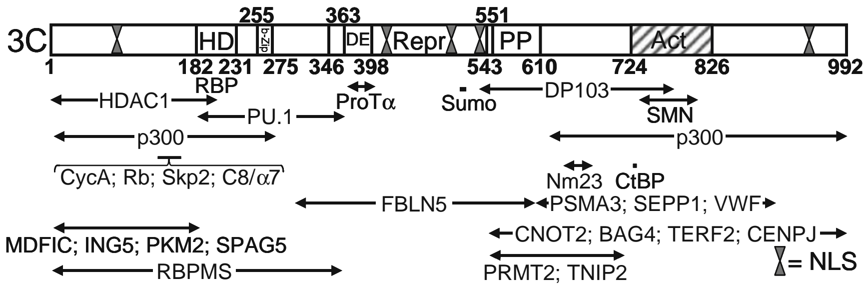

Fig. 14.7 Schematic of EBNA3C amino acids and interactive cell proteins

and PU.1 to EBNA3C has also been reported using Gst-pull down, Y2H, or co-immune precipitation assays [86, 92, 104, 106, 110, 112, 115–119, 218, 229, 237–242].

EBNA3C differs from EBNA3A and EBNA3B in co-activating the EBV LMP1p with EBNA2 in some BL cells under specific growth conditions [48, 87, 92, 95, 243]. LMP1 expression falls off in Raji cells that are arrested in G1 by overgrowth. EBNA3C is unique in restoring LMP1 expression [95, 243]. EBNA3C can also co-activate the LMP1p with EBNA2 in DG-75 BL cells placed in fresh medium after growth arrest [87, 92]. This effect is independent of the RBP/CSL-binding sites, and the EBNA3C core homology domain is dispensable for LMP1p co-activation, is dependent on the PU.1 site in the LMP1p, and may be due to a weak interaction of EBNA3C "bZIP like" domain, aa239–385, with the PU.1 ETS domain [92]. EBNA3C aa365–545 are both necessary and sufficient for this transcriptional effect in transient assays.

Overexpression of EBNA3C or EBNA3A in rat embryo fibroblasts, NIH3T3 cells, or human U2OS cells with oncogenic Ras increases transforming events [108, 111]; this effect has been attributed to an interaction with pRb [108]. However, the Ras complementation effects have been mapped to CtBP interaction sites and may be downstream of CtBP. CtBP associates with CtBP-IP, a pRb-binding protein [109–111, 230]. Although most physiologically significant CtBP effects are through transcription repression, CtBP-IP has pRb binding affects similar to those of adenovirus E1A and HPV16 E6. In cells compelled to arrest by serum withdrawal, EBNA3C overexpression can also inhibit $p27^{K1P1}$ accumulation and cause bi- and multinucleated cells by abrogating the mitotic spindle checkpoint [86, 118, 244]. Further, overexpressed EBNA3C can bind and activate cyclin A through EBNA3C aa130–190 and aa957–990 by displacing $p27^{K1P1}$ or induce $p27^{K1P1}$ and Rb proteasomal degradation by an SCF complex [86, 118]. The significance of this EBNA3C aa140–149 dependent protein interaction in LCL growth has however, not yet been directly assessed using EBNA3C-interacting domain mutants in LCL outgrowth and complementation assays. The continued growth of EBNA3A-HT and EBNA3C-HT LCL cells for 5–7 days after EBNA3A-HT or EBNA3C-HT shift to the cytoplasm

upon HT withdraw, indicates that nuclear EBNA3A or EBNA3C action is not required for continued cell growth.

In summary, current evidence favors a model that the principal EBNA3A role in LCL growth is due to EBNA3A regulation of transcription of viral and cellular genes through RBP/CSL. This is not attributable to a role for EBNA3A or EBNA3C in limiting EBNA2 interaction with RBP/CSL [71, 87–90, 179, 228, 245–250], since overexpression of EBNA3B or EBNA3C cannot replace EBNA3A; EBNA3B or EBNA3A also cannot replace EBNA3C. Although overexpression of EBNA3A and EBNA3C can complement Ras in transformation experiments, the effects map to sites of interaction with CtBP, and for EBNA3A the CtBP-binding domain is not essential for continued LCL proliferation [58]. At least in part, EBNA3C affects appear to be mediated through RBP/CSL or PU.1, since EBNA3C is specifically associated with the RBP/CSL PU.1 site in the LMP1 promoter [94]. However, the role of EBNA3C may be more complicated that that of EBNA3A, since EBNA3C inactivation is associated with the accumulation of $p16^{INK4A}$ and decreased hyperphosphorylated pRB and hypo-expression is associated with lower TCL1A levels, transcriptional effects that could affect cell growth.

14.6 EBNA1

EBV episomes persist in dividing malignant and non-malignant cells through EBNA1 interaction with multiple cognate sites in EBV oriP DNA [67, 69, 251–253]. OriP is comprised of an EBNA1-dependent enhancer element made up of a family of 20 tandem 30 bp imperfect repeats (FR) of imperfect palindromes, which are symmetrical high-affinity EBNA1 dimer-binding sites, a 980 bp spacer, and a partially EBNA1-dependent replication origin made up of a Dyad Symmetry (DS) of two pairs of EBNA1 dimer-binding sites. The centers of the dimer-binding sites in each DS pair are separated by 21 bp instead of the 30 bp separation in FR. Each pairs is bracketed by Telomere Repeat Factor 1 and 2-binding sites, which make an additional dyad symmetry around DS 3 and 4 [64, 254, 255]. An alternative, weaker, and partially EBNA1 dependent, replication origin, Rep*, is 238 bp from DS 1. [69, 252, 256–259]. Another pair of EBNA1-binding sites is downstream of the latency I Qp promoter and down modulates Qp activity in latent infections [67, 253, 260–262].

EBNA1 interaction with oriP enables EBV DNA replication once per cell cycle [263–267]. FR at the level of 5–6 copies and DS are required for efficient episome persistence and transcriptional activation in infected cells [25, 69, 120, 251, 252, 256, 268–271]. FR enables long-term retention of replication competent human DNA when EBNA1 is expressed in human cells and of replication competent rodent DNA when EBNA1 is expressed in rodent cells [272, 273] [272, 274]. FR is an EBNA1-dependent enhancer [120, 258, 271, 275–277],

whereas DS is the site of initiation of EBV episome DNA replication [275, 278]. At least two dimer DS elements are required for oriP plasmid replication [279]. DS1 and 4 are higher affinity EBNA1-binding sites than DS3 and 2. EBNA1 binding requires considerable distortion to be fully loaded with EBNA1 dimers [275, 278, 280]. Further, two copies of DS are almost as effective as oriP in replication [268]. The exact 3 base spacing between DS 4 and 3 is critical to support replication because addition or deletion of 1 base abolishes replication [281]. In vitro binding of EBNA1 to DS-bound nucleosomes occurs cooperatively [282, 283], does not require ATP or ATP-dependent chromatin-remodeling factor [284, 285], does not melt oriP [284], and destabilizes nucleosomes [283, 285]. TRF1, TRF2, and hRap1-binding sites precede DS4, separate DS3 and 2, and follow DS1 [64, 254]. Tankyrase 1 and 2 can also bind to these sites and to RXXPDG sites in EBNA1; abrogation of both EBNA1-binding sites enhances initial oriP replication [255].

EBNA1 from a prototype EBV strain is 641 amino acids [286] (Fig. 14.8). EBNA1 aa2–30 have no known function and are dispensable for replication, DNA binding, transactivation and persistence [287]. At least three EBNA1 domains are required for oriP interactions necessary for episome replication in dividing cells for enhanced episome transcription, or for long-term episome persistence; arginine–glycine (RG)1 (aa33-89), RG2 (aa328-386), and almost

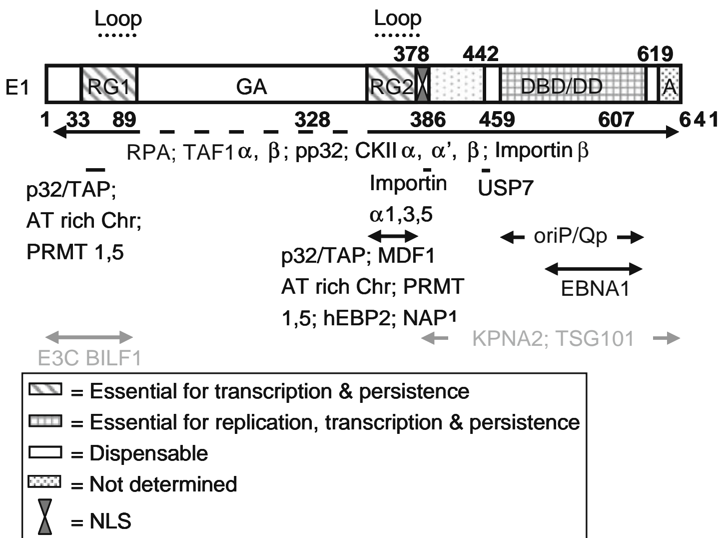

Fig. 14.8 Schematic of EBNA1 amino acids and interactive cell proteins. The EBNA1 DNA binding and Dimerization domain (DBD/DD) is necessary for EBNA1 function in DNA replication, transcription enhancement, and episome persistence. RG1 and RG2 interact with overlapping repertoire of cell proteins, mediate chromosome association, and are necessary for transcription enhancement and episome persistence

inseparable dimerization domain (DD) and oriP DNA-binding domain (DBD) (aa459-607) [62, 63, 282, 288–292].

Arginine–glycine-rich region 1 (RG1) is in aa31-89 and RG2 aa328-378. RG1 and RG2 are separated by an irregular glycine–alanine repeat domain (GA) [293–295]. Deletion of the entire GA repeat has no discernible effect on EBNA1 abundance or functional interaction with oriP. The GA domain minimizes translation [296], binds to proteasomes, and inhibits EBNA1 proteolysis [297–299]. As a consequence of both decreased synthesis and very slow degradation, EBNA1 peptides are poorly presented in the context of MHC Class I. Cells expressing EBNA1 are therefore protected from recognition by CD8 cytotoxic T lymphocytes [298–303].

RG1 and RG2 are critical domains for EBNA1 effects on OriP-mediated transcriptional enhancement and episome persistence. Either RG rich domain can mediate EBNA1 chromosome association [65, 304, 305], but both are necessary for wild-type EBNA1 chromosome association [65, 305] and for episome persistence. RG1 aa31-54 is comprised of GR repeats followed by GGRP and is similar to shorter interspersed GGRGRGGGS repeats in RG2. RG1 aa34-52 does not significantly affect episome maintenance or transcriptional activation, but aa61-83 or 65-89 are essential [306]. EBNA1 deleted for this part of the RG1 domain is a dominant negative for transactivation, but WT in episome persistence. Consistent with a special role for RG1 aa61-83 in transcription, two RG1s are superior to two RG2s in enabling transcription [66, 307]. Although RG2 aa325-376 is also required for both transcription and episome persistence in the context of wild-type EBNA1 [66, 287, 306, 308], 2 copies of RG1 can substitute for RG2 and mediate effective transcriptional enhancement and long-term episome persistence[121, 305]. Thus, proteins that interact with RG2, such as hEBP2, P32/TAP, Nap1, Karyopherin a2, PRMT5, and PRMT1 [68, 309–314] (Fig. 14.8), and are implicated in transcriptional activation (hEBP2, p32/TAP, Karyopherin, PRMT5 and PRMT1) or episome maintenance (hEBP2, Nap1) are not likely to be essential for EBNA1 functions unless they also interact with RG1.

RG1 and RG2 also mediate the ability of EBNA1 to aggregate when bound to DNA, a phenomenon called looping or linking, depending on whether the assay is by looping of FR and DS on the same molecule or linking of multiple molecules [290, 307, 315]. RG1 and RG2 can each link and 2 copies of either are also sufficient for wild-type linking [307, 316, 317]. Both aa320-355 and 351-377 can mediate looping of Gal4 DNA-binding domains with cognate DNA [315]. Deletion of RG1 does not significantly affect the fraction of protein bound DNA molecules that are looped [290].

Interestingly, replacement of EBNA1 aa1-378 or 1-450 with HMG-I or histone H1.2 results in wild-type long-term episome persistence, although transcriptional activation from oriP episomes is only 30% of wild-type EBNA1 [65, 305]. The Histone H1 C-terminus and HMGI/Y [318] bind to AT-rich scaffold regions in DNA and the RG1 and RG2 GRGR repeats are similar to the HMG-I AT hooks that bind to polydA/polydT [121]. Thus, a substantial body of data

points to the critical role of RG1 and RG2 in tethering EBNA1 to mitotic chromosomes to enable long-term episome persistence and transcription.

The EBNA1 dimerization and DNA, aa459-607, have been crystallized bound to cognate DNA sites and resolved at 2.2A [62, 63]. This domain mediates EBNA1 interaction with oriP, and Rep*, and Qp, the promoter for EBNA1 transcription in latency I and II [65, 67, 69, 273, 279, 281, 319]. While oriP-containing DNA can replicate at a low level in the absence of EBNA1 [258], EBNA1 aa379-641, which includes the NLS, the polypeptide that interacts with Ubiquitin-specific protease 7 (USP7), the dimerization and DNA-binding domain and the acidic C-terminus can increase initial oriP episome replication at least 10 fold, 50% as much as EBNA1 [65, 279, 320]. The EBNA1, EBNA1 aa378-641, or EBNA1 aa378-641 deleted for 386-450 increase in initial oriP plasmid DNA replication at 48 h in B-lymphocytes is probably due to transport of cognate DNA to appropriate nuclear sites [65, 279, 320]. Direct EBNA1 protein–protein interaction with MCM or ORC components has not been demonstrated [321–323]. EBNA1 aa379-641 is also a dominant negative inhibitor of EBNA1 interaction with cognate DNA, resulting in decreased EBNA1-dependent transcription and episome maintenance [306, 320, 324–327].

EBNA1 aa379-386 constitute a nuclear localization sequence [288]; K379 and R380 are essential components and S385 phosphorylation has an upregulatory effect on nuclear import [328]. EBNA1 aa386-450 has been in most constructs used to study EBNA1 functions and deletions of aa396-403, 407-421, and 411-437 are null for episome maintenance [287], raising the possibility that important interactions are mediated by this domain. The only demonstrated interaction is aa442-448, which bind to USP7 and likely stabilize EBNA1. These residues may also displaced p53 or Mdm2 from USP7 and affect p53 functionality [329]. However, EBNA1 RG1, RG2, and NLS, fused to 450-641 is wild type in replication, transcription, and episome maintenance [121, 305] focusing attention on RG1 and aa459-641, as the core essential domains.

Additional roles for some of these latent genes in viral transformation are described in Chapter 12.

14.7 Summary

EBNA2, EBNALP, EBNA3A, EBNA3B, and EBNA3C and to a lesser extent EBNA1 have evolved in the LCV lineage to mediate LCV effects on lymphocyte growth and survival, probably at the earliest stage of primary infection. These genes work coordinately to regulate c-myc, TCL1, and other cell genes, and LCVs have evolved similar regulatory mechanisms to enable these EBNA proteins to regulate viral promoters for EBNAs and LMPs, the other principal contributors to cell outgrowth and survival. The EBNA proteins have also evolved to be readily recognized as foreign. Thereby, latency III EBV-infected

B-lymphocyte growth is sufficient for successful seeding into lymphoid compartments, but inadequate for lymphoma development in almost all humans with normal immune function.

Acknowledgment The authors gratefully acknowledge the support of their research by the National Cancer Institute and National Institutes of Health of the USPHS.

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Chapter 15
Epstein–Barr Virus Entry

Richard Longnecker, Lindsey Hutt-Fletcher, and Theodore Jardetzky

15.1 Epstein–Barr Virus – Introduction

Epstein–Barr virus (EBV), also designated human herpesvirus 4 (HHV4), is one of eight human herpesviruses that establish latent infections in human hosts (reviewed in Pellett and Roizman 2007). Herpesviruses are organized into three subfamilies (α, β, γ) depending on biological characteristics and evolutionary relatedness. All share many similar properties including the ability to enter host cells via a multistep process that culminates with the fusion of the virion envelope with a host membrane releasing the capsid into the cytoplasm to initiate virus infection. There are two γ-herpesvirus that infect humans – EBV and the recently identified HHV8. Research on EBV has considerably expanded since its discovery and link with Burkitt's lymphoma (Burkitt 1962; Epstein et al. 1964). Along with HHV8, EBV is the only herpesvirus with an etiological role in human malignancies. It is almost universally found in endemic Burkitt's lymphoma and undifferentiated nasopharyngeal carcinoma and is an important pathogen in individuals lacking cellular immunity from genetic defects, immune suppression for organ transplantation, or HIV infection (reviewed in Rickinson and Kieff 2007). In immunosuppressed patients, EBV causes a variety of proliferative disorders including immunoblastic lymphomas, oral hairy leukoplakia, and an unusual tumor of muscle origin in children. It has also been linked to variety of other human cancers including some T-cell lymphomas, Hodgkin's disease, and gastric carcinoma (reviewed in Rickinson and Kieff 2007). Infection with EBV usually occurs early in childhood resulting in a disease that is not typically recognized as a clinical entity. If primary infection occurs in adolescence or later, B-cell proliferation and the resulting immune response more commonly results in infectious mononucleosis. After

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_15, © Springer Science+Business Media, LLC 2009

primary infection, most individuals harbor the virus for life and develop cellular immunity against a variety of viral antigens (for review (Rickinson and Kieff 2007)). Periodically, virus is shed from latently infected individuals by the induction of lytic replication in B lymphocytes. Overall, the pathologies associated with EBV infection suggest that the two primary cell types that EBV infects *in vivo* are epithelial cells and B lymphocytes.

15.2 Conserved Herpesvirus Fusion Proteins

Morphologically, the EBV virion is very similar to other herpesvirus virions consisting of an envelope containing viral glycoproteins surrounding a tegumented nucleocapsid containing the linear double-stranded viral genome. Envelope glycoproteins encoded by the different herpesvirus genomes play significant roles in a variety of important processes, including specific binding to the cell surface, fusion of viral and plasma membranes during entry, virion assembly, and egress. The nucleotide and amino acid sequences of the glycoproteins encoded by EBV range from substantially conserved to virtually unrelated when compared to those of other herpesviruses. Shown in Table 15.1 are 10 glycoproteins encoded by EBV and the subset of these that has both sequence homology and/or functional homology with glycoproteins encoded by HSV. Three EBV-encoded glycoproteins are required for epithelial cell fusion (gB, gH, and gL), whereas four are required for B-cell fusion (gp42, gB, gH, and gL) (Fig. 15.1). The three glycoproteins, gB, gH, and gL, common to fusion with both cell types are universally conserved within herpesviruses. Overall, the observations with EBV glycoproteins to date are compatible with research on other herpesviruses indicating that there are at least two discrete phases to EBV infection of target cells – binding of virions to cells followed by fusion of the virion envelope with the

Table 15.1. EBV Glycoproteins

EBV	Other Designations	HSV[1]	Known or proposed function
gB	gp110/BALF4	gB	virus maturation/fusion
gH	gp85/BXLF2	gH	complexes with gp42 and gL/binds epithelial receptor/fusion
gL	gp25/BKRF2	gL	complexes with gp42 and gH
gM	BBRF3	gM	virion maturation/expression requires gN
gN	BLRF1	gN	virion maturation/expression requires gM
gp350/220	BLLF1	gC[2]	initial virion binding to CD21
gp150	BDLF3	none	regulates epithelial fusion
gp78	BILF2	none	unknown
gp42	BZLF2	gD[2]	complexes with gL and gH, binds HLA class II/ trigger for fusion
BMRF2		none	binds integrins/infection of polarized cells

[1]The HSV glycoproteins which share sequence homology and/or functional homology.
[2]Although they have sequence homology with EBV glycoproteins, these HSV glycoproteins may serve as functional homologues.

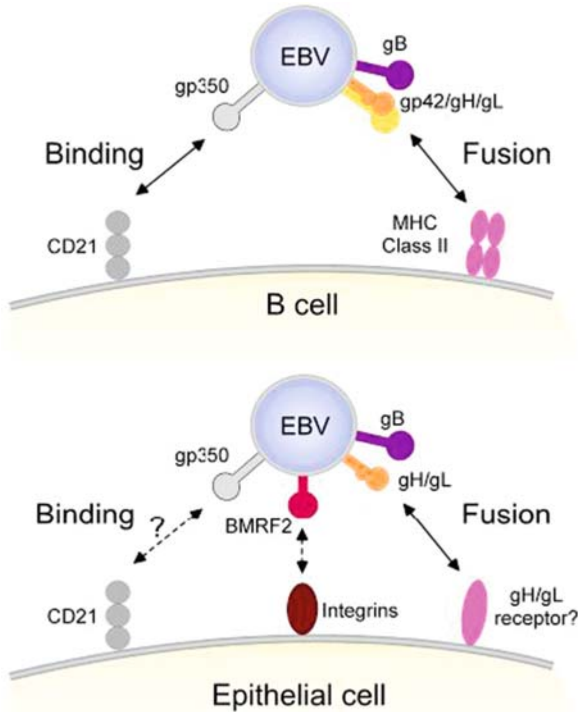

Fig. 15.1 Participants in EBV entry and EBV-induced cell fusion for B cells (top) and epithelial cells (bottom). The "binding receptor" for EBV gp350/220 for B cells is CD21. Entry and fusion in B cells require the interaction of gp42 with HLA class II ("fusion receptor"). gp42 is not required for EBV infection of epithelial cells. The EBV "binding and fusion" receptors in epithelial are not known. gH is likely the viral ligand but the cellular receptor is unknown. Recent studies have suggested a role of BMRF2 in entry of polarized epithelial cells but BMRF2 is not essential for fusion with epithelial cells thus it is likely either a binding receptor or initiates signaling important to post-fusion events in infection. For B cells, the interaction of gp42 with HLA class II is thought to activate the fusion activity of gB and gH/gL. For EBV entry of epithelial cells, activation may result from the interaction of gH with a cellular receptor

plasma membrane of the target cell. The role of each glycoprotein in EBV entry into target cells, the identified cellular receptors, and the overall mechanism of EBV entry into susceptible cells will be the subject of this chapter.

15.2.1 EBV gB

EBV gB (originally identified as gp110 or gp125) has considerable homology to its counterparts in other herpesviruses. In EBV-infected lymphocytes, gB localizes primarily to the ER and to the inner and outer nuclear membrane of B lymphocytes undergoing productive infection. The abundant, stable 110 kDa form of EBV gB has only endo H-sensitive high-mannose N-linked

oligosaccharides indicating that gB has not trafficked through the Golgi (Gong and Kieff 1990). This high mannose form is analogous to the immature form of HSV-1 gB which also accumulates in infected cells (for review (Pereira 1994)). There is only a small amount of a differentially glycosylated form of gB, approximately 125 kDa in size, in virions and in EBV-infected cell lines induced for lytic replication (Emini et al. 1987; Gong and Kieff 1990; Gong et al. 1987; Kishishita et al. 1984). Four arginine residues located in the cytoplasmic tail of EBV gB function as an ER retention signal (Lee 1999; Lee et al. 1997). Virions contain the larger form of gB both in a full-length version and in a cleaved form (Johannsen et al. 2004). Cleavage presumably occurs at a predicted furin cleavage site within the ectodomain. The role of cleavage in EBV function is not known. Interestingly, for both CMV gB and BHV-1 gB, cleavage is not essential for virion infectivity (Kopp et al. 1994; Strive et al. 2002). Recent studies of EBV gB and the related gB encoded by HHV8 indicate that within the γ-herpesvirus subfamily gB homologs have an additional function when compared to gB of HSV. For all herpesviruses where gB function has been carefully examined, gB appears to be essential for the production of virus that is infectious (Cai et al. 1988; Cai et al. 1987; Herrold et al. 1995; Pereira 1994). However, studies of EBV gB have also indicated a role of gB in assembly and budding of the virion from the nucleus of lytically infected cells compatible with the presence of gB in the nuclear membrane in cells undergoing lytic replication (Lee and Longnecker 1997). This was recently confirmed by studies in HHV8 where a similar defect in virion egress was noted in HHV8 gB mutant viruses (Krishnan et al. 2005). This is in contrast to HSV-1 gB null viruses in which virions, although non-infectious, are nevertheless made (Cai, et al. 1987, 1988). Like HSV gB, the EBV gB C-terminal tail has been shown to modulate fusion (Baghian et al. 1993; Bzik et al. 1984; Cai et al. 1988; Foster et al. 2001; Gage et al. 1993; Klupp et al. 2000; Muggeridge 2000; Pereira 1994). Compatible with these results indicating a role for EBV gB in induced membrane fusion, it was recently reported that the higher amounts of gB in EBV virions can dramatically enhance the infection of B cells and epithelial cells by EBV (Neuhierl et al. 2002). Most recently, the structure of HSV gB has been reported and from this analysis several key features of gB as a membrane fusogen of the herpesvirus family have been discerned (Heldwein et al. 2006).

15.2.2 EBV gH/gL

The EBV glycoprotein gp85 is a type I membrane protein and is a member of the gH family of glycoproteins (Heineman et al. 1988; Oba and Hutt-Fletcher 1988; Spear and Longnecker 2003). All members of this family that have been studied have been implicated in virus-directed fusion. They have little sequence similarity but if aligned at a conserved potential N-linked glycosylation site at the carboxy terminus the proteins do have a colinearity of cysteine residues that suggests some conservation of structure (Klupp and Mettenleiter 1991). Each is

also dependent on a second small type II membrane protein gL for authentic processing and trafficking in the cell that in EBV was originally referred to as gp25 (Li et al. 1995; Yaswen et al. 1993). The EBV gL homolog remains non-covalently associated with gH in a 1:1 ratio (Kirschner et al. 2006), though whether or not it retains the membrane anchor is uncertain. The HSV gH/gL complex has been modeled as a dimeric structure with the amino terminal domain of gL hidden within the amino terminus of gH (Peng et al. 1998) and such a structure would not be possible for the EBV complex unless the signal peptide of gL is cleaved.

Like the gH homologs of CMV and HSV, EBV gH contains regions predicted 0to form coiled coils which have been shown to be important for function (Omerovic et al. 2005). In addition, insertion or point mutants within a region that is recognized by a monoclonal antibody that differentially affects B-cell or epithelial fusion can alter gH function (Wu et al. 2005; Wu and Hutt-Fletcher 2007). Interestingly, substitution of glycine 594 with an alanine within this region completely abrogates fusion with both B cells and epithelial cells, whereas mutation of alanine 595 to a glutamic acid reduces epithelial fusion but enhances B cell fusion. This mutant can mediate fusion of B cells in the absence of gL. A third glycoprotein, gp42, which, among the human herpesviruses, is unique to EBV, also associates with the gH/gL complex (Li, et al. 1995). Glycoprotein gp42, a type II membrane proteins which interacts directly with gH (Wu and Hutt-Fletcher 2007) is, as will be discussed below, essential for EBV infection of B cells.

15.2.3 Other Conserved Glycoproteins

The only other glycoproteins that are conserved among all the herpesviruses are gM and gN, although the gN homolog of herpes simplex virus is not glycosylated (Pyles et al. 1992). gN is a small type 1 membrane protein and in EBV is incompletely processed in the absence of gM with which it forms a non-covalently linked complex (Lake et al. 1998). gM is a multispan membrane protein with a long highly charged cytoplasmic tail which is rich in prolines (Baer et al. 1984). If expression of EBV gN is interrupted in virus, the entire complex is lost and virus egress is significantly impaired. There is a defect in secondary envelopment and most of the virus that is released lacks a complete envelope (Lake and Hutt-Fletcher 2000). This is a striking phenotype that is at least partially recapitulated in human cytomegalovirus (Mach et al. 2007) and in some but not all alphaherpesviruses, particularly if a second non-conserved glycoprotein complex, gE/gI is lost as well (Brack et al. 1999). The very small amount of enveloped virus that is produced by a gN-null EBV can bind to cells via CD21, but has a defect in infection that cannot be repaired by addition of an exogenous fusogen such as polyethylene glycol. One interpretation of these observations is that the gM/gN complex is involved in the association of the tegumented capsid with the nascent envelope during assembly and dissociation of the capsid from the fused envelope and cell membrane during entry.

15.2.4 Non-conserved Glycoproteins

There are three additional glycoproteins that are found in the virion (Johannsen, et al. 2004) that are not conserved throughout the herpesviruses, but are confined to members of the gammaherpesvirus subfamily. These are gp150, the product of the BDLF3 gene (Kurilla et al. 1995; Nolan and Morgan 1995), the BMRF2 protein (Xiao et al. 2007) and gp78, the product of the BILF2 gene (Mackett et al. 1990). Homologs of gp150 and BMRF2 are found in both the rhadinocryptovirus and the lymphocryptovirus genera, but gp78 is unique to the lymphocryptoviruses (Rivailler et al. 2002; Rivailler et al. 2002; Russo et al. 1996). Glycoprotein gp150 is a very heavily glycosylated type I membrane protein that resembles a mucin. A virus in which its expression has been interrupted is slightly, but consistently enhanced in its ability to infect an epithelial cell, a phenotype that perhaps can be attributed to the significant loss in charge that would be effected by its absence in the virion (Borza and Hutt-Fletcher 1998). No role for glycoprotein gp78, also a heavily glycosylated type I protein, has been reported in detail, but mention of a BILF2-null virus in another context has implied that it is not impaired in infectivity (Shannon-Lowe et al. 2006). In contrast, the BMRF2 protein, predicted to be a multispan protein, may play an important role in entry or infection of an epithelial cell (Tugizov et al. 2003) as discussed below.

15.3 EBV B-Cell Entry

In vitro and *in vivo*, the cells that are most susceptible to EBV infection and most permissive for establishment of EBV latency in culture are of B cell origin. EBV-mediated fusion of B cells requires four glycoproteins (gp42, gB, and gH/gL) similar to that observed for HSV (gD, gB, and gH/gL). For both EBV and HSV, an additional glycoprotein is required for binding to target cells, which enhances infection (gp350/220 for EBV and gC for HSV). Thus, EBV entry of B cells consists of at least two discrete steps – interaction of gp350/220 with the "binding receptor" CD21 followed by interaction of gp42 with the "fusion receptor" HLA class II resulting in the triggering of fusion mediated by gH/gL and gB.

15.3.1 CD21 – gp350/220

Reflective of the specialized cellular niche of EBV in B lymphocytes, the initial binding of EBV to the B-cell surface utilizes a specific interaction between the type two complement receptor CR2 or CD21, and gp350/220 (Fingeroth et al. 1984; Frade et al. 1985; Nemerow et al. 1987; Nemerow et al. 1985; Tanner et al. 1987). gp350/220 has homologues in primate γ–herpesviruses but no known

related proteins in the other human herpesviruses (Wang 2005). Both gp350 and gp220 are encoded by the same mRNA, gp220 being spliced once, while gp350 is unspliced (Beisel et al. 1985; Hummel et al. 1984). If there is a distinct function of gp350 when compared to gp220, it is not known at present since the key gp350 domains which bind CD21 are found in first 470 amino acids of both proteins (Tanner et al. 1988).

CD21, previously designated as complement receptor type 2 (CR2), is the receptor for the C3d peptide of complement and is a 145 kD membrane glycoprotein that is a member of a large family of complement regulatory proteins (Holers 2005). Each of these family members contains one or more extracellular structural motifs known as short consensus repeats (SCR) comprised of 60–75 amino acids. CD21 is expressed on a variety of different cell types in addition to B lymphocytes, including follicular dendritic cells, certain leukemic T cell lines, immature thymocytes (CD1/CD4/CD8+), and at low levels on normal peripheral blood T cells. The extracellular domain of CD21 contains either 15 or 16 SCR, however only SCR-1 and SCR-2 are necessary for EBV and C3d binding (Carel et al. 1990; Lowell et al. 1989; Martin et al. 1991). The structure of the key gp350-binding domain on CD21 has been determined (Prota et al. 2002). In these studies, the exact region that may bind gp350 was determined based on the inclusion of a glycosylation site found in murine CD21 but not in human CD21 (Prota et al. 2002). Murine CD21 does not bind gp350. Human CD21 with this incorporated glycosylation site from murine CD21 was not able to mediate infection with EBV despite being able to bind to C3d indicating that the domain that gp350 binds on CD21 is distinct from the region that C3d binds (Prota et al. 2002). Other studies have suggested that a conformational difference between murine CD21 and human CD21 may also be important for the observed differences between murine and human CD21 (Martin et al. 1991). The crystal structure of gp350 found that the region of gp350 required for binding to CD21 is contained within a patch of gp350 that is free of glycans on an otherwise fully glycosylated protein (Szakonyi et al. 2006). In support of this conclusion, deglycosylated gp350 binds to CD21 similarly to the fully glycosylated form (Szakonyi et al. 2006).

Binding of EBV or gp350 to CD21 induces capping of CD21 followed by endocytosis (Nemerow and Cooper 1984; Tanner et al. 1987). Compatible with this observation, a variety of studies have indicated that binding of gp350 to CD21 can induce signal transduction and cellular activation suggesting that activation of B cells during EBV infection may induce cellular changes that may prime target cells for infection (Bouillie et al. 1995; D'Addario et al. 2001; Sinclair and Farrell 1995; Sugano et al. 1997; Tanner et al. 1996). The initial interaction of gp350/220 with CD21 may be very similar to the interaction of HSV gC with cell surface proteoglycans, both interactions bringing virions in close proximity to the cell surface to facilitate subsequent interactions. For both gC and gp350/220, this initial stage is not absolutely required for infection since viruses deleted for gp350/220 or gC exhibit only a reduction in infection efficiency (Herold et al. 1991; Janz et al. 2000). After binding to B cells, EBV

virions are endocytosed and fusion of the viral membrane is thought to occur via the interaction of gp42 with HLA class II followed by the induction of fusion mediated by gH/gL and gB. This leads to release of the virus nucleocapsid and tegument into the cytoplasm (Carel et al. 1990; Nemerow and Cooper 1984; Tanner et al. 1987).

15.3.2 HLA Class II-Required Domains for gp42 Binding

The role of gp350/220 in providing only a binding interaction of EBV with B cells was only fully appreciated after the identification of the importance of HLA class II in EBV entry (Li et al. 1997; Spriggs et al. 1996). The central importance of the interaction of gp42 and HLA class II in EBV B-cell entry is compatible with the observation that gp350 is not required for EBV-induced B cell membrane fusion in a cell-based fusion assay and is not required for the infection of B cells as seen in virus deleted for gp350/220 (Haan et al. 2001; Janz et al. 2000). HLA class II antigens are cell surface molecules that normally exhibit restricted expression patterns and are found predominantly, but not exclusively, on specialized antigen-presenting cells such as B cells, macrophages, and dendritic cells (Cresswell 1994). HLA class II molecules are comprised of two distinct gene products, α and β, which non-covalently heterodimerize to form the mature molecule of approximately 62 kD (Cresswell 1994). The biological function of these molecules is to bind foreign peptide antigens and form complexes that are recognized by antigen-specific T-lymphocytes (Cresswell 1994). The HLA class II genetic locus encodes three different pairs of α and β chains referred to as HLA-DR, -DP, and DQ (Cresswell 1994). These molecules are extremely polymorphic (Cresswell 1994). With the exception of DRα that only has one allele, each gene family encodes numerous alleles (Cresswell 1994).

HLA-DR was first shown to interact with gp42 in an expression library screen for proteins that bound a soluble gp42-Fc construct (Spriggs et al. 1996). This study used deletion mutational analysis to determine that the region necessary for gp42 association localized to the β1 domain. Subsequent studies have shown that the gp42/HLA-DR interaction is crucial for EBV infection in B cells, since monoclonal antibodies to both gp42 and HLA-DR can inhibit the infection of B cells in vitro (Li, et al. 1995, 1997; Miller and Hutt-Fletcher 1988). gp42 is a third component of the gH/gL complex and is essential for EBV infection of B lymphocytes, but not epithelial cells (Li et al. 1995). It has no known sequence homologues in other human herpesviruses but is found in the EBV-related primate viruses (Wang 2005). gp42 exhibits sequence features of the C-type lectin family (Mullen et al. 2002; Spriggs et al. 1996). Previous studies have shown that gp42 interacts with gH/gL via a domain contained within the amino terminus of gp42 (Wang et al. 1998). More recent studies have confirmed these observations and have shown that gp42 binds to gH/gL via two domains

in the gp42 amino terminus located outside the C-type lectin domain of gp42 (Kirschner et al. 2007). This interaction is likely essential for linking gp42 binding with the induction of fusion by gB and gH/gL. Further evidence indicating a key role of gp42 in B-cell entry includes the observation that virosomes lacking gp42 have the ability to bind, but are unable to fuse and enter into B cells (Haddad and Hutt-Fletcher 1989). In addition, virus deleted for gp42 regains the ability to enter B cells with the addition of soluble gp42-Fc, indicating that it is the interaction with gp42 and HLA class II molecules that is the limiting step in EBV entry (Wang et al. 1998). The direct mechanism by which gp42 initiates membrane fusion is uncertain, but it is hypothesized that the association of gp42 with HLA-DR triggers a change in the gH/gL complex that results in membrane fusion with the involvement of gB. The overall mechanism of EBV-induced membrane fusion will be more fully discussed in the later part of this chapter. Apart from its role in fusion, gp42 can function independently of the gH/gL complex and bind HLA-DR to inhibit peptide presentation (Ressing et al. 2003). Only recently, a cleavage site was identified in gp42 suggestive of a role of gp42 in immune evasion since this allows gp42 to be secreted from infected cells (Ressing et al. 2005).

15.3.3 gp42 Functional Domains

gp42 is most closely related to natural killer (NK) receptors such as Ly49A and shares the functional characteristic of binding to MHC superfamily members (Natarajan et al. 2002). Because of the similarity in structure of HLA class II with HLA class I, initially it was presumed that the interaction of gp42 with HLA class II would parallel that of Ly49A binding to MHC class I, but as will be discussed below, gp42 interacts very differently with HLA class II when compared to the Ly49A and MHC class I interaction. Initial studies used the high degree of polymorphism in HLA class II molecules to establish that a glutamic acid at residue 46 of the HLA class II β-chain was necessary for EBV entry into B cells (Haan and Longnecker 2000). In these same studies, it was demonstrated that an aspartic acid also functions indicating the key role of a negative charge for gp42 binding and viral entry at this site in HLA class II. Interestingly, the location of this negatively charged amino acid is homologous to a site on MHC class I that interacts with the murine NK receptor Ly49A (Tormo et al. 1999) suggesting a similar interaction of gp42 with class II. But studies of the structure of gp42 bound to HLA class II and site-directed mutants revealed that that the interaction between gp42 and HLA class II is quite different (Mullen et al. 2002; McShane et al. 2003; Silva et al. 2004). The surface region of gp42 structurally homologous to the region of Ly49 that interacted with MHC class I surprisingly did not interact with HLA class II, but rather was observed as an unoccupied hydrophobic pocket in the gp42-HLA class II structure (Mullen et al. 2002). The interaction of gp42 with HLA class II occurs

via a few key residues, paralleling what has been observed for HSV-1 gD interaction with its receptors (Connolly et al. 2003; Connolly et al. 2002; McShane et al. 2003; Mullen et al. 2002; Silva et al. 2004). Interestingly, variability of HLA class II alleles within the human population may suggest that infection of EBV may be different depending on the HLA class II alleles that an individual expresses. The key contact residue, aspartic acid 46, is conserved in all DR and DP alleles, but only a subset of DQ alleles contains the essential aspartic acid 46. There is little variability in other key contact residues indicating that it is unlikely that polymorphisms in HLA class II alter infection rates in the human population.

In addition to the gp42 domains contained within the C-type lectin, gp42 contains several additional functional domains, one of which (gH/gL binding) has been carefully mapped and functionally verified (Kirschner et al. 2007). gp42 and gH/gL binding is of high enough affinity to allow co-precipitation of the complex (Li et al. 1995). Recent structural studies have shown that the N-terminal region (residues 33–85), which is adjacent to the membrane-spanning domain of gp42, is disordered, suggesting that it may be unstructured on its own. Interestingly, this is the region that binds gH/gL suggesting that the binding of gH/gL to gp42, may induce changes in the amino terminus of gp42. gp42 deletion mutants deleted to residue 58, 90, and 122 are still able to bind HLA class II, but none associate with gH/gL (Wang et al. 1998). Interestingly, the 58 deletion had activity in an infection assay indicating that this mutant may have retained a reduced ability to bind to gH/gL since the interaction of gp42 with gH/gL is essential for the infection of B cells (Wang et al. 1998). More recent studies showed that a cleaved form of gp42 missing about 40 amino acids retains the ability to bind to gH/gL (Ressing et al. 2005) and gp42 truncated at residue 86 does not bind to purified gH/gL *in vitro* (Kirschner et al. 2006). In order to further define the gp42 sequences that determine interactions with gH/gL, site-directed and deletion mutagenesis approaches have been used. From these studies, two essential domains for fusion and gH/gL binding within the gp42 amino terminus have been identified and designated N-terminal domain 1 (NT1) and NT2 (Kirschner, et al. 2007). NT1 extends from approximately amino acid 32–36 to 52–56, whereas NT2 extends from approximately amino acid 67–71 to amino acid 87–91 of the gp42 amino terminus.

Two other potential functional domains have been identified in gp42 but at present there are no data to suggest that either constitutes an essential feature of gp42 function in EBV entry. As indicated above, a cleaved form of gp42 was recently identified which may have importance in preventing the recognition of EBV-infected cells mediated through HLA class II (Ressing et al. 2003, 2005). Cleavage of gp42 was found within the amino terminus of gp42 close to the membrane-spanning domain at amino acids 40, 41, and 42 and as would be expected the cleaved form of gp42 binds to gH/gL (Ressing, et al. 2005). How cleavage occurs is not currently known, but it is interesting to speculate that cleavage of gp42 may be required for function since soluble forms of gp42

function in fusion. If cleavage is an important feature of gp42 function in EBV entry of B cells, cleaved gp42 would be tethered to the virion by interacting with gH/gL. In addition to cleavage, gp42 forms a dimer in the crystal structure in which the N-terminal residues 86–95 from two gp42 molecules form an anti-parallel two-stranded β sheet (Mullen et al. 2002). These key residues are preserved in both rhesus and marmoset gp42. The crystallographic dimer is not very stable in solution, since only monomeric gp42 is observed during gel filtration chromatography, but might be stabilized at the virus surface or after binding HLA class II or the gH/gL complex. Structurally, the geometry of the gp42 dimer is consistent with an interaction with two cell surface bound HLA molecules and orients the N-terminal gH/gL interaction site (residues 33–91) from one monomer adjacent to the gp42 hydrophobic pocket on the second monomer (Mullen et al. 2002).

15.4 EBV Epithelial Cell Entry

B lymphocytes can be readily infected *in vitro* by cell-free virus and the implicit assumption has been that cell-free virus is responsible for infection *in vivo* as well. The parallel for epithelial cells is less clear. Saliva, which is responsible for transmission of virus from one individual to another and perhaps initiates infection of oral epithelial cells during a primary infection, contains both cell-free and cell-associated virus and several studies have suggested that, of the two, infection with cell-associated virus is the more efficient (Chang et al. 1999; Imai et al. 1998; Tugizov et al. 2003). Analyses of epithelial cell infection by cell-free virus and comparison of the parameters involved with those important to B cell infection have, however, been informative and many of the findings that have been made may be relevant to infection with cell-associated virus as well. Work on epithelial cell entry has been hampered by the inefficiency of epithelial cell infection *in vitro* and the difficulties in demonstrating infection *in vivo* in a normal healthy human host. However, these operational and psychological barriers to progress are dissolving as it becomes easier to monitor infection *in vitro* and clearer that infection is a real component of persistence of virus *in vivo*.

15.4.1 Infection by Cell-Free Virus

15.4.1.1 Attachment

Entry of virus into primary epithelial cells occurs directly at the cell surface in the absence of endocytosis (Miller and Hutt-Fletcher 1992). The nature of the receptor that initiates the process is, however, unclear. Some epithelial cells in culture express CD21, the B cell attachment receptor for EBV (Birkenbach et al. 1999), but whether all or any epithelial cells do so *in vivo* remains an unresolved

issue. Early work used monoclonal antibodies to CD21 to evaluate oral epithelial tissues (Sixbey et al. 1987; Young et al. 1986), but the antibody most commonly used was later found to cross-react with an unrelated protein (Birkenbach et al. 1992; Young et al. 1989) and no systematic analysis of CD21 expression has been done since. Three other candidates for attachment receptors have been proposed.

First, it has been shown that virus coated with immunoglobulin A can bind productively to the polymeric IgA receptor (Sixbey and Yao 1992). This is potentially an important avenue for infection in immune individuals and is perhaps particularly relevant in the context of nasopharyngeal carcinoma where high levels of EBV-specific IgA antibodies are common (Henle and Henle 1975). However, in polarized tissues virus is transcytosed without evidence of replication (Gan et al. 1997), so it may be more important for movement across epithelium than to direct infection.

Second, the BMRF2 gene product, which is a multispan envelope protein with an RGD motif in an extracellular loop, is a ligand for β1, α5, α3 and αv integrins (Xiao et al. 2007). The interaction is important to infection of polarized epithelial cells (Tugizov et al. 2003). There is, however, only a very small amount of the BMRF2 protein in the virion (Johannsen et al. 2004), so it remains possible that its primary role is in induction of downstream signaling events. Integrin signaling is important to post-fusion events in infection with the other known human gammaherpesvirus, HHV8 (Sharma-Walia et al. 2004).

Third, attachment to CD21-negative cells, such as the gastric carcinoma cell line AGS, can be mediated by gH/gL. A soluble form of gH/gL made in baculovirus can bind specifically to epithelial cells but not B cells (Borza et al. 2004), a gH-null virus loses the ability to bind (Molesworth et al. 2000; Oda et al. 2000) and a monoclonal antibody to gH/gL can reduce both gH/gL and virus binding (Borza et al. 2004; Molesworth et al. 2000). Levels of virus binding via gH/gL can be high, but infection with virus attached by these proteins is not very efficient, perhaps because, as discussed below, the cell receptor to which gH/gL binds may be the same as that used to trigger fusion in the absence of HLA class II.

15.4.1.2 Fusion

Unlike B cells, epithelial cells do not constitutively express HLA class II. Thus although gB and gH/gL are required for fusion with an epithelial cell (McShane and Longnecker 2004), gp42 is not. A gp42-null virus can infect just as efficiently as wild type virus (Wang and Hutt-Fletcher 1998). Further, not only is gp42 dispensable for infection of an epithelial cell, its presence is actually inhibitory. EBV virions carry both three part gH/gL/gp42 complexes and two part gH/gL complexes. Only three part complexes can promote infection of B cells, but only two part complexes can promote infection of epithelial cells. Addition of soluble gp42 in trans, which converts two part complexes to three part complexes, rescues the ability of a gp42-null virus to infect a B cell, but

blocks the ability of wild type virus to infect an epithelial cell (Wang et al. 1998). One interpretation of these observations is that there is a direct interaction between gH/gL and an epithelial cell surface molecule that triggers fusion in a manner analogous to the indirect trigger delivered by the interaction between gp42 and HLA class II. This direct interaction is blocked by the presence of gp42. Two observations are consistent with the cell surface molecule being the same as that used for attachment to a CD21-negative cell. First, soluble gp42 can block virus binding via gH/gL (Borza et al. 2004). Second, a monoclonal antibody to gH/gL that has no effect on B cell infection, but can block virus entry into CD21-negative epithelial cells or epithelial cells engineered to express and bind virus via CD21, inhibits binding of soluble gH/gL. The same antibody also inhibits both virus binding to and infection of a CD21-negative cell. Reactivity of another antibody that recognizes gH in the absence of gL and inhibits epithelial but not B cell infection as a Fab fragment has been mapped to residues 501–628 of gH. Mutations made within this region can either differentially affect B cell and epithelial cell fusion or block fusion with both cell types (Wu et al. 2005), consistent with a core function of the protein for both cell types, but differences in the way in which the core function is triggered.

The reciprocal use of gH/gL/gp42 and gH/gL complexes for B cell and epithelial cell entry can also impact the tropism of virus and its movement between the two cell types. Glycoprotein gp42, as discussed below, binds outside the peptide-binding groove of HLA class II (Mullen, et al. 2002) and can interact with both peptide-loaded and immature HLA class II that has not yet trafficked to the peptide loading compartment (Ressing et al. 2005). As a result, some three part complexes which interact with HLA class II inside the B cell move with it to this protease-rich compartment where they are degraded. This does not happen in an HLA class II-negative epithelial cell. Virus released from an epithelial cell therefore has a higher content of gp42 and can be as much as two orders of magnitude more infectious for a B cell than virus released from a B cell. Reciprocally, virus released from a B cell has a lower content of gp42 and is slightly more infectious for an epithelial cell than is virus released from an epithelial cell, although the phenotype is not as striking (Borza and Hutt-Fletcher 2002). Epithelial cell-derived virus binds very poorly to CD21-negative epithelial cells via gH/gL, apparently because of its high levels of three part gH/gL/gp42 complexes, whereas B-cell-derived virus binds as well to epithelial cells via gH/gL as it does to CD21 via gp350/220 (Borza et al. 2004). This second phenotype of B-cell-derived virus, is in contrast, so striking that it can be used to distinguish viruses made in each cell type (Jiang et al. 2006). Why the greatly increased binding of B-cell-derived virus does not translate into a similarly large increase in infectivity is unclear, but it perhaps suggests that if the molecule used by gH/gL to attach virus to an epithelial cell is the same as that used to trigger fusion, the efficiency of the two functions is not entirely compatible.

As discussed above, it is quite clear that gp350/220 has no role in EBV fusion. Interestingly, however, it has been suggested that its presence is not only

irrelevant but also inhibitory to infection of a CD21-negative epithelial cell (Shannon-Lowe et al. 2006). This is consistent with observations that antibodies to gp350/220 that cross-link and potentially patch the protein in the virion envelope can enhance infection perhaps by enhancing access of other more relevant proteins to the cell surface (Turk et al. 2006). What these proteins might be is not clear, but since access of gH/gL does not seem to be an issue and increased levels of gB are reported to enhance infection of an epithelial cell (Neuhierl et al. 2002), gB would seem to be a prime candidate.

15.4.2 Infection by Cell-Associated Virus

Early work proposed that fusion between virus-producing B cells and epithelial cells was responsible for epithelial cell infection (Bayliss and Wolf 1980). Certainly overlay of epithelial cells by virus-producing B cells increases the efficiency with which epithelial cells can be infected (Chang et al. 1999; Imai et al. 1998; Tugizov et al. 2003). It has never been clear whether this is because of an inherent difference in the mechanism of infection or simply because either the chances of virus making contact with the uninfected cell are increased or a virus-producing cell provides a continuous stream of virus that ultimately makes its mark. The effect is more striking when polarized cells are exposed to virus at the apical than the basolateral surface (Tugizov et al. 2003) but most recently an intriguing study of transfer of virus from a B cell to an epithelial cell has suggested that, although in some ways it may perhaps be mechanistically different, it is nevertheless reflective of the same basic processes. This study showed that most of the virus added to a CD21-positive B cell does not enter the cell but remains at the surface from where it can be efficiently transferred to an epithelial cell (Shannon-Lowe et al. 2006). An “intercellular synapse” between EBV-carrying B cells and epithelial cells was observed, no cell fusion was seen, but rather it was suggested that the CD21-gp350/220 relieved an inhibitory effect of gp350/220 on epithelial cell infection. The same proteins essential for infection with cell-free virus were implicated in infection.

15.5 Mechanistic Features of EBV Entry

A model for EBV infection is emerging as more is learned about the specific EBV glycoproteins and the cellular factors with which these viral glycoproteins interact. As with α-herpesviruses, it is apparent that sequential virion/cellular interactions are characteristic of the entry process and that some of these interactions are facilitated by conserved families of proteins. However, the mechanism by which specific recognition of cellular receptors leads to targeted entry of EBV, or other herpesviruses, into cells remains unclear. In principle, two models for EBV entry must be derived since EBV infects two cell types

using different sets of viral and cellular proteins. Although two models must differ in their initial triggering steps, they are likely to share a common membrane fusion mechanism. The hypothesis that levels of gp42 protein in the virus act as a tropism switch, with high levels promoting B-cell entry and correspondingly inhibiting epithelial cell entry, is further supported by in vitro cell:cell fusion experiments that parallel those carried out with virus (Kirschner et al. 2007; Kirschner et al. 2006). Thus the two models for EBV entry may be discriminated by the virus:cell interactions that feed into the activation of membrane fusion and thus viral entry.

Although EBV uses different sets of receptors to bind to and trigger its fusion with B and epithelial cells, the core fusion machinery is thought to be the same in both cases, and consists of gB, gH, and gL. This core fusion machinery is conserved throughout the herpesvirus family, suggesting that mechanistic features of this process will also be conserved across the family. Many viruses, such as influenza virus, HIV, flaviviruses and alphaviruses, express a single glycoprotein that is capable of driving membrane fusion during entry into cells. These fusion glycoproteins have been broadly grouped into two major classes, class I and class II, based on key structural and mechanistic features of their folding and conformational changes (Jardetzky and Lamb 2004; Kielian and Rey 2006). In contrast to viruses that carry class I and class II fusion proteins, it is not clear for the herpesviruses whether gB, the gH/gL complex, or all three proteins participate directly in fusion of the viral and cellular bilayers. In addition to the core entry glycoproteins, herpesviruses have clearly evolved unique receptor-binding proteins, such as EBV gp42 and HSV-1 gD, that are important for viral tropism and the activation of membrane fusion. However, the inherent “fusogenicity” and relative requirements for each of these glycoprotein components does seem to vary from virus to virus in reconstituted cell:cell fusion assays. For herpes simplex virus-1 (HSV-1) gB, gH/gL and gD are required for fusion in a cell:cell-based assay (Pertel et al. 2001). On the other hand, the gH/gL complexes from Varicella-Zoster virus and HHV-8 induce low levels of fusion in a similar assay when these are expressed on their own (Duus et al. 1995; Pertel 2002). A mutant of HHV-8 gB, which exhibits enhanced surface expression, also has some fusogenic activity, although less than HHV-8 gH/gL (Pertel 2002). Most strikingly, expression of a truncated variant of EBV gB that lacks the last 46 residues of the gB cytoplasmic tail can induce fusion with epithelial cells (McShane and Longnecker 2004). A link between the amount of gB in the EBV envelope and the ability of the virus to infect cells has also been observed. EBV strains that have more envelope-bound gB exhibit an increased ability to infect cells that are normally refractory to EBV infections (Neuhierl et al. 2002). The results of these functional studies suggest strongly that gB has inherent fusogenic properties, but that in some herpesviruses gH/gL may also be capable of, at least partially, driving membrane fusion. Recently, it has been suggested that HSV gH/gL can mediate hemifusion between viral and cellular membranes, while gB is required for full fusion (Subramanian and Geraghty 2007). These studies raise the interesting possibility that these two

membrane fusion steps associated with herpesvirus entry may be discretely segregated into two separate glycoprotein complexes, in contrast to what is observed for the fusion glycoproteins of other viruses. Perhaps the differing functional results implicating gH/gL or gB as the direct membrane fusogen are related to the partitioning of these intermediate steps along the membrane fusion pathway into each viral glycoprotein.

In this regard, it is also interesting that the crystal structure of the HSV-1 gB ectodomain revealed an unanticipated structural homology between gB and the vesicular stomatitis virus (VSV) fusion protein G, despite a lack of sequence similarity between the two proteins (Heldwein et al. 2006; Roche et al. 2006). Both proteins form trimers and contain structural features reminiscent of both class I and class II viral fusion proteins. However, the overall fold of the proteins is unique, and it has been proposed that gB and G proteins should be classified into a new, third class of fusion proteins (Steven and Spear 2006). The initial structures of the HSV-1 gB and VSV G proteins were suggested to represent their post-fusion conformational states and a structure of the VSV G in a potential prefusion state has also been determined (Roche et al. 2007). It remains to be established whether gB may also be capable of undergoing similarly large conformational changes to drive membrane fusion and the significant structural differences between gB and VSV G make a direct extrapolation of the prefusion state difficult. However, these structural results have clearly revealed the striking similarity between gB and VSV G, consistent with gB playing a major role in driving the full process of membrane fusion. EBV gB in particular can mediate gH/gL-independent fusion, albeit in an in vitro cell:cell fusion assay, suggesting that it could act as the primary fusogen during virus entry and that gH/gL functions may be to promote this fusogenic activity.

As discussed above, EBV entry into its target cells likely consists of at least two distinct mechanistic steps – interaction of the virus with "binding receptors" followed by interactions with "fusion receptors" that trigger gH/gL and gB-mediated membrane fusion. The binding interaction of EBV with target B cells is clearly mediated by gp350/220 with cellular CD21 and this binding may be analogous to the interaction of the α-herpesvirus gC with heparan sulfate. These binding interactions modestly enhance virus entry (Herold et al. 1991; Janz et al. 2000) and are not required for membrane fusion in cell-based fusion assays (Haan et al. 2001; McShane and Longnecker 2004). For epithelial cell entry, it remains to be established whether there is such a clear separation between binding and entry. Recent studies demonstrate gH/gL interactions with a putative epithelial receptor, which remains to be identified, which could be important both for binding and for fusion activation.

The interaction of EBV with its "triggering receptors" is clearly different for its two target cells, with B cell entry being, at least currently, better understood. For B cell entry, the binding of gp42 to HLA class II has been tightly linked to the activation of membrane fusion as described above, although mutations in the gp42 hydrophobic pocket have enabled a segregation of binding from membrane fusion (Silva et al. 2004). How might gp42 engagement with HLA class II activate

subsequent membrane fusion? While many models are potentially consistent with the available functional data, we favor one that could apply more generally to both B cell and epithelial cell entry, and even more generally to other herpesviruses. At this more general level, understanding herpesvirus entry may then really be reduced to two parts: understanding how diverse "triggering" complexes activate a common membrane fusion mechanism and understanding how this fusion reaction is mediated in detail by gH/gL and gB glycoproteins.

For EBV entry into B cells and epithelial cells, it is tempting to speculate that gH/gL acts as a common mediator of gB activation in both B cell and epithelial cell fusion, though gH/gL activation would be achieved by distinct receptor-binding events and complexes. In the case of B cell fusion the complex of gp42 and HLA class II might mimic a similar signal to gH/gL that can also be delivered through direct interactions with an epithelial cell receptor. Since gp42 binding to gH/gL can inhibit fusion with epithelial cells, there may be a multifunctional site on gH/gL that is able to receive and transmit the signal that a cellular receptor has been engaged, thereby activating gB and membrane fusion through a common mechanism. This scheme would also be consistent with observations made for HSV-1. The structure of the gD protein in the receptor bound and free states has suggested that receptor binding releases a "profusion" domain in gD that could activate the core HSV-1 fusion machinery (Carfi et al. 2001; Krummenacher et al. 2005), potentially by directly activating gH/gL-mediated hemifusion (Subramanian and Geraghty 2007). It will be interesting to understand how these initial receptor-binding interactions in different herpesviruses are used to activate membrane fusion and to see whether there are parallels in these early steps of the entry process. Other models may also equally explain the observed data and perhaps there are other key host molecules and interactions yet to be uncovered that will further clarify how EBV mediates membrane fusion and entry. However, EBV and other herpesviruses are beginning to yield tantalizing insights into the processes that direct their infection of different cell types in vivo.

Acknowledgments Research in the Jardetzky and Longnecker Laboratories was supported by Public Health Service grants CA93444 and CA117794. Work in the Hutt-Fletcher laboratory was supported by AI20662 and DE16669. The authors would like to thank current and former members of their laboratories for their contributions to the work described, as well as our many colleagues throughout the world who have contributed to understanding the entry of EBV as well as herpesviruses in general.

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Chapter 16
EBV Immunotherapy

Leslie E. Huye and Cliona M. Rooney

Abstract Epstein–Barr virus (EBV) is associated with a range of malignancies including Burkitt's lymphoma (BL), Hodgkin's disease (HD), nasopharyngeal carcinoma (NPC), and lymphoproliferative disease (LPD), and EBV proteins expressed in these malignancies provide targets for cytotoxic T lymphocytes (CTL). Adoptive immunotherapy with ex vivo-generated EBV-specific CTLs has proven safe and effective as prophylaxis and treatment for EBV-driven lymphoproliferative disease after hematopoietic stem cell transplant. EBV-specific CTLs have also shown promise as therapy for other EBV-associated malignancies, but genetic manipulation of the CTL to circumvent immune evasion strategies employed by tumors may be required before CTLs can reliably eliminate tumors in immunocompetent individuals.

16.1 Introduction

Epstein–Barr virus (EBV), a gamma herpesvirus that infects epithelial cells and circulating B cells, was the first human tumor virus identified. More than 90% of adults worldwide are persistently infected with EBV. Primary infection usually occurs in childhood when it is typically asymptomatic but if delayed until adolescence, it manifests as infectious mononucleosis in about 50% of cases. Following primary infection, EBV establishes life-long latency in resting B lymphocytes with frequent reactivation resulting in virus shedding at mucosal sites. EBV is strongly associated with several types of malignancy, including Burkitt's lymphoma (BL), Hodgkin's disease (HD), nasopharyngeal carcinoma (NPC), and EBV-associated lymphoproliferative disease (EBV-LPD), each of which has a characteristic pattern of EBV latent gene expression (Rickinson and Kieff 2001). In this chapter we discuss therapies for EBV-associated malignancies focusing on adoptive immunotherapy with EBV-specific T cells and ways to improve the efficacy of these cells.

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_16, © Springer Science+Business Media, LLC 2009

16.2 Establishment of EBV Latency

The current model of EBV latency posits that the virus, through the use of different transcription programs, mimics many features of normal B-cell biology to establish and maintain a persistent infection for the life of the host (Thorley-Lawson and Gross 2004). In vivo, EBV infects resting, naïve B cells and initiates its growth program (also called Type III latency) in which the entire spectrum of latent genes including Epstein–Barr virus nuclear antigens (EBNAs)-1, -2, -3A, -3B, -3C, and -leader protein (LP) and latent membrane proteins (LMPs)-1 and -2 are expressed. The growth program induces proliferation of resting B cells, mimicking antigen stimulation. In a naïve B cell, this promotes B-cell differentiation into quiescent memory through a germinal-center-type reaction. If EBV initiates the growth program in a B cell that cannot exit the cell cycle or if the growth program is triggered in a memory B cell, then uncontrolled growth can occur. However, since these cells are highly immunogenic, they are rapidly destroyed by cytotoxic T lymphocytes (CTLs) that circulate with high frequency in healthy seropositive individuals. Exceptions may occur in conditions of severe immunosuppression, such as after hematopoietic stem cell transplant (HSCT), solid organ transplant (SOT), or HIV infection, in which impaired CTL responses permit the continued proliferation of EBV-infected lymphoblasts leading to EBV-LPD.

The proliferation of EBV-infected naïve B lymphoblasts is usually short-lived as EBV drives infected B cells toward a memory state by switching transcription patterns from the growth program to the default program (also called Type II latency), in which only EBNA-1, LMP-1, and LMP-2 are expressed. The default program is found in germinal center B cells where LMP-1 and -2 can deliver germinal center signals that promote the transition into the memory compartment, in the absence of antigen. Oncogenic events that block this transition may result in constitutive expression of the default program producing Hodgkin's disease, -a B cell malignancy. Having completed the germinal center reaction, EBV-infected memory B cells exit the cell cycle and enter the latency program (also called Type 0 latency) in which transcription of all protein-encoding genes is shutdown except when the "memory" cell undergoes homeostatic proliferation or encounters antigen. B cells expressing the latency program are found in the peripheral circulation. It is thought that transient expression of EBNA-1 (also called Type I latency) allows for viral genome replication during homeostatic proliferation, while expression of LMP-2 may prevent B-cell activation upon encounter with antigen (Longnecker 2000). Since EBNA-1 is poorly presented to the immune response, latency I-expressing cells remain incognito to the immune system, and hence Type 0 and Type I latency-expressing B cells form the bulk of the EBV reservoir in healthy individuals. Subsequent differentiation of infected B cells into antibody-secreting plasma cells at mucosal sites reactivates the lytic cycle allowing for virus shedding and spread. Replicating EBV is also found on tonsillar epithelial cells, but the relative roles of virus shedding from epithelial versus B-cell sources are not clear (Pegtel et al. 2004).

16.3 EBV Latency in Malignancy

All EBV-associated malignancies are associated with latent infection and can be characterized by their expression of a particular latent transcription program (Table 16.1). EBV-positive BL expresses the EBNA-1 only or Type I latency program while HD, NPC, and peripheral T/NK-cell lymphomas exhibit the default or Type II latency program and express EBNA-1, LMP-1, and LMP-2. Malignancies associated with these transcription programs occur in immunocompetent individuals. Malignancies, such as EBV-LPD, that express the highly immunogenic growth or Type III latency program occur only in severely immunocompromised patients, for example transplant recipients or HIV infected individuals. Type III latency is also characteristic of lymphoblastoid cell lines (LCLs) generated by infecting B cells in vitro with EBV in the absence of functional T cells (Thorley-Lawson and Gross 2004).

Table 16.1 EBV latency

Latency type	Transcription program	Genes expressed	Normal B cells	Associated malignancy
Type 0	Latency	None	Memory	None
Type I	EBNA-1 only	EBNA-1	Memory	Burkitt's lymphoma
Type II	Default	EBNA-1, LMP-1, LMP-2	Germinal center	Hodgkin's disease, nasopharyngeal carcinoma
Type III	Growth program	EBNA-1, -2, -3A, -3B, -3C, -LP, LMP-1, LMP-2	Naïve	Lymphoproliferative disease

16.4 Immune Responses to EBV

EBV infection is accompanied by robust humoral and cellular immune responses, and while neutralizing antibodies limits the spread of infectious virus, CD4+ and CD8+ T-cell responses are critical for destroying infected cells. Memory T cells recognizing EBV antigens are present in the blood of EBV-positive individuals. These T cells consist of HLA class I-restricted CD8+ T cells and HLA class II-restricted CD4+ T cells. While CD8+ CTLs are important for elimination of infected B cells, CD4+ EBV-specific T cells may play an important role in the control of EBV persistence both in the provision of help to CD8+ CTLs and by direct cytolysis (Savoldo et al. 2001, 2002). It has been shown that CD4+ T cells are crucial in the prevention of outgrowth of newly infected B cells in vitro (Nikiforow et al. 2001) and therefore, as for CMV, different effector T-cell subpopulations may be important at different times of the virus life cycle. The EBV-specific CTL response favors early lytic cycle proteins, BZLF1, BRLF1, and BMLF1 and latency proteins, EBNA-3A, -3B,

Lytic
Latent
BZLF1
BMLF1
EBNA-3A
EBNA-3B
EBNA-3C
LMP-2
LMP-1
EBNA-1
Immunogenecity
Latency
Type III
Type II
Type I
Malignancy
Lymphoproliferative disease
Hodgkin's Disease
Nasopharyngeal carcinoma
Burkitt's Lymphoma

Fig. 16.1 Immunogenicity of EBV antigens and latent states. The lytic and EBNA-3A, -3B, and -3C antigens are the most immunogenic followed by the subdominant LMP-2 and -1 proteins and finally EBNA-1. Type III latency is the most immunogenic latent state followed by Type II and Type I. Malignancies associated with the different latent states are indicated

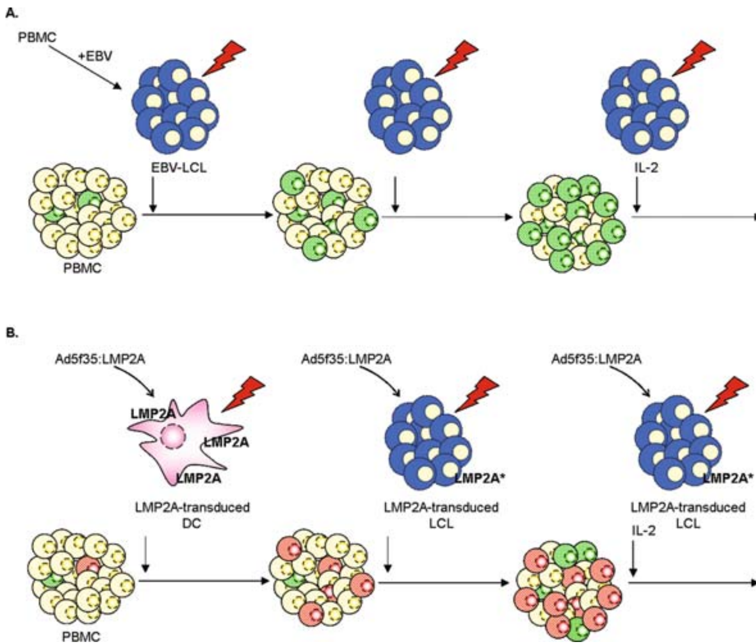

Fig. 16.2 (**A**) Ex vivo generation of EBV-specific CTL. PBMC are infected with EBV to generate EBV-LCLs. Irradiated LCLs are used to stimulate EBV-specific T cells (*green*) in autologous PBMC. The EBV-specific CTLs are expanded with subsequent stimulations with irradiated LCL. IL-2 is also added. (**B**) Ex vivo generation of LMP-2-specific T cells. Dendritic cells transduced with Ad5F35-LMP-2 and expressing LMP-2 are irradiated and used to stimulate LMP-2-specific T cells (*red*) in autologous PBMC. LMP-2-specific T cells are expanded by subsequent stimulations with LCL overexpressing LMP-2 after transduction with Ad5F35-LMP-2. Other EBV-specific T cells are also stimulated by the LCL. IL-2 is also added. (*See* Color Insert)

and -3C. Subdominant responses to LMP-2, EBNA-2, EBNA-LP, and LMP-1 are also found (Rickinson and Moss 1997; Landais et al. 2005). The hierarchy of EBV-specific CTL reactivities is depicted in Fig. 16.1. Memory EBV-specific T cells in peripheral blood can be reactivated ex vivo upon stimulation with EBV-LCL (Fig. 16.2), which allows the generation of EBV-specific CTL lines that can be used for immunotherapy of EBV-associated malignancies. Importantly for immunotherapy, LMP-2-specific T cells, while of low frequency relative to the immunodominant proteins, are present in almost all individuals, regardless of HLA phenotype (Bollard et al. 2004; Straathof et al. 2005).

16.5 EBV Lymphoproliferative Disease

In healthy individuals, EBV is efficiently controlled by cellular immune responses. However, T-cell dysfunction, such as induced by HSCT or SOT, causes an imbalance between infected B cells and EBV-specific T cells, which may lead to uncontrolled EBV-driven lymphoproliferation (EBV-LPD). After HSCT, incidences of EBV-LPD ranging from 1 to 25% have been reported with a majority of cases occurring in the first 6–9 months post-transplant. The risk of developing EBV-LPD is highest in recipients of HLA-mismatched stem cells that have been depleted of T cells to prevent graft versus host disease (GVHD) (Curtis et al. 1999; Bhatia et al. 1996). The incidence of EBV-LPD in SOT recipients ranges from 1 to 20%, with the highest incidence also in the first 6–12 months post-transplant, in EBV-seronegative children and in patients receiving intestinal grafts containing large populations of B cells. However, EBV-LPD can occur at any time in these patients due to life-long immunosuppression. Major risk factors for developing EBV-LPD for SOT patients is the level of immunosuppression and the development of a primary EBV infection post-transplant (Sokal et al. 1997; Ho et al. 1988). Early treatment of EBV-LPD is safer and more effective, but early diagnosis or predicting which patients will develop EBV-LPD is challenging. An increase in EBV DNA in the peripheral blood as detected by PCR is uniformly associated with EBV-LPD but has a low positive predictive value (Stevens et al. 2001; Rooney et al. 1995a, b; Wagner et al. 2004a, b). The interpretation of virus load is highly dependent on the underlying immunodeficiency. High virus load in a solid organ recipient is carried as latency 0, implying a large number of infected but not transformed B cells (Babcock et al. 1999). While at high risk, these patients may never develop EBV-LPD. By contrast, a persistent high virus load in a stem cell recipient is more likely to develop into EBV-LPD, implying that virus carriage may be in a different B-cell compartment. In support of this hypothesis, our studies have shown that a high virus load is sensitive to CTL infusion in stem cell but not solid organ recipients (Rooney et al. 1998; Savoldo et al. 2006; Rose et al. 2001). A high virus load is less of a concern if the patient has detectable

EBV-specific T-cell immunity, which can be quantitated using EBV-specific immunoassays, such as ELIspot or tetramer. Alternatively, the non-specific Immuknow ATP assay is clinically approved and also provides useful information on a patient's immune status (Clave et al. 2004; Lee et al. 2006a, b; Yang et al. 2000). Genetic influences on the immune response, such as the low γ-IFN secretor polymorphism, have been shown to impart additional risk for EBV-LPD (Lee et al. 2006a, b). Thus, a combination of viral load, genetic screening, and monitoring EBV-specific immune responses may improve the specificity of predictive tests so that patients can be closely monitored for early signs of EBV-LPD.

16.5.1 Immunotherapy for EBV-LPD Post-HSCT

Several options for the prophylaxis and treatment of EBV-LPD have become available in recent years. The most successful treatments include infusion of monoclonal antibodies against B cells and infusion of EBV-specific T lymphocytes. Rituximab is a humanized antibody that targets CD20 and directly lyses B cells via complement and antibody-dependent cytotoxicity. This agent has produced response rates of 70–100% in HSCT patients (Milpied et al. 2000; Kuehnle et al. 2000). In SOT recipients, malignancy frequently recurs upon B-cell recovery. By contrast, in stem cell recipients, immunity to EBV is usually reconstituted by the time B cells recover and so recurrence is not usually a problem, although outgrowth of CD20− tumors has been reported.

Infusion of unmanipulated donor lymphocytes (DLI), which contain EBV-specific precursors, is an option for the treatment of EBV-LPD in HSCT recipients and can produce complete remissions. However, DLI is limited by the frequency of EBV-specific precursors and by the risk for GVHD as a result of alloreactive T cells present in the unmanipulated product (Papadopoulos et al. 1994; Heslop et al. 1994; Lucas et al. 1998). To circumvent this limitation, a "suicide gene" approach (Bonini et al. 1997; Tiberghien et al. 2001) that involves transduction of donor T cells with the herpes simplex virus thymidine kinase gene (HSV-tk) to render the transduced cells sensitive to elimination by ganciclovir has been explored clinically. Because HSV-tk may be immunogenic, inducible human Fas or caspases have been explored in preclinical studies and may be preferable suicide genes (Straathof et al. 2003).

Our group has infused donor-derived EBV-specific T-cell lines that have been activated and expanded in vitro and lack alloreactivity, for prophylaxis and treatment of EBV-LPD (Rooney et al. 1998; Heslop et al. 1996). To reactivate EBV-specific CTL lines, donor-derived LCLs, generated by infection of peripheral blood mononuclear cells (PBMC) with a laboratory strain of EBV,

were used as APCs. Irradiated LCLs reactivate and expand EBV-specific CTL from donor PBMC (Fig. 16.2), producing polyclonal EBV-specific CTL lines that contain both CD4+ and CD8+ T cells. These CTLs prevented EBV-LPD as none of over 60 patients receiving CTL as prophylaxis developed this malignancy compared with 11% of patients who did not receive CTL as prophylaxis (Rooney et al. 1998). In addition, retrovirus gene-marked CTL administered to the first 26 patients allowed tracking of the infused cells and showed long-term persistence for over 7 years post-infusion (Bollard et al. 2004a–c).

EBV-specific CTLs were also used successfully to treat six patients with established EBV-LPD. Five of the six patients achieved complete remissions (Rooney et al. 1998). One of the five responding patients exhibited a significant inflammation at tumor sites as a result of infiltrating T cells, prior to complete tumor regression, producing the only treatment-associated toxicity. The non-responding patient was found to have a mutant virus in which the dominant CTL epitopes were deleted and thus was resistant to killing by the CTL line (Gottschalk et al. 2001). Importantly, these studies demonstrate that the infused CTLs were (1) safe and induced no significant GVHD, (2) expanded in vivo, (3) persisted long term, (4) decreased viral DNA levels, and (5) induced complete disease regression. The activity of EBV-specific CTL in HSCT recipients has been confirmed by a group from Sweden (Gustafsson et al. 2000).

Although EBV-specific CTL therapy has shown great promise, there are several limitations to this approach. Generation of EBV-specific CTL requires special facilities and takes 3–4 months. Because EBV-LPD is an aggressive disease necessitating immediate intervention, CTL lines must be initiated early. Thus, reliable tests for assessing risk for development of EBV-LPD would be beneficial in selecting patients for CTL therapy. Finally, when used as prophylaxis, this therapy protects against only one of the many viruses that can cause life-threatening diseases in transplant patients. This limitation, however, is being addressed through the generation of multivirus-specific CTLs (Sun et al. 1999; Regn et al. 2001). In fact, our group has recently shown that CD4+ and CD8+ T cells specific for CMV, EBV, and several species of adenoviruses can be produced from a single cell culture, in which EBV-LCLs are used to present antigens from all three viruses. This trivirus-specific CTL had clinical activity against all three viruses (Leen et al. 2006). Approaches to reducing the time taken to produce T cells involve selection of virus-specific T cells from peripheral blood by using HLA class I tetramers containing specific viral peptides conjugated to magnetic beads, or by selecting T cells that secrete gamma interferon after overnight stimulation with viral antigens (Miltenyi). This has been effective for the production of CMV and adenovirus-specific T cells for which specific protective antigens have been identified. However, LCLs that express all the latency antigens and early lytic cycle antigens have been used as antigen-presenting cells to produce EBV-specific CTL lines. Therefore the specific antigens that induce protective immunity are not known and must be identified before LCL production can be eliminated.

16.5.2 Immunotherapy for EBV-LPD Post-SOT

Reduction of immunosuppression is sufficient to increase EBV-specific immune responses and induce remissions of EBV-LPD in about 50% of SOT patients, but this approach is limited by the risk of graft rejection. Rituximab has also been successful in SOT recipients (Milpied et al. 2000; Ganne et al. 2003), but this therapy does not restore T cell immunity, and EBV-LPD frequently recurs when B cells recover. An alternate approach is adoptive immunotherapy using EBV-specific CTL since this strategy has shown success for the treatment of EBV-LPD in HSCT recipients. However, there are several differences between HSCT and SOT patients to consider. First, SOT recipients and donors are rarely HLA matched, and the EBV-LPD is usually of recipient origin, so that donor-derived EBV-specific CTLs are inappropriate for use in these patients. SOT recipients also receive continuous immunosuppression raising the question of whether autologous EBV-specific CTLs could be generated from these patients. This concern, however, has been alleviated by several studies showing that CTLs can readily be generated from SOT recipients, despite continued immunosuppression, even if they have active EBV-LPD (Khanna et al. 1999; Savoldo et al. 2001; Comoli et al. 2002; Savoldo et al. 2006).

Several groups have evaluated the use of autologous EBV-specific CTL for prophylaxis of EBV-LPD in SOT recipients. In one study, 10 patients with high virus load but no disease were given EBV-specific CTLs with a reduction in viral load in 8. None of the patients developed EBV-LPD (Comoli et al. 2002; Haque et al. 1998). Our group also treated eight patients with high virus load, and although there was an initial increase in EBV-specific cells in the circulation post-infusion, this increase was short-lived, and there was no consistent reduction in EBV virus load in the peripheral blood, likely reflecting continued immunosuppression, differences in the virus latency state in the two patient populations, and lack of homeostatic expansion in a replete lymphocyte compartment. EBV-specific CTLs have also been used as treatment for patients with established disease resulting in significant regressions and complete responses (Khanna et al. 1999; Sherritt et al. 2003; Savoldo et al. 2006). Generally, both reductions in virus load and complete remission of disease have required multiple CTL infusions, likely reflecting the difficulty in CTL expansion in vivo under conditions of immune suppression. While these data are promising and have alleviated concerns that CTLs might have alloreactivity and cause graft rejection, the approach is limited by the time required to generate the CTL and the failure of standard techniques to generate CTLs from EBV-seronegative recipients. Addition of cytokines, such as IL-12, at the initiation of culture or early selection of cells activated by exposure to EBV-infected cells have allowed the generation of CTL from EBV-seronegative individuals (Metes et al. 2000; Savoldo et al. 2002), while timing has been addressed by the use of allogeneic HLA-matched allogeneic EBV-specific CTLs. A bank of about 100 EBV-CTL lines that could be

accessed for immediate use was produced, overcoming timing issues for generating CTL lines (Haque et al. 2002). These CTLs were used in two studies in which 5 of 10 patients were reported to have responses, although persistence of the cells was not demonstrated (Haque et al. 2002; Sun et al. 2002). Our group is also producing a bank of HLA-typed CMV, adenovirus and EBV-specific T cell lines that can be used in allogeneic transplant recipients in response to diagnosis of disease caused by any of these three viruses.

16.6 Immunotherapy for Hodgkin's Disease and Other Type II Latency Tumors

Hodgkin's disease is a lymphoma characterized by the presence of rare malignant Reed–Sternberg (HRS) cells within a non-malignant infiltrate. In 40–50% of cases the tumor cells are infected with EBV. Although current treatments for HD are effective, they are associated with severe short- and long-term toxicities and a high risk of secondary malignancies. Moreover, a subset of patients responds poorly to current therapies. Thus safer, more highly targeted therapies are necessary to improve the quality of life for Hodgkin's patients. Current immunotherapeutic approaches for HD, which are potentially less toxic than standard therapies, focus on antibody-based therapies, such as monoclonal antibodies targeting CD30 expressed on HRS cells and the adoptive transfer of EBV-specific CTL.

In contrast to EBV-LPDs, which express all nine latent cycle EBV-encoded antigens and are highly immunogenic, the malignant HRS cells express only three viral antigens, EBNA-1, LMP-1, and LMP-2 (Fig. 16.1). Although these antigens are poorly immunogenic, they provide potential targets for CTLs. However, Hodgkin's patients demonstrate both T-cell abnormalities and monocyte dysfunction. T cells have reduced expression of the zeta chain of the T-cell receptor (TCR), resulting in aberrant signaling in response to activation. Monocytes express inhibitory molecules, like PGE2, and perform poorly in functional assays (Estevez et al. 1988; Slivnick et al. 1990; Renner et al. 1996; Roskrow et al. 1998). These defects likely result from inhibitory molecules produced by the tumor cells and the tumor infiltrate. We found however that EBV-specific CTL could be expanded ex vivo from HD patients who have had multiple relapses, although the expansion rate was lower than that of CTL generated from normal donors, and most CTL lines required allogeneic feeders at some point in their culture. However, the phenotype of the CTL lines was normal with partial recovery of TCR zeta chain expression (Roskrow et al. 1998).

Our group has administered LCL-activated, autologous EBV-specific CTL lines to patients with multiply relapsed EBV-positive HD either as treatment or as adjuvant therapy after autologous stem cell rescue (Bollard et al. 2004a–c). CTL lines, some genetically marked, given to 14 patients produced

measurable tumor responses, including two complete remissions, with no immediate toxicities. Gene marking demonstrated that the infused cells expanded in vivo and persisted for up to 12 months post-infusion. The CTLs localized to tumor sites and were functional as they reduced EBV DNA levels in peripheral blood and produced IFN-γ upon stimulation with LMP-2 peptides ex vivo.

Although this study shows that EBV-specific CTLs have activity in HD patients, the cells were less effective than for the treatment of EBV-LPD post-HSCT. One reason for this difference may be the poor specificity of the CTL lines infused for the viral antigens expressed in Hodgkin tumors. Although most CTL lines contained minor populations of LMP-2-specific T cells, LCL activation produces CTL lines dominated by clones recognizing the early lytic cycle antigens, BZLF1 and BMLF1, and the immunodominant latent proteins, EBNA-3A, -3B, and -3C, which are not expressed in HRS cells. To improve the specificity of CTL lines for appropriate EBV antigens, we initially focused on LMP-2, which is thought to be the best target because its epitopes are conserved between virus strains and among HD biopsy samples (Murray et al. 1998; Busson et al. 1995; Lee et al. 1993), whereas LMP-1 displays heterogeneity between virus strains (Khanim et al. 1996) and EBNA-1 is not processed for presentation on class I antigens (Levitskaya et al. 1995).

We and others have generated CTLs targeting LMP-2 (Gahn et al. 2001; Su et al. 2001; Redchenko and Rickinson 1999; Ranieri et al. 1999). Our strategy, illustrated in Fig. 16.2, uses an adenovirus vector Ad5f35-LMP-2 that readily transduces DCs and reactivates LMP-2-specific T cells (Bollard et al. 2004a–c). LMP-2-specific T cells could then be expanded in culture using Ad5f35-transduced EBV-LCLs as antigen-presenting cells without loss of specificity. This strategy was effective in producing CTL lines with LMP-2-specific cytotoxic function, and it overcame the problem of limiting DC numbers. LMP-2-specific CTL lines could be produced from all healthy donors tested but from only 12 of 20 patients with Type II latency lymphomas, including EBV-positive HD, EBV-positive B cell or T/NK-cell non-Hodgkin's lymphoma (NHL) (Straathof et al. 2005a, b; Bollard et al. 2006). Nevertheless, seven of eight patients who received CTLs as adjuvant therapy post-HSCT remained well up to 36 months following CTL infusion (Bollard et al. 2006), and five of six patients with active disease at the time of infusion responded, with four complete clinical responses. This study is ongoing, but has demonstrated that LMP-2-specific CTLs accumulate at tumor sites, induce clinical responses, and are well tolerated in patients with relapsed EBV-positive HD/NHL. Current studies have included LMP1 with LMP2 in the immunogen to increase the range of target epitopes that may be recognized, but LMP1 is poorly recognized by patients and healthy donors and additional targeting of EBNA1 or non-viral antigens may further improve clinical responses.

EBV-specific CTLs have also been evaluated for the treatment of NPC in three clinical trials. In one study, four patients with large tumor burdens were

treated with CTL resulting in antiviral responses but no clinical responses (Chua et al. 2001). Our group has treated 33 NPC patients. Twenty-four patients received EBV-CTLs, and nine received LMP1/2-specific CTLs. Of six patients with recurrent/refractory disease, there were two complete responses, one partial remission, and one stable disease (Straathof et al. 2005a, b). Finally, 10 patients with radiotherapy- and chemotherapy-resistant stage IV NPC were treated with a median of 10 CTL infusions resulting in 2 partial remissions and 4 stable disease (Comoli et al. 2005). These studies demonstrate the feasibility of boosting EBV-specific immune responses with clinical benefit in NPC patients. For both EBV-positive lymphoma and NPC, additional targets such as CD30 and CD70, respectively, have been evaluated preclinically, using T cells transduced with chimeric antigen receptors composed of the antigen-binding domains of antibodies with the signaling domains of the T cell receptor (Savoldo et al. 2007).

16.7 Immunotherapy for Tumors expressing Type I Latency

EBV-positive BL and other Type I tumors express only EBNA-1. Due to an internal glycine–alanine repeat, EBNA-1 is poorly processed and inefficiently presented by MHC class I molecules, and thus few CD8+ CTLs recognize EBNA-1, making CTL therapy for Type I-expressing tumors challenging (Voo et al. 2004). However, CD4+ T cells that recognize and kill EBNA-1-expressing BL cells have recently been identified (Nikiforow et al. 2003; Paludan et al. 2002), opening the field of T-cell therapy to BL and other tumors expressing Type I latency.

16.8 Resisting an Immunosuppressive Environment

Tumor-specific CTLs usually encounter an immunosuppressive environment in vivo. This may be iatrogenic due to the administration of immunosuppressive medications such as cyclosporin, FK506, or rapamycin to protect an organ graft or due to immune evasion mechanisms associated with a tumor, including (1) modulation of MHC and co-stimulatory molecules, (2) expression of inhibitory ligands, like FasL, (3) secretion of soluble factors, like IL-10 and TGF-β, (4) metabolic dysregulation through indoleamine 2,3 dioxygenase (IDO) or arginase (ARG), and (5) recruitment of regulatory T cells (Treg) (Fig. 16.3). Optimal function of infused CTLs may require genetic modification to provide resistance to tumor inhibition or strategies targeting both tumor cells and the tumor environment.

Potential strategies for rendering CTL resistant to some of these conditions have been identified and are being explored. For example, retargeting T cells to surface antigens of EBV-positive tumors by transgenic expression of CD30– or

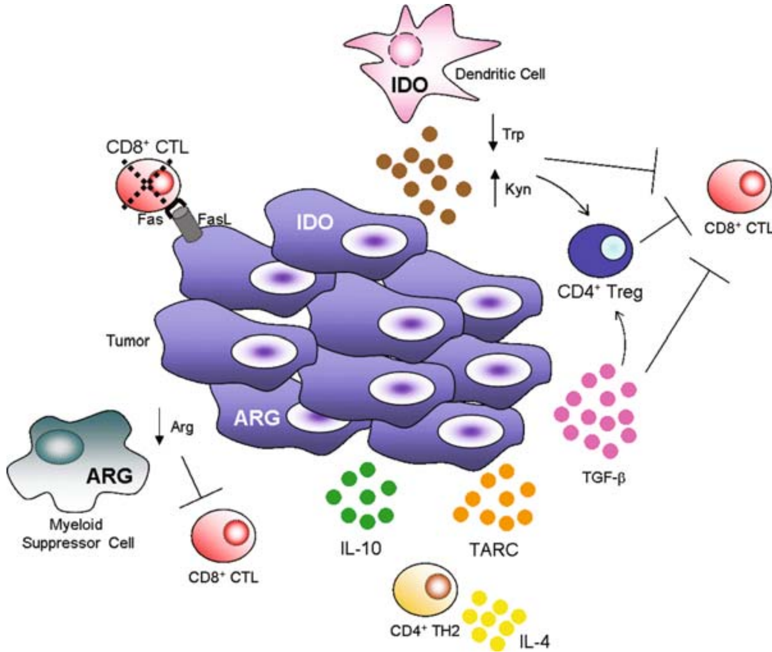

Fig. 16.3 Many tumors, including HD, use one or more strategies to evade an anti-tumor immune response. Expression of FasL causes apoptosis of Fas-expressing CTL. Secretion of inhibitory cytokines such as IL-10 and TGF-β inhibits CTL. IDO expressed by tumor cells or dendritic cells depletes tryptophan (Trp) and produces toxic kynurenines (Kyn) and inhibits T cells. ARG expressed by tumor cells or myeloid suppressor cells depletes arginine (Arg) and inhibits T cells. Tumor cells recruit CD4+ Tregs, and TGF-β and IDO promote the generation of CD4+ Tregs. Secretion of chemokines, like TARC, recruit CD4+ TH2 cells that secrete IL-4 and create a TH2 environment dampening TH1 responses

CD70– chimeric antigen receptors (CARs) in CTL, as described in section 16.9, may be able to circumvent the downregulation of MHC molecules on HD and NPC as well as provide some co-stimulatory signals (included in the transgene) lacking on tumors.

Our group has demonstrated that downregulation of Fas on T cells through stable expression of small interfering RNA (siRNA) targeting Fas renders T cells resistant to Fas-induced apoptosis, which should provide protection from FasL-expressing Hodgkin tumor cells (Dotti et al. 2005).

We also have investigated several strategies for protecting CTLs from the inhibitory effects of TGF-β, which is produced by many tumors including HD and NPC as well as by infiltrating Tregs. Many carcinomas avoid the differentiating effects of TGF-β by expressing a mutant form of TGF-β receptor II (TGF-βRII) (Park et al. 1994; Knaus et al. 1996; Wieser et al. 1993), so to determine if this strategy could be manipulated to protect CTLs from the effects

of TGF-β, we expressed a signaling-defective, dominant-negative TGF-βRII from a retrovirus vector in ex vivo-expanded EBV-specific CTLs and showed that they were resistant to TGF-β-mediated inhibition of proliferation and effector functions (Bollard et al. 2002). Transgenic expression of the dominant-negative TGF-βRII did not alter the phenotype, cytotoxic specificity, or growth factor requirements of the CTLs. This strategy has also been evaluated in murine models of safety and efficacy. Dominant-negative TGF-βRII-modified, TGF-β-resistant murine antigen-specific CTLs could be maintained for 10 months after adoptive transfer into immunized syngeneic mice by monthly DNA vaccination. When vaccination ceased, the transduced T cells disappeared with the same kinetics as GFP-transduced T cells, and lymphoproliferative disease associated with transduced T cells was not detected (Lacuesta et al. 2006). The efficacy of dominant-negative TGF-βRII-modified, murine antigen-specific CTLs was also demonstrated in a mouse model of prostate cancer. The TGF-β-resistant CTLs were able to infiltrate tumor and eliminate or significantly reduce pulmonary metastasis of TGF-β-producing tumor cells (Zhang et al. 2005). Thus, mature antigen-specific CTLs can be genetically modified to resist the inhibitory effects of TGF-β without spontaneous lymphoproliferation, providing them an advantage in targeting TGF-β-secreting tumors. This strategy has been approved for clinical application in patients with relapsed EBV-positive HD.

IDO is the initial and rate-limiting enzyme in the catabolism of tryptophan along the kynurenine pathway. It is important in the control of autoimmunity and tolerance and is expressed in the placenta where it inhibits T cells and prevents fetal rejection (Munn et al. 1998). IDO is also expressed in antigen-presenting cells and some tumor cells (Uyttenhove et al. 2003). A number of observations suggest that IDO plays a role in immune evasion in HD including (1) elevated levels of tryptophan metabolites in HD patients (Devita et al. 1971), (2) elevated levels of PGE_2, which induces IDO expression in many cell types that infiltrate Hodgkin tumors (including macrophages, dendritic cells, and eosinophils) (Passwell et al. 1983), (3) defective TCR zeta chain expression (Renner et al. 1996), a phenomenon also observed in T cells co-cultured with IDO-positive DC (Fallarino et al. 2006), and (4) our preliminary studies that demonstrate both constitutive and IFNγ-inducible IDO expression in Hodgkin tumor cell lines. IDO inhibits T cells by depleting the essential amino acid tryptophan from the microenvironment and by producing toxic metabolites (Mellor and Munn 2004). Downregulation of the TCR zeta chain can also be caused by ARG, an enzyme produced by tumors and by myeloid suppressor cells, that catabolizes and depletes arginine from the microenvironment. Small molecule inhibitors being developed against both IDO and ARG may improve the function and persistence of tumor-specific T cells in the future (Muller and Scherle 2006). Alternatively, since the T-cell inhibitory effects of both enzymes have been shown to be mediated by the stress-activated kinase, GCN2 (Munn et al. 2005; Rodriguez et al. 2007), the use of RNAi to inhibit expression of

GCN2 in CTL to provide resistance to both IDO- and ARG-mediated suppression is being explored.

Increased numbers of Tregs are found in the blood and tumors of patients with a wide variety of cancers including HD and NPC (Zou 2006; Marshall et al. 2004; Lau et al. 2007), and although the mechanisms by which Tregs suppress effector T cells are still unclear, several studies have identified factors that influence the resistance of effector T cells to suppression by Tregs (Wohlfert and Clark 2007). Cbl-b, TRAF6, or NFATc2/NFATc3 deficiency or expression of dominant-negative TGF-βRII all promote T effector cell resistance to Treg-mediated suppression, and targeting of one or more of these molecules in CTL may enhance CTL function in tumors with Treg infiltrates.

Alternatively or in addition to modifying the T cells to enhance tumor elimination, it may be necessary to target the immunosuppressive tumor microenvironment within which transferred T cells may be unable to function. For example, lymphodepletion prior to transfer of CTL may eliminate inhibitory cells both from the circulation and from intratumoral sites, allowing greater access to and continued function in tumor sites (Gattinoni et al. 2006). Depletion of the T-cell pool will also enhance the homeostatic lymphoproliferation of subsequently infused CTLs. Lymphodepletion can be achieved by chemotherapy or HSCT or more specifically using monoclonal antibodies, and these strategies have proved effective in clinical trials (Rooney et al. 1995a, b; Dudley et al. 2005). The tumor microenvironment may also be modified by targeted CTL delivery of potent Th1 cytokines such as IL-12, which may antagonize a Th2/T regulatory environment and reduce the paracrine and protective effects on tumor cells as well as protect the CTL from inhibitory cytokines like TGF-β (Wagner et al. 2004a, b). This strategy may allow the production of high concentrations of IL-12 at tumor sites, without the toxicity associated with systemic delivery of the cytokine, and may be used to target other cytotoxic molecules to EBV-positive tumors, for example, oncolytic adenovirus or retrovirus vectors. For example, adenovirus vector replication can be elicited in transduced T cells by antigen-specific activation of the E1 transactivator gene under the control of the CD40 ligand promoter (Yotnda et al. 2004). Similarly, adenovirus or retrovirus vectors can be loaded onto tumor-specific T cells and piggybacked to tumor sites, where they are released as a result of heparanase expression by activated T cells (Cole et al. 2005).

Iatrogenic immunosuppression is also a major hindrance to the function and persistence of infused therapeutic T cells and is a particular problem in patients receiving non-hematopoietic allografts, who receive long-term immunosuppression. Thus, modifying EBV-specific CTL to resist the immunosuppressive effects of these drugs could improve their long-term function in these patients. Rapamycin, a potent immunosuppressive agent commonly used in solid organ transplantation, inhibits mTor, a central regulator of cell growth, by interaction with its FKBP12–rapamycin-binding domain. A single point mutation (Ser^{2035}) in this domain renders mTor insensitive to inhibition by rapamycin, while leaving its cell regulatory functions intact (Chen et al. 1995). Therefore,

expression of this rapamycin-resistant mTor mutant in CTL may render them resistant to this immunosuppressive drug and enhance their activity in patients receiving rapamycin. Similarly, cyclosporin resistance in CTLs might be affected by disrupting expression of cyclophilins that are critical for binding to cyclosporin.

16.9 Immunotherapy for Other Cancers Using EBV-Specific T Cells

The ability of EBV-specific CTL lines to proliferate after infusion and persist long term, due to the vaccination effects of endogenous EBV-infected B cells, may be exploited for the immunotherapy of other malignancies that present tumor antigens poorly or not at all. Thus, EBV-specific CTL may be redirected to other tumors by the transgenic expression of chimeric antigen receptors (CARs), constructed by fusing monoclonal antibody-derived heavy- and light-chain variable regions as a single chain to the transmembrane and intracellular signaling domains of the TCR zeta chain (Rossig and Brenner 2003). CAR-transduced EBV-specific CTLs retain their ability to proliferate in response to and kill EBV-infected target cells in an MHC-restricted manner, while acquiring the ability to recognize and kill target cells expressing the CAR ligand in a non-MHC-restricted manner (Eshhar et al. 1993; Stancovski et al. 1993). The use of EBV-specific T cells may therefore avoid the failure of CAR-expressing, mitogen-stimulated T cells to expand, persist, or function after infusion into cancer patients (Walker et al. 2000). This strategy is currently being tested in children with relapsed neuroblastoma, who receive both EBV-specific CTL and CD3-stimulated T cells retargeted to the GD2 disialoganglioside with a GD2 CAR, with the hypothesis that EBV-specific T cells will expand and persist in vivo better than CD3-stimulated T cells. The fate of each cell population will be distinguished by quantitative PCR (Rossig et al. 2002). Alternatively, the ability of CAR-expressing T cells to proliferate and secrete cytokines in response to CAR ligand on tumor cells that do not express co-stimulatory molecules has been improved by combining the additional signaling domains from co-stimulatory molecules such as CD28 and OX40, but these modifications have yet to be tested clinically (Beecham et al. 2000; Hombach et al. 2001; Finney et al. 1998; Geiger et al. 2001; Pule et al. 2005).

This strategy can also be applied to HD by the expression of a CAR-targeting CD30 that is expressed on all HRS cells both at presentation and at relapse (Savoldo et al. 2007). CD30 CAR-expressing EBV-specific T cells should benefit not only patients with EBV-negative disease but also patients with EBV-positive tumors in which viral antigens are downregulated, mutated, or lost (Gan et al. 2002).

16.10 Concluding Remarks

Adoptive immunotherapy with EBV-specific CTL has been highly effective at preventing or treating EBV-LPD post-HSCT, and while the use of EBV-specific CTL has been less effective for other EBV-associated malignancies, the results of the clinical trials to date are promising. EBV-associated malignancies that arise in immunocompetent individuals impart additional obstacles, such as immune evasion mechanisms, that must be overcome in order to enhance the efficacy of CTL therapy in these patients. Indeed modifications to CTL, such as expression of a dominant-negative TGF-βRII or siRNA targeting Fas have demonstrated that CTL can be made resistant to some immune evasion mechanisms. A greater understanding of the molecular mechanisms responsible for suppressing CTL activity at tumor sites will provide additional targets for modification and/or modulation in CTL to render them resistant to immune evasion strategies, which would be beneficial not only for immunotherapy of EBV-associated malignancies but for other types of malignancies.

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Chapter 17
KSHV Epidemiology and Subtype Evolution

Gary S. Hayward and Denise Whitby

17.1 Epidemiology

17.1.1 Background

The etiology of Kaposi's sarcoma (KS) posed a puzzle from the first description of the disease by Moritz Kaposi in 1872 (Kaposi 1872) until the discovery of the KSHV by Yuan Chang and Patrick Moore in 1994 (Chang et al. 1994). The disease described by Kaposi, now known as Classic KS, is one of the four distinct epidemiological manifestations of KS. Classic KS is a rare, benign skin disease seen in elderly men of Mediterranean origin. A second epidemiological cluster of KS cases was reported in the 1950s in sub-Saharan Africa (Oettle 1962) and is known as African endemic KS. As with CKS, cases occurred more frequently in men but African endemic KS was reported to be more aggressive (Taylor et al. 1971). Also in contrast to CKS, in Africa KS was also reported in children in whom lymph node involvement was the definitive characteristic (Ziegler and Katongole-Mbidde 1996).

In 1970s, a third epidemiological variant of KS emerged in recipients of solid organ transplants who were iatrogenically immunosuppressed. Iatrogenic KS (IKS) occurs most often in subjects from KS endemic regions. It develops early after transplantation and often regresses if immunosuppression is removed or modified (Farge et al. 1999). In 1981, the first reports appeared of KS in otherwise healthy young men who have sex with men (MSM) in US urban centers signaling the beginning of the AIDS epidemic. AIDS-KS was more lethal than any of the other epidemiological variants of the disease and occurred

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_17, © Springer Science+Business Media, LLC 2009

commonly in HIV-infected MSM but rarely in those who had acquired HIV via infected blood products (Beral et al. 1990). Although KS could be distinguished epidemiologically into four variants, pathologically all four forms were identical, pointing to a single etiology. An infectious cause was suspected because of the association with immunosupression, while the association of AIDS-KS with MSM suggested a sexually transmitted agent. Since the discovery of KSHV many of the puzzling questions posed by the epidemiology of KS have been resolved, while new questions have arisen.

17.1.2 Diseases Associated with KSHV

The initial publication detailing the discovery of KSHV included only a few hundred base pairs of sequence data from the newly discovered herpesvirus (Chang et al. 1994). This was sufficient however to provide convincing evidence of an association with AIDS-KS based on PCR amplification of viral DNA in 90% of AIDS-KS tissues compared to only 15% of tissues from AIDS patients without KS and no detection in tissues from subjects without AIDS or KS (Chang et al. 1994). Within weeks of publication, others had used PCR to amplify KSHV sequences from biopsies of all the four forms of KS (Boshoff et al. 1995; Chang et al. 1996; Dupin et al. 1995), confirming and extending the original observations. Nested PCR amplification was used to demonstrate that KSHV DNA was detectable in the PBMC of subjects with AIDS-KS more frequently than in PBMC of AIDS patients without KS. Importantly, KSHV DNA was detected in the PBMC of HIV-infected subjects prior to the development of KS and strongly predicted disease development (Moore et al. 1996; Whitby et al. 1995).

In addition to a clear causal association between KSHV and KS, KSHV is associated with primary effusion lymphoma (PEL), a rare form of non-Hodgkin's lymphoma mostly seen in AIDS patients, and the lymphoproliferative disorder Multicentric Castleman's Disease (MCD) (Cesarman et al. 1995; Soulier et al. 1995). Associations have been proposed for numerous other diseases but the evidence has generally been poor and confirmation from other laboratories lacking. Notable proposed disease associations include multiple myeloma (Rettig et al. 1997), sarcoidosis (Di Alberti et al. 1997), and primary pulmonary hypertension (Cool et al. 2003). All have been comprehensively disproved (Henke-Gendo et al. 2004; Laney et al. 2005; Lebbe et al. 1999; MacKenzie et al. 1997; Whitby et al. 1997).

17.1.3 KSHV Prevalence

The incidence of KS varies greatly according to geography, age, gender and in the case of AIDS-KS, HIV risk group (Fig. 17.1). The extent to which these variations could be explained by variations in the prevalence of KSHV could be addressed only when serological assays for detecting antibodies to KSHV were established. Nested PCR and real time PCR could not be used for prevalence

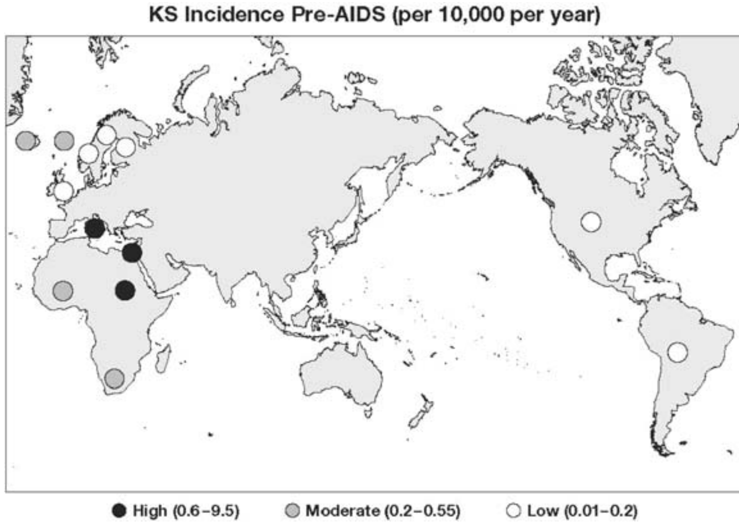

Fig. 17.1 Incidence rates of classic KS in the pre-AIDS era. Map shows reported incidence of classic KS prior to the AIDS epidemic. References from which the data was complied are cited in the text.

studies because KSHV DNA is detectable using such assays in PBMC of only 50% of KS patients and 10% asymptomatically infected subjects. The reason for this is not the sensitivity of the assays but the biology of the virus. KSHV maintains latent infection in circulating B cells but these cells are relatively rare, especially in asymptomatically infected subjects.

KSHV encodes multiple antigenic proteins, some of which are expressed only during lytic replication (Chandran et al. 1998). The major antigenic proteins are the latently expressed latency-associated nuclear antigen (LANA) encoded by ORF 73 and the lytically expressed K8.1 and ORF 65. Humoral responses to these antigens do not follow a predictable pattern and concordance between assays detecting antibodies to these antigens is moderate. First-generation serological assays were developed based on immunoflourescence of latently infected PEL cells (Gao et al. 1996; Kedes et al. 1996; Simpson et al. 1996) or PEL cells induced by TPA treatment to produce lytic antigens (Lennette et al. 1996). More recently enzyme-linked immunosorbant assays (ELISA) have been developed using recombinant proteins or peptides.

Validation of serological assays for KSHV has been problematic. Sensitivity and specificity estimates depend on the identification of gold standard positive and negative control samples. Sera from KS patients are generally used as positive controls as we can assume that 100% of KS patients are infected with KSHV. However, KS patients usually have extremely high levels of antibodies to KSHV whereas asymptomatically infected subjects often have very low antibody levels. Sensitivity estimates based on detection of KS patient sera

may therefore be too high. Identifying negative controls is more difficult and most commonly blood donors are used. However, some blood donors are likely to be infected with KSHV so specificity estimates based on blood donors may be too low. Studies comparing assay performance in multiple laboratories have shown high agreement between assays and labs in detecting antibodies in KS patients but poor agreement for detection of antibodies in blood donors (Rabkin et al. 1998; Pellett et al. 2003). Interestingly, prevalence estimates for US blood donors were similar for different assays and different laboratories, but the subjects identified as positive were rarely the same (Pellett et al. 2003).

Consequently, current KSHV serological assays are more suited to population-based studies than to definitive diagnostics. However, caution needs to be used even in viewing prevalence estimates for populations. Prevalence estimates will depend on the sensitivity and specificity of the assays used, making comparisons between studies using different assays problematic. Even when the same antigen is used for different studies, the assay performance may vary between laboratories or the cutoff used may differ. LANA IFA is considered to be the most specific but least sensitive assay while lytic IFA conversely is considered the most sensitive but least specific. This is an oversimplification however since studies in which sera from KS patients are diluted show that at the highest dilutions (i.e. the lowest level of antibody), LANA IFA shows the greatest sensitivity (Engels et al. 2000). Commercially available ELISAs also show high sensitivity and poor specificity. ELISAs based on recombinant proteins have been shown to be more sensitive than assays based on peptides (Whitby and Dollard, unpublished observations). ELISAs based on the lytic antigen ORF 65 show extreme discordance with other KSHV markers in some populations while ELISAs based on the lytic antigen K8.1 have been shown to be more reliable and have high specificity and sensitivity. LANA ELISAs have been more problematic to develop since sensitivity is very low for peptide based assays and even for assays based on bacterially expressed recombinant proteins. Assays based on baculovirus-expressed LANA have comparable sensitivity to LANA IFA however and have better reproducibility. Longitudinal studies have shown that subjects may develop antibodies to one or more KSHV antigen months or years before antibodies are developed to other antigens with no predictable pattern (Biggar et al. 2003; Laney et al. 2004). It is desirable therefore in cross-sectional studies to use assays for more than one antigen.

In addition to assay related difficulties in interpretation of KSHV prevalence data there are also problems related to study design. Prevalence estimates are often based on relatively small numbers of subjects and recruitment criteria may vary considerably between studies or may not be specified. Often one is left with the impression that the only reason for testing the sera included in a study was availability. When the study population is not well defined it is difficult to assess potential biases in recruitment that may affect the prevalence estimate obtained. For example, many studies include estimates for blood donors but do not specify whether the donors are pre-screened for other infectious agents. Other studies use hospital-based controls of case-control studies without specifying

the recruitment criteria. Another problem in assessing KSHV prevalence is that reported studies are far from comprehensive. While there are many reported studies of prevalence in the US, Italy, and parts of Africa, there are many populations where studies are lacking.

It is with some trepidation therefore that I attempt to review what is currently known about KSHV prevalence (Fig. 17.2). There have been numerous studies of KSHV prevalence in US blood donors resulting in prevalence estimates ranging from 0 to 23% (Gao et al. 1996; Hudnall et al. 2003; Kedes et al. 1996; Lennette et al. 1996). Most studies have relied on less than 100 subjects and this may contribute to the disparity in the estimates. A larger study of 1,000 blood donors tested by six independent laboratories yielded estimates from individual laboratories ranging from 0.5 to 5%. Viewed collectively, only one sample was positive in all six laboratories, and 10.7% of samples were positive in at least one. The higher estimates are all based on lytic IFA. LANA IFA and assays using recombinant EIAs generally produced estimates <5% (Pellett et al. 2003). There are no studies to date on geographic variations in the prevalence of KSHV in the US.

Little has been reported regarding the prevalence of KSHV in Canada. A study of prevalence in 42 allogeneic stem cell transplant recipients reported a prevalence of 2% (Sergerie et al. 2004), while a study of 150 renal transplant recipients reported no seropositives (Delorme et al. 2003). This sparse data suggest a low prevalence of KSHV in the Canadian general population but variations may occur in immigrant groups. A report of a cluster of Classic KS cases in Canadian Inuit suggests that this ethnic group may have an elevated KSHV prevalence (Rosen et al. 2005). A similar situation has been reported in

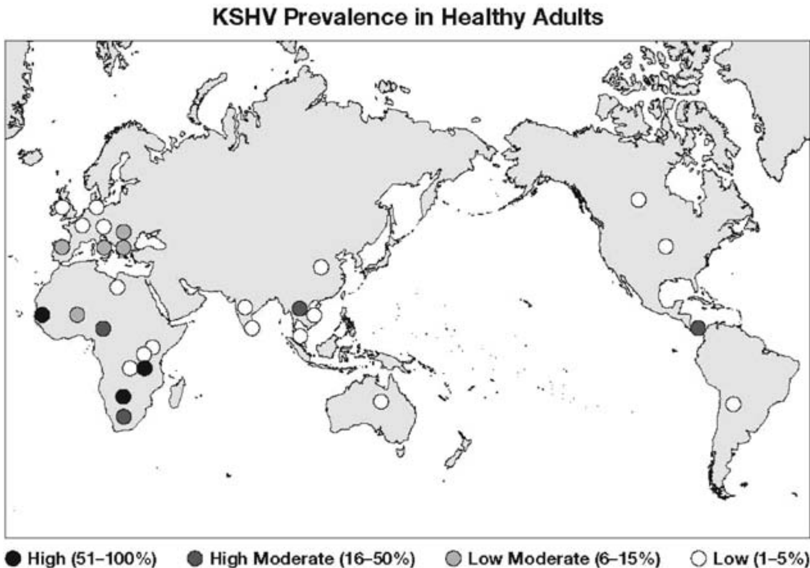

Fig. 17.2 Prevalence of KSHV. Map shows reported prevalence estimates for KSHV compiled from references cited in the text

South and Central America where prevalence estimates for blood donors in Brazil, Argentina, and Chile are around 4% (Perez et al. 2004) but much higher prevalence (>50%) is reported in Amerindians in Brazil (Biggar et al. 2000; Cunha et al. 2005), Ecuador (Whitby et al. 2004), and Peru (Mohanna et al. 2007), with slightly lower prevalence estimates reported among Amerindians in French Guiana (Kazanji et al. 2005). These observations were somewhat unexpected as KS was not reported among Amerindians. However, these populations live in very remote locations and have poor access to healthcare. Recently, a case of Classic KS was reported in a Peruvian Amerindian, and hospital records suggest that KS may be a relatively common malignancy in Amerindians (Mohanna et al. 2006).

In Europe, KSHV prevalence is reported to be low in Northern European countries: 3% in UK blood donors (Simpson et al. 1996), 2% in French population controls (Marcelin et al. 1998), 2.5% in Dutch IDUs (Renwick et al. 2002), 3% in German blood donors (Preiser et al. 2001) and 4% in Danish STD patients (Hjalgrim et al. 2001). A study of Swedish blood donors tested by multiple laboratories produced estimates of between 0.6 and 20%, the higher estimates being based on lytic IFA (Enbom et al. 2000). Most of these studies were based on fewer than 200 subjects. A larger study of blood donors in Hungary estimated prevalence in blood donors to be 1.56% (Juhasz et al. 2001).

Classic KS incidence is elevated in Mediterranean countries with considerable variation in incidence observed by geographical region (Franceschi and Geddes 1995). In Italy, KS incidence in general is higher in the South than in the North (Geddes et al. 1995; Geddes et al. 1994), but even more local differences in incidence can be observed. For example, The Po Valley region in Northern Italy has an elevated incidence of Classic KS and variations in incidence can be observed at the municipality level with the highest incidence observed in a rural area in which the Po and Oglio rivers converge (Ascoli et al. 2001). The prevalence of KSHV similarly varies geographically, mirroring the geographical variations observed in KS incidence. Prevalence estimates for Northern Italy based on LANA IFA of blood donors range from 4 to 7.5%(Gao et al. 1996; Whitby et al. 1998) with higher prevalence observed for the Po Valley (13–20%) (Calabro et al. 1998). The highest prevalence was observed in Southern Italy, particularly the Islands of Sicily and Sardinia (24–35%) (Calabro et al. 1998; Cattani et al. 2003; Serraino et al. 2006; Whitby et al. 1998). In the Po Valley, significant differences were observed in KSHV prevalence in elderly people living in the district in which the Po and Oglio rivers converged (19.4%) compared to an adjacent district without a major river (9.8%) (Tanzi et al. 2005).

Few studies of KSHV prevalence in Greece have been reported despite the high incidence of classic KS. A recent study estimated prevalence in Athens based on K8.1 EIA to be 7.6% (Zavitsanou et al. 2007). Classic KS incidence varies geographically in Greece (Stratigos et al. 1997) but studies of KSHV prevalence in different Greek regions are lacking. Prevalence estimates for Albanian and Kosovar migrants are reported to be 29 and 18%, respectively (Chironna et al. 2006) with lower estimates (8%) reported for Croatia (Rode

et al. 2005), reflecting a North to South gradient similar to that seen in Italy. A North to South gradient is also reported in Spain with a prevalence estimate of 4.5% in the Basque country compared to 8% in Catalonia (Gambus et al. 2001).

The AIDS epidemic in Asian countries has been characterized by a scarcity of KS cases suggesting that KSHV prevalence is likely to be low (Parkin 2006; Sriplung and Parkin 2004). Prevalence estimates of 4% for India and Thailand, 4.4% for Malaysia and 3.8% for Sri Lanka were reported based on a commercial WV EIA (Ablashi et al. 1999). Even lower estimates are reported for Cambodia (1.3%) (Muller et al. 2001) and Japan (0.1%) (Miyagi et al. 2002). Others have reported higher prevalences: a study of 1,201 Taiwanese subjects based on lytic IFA tested at 1:10 dilution reported 4% of children and 8–24% of adults infected, however, the titres reported in children were below cut-off used in most studies. (Huang et al. 2000). A recent report also based on lytic IFA of 992 subjects participating in an STD cohort study reported a prevalence estimate of 24.2% in Thailand, in contrast to earlier studies. (Chen et al. 2004). A cluster of classic KS cases was reported among the Ugyur ethnic group in the Xinjiang area of China associated with a prevalence estimate of 47% of 73 cancer patients based on lytic IFA suggesting that pockets of elevated KSHV prevalence may be present in parts of Asia (Dilnur et al. 2001).

In contrast to the scarcity of KS cases observed in Asia as a result of the HIV epidemic, in Africa cases of KS have increased explosively during the AIDS epidemic. In Uganda and Zimbabwe, the incidence of KS has increased 20-fold so that it is now the commonest cancer in men and the second most common in women (Bassett et al. 1995; Wabinga et al. 1993; Wabinga et al. 2000). Prior to the AIDS epidemic, considerable geographical variation was observed in the incidence of KS in Africa with the highest incidence reported in central and eastern Africa and incidence declining toward the South, West and especially North (Cook-Mozaffari et al. 1998). Reported prevalence estimates for KSHV generally mirrored these variations with an important exception. Several studies, including some with very large numbers of subjects, have estimated the prevalence of KSHV in Uganda and these have produced fairly consistent estimates of 40–50% (Gao et al. 1996; Hladik et al. 2003; Newton et al. 2003; Wawer et al. 2001). Similar estimates are reported for Kenya (43%) (Baeten et al. 2002; Lavreys et al. 2003), Zambia (40%) (Klaskala et al. 2005) and Tanzania (50%) (Mbulaiteye et al. 2003). Higher prevalence estimates are reported for Congo (82%) (Engels et al. 2000), Botswana (76%) (Engels et al. 2000; Whitby et al. 2004) and Malawi (67%) (DeSantis et al. 2002). Lower estimates are reported for South Africa (30%) (Dedicoat et al. 2004; Malope et al. 2006; Sitas et al. 1999) and some West African countries such as Burkino Faso (12.5%) (Collenberg et al. 2006) and Cameroon (25%) (Volpi et al. 2004). In other West African countries, however, most notably The Gambia, high KSHV prevalence has been reported (75%) despite a low incidence of KS before and during the AIDS epidemic (Ariyoshi et al. 1998).

17.1.4 Transmission

Prior to the discovery of KSHV, the causative agent for KS was hypothesized to be sexually transmitted because AIDS-KS was reported 20 times more frequently in MSM than in other HIV risk groups (Beral et al. 1990). Initial molecular studies provided support for this hypothesis. KSHV DNA can be detected in semen (Diamond et al. 1997; Howard et al. 1997) and in the female genital tract (Calabro et al. 1999; de Sanjose et al. 2002; Whitby et al. 1999). Early serological studies using LANA IFA in the US and the UK in HIV risk groups showed that KSHV prevalence was higher in MSM than in intravenous drug users (IDU), patients with haemophilia or blood transfusion recipients (Gao et al. 1996; Kedes et al. 1996; Simpson et al. 1996). In addition, prevalence was higher in STD clinic attendees than in blood donors (Kedes et al. 1996; Simpson et al. 1996). KSHV infection is reported to be associated with markers of sexual transmission in MSM (Martin et al. 1998; Smith et al. 1999) but attempts to identify specific sexual practices associated with transmission of KSHV among MSM have met with little success, possibly because most MSM will report multiple specific sexual practices (Grulich et al. 2005; Martin and Osmond 2000; Martro et al. 2007). KSHV is detected more frequently in saliva of infected MSM than in semen (Pauk et al. 2000) leading to the suggestion that infected saliva is the most likely source of KSHV during transmission between MSM (Martin 2003).

Reported evidence for sexual transmission between heterosexuals has been less convincing. Some groups have reported an association of KSHV infection and heterosexual sexual risk factors (Cannon et al. 2001; Greenblatt et al. 2001; Tedeschi et al. 2000), while other studies have reported a lack of evidence for heterosexual transmission (Smith et al. 1999; Engels et al. 2007; Zavitsanou et al. 2007). Studies relating to blood borne transmission have also been inconsistent with some reports suggesting that intravenous drug use does not confer increased risk of KSHV infection (Renwick et al. 1998), while others reported IDUs were at increased risk, especially with prolonged use (Atkinson et al. 2003; Cannon et al. 2001). Evidence for KSHV transmission via blood transfusion has also been reported (Dollard et al. 2005; Hladik et al. 2006; Mbulaiteye et al. 2003).

In KSHV endemic regions, infection is reported in children, and prevalence increases with age suggesting that non-sexual horizontal transmission is common (Gessain et al. 1999; Mayama et al. 1998; Whitby et al. 2000).These reports are consistent with the occurrence of KS in childhood in sub-Saharan Africa (Ziegler and Katongole-Mbidde 1996). Evidence from numerous studies suggest that KSHV transmission occurs between mothers and children but the route is usually horizontal rather than vertical (Lyall et al. 1999; Mantina et al. 2001), most likely via infected saliva. It has been shown that mothers with high levels of detectable KSHV DNA in saliva are more likely to have KSHV-infected children (Dedicoat et al. 2004). Although having a KSHV-infected mother is an important risk factor for KSHV infection in childhood

(Bourboulia et al. 1998; Dedicoat et al. 2004; Malope et al. 2006; Plancoulaine et al. 2000), infected siblings are also likely an important source of KSHV infection (Mbulaiteye et al. 2003; Plancoulaine et al. 2004; Plancoulaine et al. 2000). Children infected with KSHV are more likely to have detectable KSHV DNA in saliva than adults, and the viral load is also reported to be higher (Dedicoat et al. 2004; Mbulaiteye et al. 2004).

17.1.5 Cofactors for KSHV Transmission and Disease

The geographical variations in the prevalence of KSHV infection and of KS incidence points to cofactors for both transmission of KSHV and for KSHV pathogenesis in infected subjects. Environmental cofactors are likely to be most important in terms of transmission, although a recent report suggested an association between certain HLA types and KSHV shedding (Alkharsah et al. 2007). It has been suggested that biting insects may play an indirect role in transmission of KSHV by exposure to infected saliva as a way of soothing painful bites (Coluzzi et al. 2002). This hypothesis is consistent with the elevated prevalence of KSHV along the Po valley (Ascoli et al. 2001) and with the reported association with KSHV infection and use of surface water in Uganda (Mbulaiteye et al. 2005) and is supported by intriguing data demonstrating a decline in KSHV transmission rates associated with mosquito elimination campaigns (Coluzzi et al. 2003). Exposure to water-borne parasites has also been suggested as a cofactor for KSHV transmission (Mbulaiteye et al. 2005). A screen of natural products from KS endemic regions showed that crude plant extracts could cause KSHV reactivation in vitro suggesting that environmental exposure to such plants via traditional medicine, horticultural practices or basket making may result in increased shedding of KSHV (Whitby et al. 2007).

Such exposures may be important in explaining variations in KSHV prevalence and may also contribute to pathogenesis. Two studies have shown that the rate of KS among KSHV-infected subjects also varies geographically. In Sardinia, KS is reported to develop in 1 out of 8,114 infected subjects in Cagliari, whereas in Sassari, KS occurs in 1 out of 3,891 infected subjects (Serraino et al. 2006). Similar differences are reported in Sicily, Sardinia and Malta (Vitale et al. 2001). Such differences suggest that environmental cofactors are important determinants of disease risk. The same reports however also suggest substantial differences in disease risk between men and women with men more likely to develop KS (Serraino et al. 2006; Vitale et al. 2001). Such differences are consistent with previously reported excess risk of KS in males (Franceschi and Geddes 1995) and remain unexplained.

Host genetic factors are likely to play an important role in disease risk among KSHV-infected subjects. Classic KS is reported to be more common in those of Jewish origin in the US (Ross et al. 1985) but the prevalence of KSHV infection was reported to be similar in elderly Jews and non-Jews in a New York geriatric

clinic (Engels et al. 2002). Similarly, transplant recipients of middle eastern origin are reported to be at increased risk of KS but a study of KSHV prevalence among Saudi Arabian renal disease patients is reported to be not especially high (10%) (Almuneef et al. 2001). These studies would indicate that these ethnic groups are at an increased susceptibility to develop KSHV-related disease if infected. Polymorphisms in genes that modulate host immunity have been reported to contribute to risk of disease in KSHV-infected subjects (Brown et al. 2006a, b) and also to correlate with viral load and antibody titre (Brown et al. 2006a, b). Such studies are still very preliminary but point the way to a future in which the pathogenesis of KS will be examined in terms of the complex interactions between viral, environmental and host factors.

17.2 Strain Variability

17.2.1 Human Herpesviruses: Genome Variability and Subtype Clustering

Kaposi's sarcoma herpesvirus (KSHV) is a rhadinovirus that is the causative agent of Kaposi's sarcoma (KS), as well as of a subset of cases of primary effusion lymphoma (PEL) and of multicentric Castleman's disease (MCD). KSHV was discovered in 1994, when it first became appreciated that AIDS patients in the United States harbored a close relative of herpesvirus saimiri (HVS), a well-known T-lymphotrophic gammaherpesvirus of new world primates (Chang et al. 1994; Russo et al. 1996). Since then a great deal of information has been accumulated about genetic variability in the KSHV genomes found predominantly in KS and AIDS patients from different continents or ethnic backgrounds. The purpose of this review is to both summarize the variability patterns observed and to evaluate their origin, as well as to place them into the context of other current information about human herpesvirus evolution.

The eight known human herpesviruses fall into three sub-families classified as the alphaherpesviruses (HSV1, HSV2, VZV), the betaherpesviruses (HCMV, HHV6A/B, HHV7) and the gammaherpesviruses (EBV, KSHV). These differ at the genomic level not only by sequence divergence within common conserved genes but also, in many cases, by the acquisition or loss and replacement of as much as one-third of their total gene content. At least three distinct processes have been involved here, including individual cDNA capture events, whole spliced gene acquisitions, and formation of tandem duplications with subsequent divergence of the paralogues. Different types of processes have probably predominated at different evolutionary times in each lineage. These changes have been very long-term events occurring over the full 100–120 million years of mammalian evolution. They are also thought to have occurred in a highly stable

host-species associated manner (co-speciation) such that jumping across species barriers has been almost non-existent. Consequently, human KSHV is much more closely related to all the other primate rhadinoviruses than it is to any of the other species of human herpesviruses, with orthologous KSHV-like viruses from the most closely related hosts (eg chimpanzees and the other great apes) being the closest to the human versions. On the other hand, even the other human gammaherpesvirus, EBV, the prototype lymphocryptovirus, is hugely different from KSHV in gene content and organization, and again it is itself much more closely related to other primate lymphocrytoviruses.

When the large DNA genomes of a group of some 20 laboratory strains of herpes simplex virus (HSV) were first analyzed and compared by restriction enzyme digestion patterns in 1975 (Hayward et al. 1975), the results confirmed that indeed they fell two very different genotype groups of HSV in humans. These corresponded to previous interpretations from biological and biochemical analyses of two distinct species HSV1 and HSV2 associated with either facial or genital lesions. The patterns of different sizes of DNA fragments also revealed that many if not all strains of each species could be distinguished (or “fingerprinted”) by this type of low-resolution global genome analysis. This approach opened up new opportunities for epidemiological studies to detect and follow the transmission of individual isolates or strains during sequential infection conditions such as in hospital nosocomial outbreaks. Furthermore, these results revealed that there were no chimeric hybrids between HSV1 and HSV2 found in natural infections, despite the fact that viable HSV1/HSV2 hybrids can be generated easily by mixed infection in laboratory cell culture conditions.

Eventually, complete DNA sequence analysis of prototype strains of HSV1 and HSV2 showed that they contain exactly the same total complement of genes, and that, although some genes are more similar than others, the corresponding orthologous genes exhibit differences averaging 13% at the nucleotide level and ranging from 5 to 25% at the amino acid level. For example, the coding regions for the highly conserved HSV1 and HSV2 gB proteins differ by 6% at the nucleotide level and 8% at the amino acid level (Dolan et al. 1998). Nucleotide polymorphisms among HSV1 isolates within the gB, gE and gI genes have been reported to range from 1 to 3% (Martin et al. 2006; Norberg et al. 2004). Estimates of the comparative rate of divergence with that of other primate alpha 1 herpesviruses, including one from chimpanzees, which is much closer to HSV2 than to HSV1, suggest that HSV1 and HSV2 arose as separate entities some 8–13 million years ago, significantly before the 5–6 million years ago time frame that humans and chimpanzees diverged (Luebcke et al. 2006; McGeoch and Cook 1994; McGeoch et al. 1995).

In thinking about the process of the divergence of HSV1 and HSV2, it is obvious that, as with any other speciation event, there must have been numerous intermediate genomic forms and stages with varying degrees of change from the original common parental genome. However, just as for the human and chimpanzee hosts, all the intermediate ancient pre-Homo sapiens forms of HSV

have since been displaced or died out. Considering that HSV is a highly infectious, horizontally transmitted and nearly ubiquitous human pathogen, this is a much more difficult prospect to comprehend for the virus than for the host. Does each new virus variant that has a selective advantage simply swarm through the host population all around the world every now and then and replace the previously predominant variant? Or does the virus follow much more closely to the host evolutionary dynamics involving population bottlenecks and founder effects associated with glaciation refuges, epidemics, migrations, conquests and assimilation processes that must have frequently narrowed and refocused the genetic diversity of human and all mammalian species?

Since both HSV1 and HSV2 infect the same host (albeit in different niches) and can remain latent in post-mitotic neurons of the trigeminal or sacral ganglia over the entire lifetime of the host, there are also many additional biological issues that could have played complicating roles in this evolutionary divergence process. For example, intertypic recombination would seem likely have been common at early stages before the two (or quite possibly originally many more) variant genomes had diverged too far to allow easy homologous matching. Unfortunately, analysis of the extent of current global intratypic variability for HSV1 and HSV2 has not been pursued very extensively as yet. However, in contrast, there has been a wealth of evidence gathered in recent years about genetic diversity in several other human herpesvirus species, including EBV and HCMV, and especially for KSHV.

When individual strains, isolates or samples of human EBV, HCMV or KSHV are compared at the DNA sequence level, there are several dramatic examples of individual "hypervariable" genes that differ within humans at levels close to those found between the orthologous genes of viruses from different host species (such as between HSV1 and HSV2 for example). In EBV, this phenomenon is largely limited to the latency-associated, nuclear DNA-binding protein known as EBNA2, which comes in two flavors that differ by 45% at the amino acid level (Dambaugh et al. 1984), compared to both being 65% different from the EBNA2 protein of baboon EBV (Ling et al. 1993). This has often been used to classify individual isolates of this virus as either EBV-A and EBV-B or EBV-I and EBV-II. In KSHV, there are two such hypervariable genes known as ORF-K1 (encoding the VIP protein) and ORF-K15 (encoding the TMP protein), which are both membrane-associated tyrosine kinase-signaling proteins. In contrast, in HCMV, there is a large group of hypervariable genes dispersed across the genome that encode a wide range of different types of proteins. The latter proteins display strain-specific variations of between 20 and 60% at the amino acid level over all or large segments of their coding regions. The most variable genes of HCMV include UL144[vTNFR], UL146[vCXC1], UL09, UL37exon3, UL73[gpO], UL74[gpN], RL12[vFcR], RL13 and UL01 as well as to a lesser extent UL147[vCXC2], UL55 [gpB] and US28 [vGPCR1]. In essence, many of these genes display large protein level differences that would ordinarily justify them being rated (at the single gene level) as being representative of different orthologous viruses derived from different host species. Yet

they all fall just within the spectrum of the currently extant human versions of HCMV or KSHV. Most importantly, the amino acid sequences of these “hypervariable” proteins nearly always cluster very tightly into several distinct cladal groups, referred to as subtypes, that often show very little intratypic variation (just like HSV1 or HSV2 isolates). Finally, the actual number of distinct subtypes found of individual hypervariable genes in HCMV varies from as few as three for UL144, up through six to eight for UL09, UL37ex1 and UL73/74, and on up to 15 for HCMV UL146 and UL147. Although rare, the situation can also be complicated further by intragenic hybrids with very distinct chimeric recombination junctions, such as those described in UL144 (Arav-Boger et al. 2002).

What sense can be made of this remarkable diversity of the hypervariable genes in human herpesviruses? What drives their seemingly rapid divergence when other adjacent genes are not significantly diverged? Are they recent or ancient features of these genomes? Have the processes that led to them been the driving force for viral evolutionary selection, or are they just accidental relics of complex recombination patterns? Might they in fact have origins via the supposedly forbidden process of interspecies gene exchange? Do they contribute to possible differences in the biology and pathogenesis of various strains within a species? Finally, can they be used to validly classify and categorize distinctive strains or genotypes in epidemiologically useful and meaningful ways? The answers are likely to provide highly informative insights about the processes involved in herpesvirus evolution.

17.2.2 Patterns of Hypervariable Genes and Chimeric or Mosaic Genomes in KSHV Compared to HCMV

To address these questions, we have generated PCR DNA sequence data for between 10 and 12 variable loci each distributed across the entire 140-kb unique sequence length of KSHV genomes and the 240-kb length of HCMV genomes. In both viruses, these multiple PCR loci (each between 600 and 1800-bp in size) were derived from samples from a large variety of clinical and laboratory sources obtained from different human populations around the world. So far between 80 and 180 KSHV samples and between 40 and 120 HCMV samples have been evaluated at each locus. In most cases, this data was derived directly from tissue biopsy or PBMC samples, without any opportunity for mutational selection upon passaging in cell culture (which can and does occur for HCMV at least). The results and explanations obtained so far have proven to include highly gene-specific features, as well as being both partly very similar and partly very different for the two viruses. Furthermore, it is very evident that chimerism is a major feature of the structure of both these genomes, which tends to create significant complications for the seemingly simplistic concepts and definitions of single virus species, subtypes and strains.

Perhaps the most critical point to make initially is that one certainly cannot validly define either KSHV or HCMV genotypes on the basis of subtyping data for just one variable gene locus. Multiple loci must always be analyzed to get an accurate representation of each genome. On the other hand, there is a considerable degree of linkage found between the subtypes present at the different variable loci within individual KSHV genomes. In fact, the subtype patterns of KSHV genes and genomes found on different continents are very different and correlate closely with the ethnic origins of their infected human hosts. In dramatic contrast, there does not seem to be any residual ethnic association for the various subtypes of HCMV variable genes, and the HCMV subtype distribution patterns observed do not differ significantly in Uganda compared to the United States. Also, there is virtually no linkage at all between the subtype patterns at different loci across the HCMV genome, except for those variable genes that lie directly adjacent to one another.

We attribute these major differences between the two viruses to preferential familial transmission, rare multiple infections, and minimal levels of intertypic recombination in KSHV, compared to much higher horizontal transmission rates, higher levels of simultaneous infections with multiple strains, and rampant intertypic recombination for HCMV. These properties appear to correlate with the very low rates of infection (often just 1–3% seropositivity) in most parts of the world except sub-Saharan Africa by KSHV, compared to almost ubiquitous asymptomatic infection in all human populations by HCMV. This concept was reinforced by our studies within a cohort of 56 KS patients in Uganda, where we found no cases of multiple rather than single KSHV strain infection among 35 KSHV positive PBMCs, whereas 19 (= 65%) out of 29 of these same samples that were HCMV positive contained two or three separate strains of HCMV simultaneously.

In contrast to HCMV, where all 10 of the PCR loci analyzed represent the coding regions for hypervariable proteins, only two of the loci analyzed in KSHV encode hypervarable proteins. These encode the K1/VIP and K15/TMP proteins, which are both tyrosine kinase-signaling membrane proteins (Brinkmann and Schulz 2006; Damania and Jung 2001) and map at the extreme LHS and RHS ends the KSHV genome, respectively. The other nine KSHV PCR loci were selected as the most variable non-repetitive segments to sample across the entire remaining 135-kb length of the KSHV genome (i.e. vIL6, vMIR1/K3, ORF18/19, ORF26E, K8/K8.1, T0.7, LANA (N-term), ORF75E and UPS75). Analysis at the nucleotide rather than protein level for each of these 800–1900-bp blocks still shows polymorphism levels ranging from 3 to 8%. Although they have only minor impact on amino acid content, these nucleotide polymorphisms do show essentially the same patterns of subtype clustering as found in the VIP and TMP proteins. Therefore, we can still assess genotype and linkage patterns across the entire constant segment of the genome as well. Additional strain differences have also been reported in the central repetitive region of LANA (Zhang et al. 2000) and in the KSHV microRNA cluster (Marshall et al. 2007).

17.2.3 Modern KSHV VIP (or K1) Divergence Patterns

Unexpectedly, the situations for the KSHV K1/VIP and K15/TMP genes are remarkably different from one another. In the case of VIP, there is a rapid evolutionary process occurring that must be driven by some unknown positive biological selection process. Polymorphisms occur at more than 15% of the nucleotide positions within the VIP-coding region and show an 85% non-synonymous rate, giving rise to amino acid variations totaling 35% (Cook et al. 1999; Lacoste et al. 2000; Meng et al. 1999; Zong et al. 1999). The predominant features of the subtyping observed correlate closely with the time frame of the migrations of anatomically modern humans (= Homo sapiens) into and out of Africa (Hayward 1999; Zong et al. 1999) as is illustrated in Fig. 17.3.

We conclude that the most recent common ancestor of all human KSHV VIP proteins that we have analyzed was only 100,000 years ago. For the 289 amino acid VIP protein, this represents an extraordinarily high rate of evolutionary change of

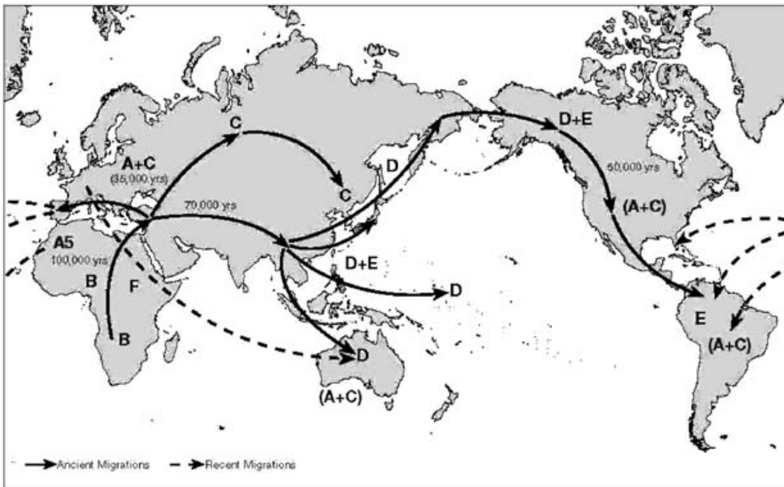

Fig. 17.3 Correlation Between KSHV VIP/ORF-K1 Subtypes and Modern Human Out-of-Africa Migration Patterns. The diagram summarizes the predominant global distribution pattern of KSHV VIP subtypes in different geographical locations. The VIP/ORF-K1 subtypes of the vast majority of KSHV genomes correlate extremely well with the ethnic ancestry of their human hosts according to the inferred evolutionary divergence associated with the migration of human populations over the past 100,000 years. With the major exception of the A5 and F subtypes in sub-Saharan Africa, almost all KSHV genomes of African origin have B-subtype VIP genes, those derived from South Asia and Pacific Rim (Austrasian, Austronesian, Polynesian and Amerindian) branch migrations have VIP-D or E subtypes, and those of European, North African the Middle East (Levant) and North Asian origin have VIP-A or C-subtypes. The A5 subtypes in Africa all derive from a single Eurasian-like clade that evidently re-entered and spread across Africa very recently (maximum of 4000 years ago) as chimeras in otherwise ancient African genome backgrounds. The KSHV VIP-E genes found in native Amerindian populations have a common origin with the Austrasian/Austronesian/Polynesian branch and diverged from the other known variants in this branch about 40,000 years ago

approximately one amino acid per 1,000 years. Thus the B-subtype VIP proteins all remained in and spread across sub-Sarahan African from their origins in East Africa about 100,000 years ago, and differ from the prototype A-subtype by 30%. A second D/E-branch migrated out of Africa to South Asia, Australia and the Pacific Rim starting 70,000 years ago and differs from both the A/Cs and Bs by 25%. Finally, the third A/C-branch split into two major subgroups represented by the A- and C-subtypes (15% diverged) that became distributed throughout Europe and North Asia starting about 35,000 years ago. The A and C branches have both in turn radiated into between 7 and 10 further distinctive clades, each roughly corresponding to the expansion of Eurasian populations from a limited number of Iberian and other ice age refuges some 10,000–15,000 years ago.

Based on the positions of nodes and the lengths of the branches in the VIP phylogenetic tree (Fig. 17.4), we judge that the precursor of the Austrasian/ Austronesian branch evidently split into the D- and E-subtypes some 50,000 years ago. Each in turn then diverged into three or four more clades beginning about 40,000 years ago, with at least one clade now resident in Australian aborigines (D3), another in Polynesians (D2), two more in the Hwalein aborigines in Taiwan (D1 and E2), one or more in the Ainu in Hokkaido, Japan (D4) and two others (E1) in South Amerindian populations. These presumably represent the spreading of the virus with its human hosts into and around the islands and coastal rim of the Pacific Ocean (Biggar et al. 2000; Meng et al. 2001; Meng et al. 1999; Whitby et al. 2004; Zong et al. 1999). Although three of the four predominant mitochondrial haplotypes in Amerindians are derived from Mongolian/North Asian haplotypes, the fourth has features in common with a novel haplotype found in Melanesian, Polynesian and other South Asian populations. Therefore, this pattern is acceptably consistent with the known origins of Amerindians, although the divergence of their KSHV subtypes (40,000 years ago) clearly occurred long before they are usually thought to have first arrived in the American continent, which is only about 13,000–20,000 years ago.

All B-subtype VIP genes appear to have had their origins in sub-Saharan Africa, but there are also three other VIP clades now found in sub-Saharan Africa. One of these is the very rare F-subtype, which is intermediate between the D/E and the A/C branch patterns (Fig. 17.4) and likely either arose independently in Africa or returned there from the Levant (Fouchard et al. 2000; Kajumbula et al. 2006; Whitby et al. 2004). There are also several other rare variants on the A/C branch such as the C7 subtype in Uganda that probably returned to sub-Saharan Africa via a Nilotic group from North Africa (Fouchard et al. 2000; Kajumbula et al. 2006; Whitby et al. 2004). Finally, there is the remarkably abundant but very narrowly diverged A5 clade found in 40% of all African samples. The A5 VIP gene clade seems to have arisen by re-entry into sub-Saharan Africa no more than about 4,000 years ago from the Mediterranean or North Africa. It has evidently then subsequently spread as a chimeric A5/B genome throughout the entire continent during the great Bantu expansions starting from Bok in Nigeria some 3,000 to 4,000 years ago (Kajumbula et al. 2006; Kasolo et al. 1997; Zong et al. 2002).

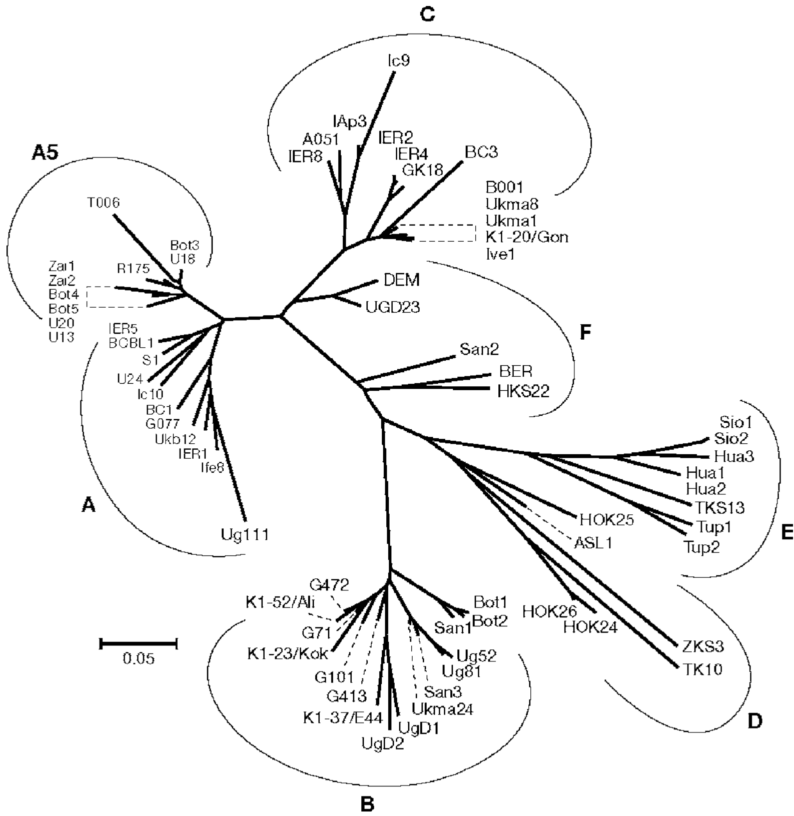

Fig. 17.4 Radial Phylogenetic Tree for Selected KSHV VIP/ORF-K1 Protein Sequences. The diagram illustrates the genetic/evolutionary relationships among all known VIP-D, E and F protein sequences relative to selected examples of the Eurasian VIP-A, C, and African B subtypes. The 12 samples of Pacific Rim origin include TK10 (D1, Taiwan aborigine), ASL1 (D2, Australian aborigine), ZKS3, 4 (D3, Polynesian), HOK24, 26 (D4, Ainu, Hokkaido, Japan), HOK25 (D5, Ainu, Hokkaido, Japan), TUP1,2 (E1, Tupi, Brazil), SIO1,2(E2, Ecuador), HUA1,2,3 (E2, Ecuador) and TKS13 (E3, Taiwan aborigine). The five anomalous (non-B1, B2, C7 or A5) African "intermediate" origin samples include 43/BER (F1, France), UDG23 (F1, Southern Uganda), 8/DEM (F2, Central African Republic), SAN2 (F3, San, Botswana) and HKS22 (F4, Gisu, Uganda). (Biggar et al. 2000; Hayward and Zong 2006; Kakoola et al. 2001; Lacoste et al. 2000; Meng et al. 2001; Whitby et al. 2004; Zong, et al. 1999)

17.2.4 Older Constant Region Subtypes and Chimerism in KSHV

The ethnic and geographical distribution of KSHV VIP subtypes fits remarkably well with the general tenets of the Out-of-Africa modern human migrations, but there is one glaring discrepancy when compared with the distribution of mitochondrial and Y-chromosome haptotypes of their human hosts. That discrepancy is the lack of much more widely diverged forms of VIP within

sub-Saharan Africa. All the humans who came out of Africa represent a single relatively narrow sub-branch from just one (L1) of the three major mitochondrial haplotype patterns (L1, L2 and L3) found within Africa. Yet for KSHV, the VIP subtypes in Eurasia cover a greater range of diversity than all the B-subtypes found in Africa, which appear to have split off from a common precursor into two branches (B1 and B2) and then both broadly diverged starting about 25,000 years ago. In essence, all the expected much older variants of VIP in Africa seem to have disappeared. However this is not so for the rest of the genome, where there are in fact up to six distinct subtypes (B, F, G, R, Q and N) of most of the constant region loci. Three of these (R, Q and N) are each at least equally diverged from the A/C versions than are the B-subtype here, and at some loci (especially on the far RHS of the genome) they are even further diverged (two- to four-fold), suggesting an origin maybe as long as 250,000–500,000 years ago.

It is also important to appreciate that chimerism is much more frequent in sub-Saharan KSHV genomes than elsewhere. In fact, many African genomes are mosaics of often two or three or even more segments that mix up B, R, Q or N derived subtype loci (Duprez et al. 2006; Hayward and Zong 2006; Whitby et al. 2004). Genomes that are predominantly B-subtype throughout the constant region are the most common pattern observed, and we have seen only a single genome that is nearly completely N-subtype throughout (except for its A5 VIP gene). Similarly, none that are completely Q- or R-subtypes throughout have been found as yet. Up to one-third of the sub-Saharan genomes also possess relatively short RHS chimeric segments that we refer to as A[B], because they are derived from a Eurasian A/C origin, although often with a distinctive minor (eg. one nucleotide) African-clade-specific polymorphic feature. On the LHS of African genotypes, both the relatively modern B1 and B2 VIP subtype loci, and especially the novel A5 VIP subtype loci, behave as being completely unlinked from the rest of the genome and are distributed virtually randomly in association with constant region genotypes containing predominantly B, R, Q or N constant region segments.

17.2.5 The Three KSHV TMP (or K15) Alleles

In contrast to the LHS K1 or VIP and central constant region patterns, the RHS K15 or TMP genes of more than one-third of Eurasian samples (including all Taiwan Chinese and Korean samples tested), have a completely different type of variant TMP gene, referred to as the M allele, rather than the standard P allele of TMP (Choi et al. 2000; Fouchard et al. 2000; Glenn et al. 1999; Hayward 1999; Kakoola et al. 2001; Poole et al. 1999). A small subset of both the African and the Pacific genomes also have TMP-M alleles. These two forms of the 500 amino acid KSHV TMP protein differ by 70% at the amino acid level, yet within each allele they show no more variation than do constant region

genes. Nevertheless, this intra-allelic variation is sufficient to reveal distinctive Eurasian, African and Pacific Rim forms of both the P and the M allele TMP proteins. This is illustrated in the phylogenetic tree of intact genomic TMP genes presented in Fig. 17.5. African TMP-P and TMP-M differ at the nucleotide level by 0.95 and 1.35% from the equivalent Eurasian forms (both corresponding to about 100,000 years divergence), whereas Pacific Rim TMP-P and TMP-M differ at the nucleotide level by 2.3 and 0.85% from the equivalent Eurasian versions (with the former judged to represent 250,000 years divergence).

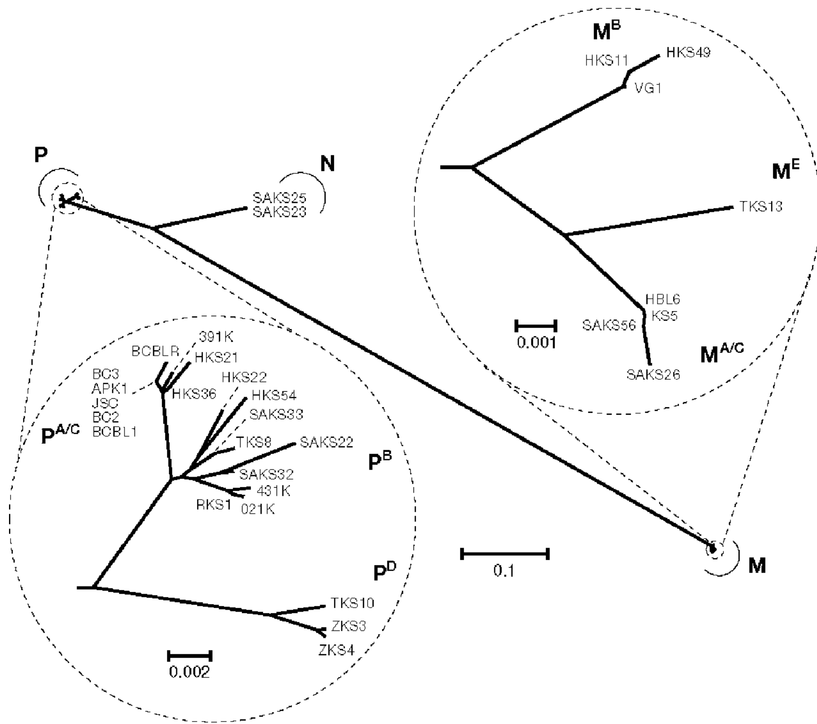

Fig. 17.5 Radial Phylogenetic Tree for KSHV TMP/ORF-K15 Genomic DNA Sequences. The complete approximately 2080-bp sequences (coding exons plus introns) for 31 different TMP-P, two TMP-N and TMP-M genes were compared. The results are displayed on a two-scale diagram with the TMP-P and TMP-M clusters each shown in 10-fold expanded detail in the inset segments. African origin samples include 431 K (P-B1), RKS1(P-B1), 021 K (P-B1), SAKS33(P-B2), FTKS8(P-B2), SAKS32 (P-B3), HKS22 (P-B4), HKS54(P-B5), SAKS22 (P-B6), VG1(M-B), HKS11 (M-B), HKS49 (M-B), SAKS56 (M-A/C), SAKS26 (M-A/CΔ), HKS54(P-AB), HKS21 (P-AB), SAKS23(N) and SAKS25(N). BCBL-R (P-A/C), BCBL1(P-A/C), APK1(P-A/C), BC3(P-A/C), BC3(P-A/C), JSC1(P-A/C), HBL6/BC1(M-A/C), ASM70/80(M-A/C) and KKS5(M-A/C) represent Eurasian samples, whereas TKS10(P-D), ZKS3(P-D), ZKS4(P-D) and TKS13(M-E) represent Pacific Rim samples (Hayward and Zong 2006; Poole et al. 1999)

There is also a rare third N-allele of the TMP protein (Hayward and Zong 2006), which has so far been found only in Southern Africa, with all four known examples three from South Africa and from Uganda being identical. The TMP-N protein differs from the TMP-P protein by 28% (21% at the nucleotide level) and from the TMP-M protein by 65% with most of the conserved functional motifs within the C-terminal tail being more similar to TMP-P than TMP-M (Fig. 17.6). Importantly, both the TMP-M and the TMP-N genes have characteristic-associated adjacent regions that we refer to as M or N-subtype constant region loci and these themselves show the greatest divergence toward the RHS. For example, at the ORF75 locus the divergence of N >> M > B, Q, R relative to A/C. Among Eurasian A/C-, J- and K-subtypes with chimeric RHS

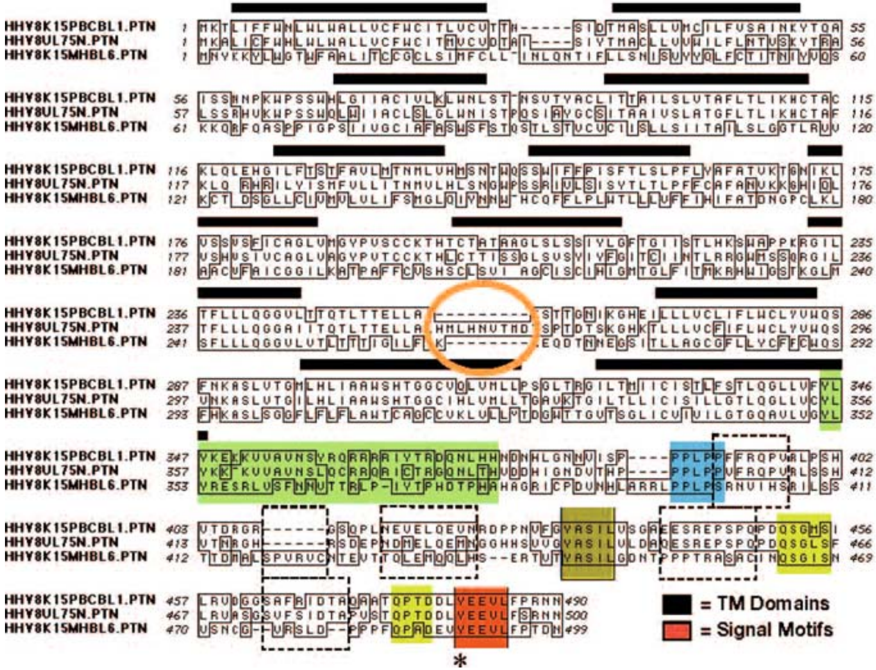

Fig. 17.6 Primary amino sequence alignment and comparison of conserved domains in the P, M and N Allelles of the KSHV TMP/ORF-K15 Protein. The 12 transmembrane domains (underlined) of the three TMP allele proteins show considerably less divergence than the intervening loop domains. Several blocks of potentially functionally relevant amino acids within the cytoplasmic tail of TMP (encoded entirely by exon 8) are highly conserved in all three versions. These include short regions implicated as SH2 or SH3 typosine kinase interaction motifs such as YEEVL and YASIL (*colored red and orange*) and PPLPP/S (*blue*), or the two potential TRAF interaction motifs QSGIS and TQPTD (*yellow*), although a possible third TRAF motif SPQPD is absent from TMP-M (Glenn et al. 1999). A postulated mitochondrial targeting motif is shown in green (Brinkmann et al. 2003). Other short sequence blocks in the cytoplasmic tail that are conserved in TMP-P and TMP-N, but not in TMP-M, or are unique to TMP-M, are indicated by solid and broken line boxes (*See* Color Insert)

M-segments, the lengths of the chimeric segments differ, ranging from the shortest such as BC1/HBL6 consisting of just the TMP gene itself (3.5 kb), up to a few with a maximum of 20-kb that are found only in a K-subtype background as in ASM70/80 (Poole et al. 1999; Zong et al. 1999). Interestingly, these adjacent M-associated sequences, although they are usually at least as divergent from the A/C-constant region pattern as are the B-subtypes, rarely differ among themselves by more than one single bp at any one locus, implying that they had a relatively recent introduction into the human population.

17.2.6 Mechanism and Evolutionary Origins of KSHV Allelic Divergence and Chimerism

The nine combined KSHV constant region loci differ between the prototype A and the B-subtypes by a total of 121 nucleotides out of 9,723 positions tested (ie. close to 1.2% over 100,000 years). Similarly, the prototype Eurasian and African versions of TMP-P and TMP-M differ by 0.95 and 1.3% at the nucleotide level or 1.3 and 2.0% at the protein level. Considering that the constant region loci analyzed were all selected as being unusually highly variable, it is probably not unreasonable for these rates to be an order of magnitude higher than the reported estimated overall average nucleotide divergence rate of $1 \times 10(-7)$ per year per site i.e. 1% per million years) for conserved herpesvirus protein coding regions (McGeoch and Cook 1994; McGeoch et al. 1995). However, it is also very noticeable that in all the KSHV constant region loci (including TMP) the intratypic polymorphism levels observed are at least 10-fold lower than the intertypic rate. In dramatic contrast, the prototype VIP-A and VIP-B genes differ by 15% at the nucleotide level and 30% at the protein level over the same 100,000 year time period, for which there obviously must be some very powerful selective pressure coming into play. We estimate that the evolutionary rates for the VIP and TMP genes are 110-fold and 7-fold faster at the nucleotide level and 250-fold and 12-fold greater at the protein level than for the HSV1 versus HSV2 gB genes.

However, as we have emphasized throughout, the situation with regard to the high KSHV RHS-end variability and alternative TMP alleles is predominantly a matter of acquisition or retention of chimeric relics of much older ancient forms of the virus, rather than because of a currently very rapidly evolving gene-like VIP. In the case of the African TMP-M subtypes, we have detected three different types of adjacent M-region sequence, one common and the other two rare, but each with a distinct recombinant junction point. There are seven examples of the commonest version all representing a significantly diverged but narrow cluster, which shares some 25 distinct African-specific polymorphic nucleotide features within the TMP-M gene. The other two rare TMP-M African versions (one in HKS56 and the other in HKS35 and FTKS2) are obviously both of recent Eurasian origin. Two of the three TMP-M

segments in B-genome backgrounds (including the common African origin one) are only 3.4 and 3.6-kb in size, and both have chimeric junctions lying just downstream from (i.e. to the left of) the C-terminus of the leftwards-oriented TMP gene itself. The other rare African TMP-M variant (4.6 kb in size) extends further into ORF75 within an R background genome. Note that all other African genomes studied that have ORF75 R-segments (nine examples) also have a single common R/A chimeric junction at about the same location, which joins otherwise African genomes to a Eurasian A/C-like ORF75 plus TMP-P RHS segment. The single example known of a Pacific Rim TMP-M gene (TKS13) forms a similar chimera in an E-subtype background that is also very short (3.4 kb), but with a more complex junction boundary.

We have proposed previously (Hayward and Zong 2006; Poole et al. 1999; Zong et al. 2002) that the TMP-M allele arose just once in evolutionary history by a two-step chimeric process. First, an exotic TMP gene from an old-world primate KSHV-like source virus, that must have diverged as much as 20 million years ago, was transferred into an ancient humanoid KSHV-like virus background (such as that of Neanderthal or Homo erectus or Homo habilus), that had itself diverged from the modern human virus by up to 500,000 years. Second, during much later contemporaneous occupation of an overlapping geographic location, presumably in the Levant or Middle East some 25,000–30,000 years ago, a segment of at least 20-kb in size from the RHS-end of this Neanderthal-like virus was recombined into a modern human P-background virus with a C2-VIP and K constant region subtype genome. Finally, the chimeric M-subtype-associated segments became steadily shorter as they were spread first to other K and J, and then a few A/C-subtype Eurasian genomes (and even occasionally into B-subtypes) by rare secondary homologous recombination events.

But then what can we make of the two other significantly diverged African and Pacific Rim-specific versions of the TMP-M allele chimeras? Could all three versions of the modern P/M genomes have arisen instead from a single chimeric event prior to the modern human migrations out of Africa, or alternatively did the Neanderthal virus itself also undergo its own founder effect branching into at least three subtypes, with the three versions of the chimeras that we see now having arising from independent human-Neaderthal virus recombination events in each of the three geographic locations? It is certainly possible that all three types of current chimeric P/M viruses are derived from a single precursor P/M chimeric virus generated before the migrationary Out-of-Africa diasporas. However, the chances that both a single prototype P/M chimeric virusand a single prototype standard P genome viruswere carried by the presumed very low population size founder groups into all three geographic branches during the human migrations seem to be extraordinarily remote. Instead, a scenario in which variants of the original chimeric M virus also independently made their way into the Middle East, Southern Africa and South Asia through earlier Neanderthal migrations and only a RHS segment was later transferred (once each) into the standard human Eurasian P-C,

African P-B and Pacific Rim P-D/E lineages seems rather more plausible. Presumably, the postulated complete intact parental Neanderthal M-genome (like its hosts) has since become extinct. Alternatively, we cannot of course completely exclude the possibilities that the intact KSHV-M virus genome actually persisted as a second virus of this type within the modern Homo sapiens lineage itself and may still exist. But if so, it would seemingly have to occupy a different biological niche than the KSHV P and P/M genomes, because it has yet to be detected and is clearly not associated with Kaposi's sarcoma samples.

Another striking observation has been that the predominant KSHV genotypes found among our South African samples are very different from those found in Uganda and most of the rest of sub-Saharan Africa (Hayward and Zong 2006). Most dramatically, six of the eight genomes that we have found that contain either or both TMP-N or N-constant region segments came from among our 14 analyzed South African KS biopsies. One of the others was from Uganda and the seventh came from a Mozambique San Bushman. These particular South African samples came from a mining area which includes a high proportion of individuals with mixed Zhosa or Khoisan ancestry, whereas the Ugandan samples came largely from individuals of Ganda or other Bantu tribal ancestry (Alagiozoglou et al. 2000; Kajumbula et al. 2006; Treurnicht et al. 2002). Furthermore, we have begun analyzing mitochondrial CRS DNA sequences from all of our samples and have found that many more from the South African set than from the Ugandan set have the most anciently diverged human African haplotype patterns, including several with the distinctive Khoisan signature pattern. Logically, KSHV N-subtype genomes and possibly some Q-subtypes (Whitby et al. 2004) have their origins within and are still largely confined to some of the most ancient of modern human populations that never left Africa. In addition, several RHS constant region segments of these genomes (e.g. the T0.7 locus Q-subtype, as well as the ORF75 N-subtype) evidently predate the origin of modern humans, and the TMP-N allele itself must also have had an exotic origin, probably diverging some 3–5 million years ago, and being acquired somewhat like the TMP-M allele by several sequential and very rare cross-species recombination events. Even the Pacific Rim version of the TMP-P allele is 2.3% diverged from both the African and the Eurasian versions (which themselves differ by only 0.95%) indicating that it too is likely to have been derived as a chimera from another hominoid version of the KSHV P virus that diverged prior to the Out-of-Africa migrations.

A similar rare interspecies chimeric event followed by a trimming down process to fix just a single small segment (as a biologically important gene that is too diverged to be able to undergo homologous recombination) presumably also accounts for the origin of the second EBNA2 allele in a subset of human EBV genomes. Therefore, this is probably indicative of a general process by which herpesviruses typically evolve over relatively long periods of time. A variation of this process is also the most likely explanation for the hypervariable genes and clustered subtypes in HCMV as well. Presumably in that case, they

represent widely diverged segments of ancient humanoid HCMV genomes that were scrambled into mosaics by recombination probably long before the origin of modern Homo sapiens. Although most of the parent-diverged humanoid HCMV genomes and their chimeric derivatives have long since been lost, unlike in the case of KSHV, we interpret that many different "strains" of HCMV were carried with each of the founder populations during the recent Out-of-Africa migrations. However, even these are still a finite and relatively small number compared to the expected number of original variants. Furthermore, and again very unlike KSHV, the much higher frequency of horizontal transmission by HCMV may in addition have since further spread most of the remaining HCMV strains across the geographic and ethnic boundaries created by the migrations.

17.2.7 Biological Selection of Chimeras

Why have only certain genes (i.e. the 10 hypervariable ones in HCMV) maintained large and variable numbers of subtype clusters, whereas many other adjacent genes do not show anywhere near this same level of diversity? Although this is not easy to rationalize or understand at present for HCMV, we believe that it is quite obvious in KSHV that both the VIP and the TMP subtypes (or more correctly certain chimeric combinations) have been undergoing extensive biological selection. In the case of the TMP genes and the RHS side of the constant region, there must have been (and possibly still is) some selective advantage for retention of chimeras with older M-allele variants in a modern P-allele genomic background within Eurasia. In contrast, for the LHS VIP genes, there has evidently been selection against retention of all the older types of VIP genes within Africa. The resulting chimeras contain modern African B1, B2 or Eurasian-derived A5 VIP genes associated with older R, Q and N constant regions that were preferentially retained compared to genomes with R, Q or N VIP genes. In fact, we have not detected a single KSHV sample that does not have a modern P-subtype VIP gene, whereas all the original R, Q, N or M-subtype K1/VIP genes have evidently been eliminated. Furthermore, the A5 VIP chimeras have seemingly spread throughout sub-Saharan Africa and been introduced into the most ancient genomic backgrounds seemingly just within the past 4,000 years. This latter process has presumably been facilitated by the rapid expansion of the host Bantu populations, with the associated displacement or assimilation of other population groups. But for the virus itself, this must also include a dramatic selective advantage for retention of the new recombinant Eurasian-like A5-subtype VIP chimeras in B, R, Q or N chimeras. Prior to that, the modern B-subtype VIP chimeras must also have been preferentially retained in otherwise more ancient KSHV R, Q and N genotype backgrounds, with an accompanying displacement of all the older previous VIP gene variants.

17.2.8 Lack of Specific Pathogenicity Associations Among Subtypes

It is necessary to emphasize that the evidence is against there being any specific disease associations for individual KSHV subtypes/genotypes or alleles. The range of KSHV subtypes found in PEL or MCD versus KS disease in the United States was described in some detail previously (Zong et al. 2002). There were distinct biases found in the subtypes of KSHV among classical KS cases versus among AIDS-associated cases in the USA. However, this probably simply reflects the tendency toward central European and Mediterranean origins for most of the classical KS cases and an evidently somewhat limited mobilization of subtypes from existing endemic sources within the United States during the early stages of the AIDS epidemic. The single B-subtype genome found so far in a PEL case (VG1) happens to be B/M chimera, but that is most likely also merely representative of the typical B genotypes found in Haiti and the United States being of that type, which were presumably introduced from West Africa where they are fairly common (Fouchard et al. 2000). The selection that we envisage for preferential spread and retention of certain KSHV subtypes or chimeric genotypes is much more likely to be related to the efficiency of transmission for example than to any particular disease associations. It could perhaps also be reflected in generally higher lytic titres and pathogenicity, or even in an enhanced ability to infect children, as occurs currently within Africa, but not elsewhere. Of course, this latter pattern may only reflect greater opportunities for exposure because of higher seropositivity, greater viral loads, or other epidemiological factors that are specific to Africa.

17.2.9 Current Global Distribution Patterns

The overall patterns of divergence, variability, and selective global distribution of KSHV genotypes can be used to offer a reasonable explanation for the unusual patterns of KSHV infection rates observed in different human populations (i.e. 1–4% seropositivity in Europe, Asia and the United States, and 10–15% in the Middle East and Mediterranean, compared to 50% or more in sub-Saharan Africa). We know that, although both HCMV and KSHV display extensive subtyping in hypervariable genes: (a) KSHV is much less likely to occur as mixed infections; (b) KSHV genomes show evidence of much lower recombination rates between variable loci; and (c) very different subtypes of KSHV are found in Africa compared to in Eurasia or in the Pacific Rim. In contrast for HCMV, there are no uniquely African-specific subtypes, and all the same sets of variants found in Africa, Europe and China are also found in the United States. This can be taken as strong evidence that KSHV is usually much more difficult to transmit in a horizontal fashion than is HCMV and is more typically transmitted in a familial fashion (at least before the advent of the

AIDS epidemic). Therefore, it would seem plausible to interpret that, during and after the modern human Out-of-Africa migrations, KSHV transmission (unlike that of HCMV) has simply not been able to keep up with the combination of human population restrictions and rapid growth. Presumably, the narrowing of European and North Asian populations into a small number of Ice Age refuges, followed by subsequent population explosions out from those refuges over just the past 12,000 years or so also contributed to this effect. In contrast, in Africa, there were no such refuge/expansion bottlenecks, and although there were recent large population shifts such as the Bantu expansion, these were likely displacing or assimilating indigenous populations that were already in contact with many older variants of the virus and had been for a much longer period of time than either the European or Asian populations.

Overall, the subject of KSHV (and HCMV) subtypes and the possible effects of genetic variability on the biology and evolution of these viruses are well worth much further study. However, the complexity of both of these very large herpesvirus genomes precludes the use of any simple uniform nomenclature to categorize different strains. Subtypes can really only be determined at the single gene level, and the entire genotypes can thus only be defined in a cumbersome, multifaceted, additive fashion. The overall genotype patterns observed for individual KSHV genomes tend to primarily reflect the ethnic and geographic ancestry of their particular human hosts. The different subtypes have their origins in ancient patterns of viral evolutionary divergence, associated with human migration and founder effects, but these are often complicated further by chimeric recombination events. Finally, it is very unlikely that there are any KSHV strain-specific features that are of direct clinical or predictive relevance to disease progression or treatment, unless therapies directed at the VIP, TMP or other hypervariable genes/proteins should become available in the future.

Acknowledgments The studies on strain variability at Johns Hopkins were funded at various times by NIH Research Grants to GSH from the National Cancer Institute and the Institute of Allergy and Infection Diseases, namely R01 CA81422, P01 CA81400, R01 CA73585 and R01 AI24576. Studies at NCI-Frederick were funded through contract N01-CO-12400 from the National Cancer Institute, National Institute of Health, Department of Health and Human Services. The authors would like to thank Rachel Bagni for phylogenetic analysis and assistance in the preparation of the figures. We would also like to thank Allen Kane for assistance with figures.

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Chapter 18
Introduction to Diseases Associated with Kaposi's Sarcoma-Associated Herpesvirus

Patrick S. Moore and Yuan Chang

Investigation of Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8) has opened new areas of research in basic cell biology, tumor virology and clinical medicine. KSHV is the cause of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and some forms of multicentric Castleman's disease (MCD), as well as some nonmalignant syndromes such as acute bone marrow failure. Studies characterizing this virus stand as a model for investigating the role of a newly discovered virus in human disease. The initial description of KSHV in 1994 was based on fundamental observations extending back over a century.

18.1 Kaposi's Sarcoma

Kaposi's sarcoma (KS) was first described by the Austro-Hungarian dermatologist Moriz Kaposi (Fig. 18.1) in 1872 as an "idiopathic multiple pigmented sarcoma of the skin" (Kaposi 1872). Kaposi was born Moriz Kohn in 1837 to a poor family in the Kaposvár region of Austro-Hungary and changed his name to Kaposi in 1871. With the support of his family, he completed university medical studies and training in obstetrics and gynecology. He took an active interest in venereal diseases after joining the medical faculty of Vienna University as a docent professor in syphilology. At the time, dermatology and venerology were intimately linked disciplines and it was a natural evolution for his career to join Ferdinand von Hebra's dermatology department. Von Hebra, a leading authority on diseases of the skin, would be later credited as the founder of the Vienna School of modern dermatology.

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_18, © Springer Science+Business Media, LLC 2009

Fig. 18.1 Moriz Kaposi, 1837–1902

Kaposi married von Hebra's daughter Martha and subsequently succeeded him as chair of the department on von Hebra's death in 1880. His rapid rise – together with a sometimes brusque personality – may have earned Kaposi jealously from his contemporaries that colors modern interpretations of Kaposi's academic career (Holubar and Frankl 1981). His name and denomination change, as well as his marriage to von Hebra's daughter, have sparked active speculation about Kaposi's integrity – criticisms that have been largely dispelled by careful historical investigation (Holubar and Fatovic-Ferencic 2001). Kaposi claimed at the time, as well as later, that he changed his name on the cusp of the publication of his most important work to avoid confusion with five other similarly named physicians. Regarding his social circumstances, the political climate for anti-Semitism in Austro-Hungary was mixed. After the 1867 emancipation, Vienna became a refuge for Jews and other ethnic minorities escaping more repressive countries. Many notable faculty at the University of Vienna were Jewish, and Sigmund Freud, Gustav Klimt and Anton Bruckner were Viennese contemporaries of Kaposi – as well as Karl Leuger, the virulently anti-Semitic Mayor of Vienna who inspired later Nazi policies. Regardless of his unknown motivations, Kaposi (Kohn) changed his name several years after marrying Martha von Hebra and their marriage was long lived and ostensibly happy.

Kaposi is remembered principally for introducing detailed pathologic descriptions of dermatologic disorders. In addition to KS, Kaposi first described xeroderma pigmentosum, a genetic disorder of nucleotide excision repair (Wood et al. 1988), and published comprehensive dermatologic descriptions of systemic lupus erythrematosis, syphilis and other disorders. Together with von Hebra, Kaposi is credited with the guiding the University of Vienna toward becoming the leading center for dermatology in the second half of the nineteenth century.

Kaposi's initial description of KS was based on five case reports of late middle-aged men. His report was prompted by earlier skin sarcoma clinical descriptions from Heinrich Köbner who described Köbner's phenomenon – in which cancers including KS arise from a site of previous scarring (Janier et al. 1990; Seckin et al. 1998). Two of the five patients presented with gastrointestinal dissemination and died within months of diagnosis. Kaposi also described a 10-year-old child with the disorder – a patient of the famous surgeon C.A.T. Billroth – who died within a year of diagnosis. These initial patients are unusual for their young age and the aggressiveness of their disease, which has led to an interesting but errant speculation that Kaposi discovered the earliest AIDS cases (Breimer 1984). KS tumor cells have a characteristic spindle cell appearance and form disorganized microvascular channels that fill with blood with hemosiderin deposition, frequently giving the tumor a pigmented or bruise-like appearance. KS is notable for generally being a mixed tumor with prominent organized neo-angiogenesis and inflammatory cell infiltration. Subsequent pathologic descriptions of KS would reveal that the tumor is a nonsarcomatous malignancy of lymphatic endothelial cell origin (Dorfman 1988).

The form of KS described by Kaposi became known as classic KS and is characterized as a relatively indolent tumor mainly affecting the elderly and most commonly occurring among persons of Mediterranean ethnicity. Classic KS has two clinical hallmarks: it is a rare tumor occurring at an estimated frequency of approximately 0.3 cases per 10,000 per year in the United States (Eltom et al. 2002) and it is a relatively benign tumor. Unfortunately, these features are not valid for KS in other settings.

By the mid-1950s, it was evident that both a more prevalent and a serious form of KS is hyperendemic in some regions of Africa. By the time of a 1961 landmark conference on Kaposi's sarcoma, KS was recognized to be common in Africa, particularly in central and eastern African countries where this tumor accounted for up to 10% of cancers reported in cancer registries (Davies and Lothe 1962; Oettle 1962). Unlike classic KS, African or endemic KS occurred in children who have particularly aggressive disease with a nearly uniform fatal course (Davies and Lothe 1962; Ziegler and Katongole-Mbidde 1996). KS in African adults also has more serious consequences than classic KS with untreated secondary complications frequently leading to disability and death.

The curious occurrence of KS in Africa first led to hypotheses that this tumor may have a viral origin (Oettle 1962). This seemed to have been confirmed by an unusual group of studies performed in the 1970s on African KS tumors by Giraldo and colleagues. This group reported in 1972 the isolation of cytomegalovirus from

tumor cell cultures explanted from KS lesions (Giraldo et al. 1972). Subsequent serologic studies among classic KS patients seem to confirm this (Giraldo et al. 1975), and cytomegalovirus became the leading candidate cause of KS. These studies, however, were eventually discounted after extensive examinations failed to confirm a role for cytomegalovirus (Ambinder et al. 1985). KSHV lytic antigens can cross-react with those of other herpesviruses (Moore et al. 1996; Simpson et al. 1996), and it is possible that Giraldo and colleagues saw molecular traces of KSHV infection using cytomegalovirus assays. KSHV, however, is rapidly lost from explanted KS tumors (Collandre et al. 1995; Flamand et al. 1996), so some of these reports are at odds with our current understanding of KSHV biology. Ironically, in 1984, Walter and colleagues reported electron microscopic detection of herpesvirus-like nuclear inclusions in spindle cells from an African KS tumor (Walter et al. 1984), but owing to the long-standing controversy over a role of cytomegalovirus in KS, this report was quickly discounted. In retrospect this seems likely to have been the first reported identification of KSHV in KS tumors.

The patterns of KS throughout the world remain perplexing because of the hyperendemicity of KS in Africa. Prior to the emergence of AIDS – which markedly skews expression of KS – the worldwide pattern of KS was consistent with an epicenter in central-eastern Africa and declining rates of disease spreading in concentric rings to Middle East and Mediterranean countries reaching very low rates of disease in Asia, Europe and the United States. This pattern is most consistent with new introduction of the KS agent into the human population in early historical times, perhaps from an African zoonotic source, and subsequent spread to neighboring populations.

After discovery of KSHV, however, this picture became much more complex: molecular epidemiologic studies by Hayward and colleagues (Hayward 1999) revealed that KSHV genetic variation closely followed the pre-historic outmigration patterns of humans from Africa. Indeed, discovery of pockets of KSHV infection among indigenous populations on Taiwan (Zong et al. 2002), Okinawa (Kamiyama et al. 2004) and the Amazonian Basin (Biggar et al. 2000) suggests that this is an ancient infection that has coevolved and migrated with humans. Discoveries of KSHV-like viruses among chimps and gorillas (Lacoste et al. 2000) and more distantly related rhadinoviruses among lower primates (Desrosiers et al. 1997; Rose et al. 1997) add to the likelihood that KSHV coevolved with humans. At present there is no parsimonious explanation to account for the worldwide patterns of KS. Potentially, KSHV, unlike other human herpesviruses, was never ubiquitous among humans in ancient times and a secondary epidemic spread of infection from central-eastern Africa took place after the African outmigration 80,000 years ago. Regardless, Africa remains hyperendemic for KSHV infection and KS, which is now the leading cause of cancer in many African countries (Bassett et al. 1995; Wabinga et al. 1993). Failure of the public health community to address African KS in a meaningful way is discouraging.

The emergence of KS as an iatrogenic complication in the 1970s, particularly among transplant patients, gave additional clues to the origin of this enigmatic

tumor (Harwood et al. 1979). As in African endemic KS, tumors arising among transplant patients occurred among a younger population and were more aggressive than classical KS. Using tumor registries of transplant patients, Penn demonstrated that KS is a common and severe complication of transplantation (Penn 1979), resulting in case-fatality rates in excess of 40% (Mendez and Paya 2000). The importance of immune surveillance for this tumor was demonstrated when it was realized that the tumor would often resolve in these patients if immunosuppression was removed, resulting in loss of the allograft. The patterns of KS among transplant patients also suggested the possibility that a viral agent was transmitted by organ transplantation – a supposition that was subsequently borne out by Parravicini and colleagues (Parravicini et al. 1997). KSHV-infected cells from the donor, rather than free virus, can generate tumors in transplant patients in some circumstances (Barozzi et al. 2003) similar to cellular transmission of transmissible canine sarcoma (Murgia et al. 2006).

The final blow to the idea that KS is both rare and benign emerged from multicenter reports of KS occurring among previously healthy men in 1981 (Haverkos and Curran 1982; Jaffe et al. 1983). Co-occurrence of KS with pneumocystis pneumonia, an infection most commonly seen among transplant patients, suggested the emergence of acquired immunodeficiency. This rapidly exploded into the current AIDS pandemic affecting every country in the world today. At early stages of the epidemic up to 40% of AIDS patients were diagnosed with KS that could frequently disseminate to internal organs and cause fatal hemorrhaging. As well-known figures in the arts and public life such as Keith Haring, Rock Hudson and Freddie Mercury died from this disease, Kaposi's sarcoma emerged from its previous status as an obscure, enigmatic medical condition.

From the earliest days of the AIDS epidemic, KS was noted to specifically afflict gay and bisexual AIDS patients, suggesting the possibility that the cause of this cancer might be sexually related. Early hypotheses on the origin of AIDS-KS included use of nitrite inhalants used as sexual stimulants (Haverkos et al. 1994), multiple antigen stimulation (Levy and Ziegler 1983) and HIV tat protein (Ensoli et al. 1990) as inducers of KS. Considerable research also focused on the possibility that KS is nonspecifically induced by inflammatory cytokines generated during the course of HIV disease (Ensoli et al. 1992; Miles et al. 1992; Nair et al. 1992).

Confusion and contradictions over the likely causes of KS were clarified once Beral (Fig. 18.2) and colleagues carefully examined the epidemiologic patterns of AIDS-associated KS (Beral et al. 1990). These authors characterized known patterns of KS and came to several important predictions. KS is likely to be caused by an exogenous infectious agent that is uncommon in the general US population and present at high frequency in African populations. The agent is likely to be highly transmissible by homosexual activity but poorly transmitted through heterosexual activity or by blood transfusion. Furthermore, they postulated that infection with the KS agent is likely to be asymptomatic in healthy persons but will cause tumors among immunocompromised persons.

Fig. 18.2 Valerie Beral, Oxford University, 1990

Importantly, HIV itself is unlikely to directly cause KS since some HIV-positive populations (e.g., hemophilics) rarely develop the tumor and other populations without HIV infection (e.g., transplant patients) do. These predictions did not match known infectious agents making it likely that an undiscovered virus was the cause of this tumor.

18.2 The Search for the KS Agent

Over 20 different infectious agents had been proposed and later discarded by 1993 as possible infectious triggers for KS. Based on the Beral studies and the assumption that KS is caused by an exogenous virus, we (Fig. 18.3) began a directed search for the agent. Although married, neither of us had previously worked together on a research project. Patrick Moore was trained as an epidemiologist and was then working for the CDC. He had recently investigated

Fig. 18.3 Yuan Chang and Patrick Moore, Columbia University 1995

a hemorrhagic yellow fever epidemic in Delta State, Nigeria, in which delays in diagnosing the viral infection led to substantial worsening of the epidemic. Successful development of molecular methods to identify unknown viral agents could potentially overcome many of the limitations of traditional viral diagnostics. He suggested to Yuan Chang that representational difference analysis (RDA) (Lisitsyn et al. 1993), a PCR-based subtractive hybrization and kinetic enrichment technique that had been recently described, might be able to identify viral nucleic acids from infected cell genomic material.

Chang was trained as a neuropathologist and had just started a faculty position at Columbia University with only a small lab bench and a $20,000 research budget. She was shortly joined by Moore who took a job in the New York City Department of Health. In the early summer of 1993, a young Hispanic gay man died of AIDS-KS and came to Columbia University's Department of Pathology autopsy service. This was unusual since by this stage of the AIDS epidemic very few AIDS patients underwent autopsies. Chang and a pathology resident, Melissa Pessin, performed RDA on this patient's KS tumor which generated four discrete DNA bands. In retrospect, these experiments were highly fortuitous: not only would this be the last AIDS-KS patient autopsied at Columbia University for over a year, but this patient's tumor would later be found to contain an unusually high copy number of the virus. From a practical point of view, there was only one chance for this technique to work given the available research resources. Although each band was cloned and sequenced, database searches at the time were too primitive to allow identification of the source of the DNA fragments.

Pessin and Chang turned to a fellow junior faculty member, Ethel Cesarman (Fig. 18.4), who maintained a collection of KS and AIDS-related tissues to confirm whether or not any of the RDA fragments were specifically associated with KS. If the DNA fragments were of human origin, all tissues should show

Fig. 18.4 Ethel Cesarman, Columbia University 1993

positivity of the DNA fragments. Initial Southern blotting revealed that two bands were of human origin whereas the provenance of the remaining two bands remained unclear. Both DNA sequences were present more commonly in KS tumors, but one control tissue sample also showed intense hybridization exceeding any of the other KS samples. The control specimens in this experiment were non-KS tissues also from AIDS patients. The positive sample belonged to an unusual AIDS-related lymphoma frequently infected with Epstein–Barr virus (EBV or HHV4) and presenting as a pleural effusion (Knowles et al. 1989).

Presence of the two RDA sequences in a non-KS tissue was intensely discouraging since this suggested that the fragments were not specific for KS. Nonetheless, the remaining results revealed that the two RDA bands, KS330 and KS631, were present in most of the KS tissues and not present in the remaining control samples.

Resolution of this dilemma only came from testing more samples, both by Southern hybridization to assure specificity and by polymerase chain reaction to achieve high sensitivity. This time, 27 KS tumors were randomized and blindly tested together with large panels of AIDS-related and non-HIV-infected tissues. The results were striking: 25 of the 27 KS tumors were positive for the RDA sequences. Re-examination of the two negative KS tumors revealed that one was a mislabeled kidney sample and the other contained degraded, non-amplifiable DNA. In contrast, KS330 and KS621 were not found in a large panel of HIV-negative tissues and were present in only a portion of lymphatic tissues taken from AIDS patients. By examining multiple tissue samples from individual KS patients, it was evident that the KS330 and KS631 sequences were not human polymorphisms and that the sequence copy numbers were highest at the tumor and decreased in more distal tissues – a crude dose–response relationship.

Despite evidence that KS300 and K631 tracked with KS, the likely agent was still unknown. Chang had trained with Frank Lee at DNAX Laboratory and worked with him to help fish out extended sequences flanking the KS330 site. More importantly, publicly available BLAST (Altschul et al. 1990) sequence alignments became available from the National Institutes of Health. When KS631 and K330 together with its adjoining sequences were examined by BLAST alignment, it was obvious that these sequences were similar to but not identical with sequences from the new world monkey herpesvirus, herpesvirus saimiri and human EBV.

A new human herpesvirus had been found infecting Kaposi's sarcoma tumors. It was given its ungainly name – Kaposi's sarcoma-associated herpesvirus, a name born from a committee – over pizza and calamari at the West End Gate Café as an attempt to state that the agent clearly was associated with KS but had not yet been shown to actually cause the cancer. After publication of these experiments, its formal name, HHV8, was prematurely used by many investigators since it was not until many years later that it could be reasonably

assumed that the DNA sequences found in KS lesions represented a single virus as opposed to a group of related viruses (e.g., HHV6A and HHV6B).

18.3 KSHV in Cell Culture

As work on confirming the association between these new herpesvirus sequences and KS was developing, attention was turned to the vexing problem of the initial control sample that had shown such strong Southern blot hybridization intensity. It was quickly confirmed body cavity-based lymphomas (later renamed primary effusion lymphomas (PEL) (Nador et al. 1996)) contained the virus at far higher copy number than KS tumors. Testing of a large panel of other lymphoid tumors failed to show evidence for infection. Early attempts to find evidence for the virus in explanted KS tumor cell lines had been consistently negative, but Cesarman recalled that a cell line had been established from the lymphoma by Giorgio Inghirami years before and then frozen away. These cells were thawed and tested for presence of the new virus. Abundant copies were present.

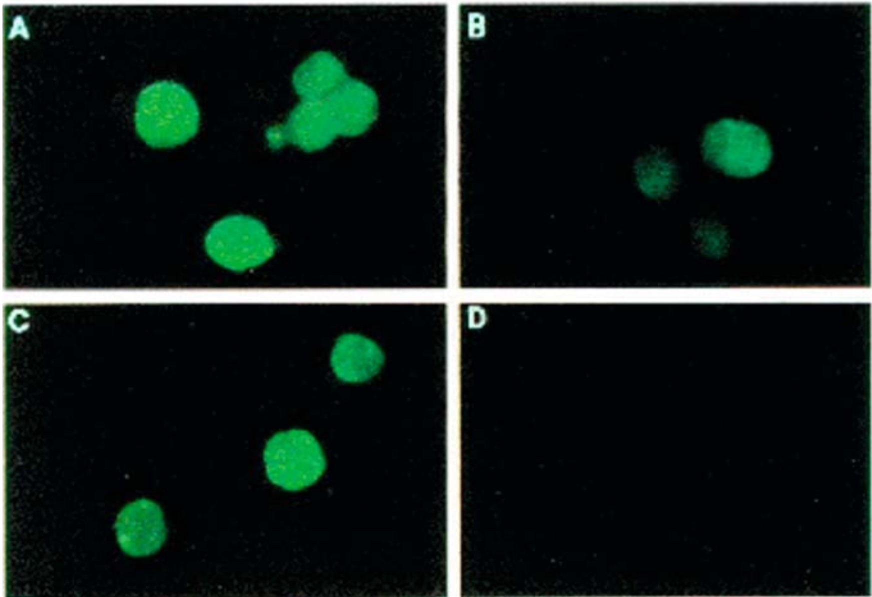

Fig. 18.5 Initial description of LANA1 (Moore et al. 1996). Both AIDS-KS and control patient sera (panels A and B, respectively) immunostain HBL-6 cells but immunoadsorption removes cross-reactive antibodies revealing specific nuclear staining for KS patients (panel C) but not control patients (panel D)

Having KSHV in culture multiplied the lines of research. Moore and Shou-Jiang Gao, a postdoctoral fellow, initially performed indirect immunofluorescence assays using KS patient sera (Moore et al. 1996) and found specific antibodies to a speckled latent nuclear antigen – LNA, later renamed LANA. A problem arose, however, that would plague investigators over the next year. Since the cell lines, like their parental tumor, were coinfected with EBV, adsorption with formalin-fixed EBV-positive cells was needed to eliminate cross-reactive antibodies (Fig. 18.5). This was resolved by Don Ganem at the University of California, San Francisco, who subsequently identified a KSHV-containing cell line free of EBV coinfection (Renne et al. 1986). Like EBV, the new virus could be induced by phorbol esters, and early transmission experiments using filtration co-culture were performed to prove that these sequences belonged to a new virus although nearly 3 years would pass before Foreman and colleagues described a practical transmission system using 293T cells (Foreman et al. 1997).

All studies performed up to this point had been on AIDS-KS patients whose immunocompromised status could lead to an opportunistic but not causal virus infection. Case-control studies of KSHV among classical HIV-negative KS tumors were performed to determine whether or not the virus was present in these samples. Similarly, collaborations were initiated with Robin Weiss (then at the Institute of Cancer Research) and Robert Newton (then at Oxford University) to see if African KS tumors contained the virus.

18.4 Virologic Characterization of KSHV and Kaposi's Sarcoma

In December 1994 the initial description of KSHV was published (Chang et al. 1994). This paper was cautious regarding the likelihood that KSHV causes KS, but substantial unpublished data was already available suggesting its role in disease: KSHV is also present in HIV-negative KS tumors (Chang et al. 1996; Moore and Chang 1995); it is found in body cavity-based lymphomas (Cesarman et al. 1995) and can be grown in body-cavity-based lymphoma, but not KS tumor, cell culture (Cesarman et al. 1995). Sera from patients with KS are reactive to LANA and control patients are not – indicating that unlike other herpesviruses, KSHV is not ubiquitous (Moore et al. 1996).

Publication of these sequences and primers for PCR detection of KSHV led to a period of intense activity in many laboratories (Fig. 18.6). In Taiwan, Su et al. were the first to publish independent confirmation of KSHV infection among AIDS-KS patients and extended these findings to HIV-negative KS (Su et al. 1995). This was rapidly repeated in HIV-negative KS patients (including transplant patients) from the United States and Europe (Boshoff et al. 1995; de Lellis et al. 1995; Dupin et al. 1995; Moore and Chang 1995) as well as KS patients from Africa (Chang et al. 1996; de Lellis et al. 1995; Eto et al. 1996; Schalling et al. 1995).

Since the most widely used detection technique involved polymerase chain reaction, inevitable false positive studies were also reported and would continue

Fig. 18.6 Conferees at the 3rd Annual KSHV and Related Rhadinoviruses Workshop, 1999, Oxford, UK. Shortly after the initial description of KSHV, numerous international groups rapidly began to define the clinical features and biologic properties of KSHV culminating in dedicated international meetings and workshops by 1996. Among the over 200 participants at the 1999 Annual Workshop are (clockwise from upper right) Don Ganem and Frank Neipel's forehead, Dharam Ablashi, Chris Boshoff and Gary Hayward, Thomas Schulz and Andrew Davison, Rolf Renne and Michael Lagunoff, Yan Yuan, and Ethel Cesarman, Dirk Dittmer, Antoine Gessain and Samuel Mbulitaye. Courtesy Dr. B. Chandran

to plague this field. When PCR contamination occurs, low-risk control samples tend to be scored as falsely positive, reinforcing the widely held notion that KSHV is a common human infection. Early reports, for example, suggested that KSHV could be found in a wide variety of non-KS skin tumors. While follow-up studies failed to confirm presence of KSHV DNA in these tumors (Adams et al. 1995; Boshoff et al. 1996), these findings were sufficient to cast doubt on the importance of KSHV in KS (Levy 1995). A major breakthrough was realized, however, when Soulier and colleagues extended the range of diseases associated with KSHV to some forms of Castleman's disease, a B-cell lymphoproliferative disorder (Soulier et al. 1995).

Although correlation between KSHV and KS was clear, formal determination that this virus is the actual infectious cause had not yet been made. Criteria for causality were proposed by the British epidemiologist Austin Bradford-Hill based on his experience investigating a role for cigarette smoking and lung cancer (Hill 1965) and delivered in a famous 1964 lecture (Table 18.1). Several criteria such as association and biological gradient had been fulfilled in the initial publication and evidence that KSHV is generalizable to all forms of KS, in different settings, by different groups rapidly followed. One key criterion for causality that remained unfulfilled was a correct temporal relationship of infection to disease. KSHV infection should precede onset of KS disease if KSHV causes KS. Alternatively, if KSHV is a noncausal passenger virus of AIDS patients, it would be likely that patients would become infected after KS

Table 18.1 The causal criteria used to weigh the evidence associating KSHV with KS and related disorders (Sarid et al. 1999)

Causal criteria	
Strength of association	How frequently is the virus found in KS lesions? Is KSHV infection associated with an increased risk for KS?
Consistency	Are these findings reproducible using different methods and different study populations?
Generalizability	Is the virus present in all forms of KS?
Specificity of association	Is KSHV uniquely associated with KS?
Temporal association	Does KSHV infection precede KS onset?
Biologic gradient	Is KSHV found more often and at higher copy number in diseased than nondiseased tissues?
Biologic plausibility	Is it biologically feasible that KSHV causes KS?
Coherence	Does the data fit with what we know about the natural history and biology of KS?
Experimental evidence	Does controlled manipulation of KSHV infection change the risk of KS?

had developed. This problem was first approached by Whitby et al. (1995) using stored peripheral blood samples from patients with sexually transmitted HIV who subsequently developed KS (Whitby et al. 1995). Similar results were obtained from a study using peripheral blood samples using multicenter AIDS cohort specimens (MACS) in which longitudinal samples were collected (Moore et al. 1996).

Shortly after the initial description of KSHV, Ambroziak et al. published that KS tumor cell cultures lack KSHV (Ambroziak et al. 1995) and that the virus is resident in circulating CD19+ B cells of KS patients consistent with its predicted behavior as a gammaherpesvirus. Evidence that KSHV infects body cavity-based lymphomas (Cesarman et al. 1995) and demonstration that the BC-1 and BC-2 B-cell lines stably harbor the virus (Cesarman et al. 1995) provided additional proof for a B-cell trophism for the virus. Meanwhile, Boshoff and colleagues localized KSHV DNA to spindle cells in KS tumors though microdissection (Boshoff et al. 1995) providing direct evidence that KSHV infects the tumor cells within KS lesions.

Characterization of the virus itself also rapidly progressed. Extended sequencing of the genome confirmed the rhadinoviral lineage of KSHV, the first member of this genus to infect humans. Episome isolation, phorbol induction, filterable transmission and antibody reactivity to LANA among KS patients also removed all doubt that KS330 and KS631 belonged to a new human herpesvirus (Moore et al. 1996). An additional critical insight was provided by Don Ganem and his postdoctoral fellow, Rolf Renne who described a KSHV-infected cell line lacking EBV, BCBL-1, providing the first unambiguous electron micrographs of the virus and also revealing its restricted gene expression profile during latency (Renne et al. 1996). This was followed by more accurate estimates of its genome size and transcriptional mapping of the genome (Renne et al. 1996; Zhong et al. 1996).

Development of a reliable and accurate serologic test for infection remained paramount for determining the importance of KSHV to KS. In the mid-1990s, a standard dogma of herpesvirology was that all herpesviruses are ubiquitous infections of humans. Despite strong molecular evidence that EBV is the infectious trigger of nasopharyngeal carcinoma, African Burkitt's lymphoma and post-transplant lymphoproliferative disorders (1997), this dogma has prevented a uniform consensus opinion from developing that this virus does indeed cause these tumors. If KSHV is similarly ubiquitous, it would not follow the epidemiologic patterns laid out by Beral et al. and hence would be highly unlikely to cause KS.

By screening BC-1 cell immunoblots with KS patient sera, Gao et al. found a unique high-molecular-weight doublet band (Gao et al. 1996) that was subsequently shown to be the latent nuclear antigen (LANA1) encoded by ORF73 (Kedes et al. 1997; Kellam et al. 1997; Rainbow et al. 1997). Immunoblotting could now be used to measure infection, and analysis of cohort samples revealed that the virus has a median incubation period of nearly 3 years before AIDS patients develop tumors (Gao et al. 1996).

Simultaneous identification of EBV-negative, KSHV-positive cell lines, first BCBL-1 and later BCP-1 (Boshoff et al. 1998) and BC-3 (Arvanitakis et al. 1996) provided a simple means to test for LANA1 antibodies without problems of cross-reactivity. This culminated in a pair of studies by Kedes et al. (Kedes et al. 1996) and Gao et al. (Gao et al. 1996) demonstrating that the virus is not ubiquitous in American populations but increases in prevalence in persons at risk for KS and in populations around the world having high rates of KS, such as Italians and Ugandans (Table 18.2). Miller et al. (Miller et al. 1996), Simpson et al. (Simpson et al. 1996) and Lennette et al. (Lennette et al. 1996) developed similar IFA assays based on lytic antigens. Like PCR-based assays, serologic

Table 18.2 Patterns of KSHV infection and non-HIV KS

KS incidence*	Regions	Population KSHV prevalence	Transmission	Risk groups
Low	North America, North Europe, Asia	0–5%	Sexual, iatrogenic	Homosexual men, STD attendees, transplant recipients
Intermediate	Mediterranean, Middle Eastern countries, Carribean	5–20%	Sexual, iatrogenic, nonsexual?	Homosexual men, STD attendees, transplant recipients, older adults
High	Africa, parts of Amazon basin	>50%	Nonsexual, sexual	Children, older adults, lower socioeconomic status

*AIDS-KS rates are highly dependent on local HIV infection rates and risk groups. From (Moore 2000).

studies also became a source of controversy. One IFA study based on cytoplasmic lytic antigens found widespread infection among low-risk adults (Lennette et al. 1996) even though earlier studies had shown that pre-adsorption was needed to eliminate cross-reactive herpesvirus antibodies (Moore et al. 1996). Eventually these controversies died away as methods to detect infection improved. The most comprehensive analysis of KSHV among American blood donors has found that 3–3.5% are infected (Pellett et al. 2003), a rate within the range of error of the earliest studies.

Almost exactly 2 years after its initial description, the long process of mapping and sequencing the KSHV genome was finished (Russo et al. 1996). This revealed that KSHV is remarkable for its molecular piracy of cellular homologs (Moore and Chang 2001). In this relatively short period, the combined efforts by many labs largely defined the basic properties of KSHV. Within 2 years, almost all of Hill's criteria for causality of KS had been examined in multiple ways, leaving little doubt that this new herpesvirus was indeed the infectious trigger for this cancer. Although experimental studies would have to await the results of large-scale clinical trials with antiherpesviral drugs (Martin et al. 1999), the overwhelming weight of scientific evidence had shown that this newly described virus was indeed the KS culprit.

The year 1996 marked an even more important sea change in AIDS-KS as widespread introduction of highly effective antiretroviral drugs started to provide near-miraculous recoveries among end-stage AIDS patients. Reports emerged of disseminated KS vanishing in patients within weeks of starting therapy. Eventually, KS rates among US and European AIDS patients would drop to 10–25% of pre-antiretroviral therapy levels (2000). Control of this epidemic cancer had been achieved by treating the underlying condition rather than the cancer itself.

18.5 Castleman's Disease

In addition to KS, KSHV is routinely found in some but not all forms of Castleman's disease, a disorder first defined by Benjamin Castleman (Fig. 18.7), Lalla Iverson and Pardo Menendez in 1956 (Castleman et al. 1956). Castleman's disease occurs as a proliferative tumor in lymph nodes or other lymphatic organs. Castleman and colleagues described 13 patients with lymphatic masses resembling thymomas with prominent germinal center formation and marked capillary proliferation that were a distinct form of lymphoid hyperplasia. Most of the patients had minimal clinical symptoms and sequelae and would now be classified as having unicentric Castleman's disease as opposed to the more severe multicentric Castleman's disease.

Pathologically, Castleman's disease can be divided into hyaline vascular (HV) or plasma cell (PC) variants, and mixed HV/PC forms (Frizzera 1988; Keller et al. 1972). The HV form is more likely to be confined to solitary site, is amenable to surgical excision, and often has a benign clinical course. The PC

Fig. 18.7 Benjamin Castleman, 1906–1982

variant can also occur as a single circumscribed lesion but tends to have a multicentric presentation involving many lymphoid sites. The distinction between unicentric and multicentric Castleman's disease (MCD) is based more on clinical than pathologic distinctions. MCD is characterized by generalized lymphadenopathy including hepatosplenomegaly, together with systemic signs and symptoms such as fevers, night sweats, increased plasma levels of acute phase proteins, hypergammaglobulinemia and autoimmune phenomenon. Unlike unicentric disease, MCD often runs a more fulminant course, with death occurring from autoimmune hemolytic anemia or secondary cancers. MCD occasionally evolves into non-Hodgkin's lymphoma and the disease has long been noted to be associated with concurrent Kaposi's sarcoma (Chen 1984; Frizzera et al. 1985). Like KS, MCD occurs among AIDS and transplant patients (Collins et al. 2006; Mandel et al. 1993).

Insight into the pathogenesis of Castleman's disease came from Yoshizaki and colleagues in 1989, who found high circulating levels of IL-6 among patients with systemic symptoms (Yoshizaki et al. 1989). The biologic effects of IL-6 include promotion of acute phase reactants, as well as B-lymphocyte proliferation and differentiation, making it plausible that dysregulated production of this pleiotropic cytokine contributes to the disease. Mouse models seem to confirm this relationship in that mice overproducing IL-6 replicate the

pathology associated with MCD (Brandt et al. 1990; De Benedetti et al. 2001). In the mouse model, systemic symptoms are alleviated by immunization of hIL-6 transgenic mice with a hIL-6 receptor agonist (De Benedetti et al. 2001). Furthermore, treatment of Castleman's disease patients with a monoclonal antibody against IL-6 or the IL-6 receptor has therapeutic benefit (Beck et al. 1994; Nishimoto et al. 2005; Nishimoto et al. 2000).

Given the long-noted association between Castleman's disease and KS, Soulier and colleagues examined Castleman tumors for KSHV and found universal infection among HIV+ MCD tumors (Soulier et al. 1995). This subpopulation presented with enlarged lymph nodes which on biopsy and pathologic examination frequently showed changes in nodal architecture consistent with Castleman's disease. Case reports prior to the 1980 s describe concurrent or sequential presentation of KS and Castleman's disease suggesting that HIV is not a required coinfection for these dual tumors (Chen 1984; De Rosa et al. 1989; Dickson et al. 1985; Frizzera et al. 1983; Kessler 1985; Rywlin et al. 1983). HIV-seropositive patients and approximately one-half of HIV-seronegative patients with MCD are infected with KSHV.

Sequencing the KSHV genome revealed that the virus encodes a functional secreted vIL-6 cytokine (Moore et al. 1996; Neipel et al. 1997; Nicholas et al. 1997) suggesting a plausible pathogenic mechanism for the occurrence of Castleman's disease in KSHV-infected individuals. This cytokine activates signaling pathways similar if not identical to the human cytokine (Osborne et al. 1999) . However, vIL-6 bypasses the IL-6-specific gp80 receptor to bind and activate the cytokine signal transducing molecule gp130 (Chow et al. 2001; Molden et al. 1997). HIV-negative Castleman's disease tumors showed prominent cytoplasmic immunoreactivity in B cells, generally restricted to mantle zone lymphocytes (Parravicini et al. 1997). This distribution of vIL-6 is in distinct contrast to hIL-6 which localizes predominantly to cells within germinal centers (Parravicini et al. 1997; Yoshizaki et al. 1989).

KSHV-positive B lymphocytes comprise only approximately 5–10% of cells in the mantle zone. Dupin and colleagues referred to these B cells as KSHV plasmablasts and found them to express high levels of cytoplasmic IgM with almost exclusive λ light-chain restriction (Dupin et al. 2000). This phenotypic profile, together with an absence of mutations in immunoglobulin genes, suggests that plasmablasts originate from naïve B cells. Although monotypic, PCR clonality studies indicate that KSHV infection invokes a polyclonal B-cell proliferation in vMCD (Du et al. 2001). When EBV infection is found in KSHV-infected lymph nodes with Castleman-like changes, coinfection of the same B cell by both gammaherpesviruses is never detected (Du et al. 2001; Parravicini et al. 1997).

The clinical course of KSHV-associated MCD (vMCD) ranges widely. Kaposi's sarcoma is the most frequently reported intercurrent disease, but severe autoimmune hemolytic anemia (Parravicini et al. 1997), fatal hemophagocytic syndrome (Li et al. 2006) and polyclonal hypergammaglobulinemia (Parravicini et al. 1997) have also been observed in association with vMCD. It

is not known whether resolution of symptoms is under-reported, but the literature suggests that vMCD in both HIV seropositive and HIV seronegative individuals carries a high degree of morbidity and mortality (83%) (Oksenhendler et al. 1996; Parravicini et al. 1997). In the setting of HIV infection, some patients have responded to conventional chemotherapy (Oksenhendler et al. 1996); however, recrudescence of disease is common and is strongly correlated with elevated serum C reactive protein, huIL-6, IL-10, and high viral loads in peripheral blood mononuclear cells (Oksenhendler et al. 2000). Unlike KS, treatment with antiviral ganciclovir can ameliorate established MCD (Casper et al. 2004) suggesting that active lytic virus replication contributes to this disease. This is borne out by immunohistochemistry studies showing expression of early and late KSHV replication protein expression in infected MCD cells (Katano et al. 2000; Parravicini et al. 2000). Treatment with anti-CD20 antibodies also has been reported to be highly effective in managing MCD (Newsom-Davis et al. 2004) but may also worsen concurrent KS (Marcelin et al. 2003).

In the setting of HIV infection, one notable sequela of vMCD is its transformation into an entity called KSHV-associated plasmablastic lymphoma/leukemia (Dupin et al. 2000; Jung et al. 2003). These lymphoma cells also show lambda restriction similar to KSHV-infected vMCD cells, but are monoclonal and are thought to arise from intermediary oligoclonal or monoclonal microlymphomas in vMCD lymph nodes (Dupin et al. 2000). Plasmablastic lymphomas cells express CD19, CD45, but not CD138/syndecan, are not coinfected with EBV and display a phenotype consistent with naïve, pregerminal center cells (Du et al. 2001; Oksenhendler et al. 2002).

18.6 Primary Effusion Lymphoma (Body Cavity-Based Lymphoma)

Primary effusion lymphomas (PEL), initially referred to also as body cavity-based lymphomas (BCBL), are a rare subtype of large B-cell lymphoma defined by the presence of KSHV. Knowles et al. first reported three of these lymphomas from AIDS patients in 1989, describing their peculiar indeterminant phenotype and speculating on a possible pathogenic role for EBV present in all three specimens (Knowles et al. 1989). As suggested by its original name, this large cell lymphoma typically forms as an effusion in body cavities; however, later reports showed that these lymphomas can not only have a more typical solid presentation but may also occur outside of serous body cavities. Three of these rare lymphomas were included in the original analysis of AIDS-related tissues in the initial publication (Chang et al. 1994). Unlike KS tumors that harbor virus generally at less than two viral copies per cell, infected PEL cells commonly maintain 30–150 viral genomes in each cell. All cell lines harboring the virus to date have been derived from PEL.

The vast majority of individuals with PEL manifest severe immunodeficiency from either AIDS or extreme old age. PELs are even less common than KS or Castleman's disease even among AIDS patients. PELs are usually coinfected with EBV, yet cases without EBV infection suggest that KSHV is the responsible primary agent with EBV contributing modulatory effects (Fan et al. 2005). Coinfection appears to be more common among AIDS-related PEL although precise estimates are difficult to determine due to the small numbers of reported patients. Among untreated AIDS patients, PEL has a rapid and progressive fatal course generally within 6 months of initial diagnosis, but complete remission has been reported for some patients receiving effective antiretroviral treatment (Hocqueloux et al. 2001; Oksenhendler et al. 1998). Just as KS is aggressive and affects younger populations among HIV patients, a similar clinical picture is seen for PEL patients. HIV-negative PEL patients tend to be elderly and their lymphomas can be indolent for long periods of time (Strauchen et al. 1996).

PEL demonstrates cellular clonality as measured through immunoglobulin gene rearrangements. It is now evident that PEL and MCD represent two extremes of the clinical spectrum of KSHV-related proliferation disorders. PEL is a monoclonal B-cell lymphoma whereas MCD is a polyclonal B-cell hyperplasia. KS is a mixed endothelial cell tumor with features of each: it is commonly polyclonal particularly at early stages but can evolve into a monoclonal neoplasia.

KSHV terminal repeat analysis also shows virus clonality as does EBV when both viruses are found together in tumor cells, suggesting that coinfection occurs at an early stage of the neoplastic process (Judde et al. 2000; Nador et al. 1996; Russo et al. 1996; Nador 1996). KSHV is latent in these tumors, with gene expression restricted largely to the latency locus that encodes LANA, v-cyclin and vFLIP, LANA2 and the kaposin locus. That PEL are rare compared to KS tumors, which develop in 25–50% of persons who are both infected with KSHV and severely immunocompromised, suggests that somatic mutations may contribute to KSHV-related lymphomagenesis. The p16Ink4a locus has been reported to be deleted or hypermethylated in most PEL cell lines (Platt et al. 2002), which have germline p53, bcl-2, c-myc and ras sequences (Nador et al. 1996).

PEL cells exhibit somatic hypermutation in rearranged immunoglobulin variable genes that the tumor has a post-germinal center B-cell derivation (Gaidano et al. 1999). This is also consistent with immunophenotyping in which PELs lack most B-lymphocyte markers but express some antigens associated with plasma cells such as CD138 and EMA (Boshoff et al. 1998; Cesarman et al. 1995; Nador et al. 1996). Gene expression profiling by Klein et al. also demonstrated that PELs have a unique post-germinal center phenotype with features intermediate between immunoblasts and plasma cells (Klein et al. 2003). PELs retain expression of some (CD39, CD30, MUM-1/IRF4) but not all (CD23) immunoblastic markers, while they have acquired most plasmacytic markers (BLIMP-1, CD138) (Klein et al. 2003). Karyotypic studies performed on

cell lines as well as on primary tumor cells generally show a hyperdiploid karyotype with numeric and structural changes. Complete or partial trisomy of chromosomes 7 and 12 and aberrations of the proximal long arm of chromosome 1 were recurring abnormalities (Gaidano et al. 1999; Wilson et al. 2002).

18.7 KSHV and Other Diseases

Several reports are found in the literature of KSHV-associated disorders not mentioned above. These are exceptionally rare and may represent unusual host responses to viral infection. Du et al. reported three cases of KSHV-associated germinotropic lymphoproliferative (GLD) disorder (Du et al. 2002) which represent polyclonal lymphoid proliferations seen in ostensibly immunocompetent individuals. Plasmablasts are detected which, in contrast to vMCD, preferentially involve germinal centers, are coinfected with EBV, and have mutations in immunoglobulin genes. Unlike both PEL and vMCD in which only small fractions (5–10%) of cells express v-IL6, most GLD cells express abundant vIL-6 (Du et al. 2002). This disorder is a localized lymphadenopathy with favorable response to chemotherapy. Other non-neoplastic disorders described include a relapsing inflammatory syndrome characterized by fever, lymphadenopathy, splenomegaly, edema, arthrosynovitis and rash (Dagna et al. 2005) and systemic plasmacytosis in post-transplantation patients (Matsushima et al. 1999). While no symptoms for primary infection have been described in immunocompetent individuals, Luppi et al. describe an acute syndrome of fever, spenomegaly and bone marrow failure with plasmacytosis among some transplant patients (Luppi et al. 2000). KSHV has been linked to numerous other diseases including multiple myeloma, primary pulmonary hypertension, sarcoidosis, pemphigus and prostate cancer, but none of these disease conditions have been confirmed to be routinely infected with KSHV on careful re-examination.

18.8 Conclusion

Since its initial discovery in 1994, over 2500 papers have been published on Kaposi's sarcoma-associated herpesvirus. In many ways more is known about its basic biology, lifecycle and clinical manifestations than for most other viruses. This wealth of published biological information on this virus can be immediately used to benefit KSHV-infected patients. Serologic tests accurately diagnose those persons infected and provide a means to prevent KSHV transmission. Antiviral therapies have been shown to be remarkably effective in preventing KSHV-related disease and in the case of Castleman's disease, treating established disease. From the clinical epidemiology of KSHV infection, it is

evident that lasting protection from KSHV might be achieved if an effective vaccine antigen is searched for and found.

Although Kaposi's sarcoma has entered the medical zeitgeist as a rare and benign tumor, overwhelming evidence indicates otherwise. KS is now the most common tumor in sub-Saharan Africa where it can be rapidly fatal especially in children (Athale et al. 1995; Bassett et al. 1995; Cook-Mozaffari et al. 1998; Wabinga et al. 1993). It remains the most common tumor of AIDS patients despite the unparalleled public health success of effective antiretroviral therapy. The decade-long respite in KS among AIDS patients in Europe and the United States may be coming to an end as AIDS patients age. Initial case reports suggest that KS will reemerge as these patients experience reduction of cytotoxic lymphocyte and immune senescence with aging even in the setting of effective HIV control. We may soon face a second wave of epidemic KS in coming years. The importance of KSHV is not restricted to the area of HIV/AIDS either. Each case of transplant-associated KSHV-related disease represents a preventable failure that existing technologies could effectively manage. Furthermore, blood-borne transmission of KSHV has been unambiguously demonstrated (Hladik et al. 2006). While this route of transmission is extremely inefficient, the large numbers of persons receiving KSHV-tainted blood make this a significant public health concern.

Intriguing questions on how KSHV causes KS and other diseases remain unanswered, but the fact that KSHV is critical for KS development is no longer in doubt. The wealth of basic information in virology, immunology, cell biology and molecular biology gained from studying this virus has not translated into effective therapy to treat or prevent KSHV-related diseases. One wonders what Kaposi would have thought about how much we now know and how little we have done about the disease that he discovered.

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Chapter 19
Organization and Expression of the Kaposi's Sarcoma-Associated Herpesvirus Genome

Yan Yuan and Rolf Renne

19.1 The Genome Organization of Kaposi's Sarcoma-Associated Herpesvirus (KSHV)

KSHV, also called human herpesvirus type 8 (HHV-8), was discovered by Chang et al. in 1994 in an effort to identify differential DNA sequences between KS lesion tissue and normal skin from a AIDS-KS patient by using representative display analysis. This assay led to isolate two DNA fragments from KS tissue, one of which showed 58% sequence homology to the minor capsid protein gene of *herpesvirus saimiri*, a γ-herpesvirus associated with T-cell lymphomas (3, 24). Soon after, many laboratories tested specimens from classical KS as well as AIDS-KS patients and found nearly 100% of samples tested positive for the presence of this novel herpesvirus DNA ((51) and reviewed in (44)). Two AIDS-associated lymphoproliferative diseases, namely body cavity-based lymphoma (BCBL) and multicentric Castleman's disease (MCD), were shown to harbor KSHV sequences. Cesarman et al. reported that BCBL, a common late-stage AIDS-associated lymphoma in the pre-HAART era, was 100% positive for KSHV DNA (22). Soulier et al. identified KSHV sequences in a subset of MCD, a rare lymphoproliferative disease frequently observed in KS patients, both in the presence and in the absence of HIV infection (112).

Permanent cell lines established from BCBL tumors, later termed primary effusion lymphomas (PEL), harbor viral episomal (circular non-integrated) DNA and were instrumental in establishing serological assays as well as in molecular cloning and sequencing of the KSHV genome (23, 98). Two complete sequences were reported: one from a BCBL cell line BC-1, which contains an

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_19, © Springer Science+Business Media, LLC 2009

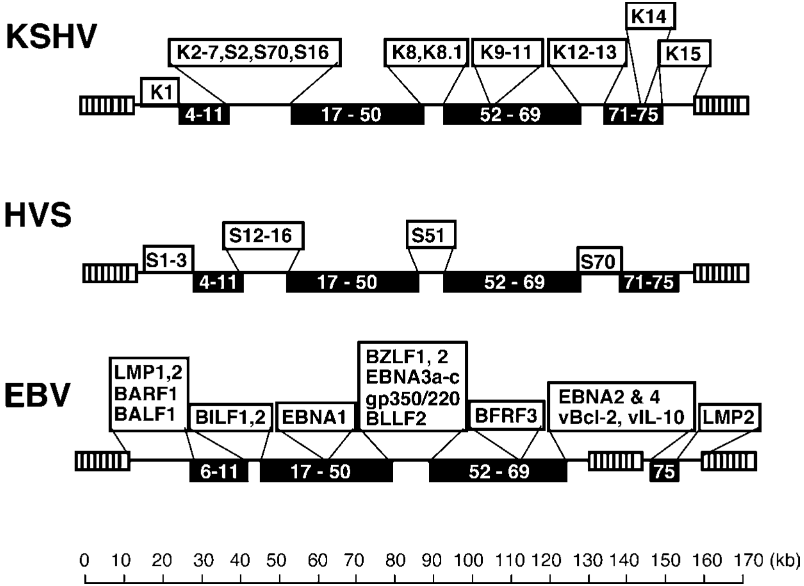


Fig. 19.1 Genome organization of γ-herpesviruses. The long unique region of all three viruses is encompassed by terminal repeat sequences. EBV, unlike KSHV and RRV, contains additional repeat sequences. Conserved gene blocks between KSHV, HVS, and EBV are labeled by numbers from left to right. Genes specific to KSHV carry the prefix K. A more detailed ORF map for KSHV is provided in Fig. 19.3

85 kbp insertion, and the other from a KS lesion (82, 101). Sequence analysis revealed an overall genome organization which classified KSHV as a member of the rhadinovirus (γ2 herpesviruses) subgroup of γ-herpesviruses (Fig. 19.1).

The characteristic feature of γ2 herpesviruses is the presence of highly GC-rich DNA (H-DNA) containing terminal repeats that encompass the coding region of low GC content (L-DNA) at either end. The GC content of the KSHV L-DNA is 53.5% while the H-DNA, compromised of TR sequences, is 84.5%. The discrepancy in GC content leads to fragmentation of viral DNA during density centrifugation, which leads to the term rhadino $\rho\eta\alpha\delta\iota\nu$o (greek) for fragile (14). The size of the KSHV genome was estimated to be around 160 kbp by pulse-field gel electrophoresis of latently infected PEL cells and purified virion DNA (97). Since the identification of KSHV, several related γ2-herpesviruses have been identified, cloned, and sequenced from Old World non-human primates (reviewed in (84)). Based on their phylogeny, the existence of two distinct lineages has been proposed. The first lineage, the rhadinovirus-1 (RV1), includes KSHV and viruses associated with retroperitoneal fibromatosis (RFHV) isolated from different macaque species. The second lineage RV-2 contains rhesus rhadinovirus (RRV) whose sequence has been completely determined by two independent groups. RRV viruses are much closely related to KSHV than to HVS and show a high degree of co-linearity in their genome organization. The presence of RV1 and RV2 lineages in Old World non-human

primates brings up the question whether RV2-like viruses also exist within the human population. This idea is further supported by the presence of both RV1- and RV2-like viruses in chimpanzees (4, 100, 108, 110).

19.2 Protein-Coding Genes

19.2.1 Common Herpesviral Genes and Genes Specific to γ-Herpesviruses

KSHV encodes more than 86 open reading frames (ORFs) and shares a somewhat co-linear genetic organization with HVS, the prototype $\gamma 2$ herpesvirus (3). A total of 68 genes are conserved between KSHV and HVS and were designated as ORFs numbered from left to right. Genes unique to KSHV were originally named K1 to K15 (Fig. 19.1) (82, 101). While most KSHV genes can be found in the genome of RRV (4, 110), the closest relatives of KSHV for which full-length sequences are available, there are some important differences in genome organization between these two viruses. With respect to co-linearity, the dihydrofolate reductase (DHFR) gene, only found in $\gamma 2$ herpesviruses, is located close to the K3 gene in the KSHV genome, while in the RRV genome the DHFR gene is located next to R1 at the left end of the genome. KSHV encodes four genes (K3, K5, K7, and K12) that are not found in RRV. Conversely, RRV encodes an expanded cluster of virally encoded interferon response factors. Interestingly, gene homologues of K3 and K5 are present in the genome of a bovine γ-herpesvirus (BHV4) (72).

A total of 39 KSHV genes in seven blocks are conserved among all herpesviruses. These encode mostly structural proteins and proteins involved in lytic DNA replication and packaging. For example, the DNA polymerase (ORF9) and its processivity factor (ORF59) display amino acid sequence identities of 67.4% or similarities of 75.5% between KSHV and RRV. The major capsid proteins (MCP/ORF25) have a similarity of 79%, while genes involved in DNA metabolism such as DNA helicase–primase (ORF40, ORF41, and ORF44), ribonucleotide reductase (ORF60), thymidylate synthase (ORF70), and maturation packaging protein (ORF29a/b) display high homology ranging from 65 to 78% amino acid similarity, indicating their close relatedness (4, 101, 110).

Surrounding the conserved gene blocks are genes specific to γ-herpesviruses including a transcriptional transactivator ORF50/RTA which is required for the switch of the virus from latent to lytic replication (74, 116) and the FGARAT motif-containing tegument protein encoded by ORF75 near the right end of the genome. Again, the homology of these ORFs is significantly higher between KSHV and RRV as compared to either HVS or EBV. Starting with K13 all the way to the K15 ORF, the genome organization at the right end is unique to KSHV and RRV (4, 101, 110). The latency-associated nuclear antigen (LANA) encoded by ORF73 is flanked on both sides by a number of viral gene products encoding cellular homologues, which will be discussed below. LANA interacts with the human tumor suppressors p53 and RB and induces S-phase entry by activating β-catenin (5, 41–43, 92). LANA also

modulates host cellular and viral transcription during latency (5, 95). In addition, LANA is an origin-binding protein required for latent DNA replication and genome segregation in dividing cells (8, 9, 34, 51, 52, 69) (see below). The most rightward gene K15, also called latent membrane protein (LAMP), encodes a membrane-associated signaling protein with limited homology to the EBV LMP proteins (16, 28, 111).

19.2.2 KSHV Encodes a Large Number of Accessory Genes Originating from Cellular Precursors

A hallmark of KSHV is the large number of accessory genes including many homologues of cellular proteins. These cellular homologues are believed to be captured by the virus from the host cellular genome and after subsequent co-evolution now provide the virus with a powerful tool kit to manipulate host environment on both cellular and organism levels. This section aims to briefly introduce these important players of KSHV biology and some of their activities (summarized in Table 19.1) and more details are discussed in several chapters throughout this book.

The first ORF in the KSHV genome encodes K1, a B-cell signaling molecule which also protects cells from *fas*-mediated apoptosis (63, 64, 122). Interestingly, K1 sequences are highly variable among viral isolates and together with observed sequence variation at the right end of the genome have been used to divide KSHV into six major subtypes (50). ORF4 encodes a complement-binding protein (CBP) homologue to cellular CD46 and complement receptors 1 and 2. K2 encodes a viral interleukin 6 (vIL-6), which has 25% amino acid sequence identity to human IL-6. vIL-6 is secreted by PEL cells and believed to be important for

Table 19.1

Viral Gene	Cellular Homolog	Function
K1	–	ITAM signaling; growth control
4	CD46, CR 1,2	complement regulation
K2/vIL6	IL6	paracrine signaling
K3/Mir3	–	Immune evasion, E3 ubiquitin ligase
K4/vMIP	cc chemokines, MIP1$\alpha\beta$	paracrine signaling regulation
K5/Mir5	–	Immune evasion, E3 ubiquitin ligase
K6/vMIP	cc chemokines, MIP1β	paracrine signaling
K7/vIAP	survivin	anti apoptotic
16	bcl-2	regulation of apoptosis
K9, 10, 11	vIRFs	gene regulation; growth control
K12/kaposin	–	signalling, stabilization of cytokine mRNAs
71/vFLIP	DED domain proteins	regulation of apoptosis
72/vCyclin	cyclinD1	growth control
73	–	OBP; transcriptional modulator
74/vGPCR	CXC chemokine receptor	signaling; G-coupled receptor
K14/vOX2	OX2	cell-cell interaction

survival of PEL cells. Four KSHV-specific genes, namely K4, K4.1, K4.2, and K6, encode proteins with significant homology to cellular CC chemokine MIP-1α and are able to signal through a variety of chemokine receptors (15, 83). K7, also called vIAP (inhibitor of apoptosis), encodes a glycoprotein homologue of survivin, a glycoprotein, which protects cells from apoptosis (126). ORF16 encodes a homologue of the cellular anti-apoptotic Bcl-2 family (27, 106). KSHV encodes four homologues of interferon regulatory factors (vIRFs) located between ORFs 57 and 58. While K9/vIRF-1 and K10.5/vIRF-3 are expressed in lytically infected cells, K11.5/vIRF-2 and K10.5/LANA2 are expressed during latency and some have been shown to have oncogenic activity when overexpressed in cells (67, 91). The presence of different classes of cell type-specific IRF homologues expressed during latent and lytic replication underlines the importance to manipulate IFN pathway in γ-herpesvirus biology. The latency-associated region encodes two cellular homologues which regulate both apoptosis and cell cycle control. ORF71, also called K13/vFLIP, shows limited sequence homology to mammalian death effector domains (DED) which mediate apoptosis through the interaction with FLICE; however, vFLIP acts as a dominant-negative inhibitor of FLICE, hence the name FLICE inhibitor protein (vFLIP). vFLIP is also a strong activator of NF-κB whose inhibition induces apoptosis in PEL cells (48, 77). Expressed from the same locus, ORF72 has 27% amino acid homology to D1 cyclin, which regulates G1 checkpoints. vCyclin interacts and signals through CDK2 and CDK4 leading to the activation of E2F-dependent transcription and subsequently cell cycle progression (68). Both vCyclin and vFlip are tumorigenic in SCID-Hu mice.

ORF74, also called viral G protein-coupled receptor (vGPCR), encodes a seven-transmembrane IL-8 receptor homologue which constitutively activates several signaling pathways including protein kinases C and B, Akt, NF-κB, and mitogen-activated kinases. vGPCR is highly oncogenic and when expressed in an endothelial cell-specific fashion induces tumors with KS-like features. However, it seems that ORF74/vGPCR is not detectable during latency but is highly induced during lytic replication (for review, see (6, 21)).

K14/vOX2 encodes a cell surface protein homologue to OX2, a member of the immunoglobulin super family; however, unlike cellular OX2 which restricts cytokine expression, vOX2 potently induces inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (29).

Lastly, three additional accessory genes, K3, K5, and kaposin, are unique to KSHV and play important roles in immune evasion and cytokine signaling. K3 and K5, also called modulators of immune recognition (MIR1 and MIR2), induce rapid endocytosis of MHC class I molecules from the cell surface; MIR2 also induces degradation of B7.2 and ICAM. MIR1 and 2 function as E3 ubiquitin ligases and have been shown to ubiquitinate lysines in the cytoplasmic tails of its target proteins; interestingly, MIR2 is the first E3 ligase to ubiquitinate non-lysine residues (18, 33, 53). Both proteins are mainly expressed during lytic replication (Fig. 19.3) but MHCI downregulation has also been demonstrated early after de novo infection (121).

The kaposin gene is located 4 kbps downstream of vFlip and encodes a family of at least three proteins (kaposin A, B, and C) that are expressed by utilizing an array of alternate CUG initiation codons situated within the repeats DR1 and DR2 upstream of ORF K12. Expression of the 48 amino acid-long K12 ORF in mice caused tumors (80). Kaposin A has also been demonstrated to signal by membrane recruitment of cytohesin-1 (61). However, the expression of this protein has not been detected in KS tumors. In contrast, kaposin proteins originating within DR1/DR2 are readily detectable in KS lesions and PEL-derived cell lines (102). More recently, McCormack and Ganem demonstrated that kaposin B activates the p38/MK2 pathway which translates into a robust stabilization of cytokine mRNAs in KSHV-infected cells (78).

In summary, KSHV has evolved to encode a fascinating array of proteins which regulate and manipulate fundamental biological processes, such as cell cycle control, immune evasion, apoptosis, and growth factor signaling within the virally infected host (for review, see (32, 38)).

19.3 Non-coding Genes

19.3.1 Polyadenylated Nuclear Transcript (PAN)

The presence of non-coding nuclear RNAs is common among herpesviruses but no unifying functions have been determined to date. All three classes of non-coding RNAs have been annotated in Figs. 19.2 and 19.3. PAN (also called T.1.1 or nut-1) is a capped and polyadenylated RNA polymerase II transcript. It exists as small nuclear ribonucleoprotein (snRNP) complexes by associating with cellular proteins. Unlike other herpesviral nuclear RNAs, PAN is expressed during lytic replication where it represents the most abundant viral RNA (115, 136). The high abundance of PAN is due in part to the RTA-responsive promoters and its long half-life time. A 79-bp long expression and nuclear retention (ENE) element has been mapped within PAN; this motif interacts with nuclear export factors and conveys both nuclear retention and extended half-life time to heterologous intron-less mRNAs (30, 31) . Whether and how PAN contributes to lytic replication is currently unknown.

19.3.2 KSHV-Encoded microRNAs (miRNAs)

miRNAs are 20- to 24-nt long non-coding small RNAs that bind to 3' UTRs of mRNA to post-transcriptionally regulate gene expression. The first virally encoded miRNAs were cloned form EBV-infected Burkitt's lymphoma cells (90). KSHV encodes a total of 12 miRNAs, 10 of which are clustered within the latency-associated region in the KSHV genome between ORF71 and the kaposin locus (20, 47, 89, 103). Although not related in sequence a similar miRNA

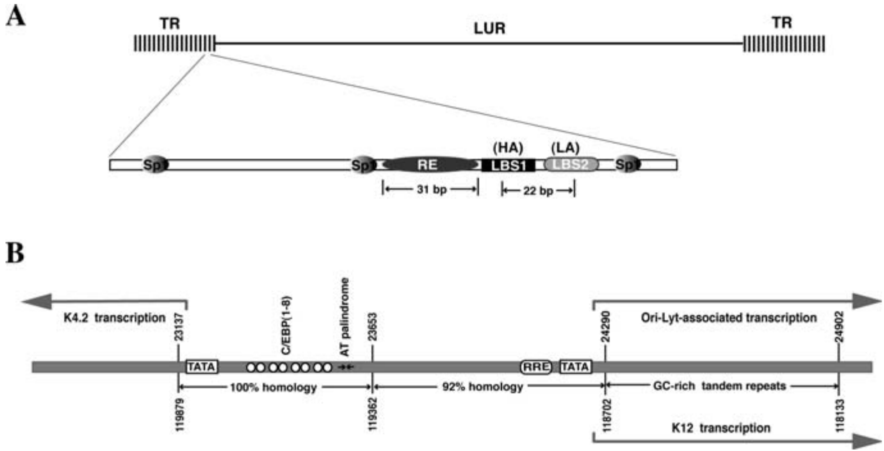
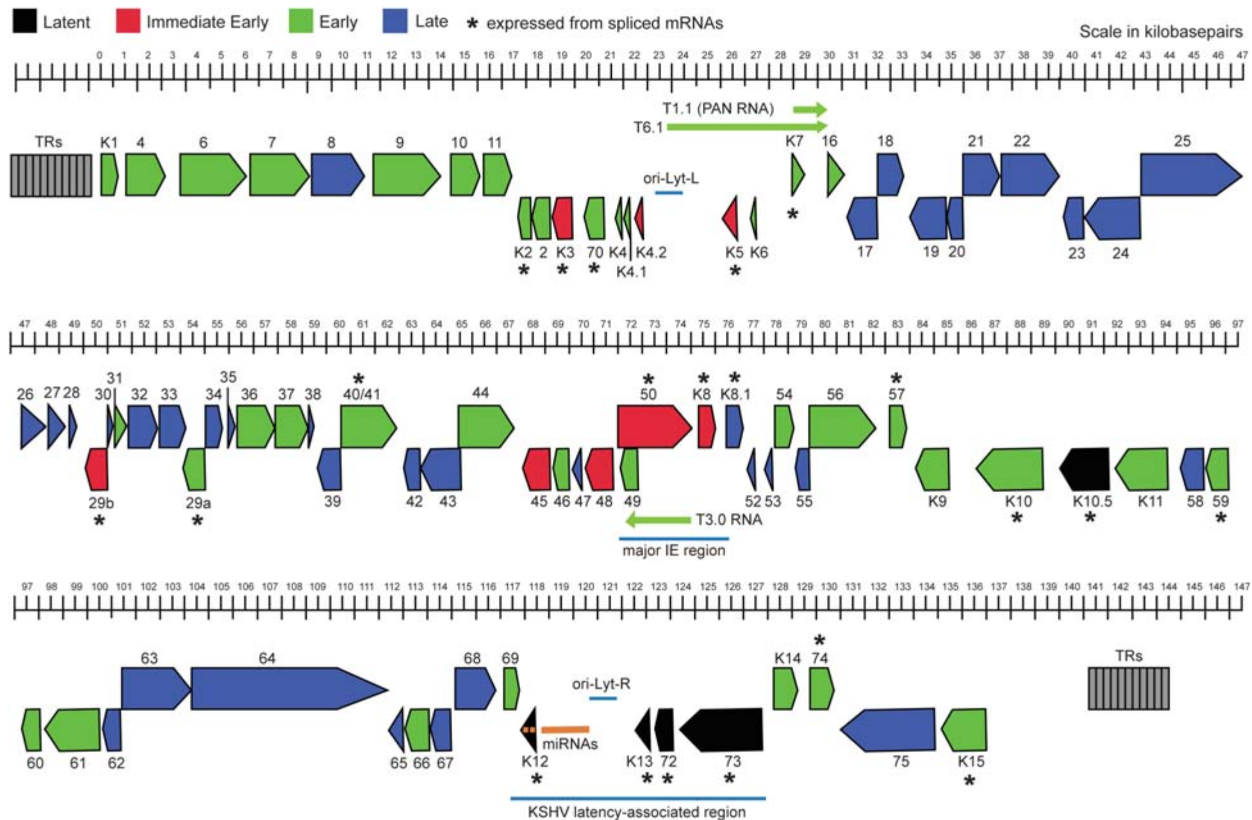


Fig. 19.2 atent and lytic origins of KSHV. (A) Genome structure of KSHV and the latent origin of DNA replication within the terminal repeats. The unit length of the TR is 801 bp. TR, terminal repeat; LUR, long unique region. It is important to note that cis-regulatory elements have been mapped within TR-containing plasmids and that the site of latent DNA replication within the context of the genome has not yet been determined. Cis-regulatory elements within the minimal replication origin are labeled as follows. LBS1/2, LANA binding site 1 and 2; HA, high-affinity binding site; LA, low-affinity binding site; RE, replication element; also indicated is the spacing between LBS1 and LBS2 and the length of the RE element. (B) Structure of the KSHV origin of DNA replication (ori-Lyt). The positions of various characteristic motifs (TATA boxes, C/EBP-binding motifs, AT palindrome, RRE, and GC tandem repeats) are as indicated. The homologies of subregions between two ori-Lyts are compared and shown on the bottom

cluster is also present in the RRV genome. KSHV encodes two additional miRNAs within the kaposin gene (47). Hence, the identification of these novel post-transcriptional regulators of gene expression has significantly increased the number of viral genes that have the potential to modulate host cellular gene expression. KSHV-encoded miRNAs may regulate cellular and/or viral genes in latently or lytically infected cells and the fact that they are highly conserved among clinical isolates of various clinical manifestations and geographical origin suggests that they may contribute to viral pathogenesis (75, 104). The identification, expression, and potential roles of these novel viral regulators of gene expression in viral biology and/or pathogenesis are discussed in detail in Chapter 25 (Non-coding Regulatory RNAs of the DNA Tumor Viruses).

19.3.3 Other Unannotated Transcripts

The KSHV genome transcribes several RNAs with low coding potentials in addition to PAN RNA (107, 120, 129). Two transcripts, T1.2 and T3.0, were


Latent
Immediate Early
Early
Late
* expressed from spliced mRNAs
Scale in kilobasepairs
T1.1 (PAN RNA)
T6.1
TRs
K1
4
6
7
8
9
10
11
ori-Lyt-L
K7
16
18
21
22
25
K2
2
K3
70
K4
K4.2
K4.1
K5
K6
17
19
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23
24
26
27
28
30
31
32
33
34
35
36
37
38
40/41
44
50
K8
K8.1
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57
29b
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55
K9
K10
K10.5
K11
58
59
T3.0 RNA
major IE region
63
64
68
69
ori-Lyt-R
K14
74
TRs
60
61
62
65
66
67
K12
miRNAs
K13
72
73
75
K15
KSHV latency-associated region

detected in the ORF50 region but complementary to ORF50/RTA mRNA. A computer analysis suggests that these transcripts encode no large open reading frame (107). Given that RTA is a critical transcription activator that controls the switch of the virus between latent and lytic life cycles, it is possible that the complementary transcripts may have a role in regulation of ORF50/RTA expression and viral life cycle. A 1.4-kb RNA is transcribed from an *ori-Lyt* region and the transcription was found to be required for *ori-Lyt*-dependent DNA replication (127, 128). It is not known whether the transcription event or the transcribed RNA product contributes to viral DNA replication. A 6.1-kb transcript, T6.1, was detected in the K4 to K7 region (120). Although the functions and roles of these annotated transcripts have not been revealed, it can be envisioned that these RNAs may play important roles in viral life cycle such as serving as anti-sense RNAs to viral protein-coding mRNA, precursors to regulatory small RNAs, and RNA components of ribonucleoprotein complexes that regulate biological processes in infected cells.

19.4 Terminal Repeats and Lytic Origins of Replication

19.4.1 The Terminal Repeats (TR) and Their Role in Latent DNA Replication and Segregation

The long unique region of KSHV is flanked by TR sequences of 801-bp unit. Like for other herpesviruses, TR sequences are crucial for genome circularization early after de novo infection. In 1999, Ballestas et al. demonstrated that cosmid clones containing either end of the KSHV genome (hence harboring TRs) could be stably replicated in B cells expressing the latency-associated nuclear antigen. This suggested that TR sequences also contain replication origins that confer DNA replication and segregation in latently infected cells (8). In contrast to lymphocryptoviruses, which contain one latent origin of replication (oriP) (65), rhadinoviruses contain as many origins as TR sequences. However, it is important to note that the exact location of DNA replication within the viral genome has not been determined. Within TR several *cis*-regulatory elements have been mapped that confer LANA-dependent replication. First, two LANA binding sites, LBS1 and LBS2, are present

Fig. 19.3 Diagram of KSHV open reading frames and kinetics of gene expression. ORFs that are conserved between KSHV and HVS are labeled by numbers from left to right. Genes specific to KSHV carry the prefix K and are numbered from K1 to K15, respectively. Kinetic classes of gene expression are indicated by colors as indicated at the top right. Marked by a star are all ORFs which are known to be expressed from spliced mRNAs. More details about the fine structure of each of these transcripts is listed at http://biochem.dental.upenn.edu/~soking/KSHV

in each TR. The first has a high affinity for LANA binding which facilitates binding to the second low-affinity binding site in a cooperative fashion (45). Adjacent to LBS1/2 is a 35-bp long GC-rich replication element (RE), which is required for LANA-dependent replication (52) (Fig. 19.2A). Both LBS1/2 and RE together constitute a minimal replicator which initiates DNA replication once per cell cycle in synchrony with the host cellular DNA during S-phase, by recruiting the host cellular origin recognition complex (ORC) in a LANA-dependent fashion (114, 123, 124). For episome segregation during mitosis the LANA binds to LBS1/2 within TR with its C-terminal domain, while the N-terminal chromosome attachment region tethers viral episomes to host chromosome via a direct interaction with histones H2A/B (10). Therefore, *cis*-regulatory elements which confer both DNA replication and segregation in latently infected cells are located within TR. The details of both *cis*- and *trans*-acting factors involved in latent DNA replication are discussed in Chapter 20 (Kaposi's Sarcoma-Associated Herpesvirus Latent Genes and Their Regulation).

19.4.2 Origins of Lytic DNA Replication

Lytic cycle DNA replication of KSHV initiates from two lytic origins (*ori-Lyt*-L and *ori-Lyt*-R) located in the genome between K4.2 and K5 and between K12 and ORF71, respectively (7, 70). The two *ori-Lyt*s share an almost identical 1.1-kb core sequence, juxtaposed to 600-bp GC-rich tandem repeats (Fig. 19.2B). Each 1.7 kb *ori-Lyt* sequence was demonstrated to be necessary and sufficient as a *cis*-acting signal for KSHV lytic replication in transient replication assays (2). However, in the context of the viral genome, *ori-Lyt*-L appears to be sufficient to propagate the viral genome, whereas *ori-Lyt*-R alone seems inert to direct amplification of viral DNA, as suggested by an investigation using recombinant KSHVs that removed one or both of the *ori-Lyt*s in the genome (133). Three essential *cis*-acting elements were identified in the 1.1-kb core domain of *ori-Lyt*: (i) an 18-bp AT-palindromic sequence, (ii) a cluster of eight C/EBP-binding motifs, and (iii) an ORF50/Rta-responsive element (RRE) linked to a TATA box (Fig. 19.2B). Substitution or deletion of any of these three core elements abolishes *ori-Lyt* function (129).

The eight C/EBP-binding motifs are organized as four spaced C/EBP palindromic pairs within a 240-bp sequence. Each palindrome contains two head-to-head CCAAT consensus motifs that are separated by a 13- or 12-bp spacer sequence (129, 132). One of the KSHV origin-binding proteins (OBPs), the K8/KbZIP protein, associates with *ori-Lyt* at the cluster of C/EBP motifs (129, 130, 132).

The RRE/TATA box motif functions as a *cis*-acting transcriptional promoter whose activity is required for *ori-Lyt*-dependent DNA replication. The viral ORF50/Rta protein binds directly to the RRE to activate transcription at

ori-Lyt. The promoter in *ori-Lyt*-L normally directs transcription of a 1.4-kb RNA containing the GC-rich repeats and a putative open reading frame of 75 amino acids, and the promoter in *ori-Lyt*-R controls the synthesis of the 2.3-kb mRNA encoding K12 (129). Transcription events controlled by these promoters are essential for DNA replication from the *ori-Lyt*, as premature termination of the transcription by inserting an SV40 polyadenylation sequence upstream of the GC-rich repeats completely abolished the transcription activity as well as DNA replication (130). But the role of the transcript in lytic DNA replication is unknown.

The 600-bp GC-rich tandem repeat sequences adjacent to the *ori-Lyt* core sequences, represented as both 20-bp and 30-bp tandem arrays in the *ori-Lyt*-L, and two types of 23-bp tandem repeats in the *ori-Lyt*-R are also essential for efficient DNA replication (130). Such GC-rich tandem repeats are also found in *ori-Lyt*s of EBV and RRV (49, 85).

19.5 Viral Gene Expression Patterns

19.5.1 Viral Gene Expression Patterns in Cultured PEL Cells

As a γ-herpesvirus, KSHV characteristically establishes latent infection in lymphoid cells. In latently infected cells, KSHV expresses a limited number of genes, which are referred to as latent genes. Five KSHV latent genes have been identified, and they encode latency-associated nuclear antigen (LANA), vCyclin, vFLIP, kaposin, and vIRF-2 (LANA-2) (17, 36, 60, 93, 102, 105). When latency is disrupted, the virus switches to lytic cycle. In primary effusion lymphoma cells, the switch from latency to lytic replication can be induced by various chemicals, such as tetradecanoyl phorbol acetate (TPA) and n-butyrate (79, 98). In lytic life cycle, herpesviruses express their lytic genes in a temporal and sequential order. A few viral genes are expressed independently of de novo protein synthesis and are classified as immediate-early genes. Early genes are expressed slightly later, and their expression is not affected by inhibition of viral DNA replication. Late genes are expressed after viral DNA synthesis, and their expression is, in general, blocked in the presence of inhibition of viral DNA synthesis.

KSHV gene expression pattern during reaction has been extensively studied in stably infected primary effusion lymphoma (PEL) cell lines. Viral gene expression patterns before and after the induction of lytic cycle by treatment of PEL cells with sodium butyrate or TPA were initially examined by Northern analyses (105, 117) and later by gene arrays covering the entire viral genome (55, 81, 87). Genes with different kinetics were distinguished with aid of protein synthesis inhibitor (e.g., cycloheximide) and viral DNA replication inhibitor (e.g., PAA) (117, 137). ORF50/RTA and a few other genes were shown to be resistant or partially resistant to cycloheximide in chemically induced PEL and

designated immediate-early (IE) genes (107, 117, 137). Although studies with cycloheximide were carried out in a viral reactivation system, rather than de novo infection as in the classic experiment with α- and β-herpesviruses, these studies practically led to identify the genes for crucial regulatory proteins that play roles in the switch of the virus from latency to lytic life cycle, such as ORF50/RTA, ORF45, and a few others (117, 137).

KSHV gene expression patterns in PEL cells were also examined by DNA array or real-time PCR array (40, 55, 81, 87). In these studies, viral gene transcripts were classified on the basis of time of first appearance and peak level of expression. Although the results do not always coincide on the expression kinetics of individual genes among these studies and with the classic experiments with chemical inhibitors described above, all of these studies generally agreed that (i) the latent genes are expressed constitutively regardless of treatment by chemical inducers and other stimuli; (ii) regulatory genes, including those for gene transactivators and modulators of cell physiological status and immune status, are rapidly induced during the switch to lytic replication; (iii) genes for DNA replication and nucleotide metabolism are expressed in the early stage (also called delayed-early stage to distinguish from immediate-early phase); and (iv) viral structural proteins necessary for virion assembly are synthesized after DNA replication. The expression of individual KSHV genes in PEL cells during latency and following reactivation is illustrated in Fig. 19.3.

19.5.2 Viral Gene Expression in Newly Infected Cultured Cells

KSHV has been demonstrated to be able to infect a wide range of cultured cells including cells of endothelial, epithelial, and mesenchymal origin. Paradoxically, the only lines which have not been able to infect in vitro are lymphoid cell lines; despite that KSHV infects lymphoid cells in vivo, as demonstrated by the presence of virus in the peripheral blood mononuclear cells of KS patients, and can cause B-cell-related diseases like PEL and MCD (11, 26, 96). In most infected lines, KSHV quickly establishes a latent infection as defined by the expression of LANA in the infected cells. Spontaneous lytic replication is rare and only a small proportion of infected cells express viral transcripts or proteins characteristic of the lytic life cycle.

Viral gene expression immediately following KSHV infection of endothelial and fibroblast cells has been investigated using real-time PCR and KSHV gene microarray analyses (62). The study revealed that the first viral transcript to be expressed following de novo infection of endothelial and fibroblast cells encodes ORF50/RTA; in contrast, comparatively low levels of LANA were expressed early after de novo infection. However, ORF50 expression declines sharply by 24 h post-infection, while the levels of LANA increase with time and maintained at a steady state until 120 h post-infection. Another important

observation in this study is that despite ORF50/RTA expression at high levels soon after infection, only a subset of RTA target genes was found to be activated during the early stage of infection. The limited number of RTA-activated viral lytic genes include K2 (vIL-6), K4 (vMIP-II), K5 (MIR2), K6 (vMIP-I), ORF11 (vIRF-2), and K7 (the surviving homologue). As discussed above, these genes all encode proteins for modulating immune responses or apoptosis. In contrast, a majority of viral genes involved in viral DNA synthesis or encoding structural proteins were not expressed in the infected endothelial and fibroblast cells. These findings revealed a unique feature of KSHV gene expression during de novo infection, that is, concurrent expression of latent and a subset of lytic genes immediately following infection and a subsequent decline or absence of lytic gene expression with the persistence of latent genes (62). This gene expression pattern suggests that KSHV quickly establishes a latent program of gene expression and only transiently expresses a set of genes to modulate host immune responses to viral infection. Given that expression of RTA in latently KSHV-infected cells has been shown to lead to the expression of full panel of viral lytic genes and virion production, the results raised important questions: Why does expression of RTA in de novo infection fail to induce a complete lytic cycle of the virus? What factor(s) determine the outcome of primary infection leading to either the establishment of latency or lytic viral replication? The answers to these questions remain elusive.

19.5.3 Viral Gene Expression in Infected Tissues and Lesions

Although a great deal of information regarding viral gene expression kinetics has been obtained in cultured cells, questions are raised if these data from cultured cells truly reflect gene expression patterns occurring in infected tissues and pathogenic lesions in vivo. To this end, studies using in situ hybridization and immunohistochemistry have been carried out with biopsy samples of KS, PEL, and MCD. Latent nuclear antigen (LANA) was detected in almost all KS spindle cells of endothelial origin by immunohistochemistry (39, 93). In situ hybridization studies of latent transcripts demonstrated their presence in the majority of spindle cells of KS (37, 59, 94). In contrast to the expression of latent genes, lytic life cycle-associated proteins, including ORF59/PPF and ORF50/RTA, were detected only in a small percentage of cells in KS tumor, indicating spontaneous lytic replication in these cells (56, 86).

Similar to the findings in KS lesions, LANA staining was also observed in the nucleus of the majority of PEL tumor cells, while expression of lytic protein ORF50/RTA was considerably rare (39, 57, 58). However, vIL-6 was detected in a significant subset of PEL cells. In contrast to ORF50/RTA, which is rarely detected, vIL-6 is present in up to 5% of PEL cells (86). The mystery of

uncoupling of vIL-6 gene expression from RTA activation was unveiled by Chatterjee and colleagues, who demonstrated that the promoter of the K2 (vIL-6) gene contains two interferon stimulating response elements (ISRE) and can be directly activated by interferon α. Thus, viruses in PEL cells can modify its cellular environment by sensing and responding to interferon signaling and induce proliferation and survival of PEL tumor cells in an autocrine manner (25).

In MCD, only a small percentage of cells located in the mantle zone surrounding the germinal centers are infected with KSHV; however, the lytic genes including ORF50, vIL-6, K8, PPF, and ORF65 were expressed more frequently in the infected cells, suggesting lytic KSHV replication in a significant subset of infected cells (86, 113). The finding that lytic KSHV infection is much more prevalent in MCD suggests that clinical symptoms of MCD are closely linked with KSHV reactivation (1). Taken together, distinct viral gene expression patterns were found in KSHV-infected tissues of different diseases. This difference may be related to either cell-specific or immune restriction of viral gene expression and may also be linked to clinical manifestation of various KSHV-associated diseases.

19.6 Regulation of Gene Expression

19.6.1 A Large Number of Spliced Genes – a Unique Feature in the KSHV Genome

Studies on KSHV transcription have revealed many interesting features of gene expression of this virus. First, many KSHV genes utilize alternative and/or temporally regulated splicing to access different protein-coding domains expressed from the same mRNA precursors (135). For example, a 5.4 kb LANA (ORF 73), a 1.7 kb vCyclin (ORF 72), and a 1.1 kb long vFLIP (ORF71) mRNAs all derive from a single latent transcript; three splicing variants of K8 are generated by alternative splicing (see below). To date, it appears that KSHV uses mRNA splicing more than any other herpesviruses. In contrast to herpes simplex virus type 1 where only 4 open reading frames (ICP0, ICP22, ICP47, and UL15) have been shown to yield spliced mRNAs (99), more than 20 KSHV genes have been found to be expressed from spliced mRNAs. Second, a significant number of KSHV mRNAs are polycistronic because there are relatively few polyadenylation signals in the genome, but usually only the 5'-proximal ORF is translated. The latter ORFs are expressed through one of the following mechanisms: (i) expression using a different transcriptional start site (e.g., K8) (71); (ii) transportation to the 5'-proximal position by splicing (e.g., ORF72/vCyclin) (36, 118); and (iii) translation via an internal ribosome entry site (IRES) (e.g., ORF71/vFLIP) (12, 46). Genes expressed from spliced mRNAs are labeled by an asterisk in Fig. 19.3.

19.6.2 The Latent Gene Cluster

The major latent genes of ORF73 (LANA), ORF72 (vCyclin), and ORF71 (vFLIP) are transcribed as a tricistronic RNA by a constitutively active promoter. The tricistronic latent transcript is processed to at least three mRNAs, designated latent transcript 1 (LT1), LT2, and LT3, by alternative splicing (Fig. 19.4A). These mRNAs are capable of translating their most 5'-proximal

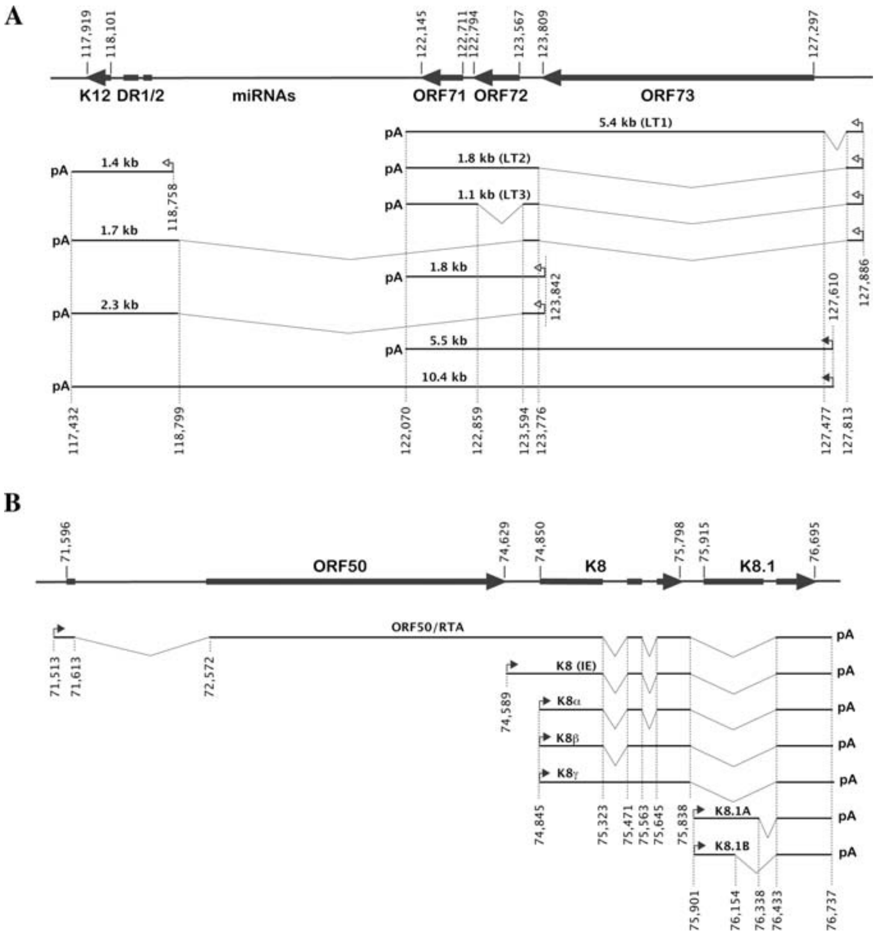

Fig. 19.4 Transcription and splicing patterns of the latency-associated region and the major immediate-early locus of the KSHV genome. In panel A, the four latent open reading frames are shown by black boxes with arrowheads. The initiation sites of latent transcription are indicated by open arrows and those of lytic transcription by solid arrows. In panel B, open reading frames for ORF50, K8, and K8.1 are presented by black boxes with arrowheads and the transcription initiation sites are indicated by solid arrows. Sequence coordinates are according to the numbering of Russo et al. (1996) (GenBank accession number U75698)

open reading frames, namely, LANA, vCyclin, and vFLIP, respectively (36, 46, 118). In addition, vFLIP is also synthesized from the second open reading frame of LT2 by using an IRES (12, 46). The IRES-dependent translation of vFLIP may allow its expression during apoptosis when normal cap-dependent translation is less efficient due to cleavage of elF4G by caspase 3 (73).

Recently, two additional promoters were identified in the latent gene region (Fig. 19.4A). One directs transcription initiation at the nucleotide (nt) position 123,842 and polyadenylation at nt 117,432. This transcript is spliced to a 2.3-kb mRNA and encodes kaposins (K12) and 11 microRNAs (19, 66, 88, 103). This promoter appears to be constitutively active. The other one, a lytic promoter, initiates transcription at 127,610, i.e., 276-bp downstream from the major latent promoter. Some of the RNAs are polyadenylated at 122,070, but a large proportion of the transcription reads through the polyadenylation signal and proceeds until the next polyadenylation site located downstream of kaposin (K12) at nt position 117,432 (19, 76). The two unspliced transcripts from this promoter, 5.5 kb and 10.4 kb, respectively, can be detected in TPA- or butyrate-induced BC-1 and BCBL-1 cells by Northern analysis (Y. Yuan, unpublished data). Both mRNAs are capable of translating LANA protein and the 10.4-kb transcript also contains all the microRNA sequences in this region. In addition, Cai and Cullen reported that part of the major latent transcription, starting at 127,886, can also read through the polyadenylation site at 122,070 and polyadenylated at 117,342. But this transcript is mainly spliced into 1.4- to 1.6-kb mRNA by removal of one or two introns extending from 127,813 to 118,799 (19). Taken together, by using such complex expression and splicing patterns, KSHV regulates the coordinate expression of at least 4 open reading frames (LANA, vCyclin, vFLIP, and kaposins) and 12 microRNA genes in this region in both the latent and the lytic life cycle.

19.6.3 The Major Immediate-Early Gene Region

The transcripts for ORF50/RTA, K8 and K8.1, share a common polyadenylation site, but driven by different promoters with distinct kinetics (71, 107, 137). A promoter located upstream of ORF50 with an immediate-early kinetics controls the transcription of ORF50 mRNA of 3.6 kb. This promoter has been reported to be activated by TPA and butyrate as early as 1 h following the treatment of PEL cell lines and by RTA itself (35, 109). An immediate-early (IE) promoter and a delayed-early (DE) promoter were identified in front of K8 open reading frame, leading to transcription of a 1.5-kb mRNA in IE stage during viral reaction and a 1.3-kb mRNA in DE stage, respectively (107). Since K8 is a multifunctional protein, acting in both IE and DE stages, the differential regulation of the gene may represent a strategy that the virus uses to fine-tune the levels of K8 protein in different stages of the life cycle or for distinct functions. K8.1 encodes an envelope glycoprotein and the expression of this

mRNA is controlled by a late promoter whose transcription activity depends on lytic DNA replication and can be inhibited in the presence of PAA (119).

K8 mRNA is highly spliced, which generates three splice variants as a result of alternative splicing. K8α is the major form, which codes for a 237 amino acid protein with a basic leucine zipper domain near its C-terminus and an acidic domain near its N-terminus. The two other variants, K8β and K8γ, encode proteins sharing the N-terminal portion with K8α, but lacking the C-terminal Zip domain. The three types of K8 mRNA, K8α, K8β, and K8γ, are expressed in TPA/butyrate-induced PEL cells at the ration of 16:4:1 (71, 137). K8α has been reported to be associated with three distinct functions in cells: (i) K8α binds to the origin of viral lytic DNA replication (*ori-Lyt*) of KSHV and recruits core replication complexes to *ori-Lyt* (70, 130); (ii) K8α causes cell cycle arrest at G1 phase through induction of C/EBP α and p21 (54, 131); and (iii) K8α interacts with RTA and represses the transcription of viral delayed-early genes by RTA (54). The former function in *ori-Lyt*-dependent DNA replication appears to be required in the DE stage, while the latter two functions are needed in the IE stage.

K8β mRNA accumulates in TPA or butyrate-induced PEL cells as a result of inefficient splicing of the second intron in the transcript. It has been reported that K8β antagonizes the ability of K8α to induce p21 and p53 and blocks interaction of K8α with CDK2 (134).

The last exon of ORF50 and K8 mRNAs overlaps with the open reading frame K8.1, which encodes an envelope glycoprotein. Two variants, namely K8.1A and K8.1B, are translated from two alternatively spliced mRNAs (13, 125) (Fig. 4B). In summary, KSHV utilizes alternative splicing to generate variants of K8 and K8.1 proteins that may have different biological properties. While the latency-associated region and the immediate-early locus illustrate the complexity of KSHV gene expression, it is important to note that there are many other spliced genes in the KSHV genome (see Fig. 19.3) and details about characterized transcripts can be found in the KSHV Gene Expression website at http://biochem.dental.upenn.edu/~soking/KSHV.

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Chapter 20
KSHV Latent Genes and Their Regulation

Dirk P. Dittmer

"Herpesviridae omnia divisa est in partes tres."

Herpesviridae are divided into three groups: alpha herpesviruses, beta herpesviruses and gamma herpesviruses. Only the two gamma herpesviruses, Kaposi sarcoma-associated herpesvirus (KSHV, or human herpesvirus 8) and Epstein–Barr virus (EBV, or human herpesvirus 4) are associated with human cancer. Herpesvirus lytic replication is also customarily divided into three phases: alpha or immediate early (IE), beta or early (E) and gamma or late (L). We base this classification on the temporal order of viral gene expression. Here, I propose that KSHV latent genes likewise may be divided into three categories based upon their pattern of transcription in KSHV-associated diseases (Table 20.1).

KSHV is associated with three proliferative malignancies in immune-compromised patients: Kaposi sarcoma (KS), primary effusion lymphoma (PEL) and the plasmablastic variant of multicentric Castleman disease (MCD) (reviewed in (Antman and Chang 2000; Ablashi et al. 2002; Dourmishev et al. 2003)). Overwhelming epidemiological evidence shows that KSHV infection is required for the disease phenotypes. Every tumor cell carries the viral genome and expresses KSHV latent proteins. Every cell that stably maintains the KSHV genome must express the latency-associated nuclear antigen (LANA), as this protein is required for latent episome maintenance (see Chapter 19). LANA is expressed in KS as well as in PEL and MCD (Kedes et al. 1997a; Kellam et al. 1997a; Dittmer et al. 1998; Dupin et al. 1999a). LANA transcription is regulated by the LANA promoter, which is constitutively active in all cell types. LANA-2/vIRF-3/K10.2 is only expressed in PEL, a tumor of B cell origin. By contrast K9/vIRF-1 is seen more prominently in KS, a tumor of endothelial origin. Another KSHV latent gene *Kaposin*/k12/t0.7 is also constitutively transcribed in all

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_20, © Springer Science+Business Media, LLC 2009

Table 20.1 The three categories of KSHV latency

	KS (in situ)	PEL, MCD (in situ)	TPA inducible	Genes
Type I	100%	100%	Minimal	LANA*, vFLIP*, vCYC*
Type II	0%	100%	Not inducible	LANA-2/vIRF-3
Type III	100%	100%	Maximal	*Kaposin**, K9/vIRF-1*

*A second, proximal promoter drives transcription in response to Rta/orf50.

KSHV-associated tumors. K9/vIRF-1 and *Kaposin* are greatly induced by phorbol ester (TPA) stimulation of PEL. The transcriptional response to TPA of LANA/orf73, vCYC/orf72 and vFLIP/orf71/K13, which are derived from differentially spliced, overlapping transcripts, is complex and LANA-2/vIRF-3/K10.5-10.6/K10.7 mRNA levels are impervious to TPA.

In sum, KSHV latent transcription seems divinely designed, or rather evolutionarily tuned, to respond to different host environments. This ensures commensal coexistence of virus and host under normal circumstances, but can lead to tumorigenesis in the setting of immune deficiency.

20.1 Profiling KSHV Transcription in Experimental Models and Primary Tumors

Herpesvirus transcription is divided into four stages: the three lytic stages (alpha, beta and gamma) and the latent stage. In KSHV, and in the related EBV, latency can be further divided into additional types, based on gene expression and host cell origin. Many excellent studies have profiled KSHV transcription genome-wide (Table 20.2). Although the KSHV transcript map is of yet incomplete (see Chapter 19 for the current transcript map), all gene expression profiling studies concur as to the identity of the KSHV latent genes (Fig. 20.1). These are LANA (orf73), vCYC (orf72), vFLIP (orf71), the viral miR cluster, *Kaposin* (orf K12) and LANA-2/vIRF-3. Except for LANA-2/vIRF-3, all other latent genes are clustered in the far right region of the viral genome. Except for LANA-2/vIRF-3, which is B-cell specific, all other latent genes are expressed in all KSHV-associated cancers and all experimental models of KSHV latency.

The kinetics of KSHV latent transcripts outside of long-term latency forms a focus of recent research. Chandran and colleagues found that LANA is expressed as an early gene upon primary infection of a HUVEC and BJAB cells and that there exists a reciprocal relationship between IE transactivator Rta/orf50 message and LANA message (Krishnan et al. 2004). Rta/orf50 mRNA peaks within minutes of infection, but then declines as LANA mRNA increases. The relative ratios depend on the host propensity to establish latent or lytic infection. In cells that support high-level KSHV lytic replication (HUVEC, HEK293), Rta/orf50 levels stay high as a significant proportion of

Table 20.2 Genome-wide profiling studies for KSHV viral gene transcription

Tissue	Platform	Citation
PEL		
BCBL-1	Reverse Northern Blot	(Zhong et al. 1996)
BCBL-1	70-mer array	(Wang et al. 2002)
BCBL-1	real-time QPCR	(Fakhari and Dittmer 2002)
BCBL-1	cDNA array	(Paulose-Murphy et al. 2001)
BCBL-1/rapamycin	real-time QPCR	(Sin et al. 2007)
BCBL-1/cidofovir	cDNA array	(Lu et al. 2004)
BCBL-1/ganciclovir	real-time QPCR	(Staudt et al. 2004)
BC-1	Northern Blot	(Sarid et al. 1998)
BC-1	Real-time QPCR	(Whitby et al. 2007)
BC-3	cDNA array	(Jenner et al. 2001)
JSC-1	real-time QPCR	Bagni et al. (submitted)
JSC-1	cDNA array	(Suscovich et al. 2004)
BCBL-1/K1	cDNA array	(Lee et al. 2002)
BCBL-1/Rta/orf50	cDNA array	(Nakamura et al. 2003)
BCBL-1/NotchIC	Real-time QPCR	(Chang et al. 2005)
Endothelial		
TIVE	Real-time QPCR	(An et al. 2006)
KS	Real-time QPCR	(Dittmer 2003)
Murine endothelial cells	Real-time QPCR	(Mutlu et al. 2007)
HMVEC-d	cDNA array	(Krishnan et al. 2004)
HMVEC-d	cDNA array	(Moses et al. 1999)
HUVEC	Real-time QPCR	(Yoo et al. 2005)

the culture enter complete lytic replication as characterized by subsequent E and L gene transcription. In cells that do not support KSHV lytic replication in the absence of external stimuli such as phorbol ester (BJAB, fibroblasts), Rta/orf50 levels rapidly decline as infected cells enter latency or loose the viral genome.

At times profiling studies also showed an increase of latent (LANA, vCYC, vFLIP) mRNA at late times after lytic reactivation (Jenner et al. 2001), which would classify LANA as a gamma2 class mRNA (Fig. 20.1). A similar observation has recently been made in EBV (Yuan et al. 2006). We did not find evidence for significant latent mRNA induction at late times in BCBL-1 cells (Fakhari and Dittmer 2002); significant in as much, as it could not be explained by genome copy number amplification or changes in PEL culture composition. Unlike EBV-infected BL cell lines, in which the majority of cells reactivate from latency upon IgM cross-linking, in any PEL cell line never more than 50% (often less than 20%) of cells reactivate the virus in response to phorbol ester. This poses no problem for the study of lytic transcription, as lytic transcripts are virtually undetectable in latent cells and only cells that do reactivate contribute to the signal. However, it makes studies of latent gene transcription in response to stimuli difficult to interpret, since these are conducted in a background of uninduced cells.

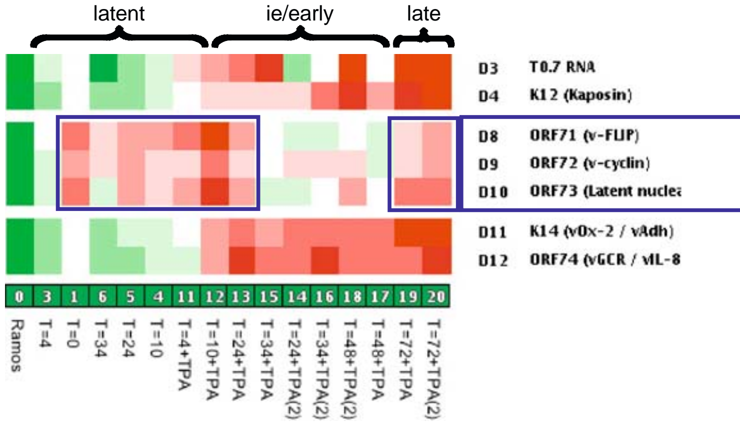

Fig. 20.1 Transcription profile of the KSHV latency locus (*See* Color Insert) Primary data from (Jenner et al. 2001) were re-analyzed using ArrayMiner™

20.2 The KSHV Latency Locus

The KSHV latent genes LANA, vCYC and vFLIP (and miRs) all cluster together within one transcription unit (Fig. 20.2). At least one nascent transcript exists that traverses the entire locus (Pearce et al. 2005; Samols et al. 2005; Cai and Cullen 2006). It initiates at the constitutive LANA promoter start site at nt 127,880 and terminates at the *Kaposin*-distal poly-A site at nt 117,432. Transcription across this locus proceeds only in one direction: from right to left. To date no evidence for mRNAs originating at the opposite strand has been found. Furthermore, latent transcription was polymerase-II dependent.

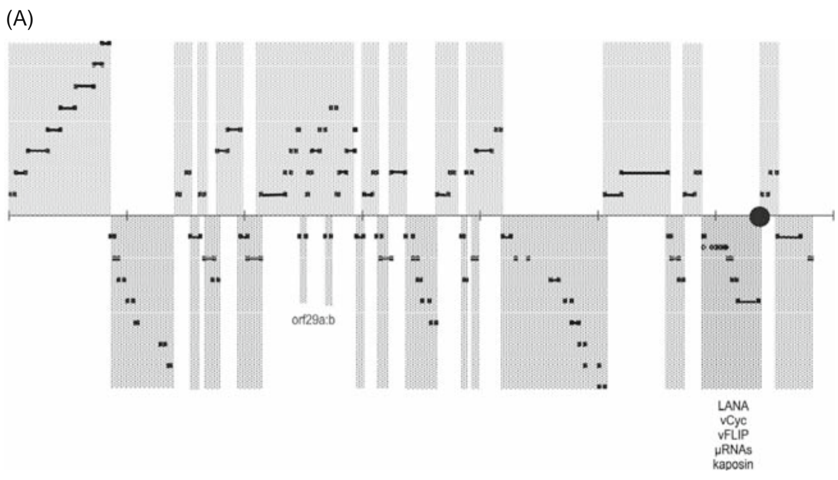

Fig. 20.2 (continued)

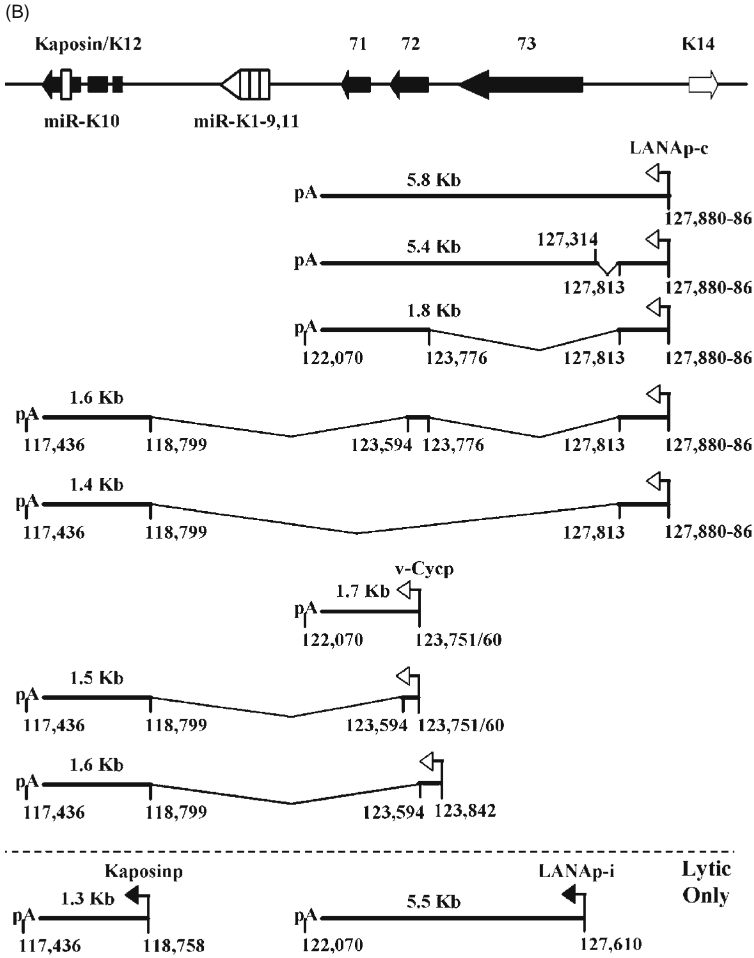

Fig. 20.2 The KSHV latency locus **(A) KSHV genome to scale.** Upper half, rightward orfs; lower half, leftward orfs, **(B) Transcript architecture of the major latency locus of KSHV (modified from Cai and Cullen (2006)).** The relative genomic position of KSHV open reading frames (ORFs) are shown along the top line as filled arrows to denote ORF directionality (*Kaposin/K12*; *ORF71/v-FLIP*; *ORF72/v-cyclin*; *ORF73/LANA*; *K14/v-Ox2*). The KSHV microRNAs (miR) are denoted as white boxes and are designated with the *K*-prefix (miR-K1-11). Transcripts expressed during latency in the absence of viral transactivators are denoted with white arrowheads; these originate at either the predominant LANAp-c or the weak v-Cycp. Transcripts dependent on the KSHV lytic-switch transactivator, Rta/ORF50, are denoted with black arrowheads and are shown below the dotted line for clarity. Lytic transcripts originate at either the LANAp-i or the *Kaposin*p. Genomic coordinates of transcript start sites and splice donors/acceptors are shown below the transcript diagram according to Russo et al. (1996a, b)

We are very confident in our assignment of the type I and type II latent genes, which show a dramatically different transcription profile that any of the other KSHV mRNAs regardless of experimental platform, PEL line or study (Fig. 20.1), and for which we have independent verification by *in situ* analysis (Dittmer et al. 1998; Dupin et al. 1999a; Katano et al. 1999, 2000; Parravicini et al. 2000; Rivas et al. 2001a). However, the PEL experimental system is far from perfect. At any given time 2–5% of PEL cells reactivate KSHV, such that highly expressed lytic mRNAs are detectable in uninduced cultures. The best example is early nut-1 RNA, which can be detected by Northern blot analysis of uninduced PEL (Zhong et al. 1996), since the few cells that express nut-1 make large amounts of RNA. *Kaposin* lytic transcripts, which originate from the gene proximal lytic promoter, likewise can be detected in latent PEL (Sadler et al. 1999), as can be those for K9/vIRF-1 (Chen et al. 2000). Yet, different latent mRNAs have been identified for K9/vIRF-1 and *Kaposin*, which map to a more distal constitutive start site. In addition, the latent genes respond to changes in the host cell. The spliced vCYC mRNA may be subjected to cell cycle regulation (Sarid et al. 1999a), or the cell cycle stage may influence the rate of spontaneous lytic reactivation (McAllister et al. 2005) thus changing the vCYC transcription pattern. In MCD, the vIL-6/K2 protein is expressed in a large percentage of cells, far more than any structural lytic protein (Staskus et al. 1999; Deng et al. 2002), suggesting that the vIL-6/K2 promoter can respond to MCD-specific transcription factors irrespective of the status of all other KSHV genes (Chatterjee et al. 2002).

Understanding KSHV transcription is a work in progress that requires careful molecular characterization of the viral regulatory elements. As we are starting to map the virus–host interactions at the transcriptional level, we gain fascinating insights into the pathogenesis of KSHV, which ultimately will lead to novel intervention targets.

20.2.1 LANA, vCYC, vFLIP

20.2.1.1 Latency-Associated Nuclear Antigen (LANA)

During latency, all KSHV-infected cells express the viral latency-associated nuclear antigen (LANA/ORF73) (Dittmer et al. 1999; Dupin et al. 1999b). LANA is the predominant target of anti-KSHV antibodies in infected individuals. LANA is necessary and sufficient for latent viral episome persistence (Ballestas et al. 1999; Cotter and Robertson 1999; Hu et al. 2002; Ye et al. 2004). Although LANA shows no homology at the DNA sequence level, its function and structural features are reminiscent of the Epstein–Barr virus EBNA-1 and EBNA-2 proteins. LANA contains a central region of acidic repeats, a leucine zipper, an N-terminal proline-rich domain and two nuclear localization sequences – one located at each termini of the polypeptide (diagrammed in Fig. 20.3). The acidic repeat regions and leucine zipper (EEDD, DE[E,Q]QQ

and LEEQEQEL between amino acids (aa) 338. . .840) and (LEEQEQEL at aa 840) are highly immunogenic and the target for the widely used commercial anti-LANA monoclonal antibody LN53 (Kellam et al. 1999).

The first function of LANA is to tether the viral episome to cellular chromatin and thus facilitate proper inheritance during cell division. The details of how LANA maintains the latent viral episome have been carefully studied. The C-terminus of LANA binds directly to two adjacent 16-bp motifs within the KSHV terminal repeats (TR), termed the LANAbinding sites 1 and 2 (LBS1 and LBS2). Both LBS share the conservation of 13 nucleotides, though nucleotides flanking this 13 nt. core are different between the two (Garber et al. 2001, 2002). LANA binds with higher affinity to LBS1 than to LBS2, suggesting the nucleotide sequence flanking the conserved 13 bp. LBS motif influences the binding affinity of LANA. This hypothesis has been directly investigated: Kaye and colleagues have found that nucleotides within and surrounding the LBS1 affect LANA's-binding affinity for that site (Srinivasan et al. 2004).

LANA has been shown to bind cellular chromatin and mitotic chromosomes through its N-terminus (Shinohara et al. 2002). In this manner, LANA tethers the KSHV episome to cellular chromatin and chromosomes – thereby ensuring proper segregation of the viral genome during host cell division (Kedes et al. 1997b; Kellam et al. 1997b; Rainbow et al. 1997; Ballestas et al. 1999; Cotter and Robertson 1999; Szekely et al. 1999) (Kelley-Clarke et al. 2007a). Experimental abrogation of LANA expression through siRNA or genomic knockout leads to loss of KSHV episomes from latently infected cells, genetically demonstrating that LANA is necessary for maintenance of latency (Ye et al. 2004; Godfrey et al. 2005). Barbera et al. (2006) demonstrated that the N-terminus of LANA docks onto cellular chromosomes by directly binding to the folded regions of histones H2A and H2B to mediate nucleosome attachment (Barbera et al. 2006). Both histones H2A and H2B were necessary for LANA to bind nucleosomes. In contrast, Robertson and colleagues have found that LANA binds histone H1 (Barbera et al. 2006) as well as a host of other proteins involved in DNA structure remodeling (Verma et al. 2006a). Cellular replication and replication-licensing factors can also bind to LANA (Stedman et al. 2004; Lu et al. 2006; Verma et al. 2006b), suggesting that the KSHV episome– host chromatin interaction is not static but responds to viral latent replication (via the latent ori in the KSHV TRs) as well as host replication.

Second to facilitating episomal attachment, LANA can mediate transcriptional suppression. In the presence of LANA, LBS1 and LBS2 repress transcription of an artificial minimal promoter when placed upstream (Garber et al. 2001, 2002). The ability of LANA to carry out these functions is directly proportional to LANA's-binding affinity for these two sites. In the context of the viral genome, LANA can repress transcription of the K1 promoter via the LBS1 and LBS2 of the TRs (Verma et al. 2006c).

Other than by direct DNA binding, the transcriptional repressor function of LANA can also be mediated by cellular methyl transferases (Shamay et al.

2006). Currently there exists no evidence that this mechanism is used to modulate viral transcription. More likely, DNA-independent transcriptional silencing provides one avenue by which LANA reprograms the latently infected host cell. Ectopic expression of LANA leads to both up- and down-modulation over 147 cellular genes (Renne et al. 2001; An et al. 2005) and this global reprogramming may provide one mechanism that mediates LANA's in vivo transforming function (Fakhari et al. 2006).

Third, LANA positively modulates the transcriptional activity of its own promoter (Jeong et al. 2001; Renne et al. 2001; Jeong et al. 2004). Although the LANA promoter (LANAp) is constitutively active in the absence of viral proteins, expression of LANA leads to auto-activation of its own promoter to maintain a positive-feedback loop. The C-terminus of LANA protein is required for auto-activation of the LANAp since deletions of amino acids (aa) 1002–1062 or 1113–1162 reduced LANAp auto-activation (Jeong et al. 2004). This comprises the DNA-binding region of LANA (Kelley-Clarke et al. 2007b) and is also required for binding to KSHV TR sequences LBS1 and LBS2 (Garber et al. 2001, 2002). The central repeat domains of LANA that span aa 214–750 (including a proline-rich, a DE acidic repeat, and a Q-rich domain) are dispensable for auto-activation of the LANAp since expression of these deletion mutants still transactivated the LANAp to wild-type/full-length levels (Jeong et al. 2004). This is not surprising since the central acidic repeat region of LANA is highly variable among KSHV isolates and is missing entirely from the rhesus rhadinovirus (RRV) LANA (Zhang et al. 2000; DeWire and Damania 2005).

LANA protein binds to nt. 127,903–127,923 of the core LANAp as demonstrated by EMSA (Jeong et al. 2004). Sequence analysis of nt. 127,908–127,915 revealed the presence of an 8 bp motif that is centrally present with LBS1 within the KSHV terminal repeats (TRs). In contrast to each TR where two 16 nt. LBS exist in tandem, the LANA promoter contains only a single core LANA-binding site motif upstream of the latent 127,880 start site.

A detailed investigation has reported on LANA's transactivation of the cellular human telomerase reverse transcriptase (hTERT) promoter through interaction with cellular Sp1 protein (Verma et al. 2004). Verma et al. showed that the C-terminus of LANA is necessary and sufficient to bind Sp1 protein. LANA was shown to bind to Sp1 via Sp1's glutamine-rich "B" domain, which is one of two (A and B) domains required for transcriptional activation (Courey et al. 1989; Verma et al.) but not DNA binding (Kardassis et al. 1999). This report implicates synergism between LANA and Sp1 to activate transcription on the cellular hTERT promoter. Of interest to this idea is the location of the core LANA-binding site within the core LANAp (127,908–920), which is adjacent to the Sp1-binding site (127,928–933) (Jeong et al. 2004). Based on these observations, it is likely that LANA and Sp1 synergize to activate LANAp transcription.

In addition to specific DNA binding, LANA can act as a promiscuous transcription co-factor on other promoters independent of its own DNA-binding recognition element through interaction with cellular proteins including:

Sp-1 (Verma et al. 2004), RBP-jkappa (also known as CSL) (Lan et al. 2005a), p53 (Friborg et al. 1999), Rb (Radkov et al. 2000a, b), GSK-3beta (Fujimuro et al. 2003), CBP (Lim et al. 2000, 2001), ATF4/CREB2 (Lim et al. 2000, 2001), Ring3 (Platt et al. 1999) (Mattsson et al. 2002) (Viejo-Borbolla et al. 2005; Ottinger et al. 2006) and KSHV Rta/orf50 (Lan et al. 2005b). Chromatin-modifying factors SAP30, mSin3A and CIR (Krithivas et al. 2000, 2002), meCP2, DEK (Krithivas et al. 2000, 2002), Histone H1 (Cotter and Robertson 1999) and Histones H2A and H2B (Barbera et al. 2006) also can mediate these effects.

Finally, LANA binds to and inhibits Rb (Radkov et al. 2000b) as well p53 function in reporter assays (Friborg et al. 1999; Wong et al. 2004). Conversely, p53 can inhibit the LANA promoter (Jeong et al. 2001). This initially led to a model, in which LANA behaved very much like the small DNA tumor virus transforming proteins. In fact, because of its ability to decorate host chromosomes LANA can induce chromosome instability phenotypes that are akin to p53 inactivation (Pan et al. 2004; Si and Robertson 2006). However, the situation is more complex. At least one PEL cell line (BC-3) has lost Rb protein expression (Platt et al. 2002), which seems unnecessary if LANA efficiently counteracted all Rb functions, but it leaves open the possibility that LANA may interact with and inactivate other RB family members. LANA, of course, has multiple binding partners ($\geq$10) and functions (Si et al. 2006; Cai et al. 2006). These include Ku70, Ku80 and PARP-1, which can also be in complexes containing p53. Hence, it is easy to rationalize how some LANA can be found in complex with p53. Despite being in complex with LANA, p53 is fully functional in PEL (Petre et al. 2007) and can be activated by doxorubicin. Moreover, the LANA-p53 complex can be destroyed by the mdm-2/p53 interaction inhibitor nutlin (Petre et al. 2007; Sarek et al. 2007), which leads to p53-dependent apoptosis in PEL.

20.2.1.2 v-CYC/orf72

v-cyclin (orf72) represents another candidate KSHV oncogene because of its homology to the human cyclin-D/Prad oncogene. In general, cyclin-D proteins (D_1, D_2, D_3) associate with specific cyclin-dependent kinases (CDKs) and these complexes phosphorylate Rb family members (reviewed in Sherr (1996)). This in turn liberates E2F/DP-1 transactivation functions that are necessary and sufficient for S-phase entry. Importantly, the human cyclin-D_1 gene is amplified in parathyroid tumors, a subset of prostate and breast cancers as well as human mantle cell lymphomas. It can complement *ras* in transforming low passage rodent cells in culture (Hinds et al. 1994; Lovec et al. 1994) as well as *c-myc* in transgenic mice (Bodrug et al. 1994). An oncogenic cyclin-D homolog is also present in other gamma herpesviruses (reviewed in Neipel et al. (1997)). Ectopic expression of the murine herpesvirus 68 (MHV68) cyclin in T cells causes T-cell lymphomas in transgenic mice (van Dyk et al. 1999).

The mechanism of transformation by KSHV v-cyclin is most likely novel and unique, since it phosphorylates pRb but, unexpectedly, also histone H1, $p27^{KIP1}$ and bcl-2 (Chang et al. 1996; GoddenKent et al. 1997; Li et al. 1997; Ojala et al. 2000; Laman et al. 2001). Unlike human cyclin-D, v-cyclin/cdk6-mediated phosphorylation of Rb is resistant to inhibition by the cyclin-dependent-kinase-inhibitors (CDKIs) $p16^{INK4}$, $p21^{CIP1}$ and $p27^{KIP1}$ (Swanton et al. 1997). Moreover, v-cyclin/cdk6 induces the degradation of $p27^{KIP1}$ (Ellis et al. 1999; Mann et al. 1999). Yet, the results of these transient expression studies remain controversial: v-cyclin can overcome a $p16^{INK4}$ G_1 arrest (Swanton et al. 1997), but its activation of the E2F-responsive cyclin A promoter is inhibited by $p16^{INK4}$(Duro et al. 1999). Depending on the cell line used, v-cyclin binds exclusively to cdk6 (GoddenKent et al. 1997), to cdk4 and cdk6 (Li et al. 1997) or to cdk4, cdk6 and cdk2 (Mann et al. 1999).

Despite significant overall sequence identity, key residues required for cyclin D1 nuclear export and degradation are lacking in the K-cyclin C-terminus . As a result, K-cyclin possesses a longer half life than cyclin D1 and displays more pronounced nuclear accumulation. In the case of human cyclin D, a mutant allele (K112E or K114E) has been generated, which is incapable of activating CDKs. Mutation of the homologous K-cyclin residue (K106 to E) significantly (~50%) reduced CDK6 interaction as well as RB phosphorylation. Recent evidence (Upton and Speck 2006) suggests that the homologous, cdk-binding deficient mutant in the murid herpesvirus 68 (MHV-68) viral cyclin D homolog was able to replicate in culture, but was attenuated for replication *in vivo*.

V-cyclin over-expression induces transient proliferation (Swanton et al. 1997), as well as apoptosis (Ojala et al. 1999; Hardwick 2000; Ojala et al. 2000). To date, no stable cell lines that express v-cyclin have been reported, suggesting that high-level expression of v-cyclin is not compatible with continued cell growth. However, loss of p53 uncovered the transforming potential of vCYC *in vivo*. While KSHV vCYC single transgenic mice did not develop tumors, lymphomas developed rapidly in a p53null background (Verschuren et al. 2002, 2004). An analogous phenotype has been observed in at least one transgenic model for human cyclin D1, where either deletion or targeted overexpression of wild-type cyclin D1 in photoreceptor cells was associated with apoptosis (Fantl et al. 1995; Ma et al. 1998; Skapek et al. 2001). Presumably, loss of p53 counteracted the pro-apoptotic signals that were associated with forced KSHV vCYC expression. In contrast to KSHV, 60% of transgenic mice expressing the MHV-68 cyclin D homolog in T cell developed lymphoma within 12 months (van Dyk et al. 1999). This suggests that cell lineage and the differentiation state of the host cell and cyclin needed to be in the right balance. This data suggest a model that requires multiple events initiated by the concerted action of all KSHV latent proteins for KSHV-dependent lymphomagenesis.

20.2.1.3 vFLIP/orf71

v-FLIP/orf71 is transcribed from the LANA promoter and translated from an internal ribosome entry site located within the vCYC coding region (Grundhoff

and Ganem 2001) (Low et al. 2001). The vFLIP protein has sequence homology to equine herpesvirus-2 E8 and herpesvirus Saimiri (HVS) orf71 (Hu et al. 1997). It inhibits CD95/FAS-induced apoptosis in vitro by blocking caspase-3, -8 and -9 (Djerbi et al. 1999). Both CD95/Fas-L and TRAIL/TNF-alpha induce apoptosis through a similar mechanism (Muzio et al. 1996; Medema et al. 1997). Clustering of the receptor upon binding of the ligand recruits an adapter molecule (FADD and TRADD, respectively) with a binding domain (DD) for the receptor and a conserved "death-effector-domain" (DED) that binds and triggers the activation of caspase-8. The death signal is then transduced through a number of cellular caspases resulting in the commencement of cellular apoptosis (for review see Hu et al. 1997). A possible mechanism for viral FLIPs postulates competition with the adapter molecule for binding to caspase 8 via its DED domain.

A more recent line of inquiry found vFLIP to be involved in NFkappaB signaling. Here, vFLIP uses its TRAF-binding domain to activate NFkappaB signaling (Guasparri et al. 2006). vFLIP activated IkkappaB-kinase (An et al. 2003; Field et al. 2003) and thereby increases NF-kappaB activity, which is anti-apoptotic in PEL cell. In addition, vFLIP induced MHC-I expression through NF-kappaB in KSHV-infected lymphatic endothelial cells (Lagos et al. 2007), which underscores the physiological importance of the vFLIP-NF-kappaB interaction. Moreover, vFLIP transgenic mice develop lymphoma (Chugh et al. 2005). Eliminating either vFLIP or NF-kappaB activity from PEL induces apoptosis (Keller et al. 2000; Guasparri et al. 2004; Godfrey et al. 2005), demonstrating that this pathway is essential for lymphomagenesis.

20.2.2 KSHV miRNAs

Micro RNAs (miRNAs) are a novel class of mammalian genes. They regulate the transcription and translation of many target proteins and have been implicated in normal development as well as carcinogenesis. Viruses also encode miRNAs. In KSHV, the miRNAs are conserved among different isolates (Marshall et al. 2007) and grouped together in the viral latency region (nucleotide 119305–121911) (Cai et al. 2005; Pfeffer et al. 2005; Samols et al. 2005). This organization is similar to mammalian miRNA gene organization where clustering has been observed for 50–70% of miRNA genes (Altuvia et al. 2005). The maturation of miRNAs is the subject of active research (reviewed in (Cullen 2004)). First, a primary miRNA, or pri-miRNA, is transcribed by RNA polymerase II. It is capped and polyadenylated in the nucleus (Cai et al. 2004). The pri-miRNA can be of any length and contain any number of clustered miRNAs. The KSHV miRNAs are derived from a common precursor molecule (Pearce et al. 2005; Cai and Cullen 2006), comparable to the stable HSV-1 lat intron (Cui et al. 2006). They share a common leftward-orientation and are regulated by multiple splicing events, multiple

termination sites and multiple transcription initiation sites (at nt 127,880, 123,848, 118,758) (Dittmer et al. 1998; Sadler et al. 1999; Sarid et al. 1999a; Talbot et al. 1999; Li et al. 2002; Marshall et al. 2007). Promoter–reporter constructs encompassing these three latent start sites all exhibited activity after transient transfection, but by comparison the constitutive LANA promoter at nt 127,880 exhibited 20-fold higher basal activity then the vCYC promoter (O'Hara and Dittmer, unpublished observation). This pattern of promoter activity is consistent with a model whereby the common LANA promoter regulates all KSHV latent RNA species, giving rise to mRNAs as well as all miRNAs in KSHV-associated cancers.

The pri-miRNA serves as substrate for the Drosha nuclease complex (Zeng et al. 2005). Through Drosha the precursor miRNAs, or pre-miRNAs, are generated, each serving as the precursor of one or two mature miRNAs. The pre-miRNAs reside in the nucleus and are ~70 nucleotides in length. The stability of the pre-miRNAs can vary (Schmittgen et al. 2004; Pfeffer et al. 2005). In KSHV, the pre-miRNAs are stable as they can be detected by Northern hybridization. Overall their levels correlate with the level of the mature KSHV miRNAs (Cai et al. 2005; Pfeffer et al. 2005); Samols et al. 2005) (see reference (Gottwein et al. 2006) for an exception) and can be detected in all PEL cell lines as well as in primary KS biopsies (O'Hara and Dittmer, submitted). The pre-miRNAs are subsequently exported out of the nucleus with the help of Exportin 5 and serve as a substrate for Dicer in the cytoplasm. Mature miRNA levels can be regulated by modulating exportin-5 expression (Yi et al. 2005). In the cytoplasm, Dicer processes the pre-miRNA into the mature miRNA and complementary strand, each comprising ~22 nt in length. For some miRNAs, both the sense and the anti-sense pre-miRNA strands serve as template for mature miRNAs. For KSHV, this has been demonstrated for miR-K4, miR-K6 and miR-K9. The mature miRNAs are then incorporated into the RISC complex, which carries out the enzymatic function.

The elucidation of miRNA targets and the function that the KSHV miRNAs play in the viral life cycle is the subject of active research and covered in detail in Chapter 25.

20.2.3 Kaposin/K12

Kaposin is located immediately downstream of LANA, vCYC and vFLIP and in addition to the common promoter can be regulated by a promoter located between LANA and cyclin (Li et al. 2002) and during lytic reactivation yet another, orf-proximal promoter (Sadler et al. 1999). Like LANA, *Kaposin* too is expressed in every tumor cell (Staskus et al. 1997). In fact, *Kaposin* mRNA is the most abundant mRNA in latently infected PEL. It gives rise to an interesting group of alternatively translated proteins (Sadler et al. 1999), at least some of which can transform NIH3T3 cells in culture (Muralidhar et al. 1998). *Kaposin*

interacts with cytohesin-1 (Kliche et al. 2001). In addition, it has been shown to stabilize cellular cytokine mRNAs through the p38 mitogen-activated protein kinase (MAPK)/MK2 kinase pathway (McCormick and Ganem 2005; McCormick and Ganem 2006). Interestingly, and perhaps because of its high expression and protein repeats, *Kaposin* provides target peptides for the human CD8 cytotoxic T-cell response (Brander et al. 2001; Micheletti et al. 2002).

20.3 LANA-2/vIRF-3 and K9/vIRF-1

Profiling of KSHV mRNAs in PEL revealed one new latent orf that was not included within the LANA latency locus (Fakhari and Dittmer 2002). It belonged to a KSHV vIRF homolog, also called LANA-2/v-IRF-3, which is not expressed in KS, but is expressed in 100% of PEL and MCD in a pattern similar to LANA (Lubyova and Pitha 2000; Rivas et al. 2001a). LANA-2/vIRF-3 counteracts cellular IRFs, but also p53 function (Rivas et al. 2001a). LANA-2/vIRF-3 is a member of several KSHV IRF homologs. Their function in immune evasion is described in detail in elsewhere (see Chapter 24). The viral IRFs, just like the viral latent genes are clustered and oriented as repeats of leftward orfs (see Table 20.3). There transcription is complex and not fully understood. LANA2/vIrf-3 is only expressed in B lineage cells, but here in every cell, whereas for K9/vIRF-1 has both latent and lytic transcriptional start sites (Chen et al. 2000) have been described in PEL. More important, by cluster analysis, K9/vIRF-1 clustered with the other latency genes in endothelial-cell lineage KS tumors (Dittmer 2003), as if at least one vIRF has to be expressed during viral latency.

Table 20.3 KSHV encodes multiple viral interferon regulatory genes as clustered leftward orfs

Name	Location based on (Russo et al. 1996b)	Transcriptional class
orf57/Mta/Sm (right)		IE
	polyA-termination: 83637	
K9/vIRF-1	83860–85209	E
	Upstream start (-84)	LATENT
	polyA-termination: 86006	
K10 s	88085–86074	LATENT
K10:	88164–86074	LYTIC
K10-K10.1/vIR-4	88910–86076 (intron: 88443–88343)	LYTIC
K10-K10.1	88910–86076 (intron: 88443–88343 intron: 89034–88799)	LATENT
K10.5-K10.6 (or 10.7)/ LANA-2/ vIRF-3	91393–89599 (intron: 90938–90847)	LATENT
K11:	91964–93367	LYTIC
K11-K11.2:	94123–91964 (intron: undefined)	LYTIC
K11.2/ vIRF-2	94123–93623	LYTIC

20.4 Architecture of the KSHV Latency Locus Promoter (LANAp)

LANA transcription is regulated by the LANA promoter (LANAp), depicted in Fig. 20.4 (Dittmer et al. 1998; Sarid et al. 1999; Talbot et al. 1999). In its initial characterizations, the LANAp was found to direct transcription of poly-cistronic mRNAs encoding either LANA/ORF73, v-cyclin/ORF72, and v-FLIP/ORF71 or only v-cyclin/ORF72 and v-FLIP/ORF71 through alternative splicing out of LANA/ORF73 (Dittmer et al. 1998; Sarid et al. 1999) (diagrammed in Fig. 20.2, *top three transcripts*). Transcriptional profiling of viral gene expression showed that during viral latency in PEL and in primary KS biopsies, LANA, v-cyclin, v-FLIP and *Kaposin* were constitutively expressed (Jenner et al. 2001; Paulose-Murphy et al. 2001; Fakhari and Dittmer 2002; Dittmer 2003). Therefore, under conditions where other KSHV promoters were silenced, the LANA promoter remained constitutively active. GpC islands within the LANAp are constitutively unmethylated in both PEL and KS (Chen et al. 2001) and are associated with an "open" chromatin environment. This is in contrast to, for instance, the promoter for the KSHV lytic-switch protein Rta/ORF50. Treatment with sodium butyrate, an inhibitor of histone deacetylases (HDACs), did not change the acetylation status of histones H3 and H4 on the LANAp since the promoter was already de-repressed, contrasting the response observed on the Rta/ORF50 promoter (Lu et al. 2003).

Left of the LANA/v-cyclin/v-FLIP locus is another latently expressed gene, *Kaposin/K12*. While *Kaposin/K12* has its own promoter that is highly responsive to the lytic-switch protein, Rta/ORF50. Recent work has discovered transcripts containing *Kaposin*/K12 message originating from a weak promoter located in front of v-cyclin/ORF72 (Pearce et al. 2005; Cai and Cullen 2006) and also originating from the latent LANAp (Cai and Cullen 2006). Importantly, the newly discovered KSHV microRNAs (miRNAs) are located within the intergenic region between the v-FLIP/ORF71 and the *Kaposin*/K12 open reading frames (Cai et al. 2005; Pfeffer et al. 2005; Samols et al. 2005). Therefore, transcripts that

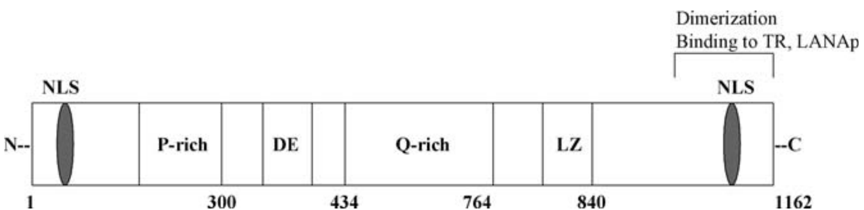

Fig. 20.3 Domain structure of LANA **Diagram of the KSHV Latency-Associated Nuclear Antigen, LANA (with permission from M. Staudt, 2006).** The gene product of *ORF73* is the KSHV latency-associated nuclear antigen (LANA). LANA is an 1162 amino acid (aa) protein that contains a nuclear localization sequence (NLS) at both the N- and C-termini, a proline-rich domain (P-rich), an acidic repeat domain of aspartic and glutamic acid (DE), a glutamine-rich domain (Q) and a leucine zipper domain (LZ). The C-terminal 231 aa facilitates binding to genomic terminal repeat (TR) DNA and to LANA promoter DNA

originate from the latent LANAp and encode the *Kaposin*/K12 ORF also encode the miRNAs, and as such can explain the constitutive expression of the KSHV miRNAs during latency (Cai and Cullen 2006) (see Fig. 20.2 for diagram of transcripts). These novel reports demonstrate that despite its name, the LANAp can also direct expression of every viral gene expressed during latency. An exception to this is the latent PEL-specific LANA-2/vIRF3, which is located in a distant genomic location outside of the LANA latency locus (Rivas et al. 2001a, b). Spatial clustering and 5′-co-terminal regulation underscores the importance of this region and sets the latency-associated locus and the LANAp apart from all other viral transcription regions.

The LANAp is constitutively active in the absence of viral proteins in all cell lines tested, including KSHV-positive and -negative B cells, HEK293 epithelial cells and SLK endothelial cells (Jeong et al. 2001; Jeong et al. 2004). Moreover, a 1,861 bp DNA fragment originating at the LANA AUG at position 127,300 and extending to position 129,161 (–1299 bp relative to the latent transcription start site) was able to direct B cell-specific reporter gene expression in transgenic mice (Jeong et al. 2002). This demonstrated that host cell transcription factors in the absence of any viral transactivators suffice to direct LANAp activity and, by inference, LANA, v-cyclin, v-FLIP, *Kaposin*/K12 and miRNA transcription during viral latency.

Previous reports on LANAp deletion analyses mapped the core promoter region from + 10 to –88 (nt. 127,870–127,968) relative to the latent transcription start site at 127,880 (Dittmer et al. 1999; Jeong et al. 2001). While the core promoter mediates minimal LANAp activity, the presence of additional promoter sequence both up- and downstream of the core promoter significantly contributed to LANAp activity (Jeong et al. 2004). The presence of distal sequences from –88 up to –279 (nt. 127,968–128,159) as well as sequences within the 5′UTR from + 10 down to + 271 (nt. 127,870–127,609) enhanced reporter activity more than 10-fold relative to the minimal core promoter (Jeong et al. 2004).

Although the LANAp exhibits constitutive activity in the absence of viral proteins, expression of LANA protein leads to an increase in promoter activity (Jeong et al. 2001; Renne et al. 2001). Presumably central to this function, LANA has been shown to directly bind within its own promoter to a region that contains a small 8 bp consensus motif that is centrally located within the larger 16 bp. LANA-binding site 1 (LBS1) of the KSHV terminal repeats (TRs) (Garber et al. 2001, 2002). Enhancement of LANAp activity by LANA protein establishes a self-stabilizing feedback loop to maintain KSHV latency (Renne et al. 2001; Chiou et al. 2002; Wong et al. 2004).

The mammalian core promoter is generally defined as the minimal stretch of contiguous DNA sequence that is sufficient to direct accurate initiation of transcription by the RNA polymerase II machinery. They typically encompasses DNA sequences between approximately + 50 and –40 relative to a transcriptional start site (Weis and Reinberg 1992; Javahery et al. 1994; Smale 1997; Smale 2001). Several sequence motifs are commonly found in mammalian core promoters: these include the TATA box, initiator (Inr), the TFIIB

recognition element (BRE) and the downstream core promoter element (DPE). Each core promoter element can be found in some but not all core promoters, and it is a misconception that all promoters must contain each element. The core LANAp has been defined as +10 to –88 (Jeong et al. 2001; Jeong et al. 2004) and it contains the expected mammalian core promoter elements as well as an essential Sp1 site. These elements are independently required as shown by site-directed mutagenesis (Staudt and Dittmer 2006).

Other than during asymptomatic latency in B cells (Mesri et al. 1996; Dittmer et al. 1999) or in KSHV-associated tumors (Dittmer et al. 2003; Dupin et al. 1999b), LANA protein and LANAp originating mRNAs have also been detected immediately after de novo infection of permissive endothelial cells and non-permissive fibroblasts (Krishnan et al. 2004; Yoo et al. 2005). In these experiments, KSHV rapidly establishes latency but can be reactivated by TPA. LANA and Rta/ORF50 mRNAs were described as immediate early mRNAs upon de novo infection. This prompted the discovery of a novel lytic-phase LANA promoter (Lan et al. 2005b; Matsumura et al. 2005, Staudt and Dittmer 2006).

The LANAp-c (127,880) is constitutively active during all forms of latency; its activity is enhanced by LANA (Jeong et al. 2004) and independent of Rta/ORF50. A second, novel, downstream start site is only active in the presence of Rta/ORF50. Nucleotides 127,607–127,675 are sufficient and required for Rta/ORF50-responsiveness and encompass the core elements of the LANAp-i.

Interestingly, the LANAp-i was significantly more responsive to Rta/ORF50 in isolation than when linked to the LANAp-c as in the LANA-FL reporter. This effect could be a result of transcript elongation ensuing from the LANAp-c through the *LANA/ORF73* 5′ -UTR (containing the LANAp-i), which might prevent initiation events on LANAp-i *cis* regions. Such a mechanism was previously reported for the *GAL10* and *GAL7* promoters of *Saccharomyces cerevisiae* and for transcription through tandem HIV-1 promoters (Greger et al. 1998; Greger and Proudfoot 1998). In support of this notion, deletions of core LANAp-c regions that decreased basal promoter activity were associated with increased Rta/ORF50-responsiveness.

In the opposite direction of LANA and the latent transcripts is K14 and the vGCPR (see Fig. 20.3). The vGPCR promoter is absolutely dependent on Rta/

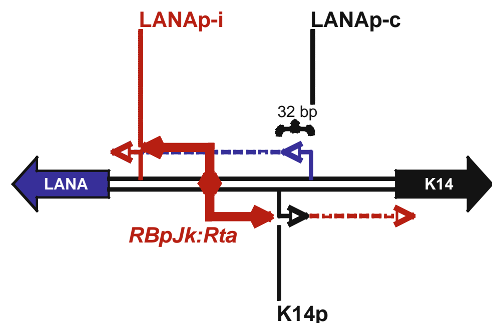

Fig. 20.4 The LANA promoter

orf50 (Liang and Ganem 2004; Liang et al. 2002). Mutation of a shared consensus RBP-jκ site at 127,736–127,740 reduced the ability of Rta/ORF50 to transactivate both the LANAp-i and the K14 promoters (Staudt and Dittmer 2006). These data suggest a mechanism whereby LANA transcripts derived from the LANAp-i can be transcribed during lytic reactivation without polymerase interference by K14/vGPCR transcripts that are simultaneously being transcribed on the complementary strand in the opposite orientation as a result of bi-directional transactivation from the KSHV lytic-switch protein, Rta/ORF50.

During *de novo* infection, Rta/ORF50 is present within KSHV virions (Bechtel et al. 2005; Lan et al. 2004) and as such is delivered into the host cell upon infection in the absence of LANA protein expression. Therefore, based on the data reported herein we speculate that Rta/ORF50 protein could initially transactivate the LANAp-i and K14/vGPCR promoters through direct DNA binding or via the shared consensus RBP-jκ site during de novo infection. As LANA protein expression ensues and LANA accumulates within the cell, expression of Rta/ORF50 protein is silenced as a result of LANA repression of the Rta/ORF50 promoter and LANA's inhibition of Rta/ORF50's transactivation function (Lan et al. 2004). As a result, the LANAp-i and K14/vGPCR promoter activity would cease and LANA-coding mRNA could be transcribed from the latent LANAp-c, which is auto-regulated by LANA protein. This sequence of events can establish a positive-feedback loop that is sufficient to initiate and maintain viral latency within a permissive cellular environment.

Acknowledgment Work in the authors group is supported by funding from the Leukemia and Lymphoma Society and NIH (CA109232). Thanks to Drs. Blossom A. Damania and Michelle R. Staudt for illuminating discussions.

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Chapter 21
Regulation of Expression, Mode of Action and Downstream Targets of ORF50 Protein in KSHV Lytic Cycle Activation

Pey-Jium Chang, Jianjiang Ye, and George Miller

Abstract Kaposi's sarcoma-associated herpesvirus (KSHV), human herpesvirus 8, is a gamma class herpesvirus associated with at least three human malignancies which are increased in prevalence in patients with HIV/AIDS: Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. In common with all herpesviruses, KSHV can exist in a latent or a lytic state. Since both latent and lytic viral gene products play essential roles in the viral life cycle and in oncogenesis by KSHV, understanding the control of the latent to lytic switch provides important insights into KSHV pathogenesis. The switch between latency and lytic replication of KSHV is initiated by a single multifunctional protein encoded by open reading frame 50 (ORF50) of the viral genome. During latency the ORF50 gene is repressed. When the ORF50 protein, also called RTA (replication and transcription activator) is expressed, it is sufficient to induce the complete viral lytic replication cycle. Here we discuss the regulation of the ORF50 promoter, summarize recent efforts to characterize the molecular actions of ORF50 protein, and describe how viral targets of ORF50 protein may contribute to KSHV replication and pathogenesis.

21.1 Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) or Human herpesvirus-8 (HHV-8) was discovered in 1994 by use of the technique of representational difference analysis (Chang et al. 1994). The complete KSHV genome was sequenced (Russo et al. 1996), and the virus was classified as a gamma herpesvirus. The gamma herpesviruses can be subdivided into two genera: the gamma-1 or lymphocryptovirus genus and the gamma-2 or rhadinovirus genus. Epstein–Barr virus (EBV) is the prototype member of the gamma-1 herpesvirus family (Baer et al. 1984). Members of the gamma-2 genus include KSHV, rhesus

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_21, © Springer Science+Business Media, LLC 2009

rhadinovirus, herpesvirus saimiri, and murine herpesvirus-68 (Albrecht et al. 1992; Alexander et al. 2000; Chang et al. 1994; Virgin et al. 1997). KSHV is associated with all forms of Kaposi's sarcoma (KS). Viral DNA sequences can be detected in classical and endemic forms of KS, as well as transplant-related and AIDS-associated KS (Boshoff and Weiss 1998). KSHV is also associated with several types of lymphoproliferative diseases, including primary effusion lymphomas (PELs) or body-cavity-based lymphomas (BCBL) and multicentric Castleman's disease (MCD) (Boshoff et al. 1995; Cesarman et al. 1995; Soulier et al. 1995).

Like all herpesviruses, KSHV has two distinct phases of its life cycle, latency and lytic replication (Dourmishev et al. 2003). Both the latent and the lytic cycles are essential for the long-term persistence of the virus. Gene products expressed from both programs of the life cycle have been implicated in the pathogenesis of KSHV-associated disease (Dourmishev et al. 2003). Latency is characterized by persistence of the viral genome with expression of a limited set of viral genes (Dittmer et al. 1998; Kedes et al. 1997). The viral genes expressed during latency include latency-associated nuclear antigen (LANA), v-cyclin, v-FLIP, kaposin, and vIRF-2 or LANA-2. The latency-associated viral products have been shown to have transforming and cell cycle-deregulating properties which contribute to KSHV-associated malignancies. Once the virus is reactivated from latency and enters the lytic cycle, viral lytic genes are expressed in an ordered fashion, ultimately leading to production of infectious virions (Gradoville et al. 2000; Sun et al. 1999).

Active KSHV lytic replication is strongly correlated with progression to KS and severity of the pathogenic stage of KS (Goudsmit et al. 2000). Treatment with ganciclovir, a drug that inhibits KSHV lytic but not latent DNA replication, markedly reduces the incidence of KS development in AIDS patients (Martin et al. 1999). Molecular studies support the hypothesis that expression of several lytic genes, encoding growth-regulatory and immunomodulatory proteins, are crucial for KS development. These lytic genes include three macrophage inflammatory factors (vMIP-1, vMIP-II, and vMIIP-III), interleukin-6 (vIL-6), interferon regulatory factor (vIRF), G-protein-coupled receptor (vGPCR), and vBcL-2 (Neipel et al. 1997). Thus, it is important to understand the control of the latent-lytic switch for providing further insights into KSHV pathogenesis.

At the earliest stages of KSHV reactivation, the lytic genes that are expressed first and are resistant to cycloheximide treatment are known as immediate-early (IE) genes. Viral IE genes usually encode regulatory proteins that possess the capacity to modulate expression of viral and host genes which facilitate later stages of lytic replication. Among these IE genes, the protein product encoded in open reading frame 50 (ORF50) of the viral genome is unique in its ability to trigger the viral lytic cascade to completion in latently infected cells (Gradoville et al. 2000; Sun et al. 1998). This review focuses on the transcriptional regulation of the ORF50 gene, functional modulation of ORF50 protein, and target genes of ORF50 action during the KSHV lytic phase.

21.2 Evidence that ORF50 Protein is a Molecular Switch for the KSHV Lytic Cycle

The ORF50 protein is encoded from a multicistronic 3.6 kb mRNA that also contains two other open reading frames, namely, K8 and K8.1 (Sun et al. 1998). The K8 protein is a homologue of EBV ZEBRA protein, which plays a key role in the replication and reactivation of EBV (Lin et al. 1999). The K8.1 ORF encodes a glycoprotein that is a structural component of the envelope of KSHV particles (Luna et al. 2004). Several lines of evidence prove that ORF50 protein serves as the key regulator of the switch from viral latency to lytic replication. Exogenous expression of ORF50, but not K8, in latently KSHV-infected PEL cells, was sufficient to disrupt latency, to result in a cascade of expression of viral lytic genes and, ultimately, the release of virus particles (Gradoville et al. 2000; Lukac et al. 1998; Sun et al. 1999). A dominant-negative ORF50 mutant which lacked the C-terminal activation domain significantly reduced viral reactivation triggered by multiple stimuli (more detail in Sect. 21.3), including TPA or sodium butyrate treatment or ORF50 protein expressed by DNA transfection (Lukac et al. 1999). These results also indicated that the level of ORF50 expression induced by these exogenous stimuli is sufficient for lytic induction of KSHV; overexpression of ORF50 protein is not required. The importance of ORF50 protein in lytic induction is emphasized by experiments using a more efficient strategy for knocking down ORF50 gene expression (Zhu et al. 2004). Inhibition of ORF50 expression by RNase P-based gene interference significantly abolished viral lytic gene expression and virion production. Lastly, genetic knockout of the ORF50 gene in a bacmid containing the KSHV genome clearly demonstrated the importance of ORF50 protein in viral reactivation (Xu et al. 2005). An ORF50-deleted recombinant KSHV genome, BAC36Δ50, remained latent in 293 cells, but this genome could not be induced into lytic gene expression or triggered to release infectious virus after treatment of the cells with TPA. The defective phenotype in viral reactivation observed in the BAC36Δ50-containing cells could be rescued by exogenous expression of ORF50 protein provided in trans (Xu et al. 2005). Taken together, these studies show that the ORF50 protein is the “molecular switch” for lytic reactivation of KSHV.

21.3 KSHV Reactivation and ORF50 Gene Regulation

Although the physiological signals that trigger the switch between latency and lytic replication in infected patients are poorly understood, treatment of latently KSHV-infected primary effusion lymphoma cell lines in cell culture with various environmental or chemical inducers activate viral lytic replication. These stimuli, summarized in Fig. 21.1, include 12-*O*-tetradecanoylphorbol-13-acetate (TPA), sodium butyrate, epinephrine, norepinephrine, proinflammatory

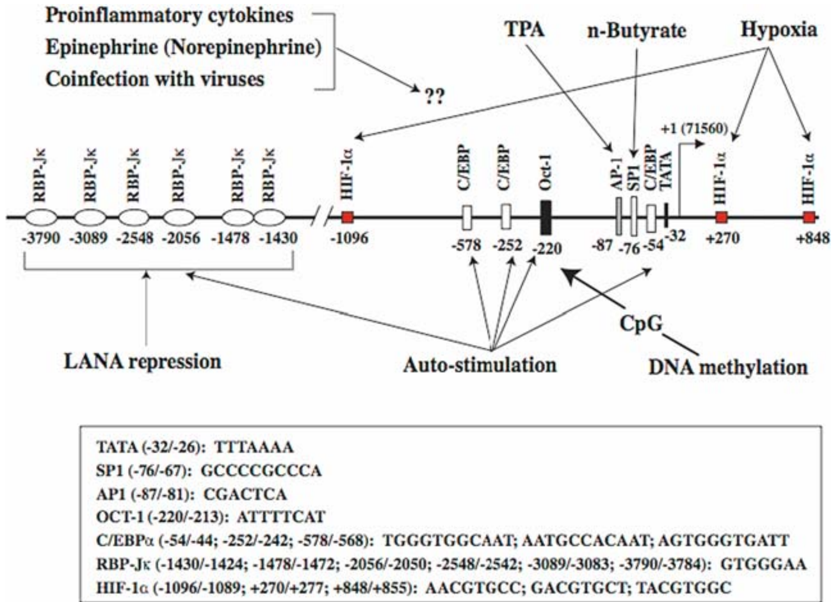

Fig. 21.1 Schematic depiction of the ORF50 promoter region. The start site of the ORF50 transcription is at nucleotide position 71560 of the KSHV genome. The location and detailed sequence of transcription factor binding sites in ORF50 promoter are shown in the open box. The association between various inducers and their responsive elements is indicated

cytokines, hypoxia, and superinfection with other viruses (Chang et al. 2000; Chang et al. 2005b; Davis et al. 2001; Lu et al. 2005a; Lukac et al. 1999; Miller et al. 1997). Since the ORF50 protein is the master controller of KSHV lytic reactivation, much attention has focused on transcriptional activation of the ORF50 gene by these stimuli. Several important *cis*-elements in the ORF50 promoter conferring the responses to these stimuli have been identified (Fig. 21.1), and several possible signaling mechanisms have been proposed. Additionally, ORF50 protein functions to auto-stimulate its own promoter thereby augmenting the abundance of its mRNA. On the other hand, repressive signals are also present on the ORF50 promoter to maintain low-level ORF50 expression during latency. Such repressive signals may include DNA methylation and repression of the ORF50 promoter by LANA.

21.3.1 Positive Stimuli for Activating the ORF50 Gene

21.3.1.1 TPA

TPA, a well-known activator of protein kinase C, can experimentally induce reactivation of KSHV in several PEL cell lines (Renne et al. 1996; Wang et al.

2004a). Evidence suggests that TPA induces KSHV lytic replication via the ERK (extracellular signal-regulated kinase)/MAPK (mitogen-activated protein kinase) pathway. Inhibitors of this pathway block expression of ORF50 and other viral lytic-genes in PEL cells treated with TPA (Cohen et al. 2006). The Raf/MEK/ERK-signaling pathways have been implicated in KSHV reactivation by TPA (Ford et al. 2006). Experiments with PKC inhibitors suggest that TPA induces a bifurcated signaling pathway to regulate KSHV reactivation. One limb involves PKCdelta, extracellular-regulated kinase (ERK) phosphorylation and c-Fos expression; the other limb of the pathway requires another PKC isoform that induces c-Jun phosphorylation (Cohen et al. 2006). Under these proposed pathways, the active AP-1 complex formed by c-Fos and c-Jun functions to activate ORF50 gene expression. This model is consistent with the observation that ectopic expression of c-Jun and c-Fos activated the ORF50 promoter in transient transfections and promoted expression of ORF50 protein in PEL cells (Wang et al. 2004a). An AP-1-binding site, mapping at −87 to −81 in the ORF50 promoter, serves as a target element for mediating this signaling (Wang et al. 2004a).

MEK/ERK signaling is also required for productive lytic replication during primary KSHV infection (Pan et al. 2006; Sharma-Walia et al. 2005). MEK/ERK inhibition diminishes the activities of c-Fos and c-Jun and reduces AP1 binding to the ORF50 promoter during primary infection (Sharma-Walia et al. 2005). Activation of MEK/ERK signaling is proposed to create an environment which facilitates virus infection. In addition to the MEK/ERK pathway, JNK and p38 MAP kinases are also required for optimal KSHV infection and replication during primary infection (Pan et al. 2006). These MAPK pathways modulate activation of the ORF50 promoter activity through AP-1 (Pan et al. 2006).

21.3.1.2 Sodium Butyrate and Trichostatin A (TSA)

Butyrate efficiently induces KSHV lytic reactivation in BC1 cells (Miller et al. 1997). Both sodium butyrate and TSA are inhibitors of histone deacetylases (HDACs). Treatment with sodium butyrate or TSA may directly affect chromosome structure and thus activate the ORF50 promoter. Chromatin remodeling of the ORF50 promoter correlates with lytic reactivation in response to butyrate and TSA (Lu et al. 2003). The element in the ORF50 promoter responsive to butyrate and TSA was mapped to a consensus Sp1 site (Lu et al. 2003). By micrococcal nuclease mapping, a nucleosome was found to be positioned across the transcriptional initiation and the Sp1 sites. Butyrate and TSA treatment rendered this region more susceptible to micrococcal nuclease digestion, suggesting that the HDAC inhibitors opened the chromatin. This region is bound by histone deacetylases 1, 5, and 7 when KSHV is latent. Butyrate treatment induces association of chromatin-remodeling proteins Ini1/Snf5. Over expression of the histone acetyltransferase, CBP, activates the ORF50 promoter. Thus, it is proposed that nucleosome remodeling at the ORF50 promoter regulates KSHV latency to lytic cycle switch (Lu et al. 2003). Similarly, Ye et al. showed that an Sp1-binding element in the ORF50 promoter

mediates lytic cycle induction by butyrate (Ye et al. 2005). By means of electrophoretic mobility shift assays, both Sp1 and Sp3 were shown to form complexes in vitro with the ORF50 promoter at the Sp1 site. Chromatin immunoprecipitation experiments in a PEL cell line showed that butyrate induces Sp1, CBP, and p300 binding to the ORF50 promoter in vivo in an on-off manner. These results indicate that induction of the KSHV lytic cycle by butyrate is mediated through enhanced binding of Sp1 at the ORF50 promoter (Ye et al. 2005).

21.3.1.3 Epinephrine and Norepinephrine

Physiological concentrations of epinephrine and norepinephrine efficiently induce the entire lytic replication cycle of KSHV in some latently infected PEL cells, including KS-1, BC3, and BCBL-1 (Chang et al. 2005b). The receptor for epinephrine and norepinephrine responsible for inducing KSHV reactivation is the β-adrenergic receptor, which modulates the activity of the adenylyl cyclase/cAMP/protein kinase A (PKA)-signaling pathway. PKA antagonists efficiently blocked induction of expression of ORF50 and other lytic-genes induced by norepinephrine. Direct activation of cAMP signaling with a cell-permeable cAMP analogue or a cAMP inducer also activated KSHV lytic gene expression. Furthermore, overexpression of the PKA catalytic subunit upregulated ORF50 gene expression and enhanced activity of the ORF50 promoter (Chang et al. 2005b). Although there are six putative cAMP response elements (CREs) present in the ORF50 promoter, the ORF50 promoter still continued to show significant induction by the cAMP analogue even when all putative CRE sites were eliminated (Chang et al. 2005b). Thus, transcription factors binding to these CREs, such as CREB/ATF, may only quantitatively enhance the PKA effect on the ORF50 promoter. Other unknown transcription factors may play a more significant role.

21.3.1.4 Proinflammatory Cytokines

Since both clinical and experimental observations have pointed to a potential role for inflammatory cytokines as permissive factors for KS development, several studies have focused on the relationship between inflammatory cytokines and KSHV replication (Monini et al. 1999). Interferon gamma has been demonstrated to have a weak, but consistent capacity to activate KSHV replication (Chang et al. 2000; Mercader et al. 2000; Monini et al. 1999). Other cytokines, such as oncostatin M and hepatocyte growth factor, were also found to induce KSHV lytic replication (Mercader et al. 2000). The detailed mechanisms by which cytokines mediate KSHV lytic replication are poorly defined.

21.3.1.5 Hypoxia

Based on the observation that KS lesions commonly occur in tissues with relatively low oxygen (O_2) saturation, the relationship between hypoxia and

lytic replication of KSHV has been explored (Davis et al. 2001). In these experiments, hypoxia or hypoxia mimetic agents induced lytic replication of KSHV. Moreover, hypoxia enhanced lytic replication initiated by TPA. Under hypoxic conditions, the level of certain hypoxia-induced transcription factors (HIF), including HIF-1α and HIF-2α, were increased (Haque et al. 2003). In transient reporter assays the ORF50 promoter was preferentially activated by HIF-2α (Haque et al. 2003). However, in other reporter assays HIF-1α activated the ORF50 promoter by interacting with LANA protein (Cai et al. 2006). Several putative hypoxia response elements (HRE) were identified in the ORF50 promoter. Three of the putative HREs in the ORF50 promoter were demonstrated to be critical for the HIF-1α-dependent activation (Fig. 21.1).

21.3.1.6 Superinfection with KSHV or Coinfection with Other Viruses

Superinfection by human immunodeficiency virus (HIV), human cytomegalovirus (HCMV), HHV6, and KSHV induces lytic replication of KSHV (Lu et al. 2005a, b; Varthakavi et al. 1999; Vieira et al. 2001). The detailed mechanisms by which these viruses promote KSHV reactivation are not fully understood. Induction of lytic KSHV replication required active replication of HIV (Varthakavi et al. 1999). Addition of antiretroviral drugs that prevented HIV replication also inhibited induction of KSHV lytic replication. Of interest, KSHV lytic replication occurred in both HIV-positive and HIV-negative cells following HIV infection. These data suggest that soluble-inducing factors, such as HIV-related proteins or cytokines, produced from HIV-infected cells may be responsible for KSHV reactivation (Varthakavi et al. 1999). Superinfection by live HCMV, but not UV-inactivated HCMV, also resulted in the induction of KSHV lytic cycle (Vieira et al. 2001). By co-culture of HHV-6-infected T cells with the BCBL-1 cell line in which the KSHV is latent, it was shown that HHV-6 played a critical role in KSHV reactivation (Lu et al. 2005a). Since AIDS KS patients are often immunosuppressed and liable to infection by other viruses, HHV-6 may promote KSHV replication in vivo (Lu et al. 2005a). KSHV virions have also been shown to stimulate the ORF50 promoter (Lu et al. 2005b). The KSHV virion-responsive element was mapped to a region (–150 to + 1) relative to the ORF50 ATG. UV inactivation of KSHV and cycloheximide treatment of recipient cells did not block ORF50 induction, suggesting that interaction between virions and infected cells is primarily responsible for stimulating ORF50 expression (Lu et al. 2005b).

21.3.2 Negative Signals Involved in Repressing the ORF50 Gene

Besides modulation by specific stimulators, the ORF50 promoter is also subject to repressive regulation. TPA treatment of BCBL1 cells leads to demethylation of the highly methylated ORF50 promoter (Chen et al. 2001). This demethylation

step is essential for ORF50 expression (Chen et al. 2001). Methotrexate (MTX), a widely used potent anti-neoplastic and anti-inflammatory agent, also inhibits activation of the ORF50 expression (Curreli et al. 2002). Although it is not clear how MTX downregulates ORF50 expression, this compound may have therapeutic value since some of the lytic genes downstream of ORF50, including vGPCR, vIL-6, and K12/kaposin, manifest cell-transforming activities (Curreli et al. 2002).

KSHV latency-associated nuclear antigen (LANA) also represses ORF50 expression during latency (Lan et al. 2004, 2005; Lu et al. 2006). LANA represses the ORF50 promoter in transient reporter assays and inhibits production of KSHV progeny virions in PEL cells treated with TPA and butyrate (Lan et al. 2004). LANA was shown to interact with RBP-Jκ protein, a transcriptional repressor and the target of Notch-signaling pathway (Lan et al. 2005). The physical interaction between LANA and RBP-Jκ directly mediates the repression of the ORF50 promoter (Lan et al. 2005). Overexpression of LANA blocks ORF50 expression in BCBL1 cells, while LANA depletion by small interfering RNA activates ORF50 expression. Additionally, ORF50 transcription was elevated in cells infected by LANA-deficient KSHV viruses (Lan et al. 2005). Thus, LANA acts as a repressor of KSHV lytic replication during latency.

21.3.3 Autoregulation of the ORF50 Gene

ORF50 protein possesses an ability to auto-stimulate its own promoter (Deng et al. 2000; Gradoville et al. 2000). Ectopic expression of ORF50 activates endogenous ORF50 message in PEL cells and increases the activity of the ORF50 promoter in reporter assays (Deng et al. 2000; Gradoville et al. 2000). This auto-stimulation strategy utilized by ORF50 may amplify a weak environmental cue to promote virus replication. Three groups of important *cis*-elements in ORF50 promoter, including Oct1, C/EBPα, and RBP-Jκ-binding sites, appear to mediate ORF50 autoregulation (Liang and Ganem 2003; Sakakibara et al. 2001; Wang et al. 2003c). Interaction of octamer-binding protein 1 (Oct-1) with the ORF50 promoter is critical for ORF50 auto-stimulation (Sakakibara et al. 2001). Three C/EBPα-binding sites are found in the ORF50 promoter; C/EBPα and ORF50 cooperatively stimulate the ORF50 promoter (Wang et al. 2003c). In addition, there are six RBP-Jκ-binding sites found in the ORF50 promoter (Chang et al. 2005c; Liang and Ganem 2003). Binding of the RBP-Jκ protein to the ORF50 promoter may play a critical role to mediate ORF50 autoregulation. In an RBP-Jκ null cell line, ORF50 auto-stimulation was dramatically reduced (Liang and Ganem 2003). Deletion of the putative RBP-Jκ-binding sites in the ORF50 promoter reduced autoactivation (Chang et al. 2005c). Interestingly, RBP-Jκ, the major effector of the Notch pathway has also been shown to mediate activation of several downstream viral genes by ORF50 protein (more detail in

Sect. 21.4). Recently, it has been found that constitutively active Notch1 induces KSHV lytic replication (Lan et al. 2006). Thus, the downstream effector of Notch-signaling pathway, RBP-Jκ, is a key cellular modulator of ORF50 expression and ORF50 function (also see Sect. 21.4.3).

21.4 Modulation of ORF50 Functions

21.4.1 Functions of ORF50 Protein

ORF50 protein is a potent transcriptional activator that activates both viral and cellular genes (summarized in Fig. 21.2). Transient transfection experiments have demonstrated that ORF50 protein activates a number of viral genes that

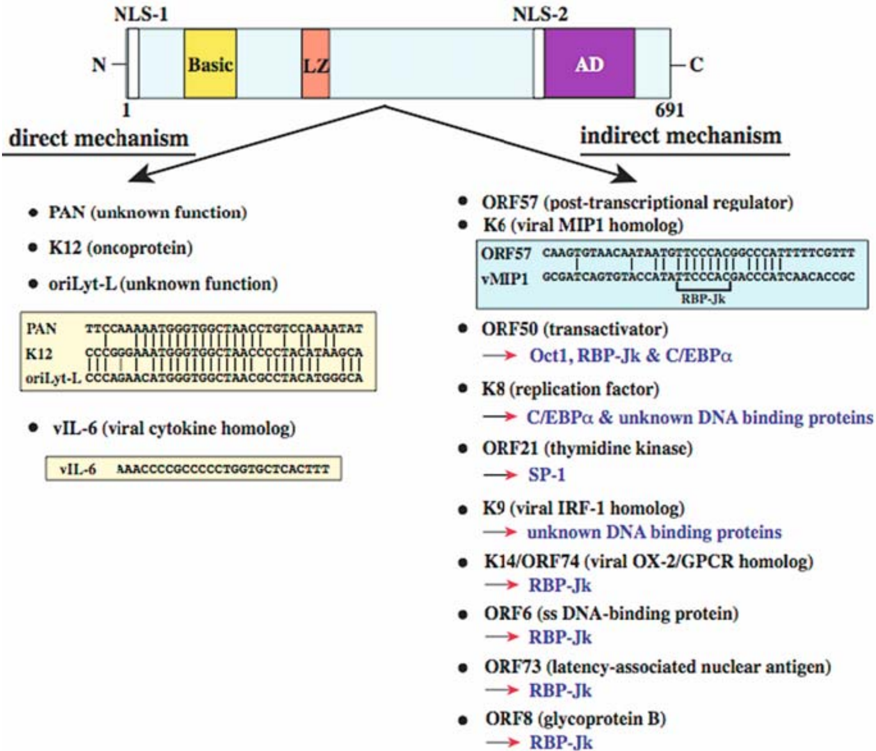

Fig. 21.2 Domain structure of ORF50 protein and its responsive genes. The diagram at the top of the figure represents characteristic domains of ORF50 protein. Putative domains and motifs are indicated as follows: NLS, nuclear localization signal; Basic, basic-rich region; LZ, leucine zipper; AD: activation domain. The bottom of the figure indicates the identified ORF50-responsive genes. The molecular mechanisms of transactivation by ORF50 protein are described in the text

are involved in lytic replication, including its own gene (Sect. 21.3.3), polyadenylated nuclear (PAN) RNA, K12 (kaposin), ORF57, K6 (vMIP-1), K8 (K-bZIP), K9 (vIRF), ORF21 (thymidine kinase), K5, ORF6 (single-stranded DNA binding protein), K14 (vOX-2), ORF74 (vGPCR), and K2 (vIL-6) (Chang et al. 2002; Chen et al. 2000; Deng et al. 2000, 2002; Jeong et al. 2001; Lukac et al. 2001; Lukac et al. 1999; Song et al. 2001; Wang et al. 2001b; Zhang et al. 1998). In addition to activation of early-lytic genes, ORF50 protein has been shown to activate a late-lytic gene (glycoprotein B) and latency-associated genes (LANA, v-cyclin and v-FLIP) under certain conditions (Damania et al. 2004; Matsumura et al. 2005). These studies highlight the fact that ORF50 protein possesses diverse functions in initiating lytic replication and in controlling establishment of latency during de novo viral infection.

In order to provide a favorable milieu for viral production, the ORF50 protein also stimulates expression of specific cellular genes. Two cellular genes, CD21 and CD23, respond directly to ORF50 protein (Chang et al. 2005a). ORF50 protein also selectively activates cellular interferon-stimulated genes, including ISG-54, MxA, and STAF-50, in primary endothelial cells (Zhang et al. 2005). The biological relevance of the ORF50 protein in the KSHV life cycle has been extended by the finding that the ORF50 protein is a component of the tegument of KSHV virions (Bechtel et al. 2005). The incoming ORF50 from the virus tegument may support productive infection or initiate a burst of aberrant lytic-cycle gene expression, a phenomenon that was observed during early viral infection of primary endothelial and fibroblast cells (Krishnan et al. 2004).

Besides functioning as a transcriptional activator, ORF50 protein also participates in directing the assembly of replication complexes required for initiation of viral DNA replication. Two independent studies revealed that an ORF50-binding site present in the lytic origin of DNA replication (oriLyt) is indispensable for viral DNA replication (AuCoin et al. 2004; Wang et al. 2004b).

A recent study showed that the ORF50 protein promotes the proteasome-mediated degradation of polyUb-conjugated interferon regulatory factor 7 (IRF7) in cotransfected cells (Yu et al. 2005). In a cell-free ubiquitination assay, the ORF50 protein was shown to directly add polyUb to IRF7, suggesting that ORF50 protein possess ubiquitin E3 ligase activity. The ORF50 protein may also autoregulate its own stability through polyubiquitination (Yu et al. 2005).

21.4.2 Functional Domains of ORF50 Protein

The ORF50 protein is conserved among gamma herpesviruses (Lukac et al. 1998; Sun et al. 1998). However, no significant sequence homology between ORF50 and cellular proteins has been identified until now. The ORF50 protein is a 691-amino-acid (aa) polypeptide. Its predicted molecular mass is 73.7 kDa.

When the ORF50 cDNA is transcribed and translated in vitro, the molecular mass is approximately 90 kDa in SDS–PAGE gels (Lukac et al. 1999). By contrast, ORF50 protein expressed in mammalian or insect cells migrates at 110 kDa mainly due to hyperphosphorylation (Lukac et al. 1999). Little is known about the phosphorylation positions in ORF50 protein or their potential significance on ORF50 function. There are two putative nuclear localization signals (NLSs) present in ORF50 protein located at aa 6–12 and at aa 516–530 (Lukac et al. 1998). Deletion analysis has shown that the transport of the protein into the nucleus is predominantly dependent on the N-terminal NLS (Chen et al. 2000).

The ORF50 protein is a typical transcriptional activator that contains a sequence-specific DNA-binding domain and a transactivation domain (Chang and Miller 2004; Chang et al. 2002; Lukac et al. 1999; Wang et al. 2001a). Some poorly conserved DNA sequences bound by ORF50 in vitro consist of tandem arrays of phased A/T trinucleotide motifs (Liao et al. 2003). However, two known targets found in the PAN and K12 promoters, share a highly conserved core element, AAATGGGTGGCTAACCCCTACATAA. (The conserved sequences are underlined). The DNA-binding domain of ORF50 protein, located within the first 390 amino acids, displays strong affinity to the PAN and K12 elements (Chang et al. 2002). In the DNA-binding domain, a cluster of basic residues that include R160, R161, R166, and R167 appears to be directly involved in the interaction of ORF50 protein with its target DNA. Single point mutations of these basic residues dramatically reduce the DNA-binding activity of the protein (Chang et al. 2005c). A potential leucine zipper motif located between aa 247 and 268 is also present in the DNA-binding domain. This domain may mediate oligomerization of ORF50 protein or contribute to heterodimerization with other proteins (Lukac et al. 1998).

The C-terminal region (aa 486–691) of ORF50 protein contains a domain that can activate transcription when fused to another DNA-binding protein, GAL4 (Lukac et al. 1999; Wang et al. 2001a). Deletion of the activation domain resulted in loss of normal activation function and generated a dominant-negative mutant (Lukac et al. 1999). By creation of chimeric proteins that fuse the DNA-binding domain of the yeast transactivator GAL4 (residues 1–147) to various region of ORF50 protein, the region between aa 527 and –634 of ORF50 protein was found to be most important for conferring the transactivation function (Wang et al. 2001a). A proline-rich region and a serine/threonine-rich region found in the central part of ORF50 protein may be crucial for mediating protein–protein interactions or regulating ORF50 post-translational modification (Gwack et al. 2003b). Additionally, a multifunctional regulatory region present in amino acids (aa) 520–535 of ORF50 protein controls DNA binding and protein stability (Chang and Miller 2004). In this region, seven out of 15 amino acids are basic. Deletion or mutation of a basic motif (KKRK) in this region dramatically enhances DNA-binding activity and leads to abundant expression of an ORF50 variant, ORF50B (see more detail in Sect. 21.4.4).

These diverse regulatory domains and motifs strongly emphasize the complex function of ORF50 protein in lytic replication.

21.4.3 Direct and Indirect DNA-Binding Mechanisms of ORF50 Transactivation

Despite extensive studies of ORF50-mediated activation of viral gene expression, many details of ORF50 action on individual target promoters are not fully understood. In order to improve understanding of the detailed molecular actions of ORF50 protein, considerable efforts have been expended to identify and to classify the *cis*-acting regions from target promoters responding to ORF50 (Fig. 21.2). A DNA motif conferring ORF50 responsiveness is termed an ORF50 response element (ORF50 RE) or an Rta response element (RRE) (Chang et al. 2002; Lukac et al. 2001; Song et al. 2001). A *bona fide* ORF50 RE should respond to ORF50 protein even when fused to a heterologous promoter. The methods for characterizing the ORF50 REs usually include a nucleotide sequence homology search, electrophoretic mobility shift assays (EMSA), and functional transcriptional reporter assays. The correlation among these several assays is used to assess the potential transcriptional mechanism of ORF50 protein. Despite the several limitations of EMSA experiments, the binding of ORF50 protein to target elements in vitro generally reflects its behavior in vivo. Different sources of ORF50 protein used in EMSA experiments may be prepared from *E. coli*, insect cells, or mammalian cells as well as from in vitro translation (Chang et al. 2002; Lukac et al. 2001; Song et al. 2001; Wang et al. 2003b). It is noteworthy that in some instances highly purified and concentrated ORF50 protein used in EMSA may lead to non-specific but preferential binding to DNA elements in vitro (Ziegelbauer et al. 2006). This non-specific binding is probably due to the basic property of ORF50 protein. Such false binding by ORF50 protein is not usually accompanied by appreciable ORF50 responsiveness (Ziegelbauer et al. 2006).

Several lines of evidence indicate that there are at least two different molecular mechanisms by which ORF50 protein activates its target genes during the lytic cycle: direct DNA binding and interaction with cellular proteins bound to promoter DNA. Although ORF50 protein can behave as a specific DNA-binding protein, only a few ORF50 REs can be bound directly by ORF50 protein (Chang et al. 2002; Chang et al. 2005c; Ueda et al. 2002). In Fig. 21.2, we summarize and classify the identified ORF50 REs into two groups based on published studies. Since both direct and indirect actions of ORF50 protein may operate simultaneously on the same or overlapping elements in promoters, here we select the predominant mechanism by which ORF50 activates specific promoters.

Activation of one class of target genes, including PAN and K12, by ORF50 protein operates mainly through a direct DNA-binding mechanism (Chang et al. 2002; Chang et al. 2005c; Song et al. 2001). PAN RNA is a 1.2-kb non-coding

polyadenylated transcript that is the most abundant transcript expressed during the KSHV lytic phase (Sun et al. 1996). The biological function of PAN RNA in KSHV replication and pathogenesis remains unclear. The K12 gene may encode an oncoprotein involved in cellular transformation (Muralidhar et al. 1998; Sadler et al. 1999). The ORF50 REs identified in the PAN and K12 promoters share similar DNA sequences (Chang et al. 2002). These conserved sequences are also found in the second lytic origin of DNA replication, oriLyt-L, located between ORFs K4.2 and K5 (Wang et al. 2004b). The element in oriLyt-L also confers strong responsiveness to ORF50 protein (Wang et al. 2004b). The ORF50 RE in the vIL-6 promoter has been reported to bind to ORF50 protein (Deng et al. 2002). However, the ORF50 RE in the vIL-6 promoter does not share significant homology with the conserved sequences found in the PAN and K12 promoters. Why ORF50 protein would have such diverse DNA-binding targets remains unknown.

On the other hand, ORF50 activates many more target genes through a mechanism involving in indirect DNA binding. A cellular protein, RBP-Jκ (also known CSL or CBF-1), binds ORF50 protein and mediates the ORF50 activation of two target genes ORF57 and ORF6 (Liang et al. 2002) (Fig. 21.2). This finding has been exploited to investigate another transactivation mechanism utilized by ORF50 protein. RBP-Jκ is a sequence-specific DNA-binding protein which is a downstream effector of Notch signal transduction (Lai 2002). Under normal conditions, RBP-Jκ binds to target DNA in promoters and acts as a repressor until stimulating signals arrive. The interaction between ORF50 protein and RBP-Jκ leads to the conversion of RBP-Jκ from a repressor to a transactivator of target genes. This mechanism of transactivation by ORF50 protein has been demonstrated for the ORF57, ORF6, K14/ORF74, K6 (vMIP-1), ORF50, and LANA (LTi) promoter (Chang et al. 2005c; Liang and Ganem 2003, 2004; Matsumura et al. 2005). All the ORF50 REs in these promoters contain a conserved or related RBP-Jκ binding site, GTGGGAA. In the case of the ORF57 and vMIP-1 promoters, mutation of the RBP-Jκ-binding site dramatically abolished the response to ORF50 protein (Chang et al. 2005c). Furthermore, the ORF50 protein failed to activate the ORF57 and ORF6 promoter in RBP-Jκ-null cells, while such activation was restored by co-transfection with an RBP-Jκ expression vector (Liang et al. 2002). These observations clearly demonstrated that activation of the ORF57 and ORF6 promoters was dependent both on an intact RBP-Jκ-binding site in these promoters and on expression of RBP-Jκ protein. The indirect mechanism of action of ORF50 protein, involving protein–protein interaction with RBP-Jκ, may be independent of the DNA-binding function of ORF50 protein. This hypothesis was addressed by characterizing the transactivation function of DNA-binding-deficient mutants of ORF50 protein. Several DNA-binding-deficient mutants of ORF50 protein could selectively activate the ORF57 and vMIP-1 promoters but were markedly defective at activating the PAN and K12 promoters (Chang et al. 2005c).

The diversity in DNA sequences of ORF50 REs from other ORF50-responsive promoters implies that ORF50 protein may use other indirect mechanisms for activating these promoters. In addition to RBP-Jκ, several other cellular proteins have been found to bind to ORF50 responsive promoters. These include C/EBPα, OCT1, SP1, and some unidentified proteins (Sakakibara et al. 2001; Ueda et al. 2002; Wang et al. 2003b; Wang et al. 2003c; Zhang et al. 1998). These other proteins may also serve as mediators for recruiting ORF50 protein to the promoters to initiate transcriptional activation.

21.4.4 Regulation of DNA Binding and Stability of ORF50 Protein

ORF50 is a multifunctional protein which activates numerous viral and cellular genes and promotes viral lytic DNA replication. Although ORF50 protein binds DNA specifically, most target genes seem to be activated by ORF50 protein via a mechanism which is independent of DNA binding (Fig. 21.2). Therefore, in cells lytically infected with KSHV there must exist an elaborate control mechanism for regulating the DNA-binding function of ORF50 protein. A non-DNA-binding conformation of ORF50 protein is likely to be predominant in cells. This hypothesis is consistent with our observation that wild-type ORF50 protein, expressed in mammalian cells, displays little detectable DNA-binding capacity in vitro (Chang and Miller 2004). By using truncated ORF50 proteins, we identified an inhibitory region, located between aa 520 and 535, that represses DNA-binding activity (Chang and Miller 2004). The amino acid residues in this regulatory region are QRSKERSSKKRKALTVP. A detailed analysis of this regulatory region revealed that a centrally located basic motif, KKRK, but not the upstream three serines play a critical role in inhibiting the DNA-binding activity of ORF50 protein. Deletion or mutation of the basic motif (KKRK) in this region dramatically increased the DNA-binding capacity of ORF50 protein (Chang and Miller 2004). The enhancement in DNA-binding capacity of ORF50 mutants has been extensively examined by EMSA, DNA-affinity chromatography, and chromatin immunoprecipitation. Independently, the same region has also been shown to contribute to the stability of ORF50 protein. Mutations of the KKRK motif in the full-length ORF50 protein led to abundant accumulation of an electrophoretic mobility variant, ORF50B, which appears to be an unmodified form of ORF50 protein (Chang and Miller 2004). Since mutations in the regulatory region did not alter the level of ORF50 mRNA, it is more likely that these mutations stabilize the protein. In addition to the KKRK motif, another region located between aa 590 and 650 also plays a role to regulate the stability of ORF50 protein (Chang and Miller 2004). ORF50 protein also serves as an ubiqutin E3 ligase and autoregulates its own polyubiquitination and stability (Yu et al. 2005). The critical region for regulating ORF50 stability was mapped to a Cys/His-rich N-terminal region (aa 118–207). This region does not overlap with the region that our lab

has found to regulate protein expression. Taken together, it is possible that multiple regions in ORF50 protein may coordinately regulate protein stability via the ubiquitin-proteasome pathway. Notably, the lysine residues found in the KKRK motif could serve as acceptor sites for polyubiquitination.

Despite the fact that several specific mutations could enhance the DNA-binding activity or increase the protein stability, all ORF50 mutants with enhanced DNA binding or increased stability appear to be partially defective in activating downstream target genes (Chang and Miller 2004). This subtle autoregulation is likely to represent an essential strategy to allow ORF50 protein to carry out multiple functions by distinct mechanisms in controlling lytic replication of KSHV.

21.4.5 Cellular Proteins that Interact with ORF50 Protein to Modulate its Actions

Proteins that interact with ORF50 protein may influence the DNA-binding activity, protein stability, post-translational modification, and transactivation function of ORF50. Several cellular DNA-binding proteins, transcriptional cofactors, enzymatic proteins, or viral proteins that directly associate with ORF50 protein have shown to be crucial for regulating lytic gene expression. A summary of the ORF50-associating proteins is presented in Fig. 21.3. These

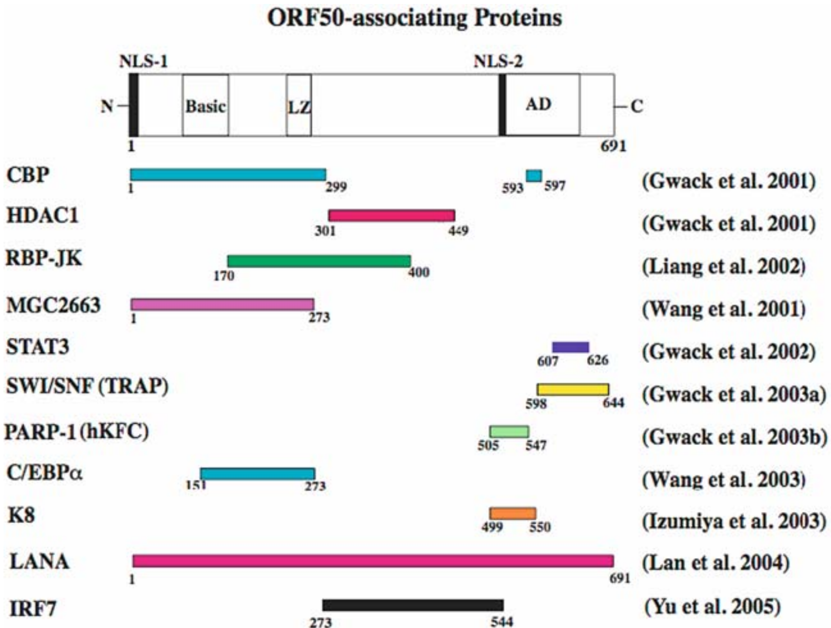

Fig. 21.3 Regions of protein/protein interactions between ORF50 and other proteins. The numbers under the shaded boxes correspond to residue numbers of ORF50 protein

cellular and viral proteins include CREB-binding protein (CBP), histone deacetylase-1 (HDAC1), RBP-Jκ, MGC2663, STAT3, SWI/SNF complex, TRAP/mediator complex, poly(ADP-ribose) polymerase 1 (PARP-1), Ste20-like kinase (hKFC), C/EBPα, interferon regulatory factor 7 (IRF7), KSHV ORF K8, and KSHV ORF73 (LANA). Most of these ORF50-interacting proteins were identified using either protein co-purification or a yeast two-hybrid system.

The best-characterized ORF50-interacting protein is RBP-Jκ which was found by a yeast two-hybrid screen (Liang et al. 2002). RBP-Jκ protein interacts with a central region of ORF50 protein located between aa 170 and 400 (Fig. 21.3). Increasing evidence suggests that RBP-Jκ acts as a major cellular mediator involved in ORF50-activated viral transcription (see Sect. 21.4.3). In addition to RBP-Jκ, two cellular DNA-binding proteins, C/EBPα and STAT3, also interact with ORF50 protein and mediate the activation of certain viral and cellular promoters (Gwack et al. 2002; Wang et al. 2003b, c). Physical association between ORF50 and C/EBPα required the N-terminal DNA-binding domain of ORF50, while binding to STAT3 requires the C-terminal transactivation domain of ORF50. Potential C/EBPα-binding sites present in the promoters of K8, ORF50, PAN, and ORF57 could mediate the cooperative activation by ORF50 and C/EBPα (Wang et al. 2003b, c). STAT3 is a latent cytoplasmic protein that can be activated by various cytokines and growth factors (Schindler and Darnell 1995). Upon stimulation, STAT3 undergoes phosphorylation and dimerization, and then transport into nucleus. ORF50 recruits STAT3 to the nucleus and induces the dimerization of STAT3 monomer in the absence of STAT3 phosphorylation (Gwack et al. 2002). This interaction between ORF50 and STAT3 leads to stimulation of STAT3-mediated transcription.

An uncharacterized protein, MGC2633, that interacts with ORF50 protein has been identified using a yeast two-hybrid screen (Wang et al. 2001b). MGC2633 is 554-aa polypeptide which displays sequence similarity with members of the Krueppel-associated box-zinc finger proteins. However, little is known about the biological function of MGC2633. MGC2633 binds to the N-terminus of ORF50 protein and can activate viral promoters in synergy with ORF50 protein.

Several other cellular proteins, including CBP histone acetyltransferase, the Brg1 subunit of SWI/SNF chromatin remodeling complex, and the TRAP230 subunit of TRAP/mediator complex, that interact with the C-terminal transactivation domain of ORF50 protein may be essential to form an active transcriptional complex (Gwack et al. 2001, 2003a). The CBP and SWI/SNF complexes are transcriptional cofactors that cooperatively disrupt nucleosome structure and facilitate transcription factor binding. The TRAP/mediator complex functions as an interface between sequence-specific transcription factors and the general transcriptional apparatus. Recruitment of these cellular transcriptional cofactors by ORF50 protein may be a general way for mediating transcriptional activation of all target genes.

The function of ORF50 protein can be repressed by interaction with HDAC1, PARP1, and hKFC (Gwack et al. 2001, 2003b). The HDAC1-binding domain in

ORF50 is located between aa 301 and 449, which contains several proline residues (Gwack et al. 2001). PARP1 and hKFC interact with the serine/threonine-rich region of ORF50 protein (Gwack et al. 2003b). Poly(ADP-ribosyl)ation and phosphorylation of ORF50 protein by PARP1 and hKFC repress ORF50-mediated transcriptional activation. IRF7, a key mediator of type I interferon induction, was found to interact with ORF50 protein by a yeast two-hybrid screen (Yu et al. 2005). The critical residues in ORF50 protein for IRF7 binding were mapped between aa 273 and 544. In such a case, ORF50 protein acts as an ubiqutin E3 ligase and specifically inhibits the biological function of IRF7. Thus, ORF50 protein may possess immunomodulatory properties.

KSHV-encoded proteins, including K8 and LANA, interact with ORF50 protein (Izumiya et al. 2003a). The association between ORF50 and K8 requires a specific region (aa 499–550) of ORF50 protein. The K8 protein functions as a coregulator of ORF50 protein to selectively repress the activation of the ORF57 gene, but not the PAN gene (Izumiya et al. 2003a). LANA also inhibits the ORF50-mediated transactivation in transient reporter assays (Lan et al. 2004).

21.5 Biological Relevance of the Lytic Targets of ORF50 Protein in the KSHV Lytic Cycle

Most of the lytic cycle KSHV genes which are targets of ORF50 protein play essential roles in viral DNA replication, regulation of viral and cellular gene expression, and viral pathogenesis (Choi et al. 2001; Dourmishev et al. 2003). Based on their identified functions, these KSHV lytic genes downstream of ORF50 protein can be divided into different categories, including replication proteins (ORF6, K8, and ORF21), transcriptional modulators (K8, ORF57, and vIRF1), signal ligands, receptors and transducers (vOX2, vGPCR, and Kaposins), cytokines (vIL-6 and vMIP-1), immunomodulatory proteins (K5, K3), viral structural protein (gB), as well as products of uncertain function (PAN RNA and oriLyt-associated transcript). Here we review how these target genes of ORF50 protein contribute to viral replication and viral pathogenesis.

21.5.1 Viral Replication Proteins: ORF6, K8, and ORF21

ORF6 (ss DNA-binding protein) is a component of the prereplication complex involved in lytic synthesis of KSHV DNA (AuCoin et al. 2004; Wu et al. 2001). In addition to ORF6, the core replication proteins encoded by KSHV include ORF9 (DNA polymerase), ORF40-41 (primase-associated factor), ORF44 (helicase), ORF56 (primase), and ORF59 (polymerase processivity factor) (AuCoin et al. 2004; Wu et al. 2001). Among these core replication proteins, only the promoter of ORF6 has been shown to respond to ORF50 protein

independently of other viral proteins (Liang et al. 2002; Lukac et al. 1998). A transient cotransfection–replication assay showed that these core replication proteins, together with ORF50 and K8, are sufficient for oriLyt-dependent DNA replication (AuCoin et al. 2004).

K8 (also called K-ZIP or RAP), a basic region-leucine zipper (bZIP) protein, is a homolog of EBV ZEBRA (Lin et al. 1999). Both ORF50 and K8 may function as origin-binding proteins or initiator proteins in viral DNA synthesis (Lin et al. 2003; Wang et al. 2004b, 2006). Palindromically arrayed C/EBP motifs within oriLyt are absolutely required for binding K8 protein and for lytic DNA replication (Lin et al. 2003). Since K8 alone cannot directly bind to the region that harbors the C/EBP motifs, it is suggested that K8 binds to KSHV oriLyt through interaction with C/EBPα bound to DNA (Wang et al. 2004b). Similarly, the ORF50 response element in oriLyt was also indispensable for lytic DNA replication (AuCoin et al. 2004; Wang et al. 2004b). ORF50 protein may also be a component of the prereplication complexes and allow the complexes to bind oriLyt DNA (Wang et al. 2006).

The ORF21 (thymidine kinase) is not absolutely essential for lytic KSHV DNA synthesis. However, this enzyme may increase the replication rate of viral DNA (Zhang et al. 1998). KSHV thymidine kinase, like that of other herpesviruses, plays an essential role in metabolism of nucleoside analogues which function as antiviral drugs (Morin et al. 1997).

21.5.2 Viral Proteins which Influence Gene Expression: K8, ORF57, and vIRF1 (K9)

K8 regulates gene expression and influences the cell cycle. K8 interacts with many viral and cellular proteins, including ORF50, viral protein kinase (ORF36), Ubc9 (E2 SUMO conjugation enzyme), p53, and cyclin-dependent kinase CDK2 (Izumiya et al. 2003a, b, 2005, 2007; Park et al. 2000). Binding of K8 to ORF50 protein negatively regulates the transcription function of ORF50 on the K8 and ORF57 promoters but not on the PAN promoter (Izumiya et al. 2003a; Liao et al. 2003). Sumolylation and phosphorylation of K8 are critical in regulating its transcriptional repression activity (Izumiya et al. 2005, 2007). Phosphorylation of K8 by viral protein kinase (ORF36) attenuates the transcriptional repression activity of K8, while sumolylation of K8 through its interaction with Ubc 9 promotes K8's repression function (Izumiya et al. 2005, 2007). K8 also physically interacts with p53 and functionally represses the transcriptional activity of p53, which is required for apoptosis of the host cells (Park et al. 2000). Expression of K8 also causes cell cycle G1 arrest by binding to the cyclin-CDK2 complex and downregulating its kinase activity (Izumiya et al. 2003b).

KSHV ORF57 encodes a nuclear protein of 455 amino acid residues, which is a homolog of the EBV BMLF1 gene product (SM, Mta, EB2) and herpes

simplex virus (HSV) ICP27 gene (Bello et al. 1999). ORF57 protein is essential for virion production. It enhances expression of a subset of lytic cycle genes (Han and Swaminathan 2006; Majerciak et al. 2007). In transient reporter assays, ORF57 shows little effect on several KSHV lytic promoters; thus the ORF57 product is not a "real" transcriptional activator (Kirshner et al. 2000). However, ORF57 acts at several post-transcriptional levels to modulate the accumulation of certain viral transcripts, including cytoplasmic mRNAs (e.g., ORF59 and gB) and nuclear RNA (PAN) (Kirshner et al. 2000). ORF57 is known to interact with the cellular export factor Ref/Aly and with RNA (Malik et al. 2004b), which may be critical to facilitate nuclear RNA export. In addition to enhancing nuclear RNA transport, ORF57 may act on messenger RNA accumulation by export-independent effects on RNA stability or transcript elongation (Nekorchuk et al. 2007). Furthermore, ORF57 may synergize with ORF50 protein to enhance transcription of ORF50-responsive promoters (Kirshner et al. 2000; Malik et al. 2004a). To study KSHV ORF57 function in the context of the viral genome, recombinant KSHV genomes have been generated in which the ORF57 gene was disrupted (Han and Swaminathan 2006; Majerciak et al. 2007). Consistent with the results described above, mutant KSHV genomes lacking ORF57 expression were unable to express a subset of viral genes, were not induced to a higher level of PAN RNA expression after butyrate treatment, and did not produce infectious viruses (Han and Swaminathan 2006; Majerciak et al. 2007).

vIRF1 (K9) is a 449-amino acid protein with significant homology to cellular IFN regulatory factors (IRFs) (Russo et al. 1996). vIRF-1 was the first KSHV protein shown to have cell transformation potential (Gao et al. 1997). Expression of vIRF-1 in rodent fibroblast cells leads to morphological changes, focus formation, and tumor induction in nude mice (Gao et al. 1997; Li et al. 1998). One component of its transforming capacity seems to result from v-IRF1 interfering with IFN signaling. v-IRF1 inhibits cellular IRF-1 and IRF3-mediated transcription (Lin et al. 2001; Zimring et al. 1998). Despite the homology of vIRF1 with the DNA-binding region of cellular IRFs, v-IRF1 does not seem to bind directly to DNA (Roan et al. 1999). Several mechanisms, including direct binding of vIRF-1 to its cellular homologs and functional inhibition of a required co-activator, p300, are thought to serve as critical events in the capacity of v-IRF1 to inhibit IFN-mediated gene expression (Burysek et al. 1999; Li et al. 2000; Lin et al. 2001; Zimring et al. 1998). Additionally, vIRF1 synergizes with CBP to induce expression of the c-myc oncogene at levels that are required for cell transformation (Jayachandra et al. 1999). Furthermore, vIRF1 interacts with the cellular p53 tumor suppressor gene and with ATM kinase (Nakamura et al. 2001; Seo et al. 2001; Shin et al. 2006). Interaction between vIRF1 and p53 inhibits the transcriptional activation by p53 and enhances degradation of p53 (Nakamura et al. 2001; Seo et al. 2001), while binding of vIRF1 to ATM kinase inhibits ATM kinase activity (Shin et al. 2006).

21.5.3 Viral Proteins which Modulate Cell Signal Transduction: vOX2 (K14), vGPCR (ORF74), Kaposin (K12), K1, and K15

vOX2 and vGPCR are encoded from a ORF K14-ORF74 bicistronic mRNA; the K14 ORF is located in the 5′ position (Kirshner et al. 1999). vOX2 is a glycosylated cell-surface protein with an apparent molecular mass of 55 kDa (Chung et al. 2002). Unlike cellular OX2, which delivers a repressive signal to myeloid-lineage cells, vOX2 provides an activating signal to induce inflammatory cytokine production in myeloid-lineage cells (Chung et al. 2002). The stimulus provided by vOX2 may potentially promote cytokine-mediated angiogenic proliferation of KSHV-infected cells.

vGPCR is a member of the family of CXC G-protein-linked chemokine receptors, with significant homology to CXCR1 and CXCR2 (Russo et al. 1996). Most studies in cultured cells or in animal models agree that vGPCR is the predominant oncogene of KSHV which initiates Kaposi's sarcoma (Arvanitakis et al. 1997; Bais et al. 1998; Montaner et al. 2003; Sodhi et al. 2006; Yang et al. 2000). The viral G protein-coupled receptor has been shown to be ligand independent, constitutively active and capable of stimulating signals which regulate the PI3K/AKT/TSC/mTOR and MAPK/ERK pathways (Cannon et al. 2003; Sodhi et al. 2006, 2004). vGPCR induces secretion of angiogenic growth factors, including VEGF, IL-8, and Gro-α, from cells in which it is expressed. Thus, vGPCR may serve a role both in direct cell transformation and in indirect (paracrine) cell transformation (Polson et al. 2002; Shepard et al. 2001; Sodhi et al. 2000).

Investigation in the BCBL1 PEL cell line showed that the K12 locus exhibited a complex translational program. The K12 transcript generates a variety of K12 isoforms (Sadler et al. 1999). These isoforms are designated kaposin A, B, and C. In contrast to kaposin A (ORF K12), kaposin B and C use non-ATG start codons (CUG), located upstream of ORF K12, to initiate polypeptide synthesis (Sadler et al. 1999). Kaposin A is a 60-aa hydrophobic protein that has been shown to induce focus formation of rodent fibroblast cells and tumor formation in nude mice (Muralidhar et al. 1998, 2000). Cell transformation induced by kaposin A may be mediated through its direct interaction with cytohesin-1, a guanine nucleotide exchange factor of ARF GTPases (Kliche et al. 2001). Functional activation of cytohesin-1 by kaposin A results in stimulation of the ERK2/MAP kinase pathway (Kliche et al. 2001). Kaposin B binds to and activates the kinase MK2, a target of the p38MAP kinase pathway (McCormick and Ganem 2005, 2006). The kinase MK2 has been well characterized to play a critical role in the stabilization of cytokine transcripts and other mRNAs that contain AU-rich elements (AREs) in their 3′ noncoding regions (Winzen et al. 1999). Thus, it was suggested that functional activation of MK2 by kaposin B may increase the expression of cytokines by preventing the degradation of their mRNAs (McCormick and Ganem 2005). So far, the precise role of kaposin C in viral replication and pathogenesis is not well understood.

ORF50 has also been shown to activate gene expression of the K1-transforming protein of KSHV (Bowser et al. 2002, 2006) and the KSHV K15-signaling protein (Wong and Damania, 2006). Further information about these proteins can be found in Chapter 22.

21.5.4 Viral Cytokines: vIL-6 (K2) and vMIP-1 (K6)

vIL-6, encoded by ORF K2, shows 25% sequence homology to human IL-6 (Neipel et al. 1997). Functional studies with a cloned vIL-6 gene have demonstrated that it stimulates proliferation of an IL-6-dependent mouse myeloma cell line and promotes the growth of KSHV infected PEL cells (Moore et al. 1996; Nicholas et al. 1997). vIL-6 also promotes hematopoiesis and acts as an angiogenic factor through the induction of VEGF (Aoki et al. 1999). Several signal transduction pathways are activated by vIL-6; these include the JAK/STAT and Ras/MAP kinase pathways (Chatterjee et al. 2002; Molden et al. 1997; Osborne et al. 1999). Despite their similarities in sequence and function, human IL-6 and vIL-6 display differences in receptor usage (Molden et al. 1997; Wan et al. 1999). Human IL-6 requires both IL-6Rα and gp130 for intracellular signaling; however, vIL-6 seems to require only gp130. Furthermore, N-linked glycosylation is absolutely required for optimal function of vIL-6, but not human IL-6 (Dela Cruz et al. 2004). Although vIL-6 has been implicated to play an important role in KSHV-associated diseases, different levels of expression of vIL-6 have been observed in KSHV-associated tumor lesions (Aoki et al. 1999; Parravicini et al. 2000). Immunohistochemistry has shown that only a minor population of virus-infected cells in KS lesions expressed vIL-6, whereas a high level of vIL-6 expression is detected in tissue from multicentric Castleman's disease.

vMIP-1 is a homolog of human MIP-1α, a βCC chemokine (Nicholas et al. 1997). vMIP-1 acts as specific agonist for CCR8, which is preferentially expressed on Th2 type T cells (Dairaghi et al. 1999). In an assay in the chick chorioallantoic membrane, vMIP-1 protein demonstrated strong angiogenic properties (Boshoff et al. 1997). In addition, vMIP-1 mediates a protective effect against dexamethasone-induced apoptosis (Liu et al. 2001). In PEL cell lines, vMIP-1 and vIL-6 have been shown to induce the expression of VEGF-B and VEGF-A, respectively (Liu et al. 2001). Since VEGF receptor 1 (Flt-1) is expressed in PEL cells, induction of VEGF expression by vMIP-1 and vIL-6 may provide autocrine regulation in vivo. Additional information about these proteins can be found in Chapter 22.

21.5.5 Immune Modulation: K5 and K3

K5 is an endoplasmic reticulum protein that contains ring finger motifs, similar to those found in cellular E3 ubiquitin ligases, a single transmembrane

domain, and a tyrosine-based sorting motif (Coscoy and Ganem 2000, 2001; Haque et al. 2000; Haque et al. 2001). K5 downregulates surface expression of MHC class I molecules through enhancement of their endocytosis and degradation (Haque et al. 2000; Haque et al. 2001). The KSHV protein K3, related to K5, also targets MHC class I for degradation (Coscoy and Ganem 2000). K3 and K5 exhibit 40% amino acid identity to each other and both are expressed early during the lytic cycle (Coscoy and Ganem 2000). Despite their similarity in sequence and function, K3 and K5 differ in their specificity. While K3 has been shown to efficiently target HLA-A, -B, -C and -E, K5 is only active against HLA-A and -B (Ishido et al. 2000b). Furthermore, K5 also downregulates ICAM-1 and B7.2, which are ligands for NK cell-mediated cytotoxicity receptors (Ishido et al. 2000a). As a consequence, K5 expression inhibits NK-cell-mediated cytotoxicity. The molecular mechanisms underlying downregulation of these cell-surface proteins are not completely clear, but have been thought to depend on the ability of K5 to act as an E3 ubiquitin ligase (Means et al. 2007). K3 and K5 also specifically target gamma interferon receptor 1 (IFN-γR1) and induce its ubiquitination, endocytosis, and degradation (Li et al. 2007). Further information about these proteins can be found in Chapter 24.

21.5.6 gB

KSHV-gB, encoded by ORF8 of the viral genome, is a virion envelope-associated glycoprotein (Russo et al. 1996). The gB protein possesses a putative heparan-binding domain and an integrin-interacting RGD (Arg–Gly–Asp) motif. These components of gB interact with heparan sulfate-like moieties and $\alpha 3\beta 1$ integrin on the cell surface (Akula et al. 2001; Wang et al. 2003a). Those interactions allow KSHV virions to enter target cells and to induce host cell signal transduction pathways. Expression of KSHV-gB, gh, and gL in CHO cells is sufficient to mediate the fusion of CHO cells with two human cell types, embryonic kidney cells, and B lymphocytes (Pertel 2002). The embryonic kidney cells and B lymphocytes which served as targets for cell fusion in the assays are known to be susceptible to HHV-8 entry. Additionally, soluble gB can induce cytoskeletal rearrangement in target cells as the result of activation of a focal adhesion kinase, Src, phosphatidylinositol 3-kinase, and rho GTPase (Krishnan et al. 2006; Sharma-Walia et al. 2004). A recombinant KSHV genome lacking the gB open reading frame has been constructed to study the function of gB in the context of viral infection (Krishnan et al. 2005). Besides its role in virus binding and entry into target cells, KSHV-gB also plays a role in maturation and egress of virus from the infected cells (Krishnan et al. 2005). Further information about this gene can be found in Chapter 23 by Bala Chandran and Neelam Sharma-Walia.

21.5.7 PAN and oriLyt-Associated Transcript

PAN (also called T1.1 or nut1) is the most abundant lytic phase transcript, which comprises as much as 80% of polyadenylated RNA in cells lytically infected with KSHV (Sun et al. 1996). Unlike most mRNAs, PAN is exclusively nuclear and does not encode polypeptide (Sun et al. 1996; Zhong and Ganem 1997). Despite the abundant expression of PAN in the lytic phase, its function in KSHV replication and pathogenesis remains unclear. Similarly, the function of the oriLyt-associated transcript is still unclear. Northern analysis showed that the oriLyt-associated transcript (oriLyt-T) is about 1.4 kb in length. oriLyt-T RNA contains GC-rich tandem repeat sequences in the 5′ half of the molecule which are followed by an ORF of 75 amino acids. So far, there is no evidence to show that any products are translated from this transcript (Wang et al. 2004b).

In summary, in this chapter we have described the complex regulation of expression and regulation of function of KSHV ORF50 protein, the key initiator of the lytic cascade. ORF50 expression is regulated by specific transcriptional activators and repressors and epigenetic mechanisms (Fig. 21.1). ORF50 protein itself activates downstream genes by direct binding to DNA and by interactions with other cellular proteins (Fig. 21.2). The DNA-binding activity and protein expression level are tightly regulated in cells. The many activities of ORF50 protein, including regulation of viral and cellular gene expression and viral lytic DNA replication, are influenced by many protein– protein interactions (Fig. 21.3). In a final section we describe the function of the many downstream lytic cycle genes of KSHV which are under control of the ORF50 protein. Each of these subtle exquisitely complex regulating systems represents a fertile field for further exploration.

Acknowledgments Work in the authors' laboratories was supported by grants CA16038 and CA12055 from NIH and by a grant CMRPD650012 from Chang-Gung Memorial Hospital (Taiwan) and by a grant NSC95-2320-B-182-054-MY3 from National Science Council (Taiwan).

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Chapter 22
HHV-8/KSHV Proteins Involved in Signaling and Transformation

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22.1 Introduction

When the first partial and complete sequences of Kaposi's sarcoma herpesvirus were obtained (Neipel et al. 1997; Nicholas et al. 1997; Russo et al. 1996), it became apparent that the presence of multiple viral genes with low but significant homologies to mammalian proteins involved in signal transduction is one of the key features of this virus. While equivalents of some of these homologues had been found in other herpesviruses or other large DNA viruses, for example chemokines and chemokine receptors, others such as interleukin-6 had not previously been identified in a virus. Given the low sequence homology to their cellular counterparts and the fact that some of the cellular gene homologues occur in different large DNA viruses, it seems that such homologues have entered their respective viral genomes a long time ago in the early phases of DNA virus evolution. Their continued presence, however, points to a selective advantage during virus evolution, probably by facilitating the spread and persistence of these viruses in their hosts and by counteracting the host's defense mechanisms. In the context of HHV-8/KSHV, a particularly interesting question is whether some of these homologues, and other viral proteins capable of activating intracellular signaling pathways, may contribute to oncogenesis. This review discusses the evidence available for individual HHV-8/KSHV genes.

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_22, © Springer Science+Business Media, LLC 2009

22.2 HHV-8/KSHV Membrane Proteins

22.2.1 vGPCR

The vGCPR encoded by ORF74 of HHV-8/KSHV is a constitutively active (ligand-independent) G protein-coupled receptor that is most related structurally to the CXCL8 (IL-8) receptor CXCR2 (Russo et al. 1996). The receptor is promiscuous in its Gα coupling, activating i, q and 12/13 classes of Gα proteins that lead to a broad range of signaling pathway activation (Cannon et al. 2003; Couty et al. 2001; Liu et al. 2004; Shepard et al. 2001). Kinases and transcription factors activated by vGPCR include MAPK (Erk, p38 and Jnk), Akt, NFAT, CREB, NF-κB, AP-1 and HIF-1α, and these are relevant to promotion of cell proliferation and survival and to induction of angiogenic responses via cytokine gene activation (Arvanitakis et al. 1997; Bais et al. 1998; Montaner et al. 2001; Pati et al. 2001; Schwarz and Murphy 2001; Shepard et al. 2001; Sodhi et al. 2000) (see below). Although constitutive, HHV-8/KSHV activity can be modulated both positively and negatively by cellular chemokines such as agonist GROα and IL-8 and inverse agonist IP-10 and SDF-1α (Geras-Raaka et al. 1998; Geras-Raaka et al. 1998; Gershengorn et al. 1998). In addition, the HHV-8/KSHV-encoded chemokine vCCL-2 (specified by ORF K4) acts as an inverse agonist (Geras-Raaka et al. 1998). Several "neutral" ligands bind to vGPCR without affecting signal transduction; these include CC ligands CCL1 (I-309), CCL5 (RANTES) and CCL3 (MIP-1a) and CXC ligands CXCL7 (NAP-2), CXCL5 (ENA-78) and CXCL4 (PF-4) (Arvanitakis et al. 1997; Rosenkilde et al. 1999). The biological relevance of vGPCR-chemokine functional and neutral interactions is unresolved, as is the function of vGPCR in HHV-8/KSHV biology. It is known, however, that agonist stimulation of vGPCR leads to a switch to predominantly Gα_q-initiated signaling, and this is likely to be relevant to KS pathogenesis (see below).

Of all the HHV-8/KSHV-encoded proteins vGPCR is probably the one for which there is most evidence for a role in virus neoplasia, specifically KS. While the first functional reports on vGPCR were focused on its pro-proliferative and transforming activity in experimental systems (Arvanitakis et al. 1997; Bais et al. 1998), subsequent evidence demonstrating the expression of the viral receptor only during productive replication (in cultured PEL cells) suggested that "autocrine" signaling was unlikely to be relevant to the postulated contribution of vGPCR to virus-induced pathogenesis (Kirshner et al. 1999). Rather, paracrine mechanisms, relying on cellular cytokine production induced by vGPCR in lytically infected cells, is more likely. Indeed, the seminal studies of Yang et al. (2000) investigating pathology in vGPCR transgenic mice identified the viral receptor as an inducer of KS-like lesions but in which expression of vGPCR was restricted to only a small minority of cells. Thus, evidence of vGPCR-induced paracrine mechanisms of KS pathogenesis was provided. These and subsequent studies using similar murine model systems verified the

induction of angiogenic cytokine release in the endothelial tumors (Guo et al. 2003; Holst et al. 2001; Montaner et al. 2003). Significantly, the elegant studies of Holst et al. (2001), utilizing a constitutively active but agonist-refractory variant of vGPCR, demonstrated convincingly that chemokine stimulation of vGPCR was necessary for efficient induction of KS tumors in the murine model. This coupled with the findings of others that vGPCR agonist CXCL1 activated $G\alpha_q$ signaling specifically, that the activation of MAPK signaling was predominantly $G\alpha_q$ mediated in endothelial cells and that VEGF-inducing HIF-1α activation by vGPCR was mediated via p38/Erk implicated $G\alpha_q$/MAPK-activated, cytokine-mediated paracrine signaling as a possible mechanism of vGPCR paracrine pathogenesis (Couty et al. 2001; Sodhi et al. 2000; Verzijl et al. 2004).

More recent studies have highlighted other potential mechanisms of vGPCR-induced KS pathogenesis. For example, Akt-mediated signaling has been identified as important for murine sarcomagenesis in transgenic animals and for tumorigenicity of vGPCR-expressing endothelial cells in recipient athymic mice (Sodhi et al. 2004). Furthermore, mTOR activation via inactivation of the negative regulator TSC2 by Akt-mediated phosphorylation appears to be centrally important to vGPCR-induced endothelial cell growth in culture and to tumorigenesis in the allograft model (Sodhi et al. 2006). In this study, the mechanism of sarcomagenesis via mTOR was shown convincingly to be mediated via paracrine mechanisms. Independent studies utilizing transgenic mice conditionally expressing vGPCR demonstrated that continued vGPCR expression is required for sustained KS-like pathogenesis and the production of secreted angiogenic factors in vGPCR-expressing cells, consistent with a cytokine-driven model of pathogenesis rather than a model in which immortalization and transformation are mediated via direct effects of vGPCR in the cell in which it is expressed (Jensen et al. 2005). Also worthy of note with respect to the role of paracrine signaling in vGPCR-induced murine KS, especially considering the association of HIV infection with KS, is the augmentation of this process by the HIV Tat protein (Guo et al. 2004). With regard to vGPCR-induced murine sarcoma development and progression, Grissotto et al. (2006) recently reported that vGPCR-expressing endothelial progenitor cells were abundant in murine KS-like lesions at early stages, and produced angiogenic cytokines, but that these cells constituted a smaller fraction of tumor cells at later stages of tumor development. These results suggest that vGPCR might play both a direct role in cellular growth, differentiation and angiocytokine production and a paracrine role with respect to tumorigenesis.

Notwithstanding the lack of available evidence that vGPCR can be expressed during viral latency, or at least in the absence of full productive replication (that would lead to cell death), there is the possibility, as shown for vIL-6, K3 and K5 (Chang et al. 2005), that vGPCR may be regulated independently of lytic cycle and that it may therefore contribute to pathogenesis in an "autocrine" manner. Indeed, it is known that RTA responsiveness of the vGPCR promoter is mediated via *cis* elements that bind CSL (RBP-Jκ and CBF1), and this suggests that RTA-independent (Notch signaling) mechanisms of transcriptional induction via alleviation of CSL inhibition could perhaps

operate to induce vGPCR expression in the absence of lytic replication (Liang and Ganem 2004). It should be noted, however, that in PEL cells conditionally expressing NIC, the truncated active form of Notch, ORF74, was not one of the HHV-8/KSHV genes detectably induced by NIC (Chang et al. 2005). Of relevance to KS is the demonstration by Bais and colleagues of endothelial cell immortalization induced by vGPCR transduction of primary endothelial cells, a process which involves vGPCR-induced VEGF–VEGFR2 autocrine signaling (Bais et al. 2003). There is also evidence of direct cell transformation by vGPCR, albeit in non-endothelial/B cell NIH–3T3 cells that do not reflect accurately normal HHV-8/KSHV-induced neoplasia (Bais et al. 1998; Burger et al. 1999). In this context, Burger and colleagues (2005) found that vGPCR activation of JAK2, which leads to STAT3 signaling, is essential for virus receptor-induced transformation, as measured by soft-agar/focus-forming assay. In a separate study again using NIH–3T3 cells in in vitro transformation assays, heme oxygenase-1 (HO-1) was found to be induced by vGPCR and to be involved in receptor-mediated transformation and VEGF induction; shRNA and chemical inhibition of HO-1 expression and activity inhibited the transformation by vGPCR (Marinissen et al. 2006). Furthermore, stable transduction of primary human endothelial cells with vGPCR or HO-1 expression cassettes led to increased cell proliferation and survival, sensitive in the case of both cell types to chemical inhibition of HO-1. These data suggest that both endothelial cell immortalization and cell line transformation by vGPCR are mediated via HO-1 induction, possibly in part via HO-1-stimulated expression of VEGF. While there are many unanswered questions regarding the mechanisms of vGPCR-induced cell immortalization and transformation, it is apparent that they are independent of agonist stimulation of vGPCR as the viral receptor can transform a variety of cell lines in the absence of added agonist. In contrast, vGPCR-induced sarcomagenesis is dependent on chemokine responsiveness of vGPCR (Holst et al. 2001). This difference suggests that these two activities of vGPCR, direct "autocrine" cell transformation and induction of sarcomagenesis, are dissociable and distinct.

22.2.2 K1

The K1 open reading frame (ORF) is located at the left end of the HHV-8/KSHV genome. Genes at the equivalent locus in other gammaherpesviruses encode proteins involved in signaling and transformation. These include the herpesvirus saimiri (HVS) transforming protein (STP) (Murthy et al. 1989), the latent membrane protein-1 (LMP-1) of Epstein–Barr virus (EBV) (Kaye et al. 1993) and the R1 protein of rhesus monkey rhadinovirus (RRV) (Damania et al. 1999). Like these proteins, K1 activates intracellular signaling cascades and induces proliferative, anti-apoptotic and inflammatory responses.

Structurally, K1 is a type I transmembrane protein of 46 kDa. Its extracellular domain (228 aa) features regional homology with the immunoglobulin (Ig)

superfamily and consists of two conserved regions (C1 and C2) as well as two highly variable regions (V1 and V2). The cytoplasmic tail (38 aa) contains an immunoreceptor tyrosine-based activation motif (ITAM) (Lee et al. 1998), analogous to motifs within the Igα and Igβ chains of the B-cell receptor (BCR). Although the K1 cytoplasmic tail is highly variable among different HHV-8/KSHV isolates around the world, the Src homology 2 (SH2)-binding motifs comprising the ITAM are always conserved (Zong et al. 1999).

Unlike the BCR, K1 signaling activity is constitutive in the absence of exogenous ligands, presumably through multimerization of its cysteine-rich extracellular domain, which then results in phosphorylation of the tyrosine residues in the ITAM. The ITAM is necessary for phosphorylation and activation of SH2-containing signaling proteins, such as Syk, Lyn, Cbl, Vav and the p85 subunit of phosphatidylinositol-3′-OH kinase (PI3K) as well as for K1-mediated upregulation of PI3K pathway signaling and NF-κB and NFAT promoter-dependent activity (Lagunoff et al. 1999; Lee et al. 1998; Prakash et al. 2005; Prakash et al. 2002; Tomlinson and Damania 2004). Activation of PLCγ2, RasGAP, SH2 domain-containing protein tyrosine phosphatase 1/2 (SH2-PTP1/2) and Grb2 has been observed upon stimulation with a monoclonal antibody directed against the extracellular domain of K1 (Lee et al. 2005). Recent studies performed by Tomlinson and Damania provide evidence that K1's signaling function is associated with its internalization via clathrin-mediated endocytosis. The group suggests that K1 signaling from endosomes might allow spatial and temporal regulation of its signal transduction (Tomlinson and Damania 2008).

K1 is expressed as an early gene during the HHV-8/KSHV lytic cycle (Bowser et al. 2002; Fakhari and Dittmer 2002; Jenner et al. 2001; Nakamura et al. 2003; Paulose-Murphy et al. 2001; Sarid et al. 1999). Although low-level expression has been reported in latently infected PEL cell lines (Samaniego et al. 2001), this could have been due to a small proportion of cells in these cultures undergoing lytic (productive) replication. K1 expression has been observed in tumor tissues of KS and MCD (Lee and Merchant 2003; Samaniego et al. 2001), and in KS cells expressing K1, the induction of secretion of inflammatory cytokines implicated in KS lesion formation, such as IL-6, IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF), was observed (Prakash et al. 2002; Samaniego et al. 2001).

The first evidence of the ability of K1 to transform cells emerged from studies in rodent cells: K1 expression transformed rodent fibroblasts in vitro, and recombinant herpesvirus saimiri strains in which the STP had been replaced with the K1 gene induced lymphomas in vivo (Lee et al. 1998). Further experiments indicated that a minority of K1 transgenic mice develop tumors with features of a spindle cell sarcomatoid tumor and malignant plasmaplastic lymphomas (Prakash et al. 2002). In these mice, IL-12 levels were severely decreased and basic fibroblast growth factor (FGF2) expression was upregulated in lymphocytes and tumors (Prakash et al. 2002). Interestingly, FGF2 has been described as an endothelial cell autocrine growth factor that promotes growth and angiogenesis of AIDS-KS cells (Samaniego et al. 1995).

Lymphocytes isolated from transgenic mice expressing K1 showed constitutive activation of transcription factors NF-κB and Oct-2 as well as enhanced Lyn kinase activity (Prakash et al. 2002). Biochemical and in vitro studies confirmed that NF-κB-dependent promoter activity is mediated by K1, and for B cells, Prakash et al. (2002) demonstrated that this activation further depends on the interaction of ITAM with Lyn kinase. Additionally, the transcription factor NFAT was identified as a downstream target of K1-induced PI3K, Syk and PLCγ signaling (Lagunoff et al. 1999; Lee et al. 2005; Lee et al. 1998; Prakash et al. 2005; Samaniego et al. 2001), and Wang et al. (2004) provided evidence that the transcription factor AP-1 is activated by K1-mediated activation of PI3K-MAPK signaling. NF-κB and NFAT are known to increase the expression of cytokines associated with inflammation and proliferation, and Lee et al. (2005) identified numerous inflammatory cytokines (MDC, IL-8, IL-10, VEGF, IL-1α/β and RANTES), which all contain NF-κB- and/or NFAT- and/or AP-1-binding sites in their promoter sequences and were induced by K1-mediated signal transduction. K1 may therefore activate uninfected or latently infected cells in a paracrine manner through activation of NF-κB-, NFAT- and AP-1-dependent promoters and secretion of inflammatory cytokines. Co-expression of the HIV-1 transactivation gene tat with K1 results in an additive effect on NF-κB-dependent transcription, suggesting cooperative signal activation of K1 and Tat. It has therefore been postulated that these two proteins might converge to reach an enhanced level of inflammation that may underlie progressive KS in AIDS patients (Prakash et al. 2000).

K1 utilizes several mechanisms to prevent cells from undergoing apoptosis and to support cell proliferation. These processes are mediated by increased anti-apoptotic and survival signaling via direct inhibition of proteins involved in apoptosis or by expression and secretion of growth factors such as VEGF. A role for K1 in the survival of HHV-8/KSHV-infected cells was first described by Lee et al. (2000), who found that the N-terminal extracellular domain of K1 interacts with the μ (heavy) chains of BCR complexes to retain them in the endoplasmic reticulum (ER), resulting in downregulation of their surface expression. Later, Tomlinson & Damania (2004) demonstrated that K1-mediated upregulation of the PI3K signaling cascade in B cells results in the phosphorylation of Akt and PTEN, which further leads to increased phosphorylation and thereby inactivation of forkhead transcription factors (FKHR). This would enhance cell survival signals and protect cells from apoptosis. Wang et al. (2006) showed that K1 can immortalize primary human umbilical vein endothelial cells (HUVEC) in culture and suggested that this is a consequence of the activation of the PI3K pathway. In addition to the PI3K downstream signals in B cells, this group observed K1-induced activation of mTOR and inactivation of GSK-3 and Bad, which are all events that would promote cell survival. In addition, Wang et al. (2007a,b) showed that Fas is an interaction partner of K1 in vitro and in vivo suggesting that K1 may directly inhibit Fas-mediated apoptosis to promote cell survival.

Inhibition of pro-apoptotic factors within the cell represents a direct effect of K1 on the infected cell, which may ensure host cell survival during the lytic cycle

of HHV-8/KSHV for efficient virus production. In addition, paracrine mechanisms apparently mediated by K1 may also contribute to HHV-8/KSHV maintenance and pathogenesis. It has been demonstrated that K1 induces the expression of vascular endothelial growth factor (VEGF), matrix metalloproteinase-9 (MMP-9) and basic fibroblast growth factor (FGF2) (Prakash et al. 2002; Wang et al. 2004), and VEGF is secreted in the surrounding matrix (Prakash et al. 2005; Wang et al. 2004). Angiogenesis is an essential feature of KS, and VEGF and FGF2 have been described to be involved in this process (Samaniego et al. 1998; Sgadari et al. 2002). Thus, the signaling activity of K1 may play an important role in VEGF-mediated angiogenesis in KS tumors.

22.2.3 K15

ORF K15 is located at the right end of the HHV-8/KSHV genome, adjacent to the terminal repeat region. Multiple and alternative splicing of its eight exons gives rise to a family of proteins that share a common C-terminal cytoplasmic domain but vary in the number of membrane anchor domains. So far, only the largest K15 protein with predicted 12 transmembrane domains linked to the cytoplasmic region has been shown to activate signaling pathways within host cells. Signaling motifs found in the cytoplasmic tail include two potential SH2- and one SH3-binding sites as well as a TRAF-binding region (Brinkmann et al. 2003; Choi et al. 2000; Glenn et al. 1999). These motifs are conserved among the two highly divergent P (predominant) and M (minor) genotypes of K15 (Glenn et al. 1999; Poole et al. 1999), suggesting the conservation of associated functional properties.

Biochemical studies with a CD8-K15 (C-Tail) chimera expressed in HHV-8/KSHV-negative BJAB cells performed by Choi et al. (2000) showed that the tyrosine residue (Y_{481}) within the putative SH2-B domain Y_{481}EEVL is the major site of phosphorylation by cellular tyrosine kinases and that this tyrosine phosphorylation is independent of antibody stimulation.

Structurally and functionally, K15 shows similarities to LMP1 and LMP2A of Epstein–Barr virus (Brinkmann and Schulz 2006; Nicholas 2003). Like LMP1, K15 interacts with TRAF-1, TRAF-2 and TRAF-3 and activates the MAP kinases ERK2 and JNK as well as the transcription factors NF-κB and AP-1 (Brinkmann et al. 2003; Glenn et al. 1999). Reminiscent of LMP2A, K15 interacts with members of the Src family of protein tyrosine kinases, which phosphorylate the tyrosine residue Y_{481} in a YEEV SH2-binding domain (Brinkmann et al. 2003). In a further parallel to LMP2A, K15 is able to inhibit B-cell receptor (BCR) signaling, as shown by the suppression of tyrosine phosphorylation and intracellular calcium mobilization in the B-cell line BJAB (Choi et al. 2000). Inhibition of BCR signaling involves the SH2-B (Y_{481}EEV) and the SH3-B (P387PLP) domains, as K15 variants with point mutations in these motifs do not suppress mobilization of intracellular free calcium upon anti-IgM stimulation (Choi et al. 2000). The second putative

SH2-B motif (Y_{431}SIL) is not significantly phosphorylated and may therefore not serve as a SH2-B domain but provide other functions. It has been shown that K15 interacts via its PPLP-motif with the SH3-C domain of Intersectin 2 (ITSN2) (Lim et al. 2007), a large scaffolding protein responsible for the assembly of protein complexes involved in the clathrin-mediated endocytic machinery (Pucharcos et al. 2000). This interaction appeared to link K15 to the regulation of BCR surface expression by increasing its rate of internalization upon stimulation (Lim et al. 2007). The ability of K15 to inhibit BCR signaling might therefore involve effects on intracellular signaling.

K15 is expressed during the lytic cycle of HHV-8/KSHV with low expression levels observed in unstimulated PEL cells (Choi et al. 2000; Glenn et al. 1999; Poole et al. 1999). This is reminiscent of the K1 expression pattern (see above), and clustering of gene expression array data grouped K15 together with K1, indicating a similar expression pattern for these two proteins (Jenner et al. 2001; Nakamura et al. 2003; Paulose-Murphy et al. 2001). Recently, it was shown that TPA-induced lytic infection and the HHV-8/KSHV ORF50/RTA protein activate K15 promoter elements (Wong and Damania 2006). Furthermore, a 45 kDa K15-derived protein has recently been shown to be expressed after the activation of the lytic (productive) replication cycle in 293 cells carrying a recombinant viral genome (Brinkmann et al. 2007). These findings indicate a predominant role for K15 during the HHV-8/KSHV lytic cycle.

K15 is capable of inducing the expression of multiple cytokines, including in particular IL-8, IL-6 and CXCL3 (Gro-γ), which have been shown to play a role in HHV-8/KSHV-associated pathogenesis (Brinkmann et al. 2007). While strongly inducing the expression and secretion of the angiogenic chemokine IL-8, K15 does not, unlike K1, show a marked effect on VEGF expression, but activates a number of VEGF target genes directly, e.g., dscr1 and cox-2 (Brinkmann et al. 2007). Expression of the majority of K15-induced genes depends on an intact YEEV SH2-binding site, underlining the importance of this motif, and presumably the recruitment of members of the src kinase family, for K15-mediated signaling (Brinkmann et al. 2007).

Therefore, as suggested for K1, K15 signaling may contribute to the hypothesized paracrine effects of lytically infected cells in KS lesions on latently infected or uninfected neighboring cells. In addition, K15 may also have an anti-apoptotic role. Sharp et al. (2002) observed that K15 interacts with HAX-1 (HS1-associated protein X-1), a protein with anti-apoptotic functions. A K15 protein and HAX-1 were shown to co-localize in the ER and, consistent with apoptotic regulatory activity, in mitochondria (Sharp et al. 2002). However, there is as yet no functional evidence that K15 association with HAX-1 affects cell survival. In addition, K15 has recently been shown to upregulate the expression of anti-apoptotic genes such as *tnfaip3/A20, bf, birc3, birc2* and *bcl2a1*, of which, notably, the last shows sequence homologies with HAX-1 (Brinkmann et al. 2007). Another interesting factor shown to be upregulated through K15-induced signaling pathways is Cox-2 (Brinkmann et al. 2007). Cox-2 expression after HHV-8/KSHV infection had already been reported

(Naranatt et al. 2004) and its association with other herpesviruses, including EBV, CMV and HHV-6, has been observed (Janelle et al. 2002; Murono et al. 2001; Speir et al. 1998; Zhu et al. 2002). Cox-2 may contribute to viral replication by increasing prostaglandin E_2 synthesis, which has been shown to induce multiple gene products of the HHV-8/KSHV-related γ-herpesvirus MHV68 (Symensma et al. 2003).

At least three allelic genotypes of K15 have so far been identified in different virus genomes and are thought to have arisen from recombination events with related γ_2 herpesviruses (Glenn et al. 1999; Kakoola et al. 2001; Poole et al. 1999). The K15 genotypes are highly divergent but share several of the functionally important sequence motifs listed above, e.g., the YEEV SH2-binding site, the PPLP SH3-binding site and the YASIL motif. So far, most studies on K15 function have been carried out with the "predominant" K15-P allele (Brinkmann et al. 2003; Brinkmann et al. 2007; Choi et al. 2000; Sharp et al. 2002). A recent study showed that the K15-M allele, which is to date the most divergent K15 variant known, shares most functional characteristics of K15-P (Wang et al. 2007a). In particular, K15-M activates the MEK/Erk, JNK and NF-κB signal cascades and induces a similar range of cellular genes. There may be minor differences between K15-P and K15-M in that the activation of the JNK pathway by K15-M appears to be less dependent on an intact YEEV SH2-binding site than in the case of K15-P (Wang et al. 2007).

22.2.4 Kaposin

The kaposin locus comprises a small open reading frame initiated by a conventional ATG start codon (ORF K12) preceded by two families of 23 nucleotide GC-rich direct repeats termed DR1 and DR2 and is transcribed as mRNAs encompassing all three components. A complex translational program, including initiation from non-AUG (i.e., CUG) codons, generates a variety of proteins from this mRNA (Sadler et al. 1999), namely kaposin A, B and C, with apparently different functions in HHV-8/KSHV biology. mRNAs encoded by the kaposin locus are detectable in most spindle cells of all stages of KS and in PEL cells during latency and lytic replication (Jenner et al. 2001; Nakamura et al. 2003; Paulose-Murphy et al. 2001; Staskus et al. 1997; Sturzl et al. 1997). The relative amount of each kaposin isotype varies among different cell types, but it seems that kaposins A and B dominate over kaposin C.

Kaposin A is the actual product of ORF K12 with 60 amino acids in length and a predicted molecular weight of 6 kDa. Previously called T0.7, its transcript was the first to be described among the kaposin isoforms and found to be highly abundant in KS and PEL cells (Kliche et al. 2001; Russo et al. 1996; Zhong et al. 1996). Early studies by Muralidhar et al. (1998; 2000) showed kaposin A expression to be localized to the cytoplasm, and it was suggested that the protein was Golgi associated. More recent data derived from confocal microscopy, subcellular fractionation studies and flow cytometry indicate that

kaposin A localizes to perinuclear regions as well as to the plasma membrane (Kliche et al. 2001; Tomkowicz et al. 2002). These results coupled with secondary structure predictions and hydrophobicity plots characterized the protein as a type II transmembrane protein with two hydrophobic motifs of approximately 20 amino acid residues (Kliche et al. 2001). Expression of kaposin A leads to activation of cellular serine–threonine kinases with important roles in cell proliferation, such as PKC, CAM kinase II and cdc2-kinase, induction of Erk1/2 signaling and AP-1 activity as well as upregulation of myosin light chain kinase (MLCK) and cGMP-dependent protein kinase activity (Kliche et al. 2001; Muralidhar et al. 2000; Tomkowicz et al. 2002). However, the molecular mechanisms behind these effects are still not known as no direct interaction between kaposin A and any of these proteins has yet been demonstrated and no signaling motifs have been described in the kaposin A sequence.

The transforming capacities of kaposin A were first shown in Rat-3 cells (Muralidhar et al. 1998), in which transfected kaposin A induced focal transformation. Upon subcutaneous injection into athymic *nu*/*nu* mice, all transformed Rat-3 cell lines containing kaposin A sequences produced high-grade, highly vascular, undifferentiated sarcomas. Later, Kliche et al. (2001) confirmed the oncogenic potential of kaposin A in NIH3T3 fibroblasts. Furthermore, the group could demonstrate that the protein induces cell adhesion and aggregation of lymphocytes. Kliche et al. identified activation of cytohesin-1, an ADP-ribosylation factor (ARF) guanine nucleotide exchange factor (GEF), to be mediated by kaposin A through recruitment to tubulovesicular membranes. Functional interaction of cytohesin-1 and kaposin A may coordinate downstream events such as actin remodeling and gene activation. It was shown that a cytohesin-1 mutant, which was unable to catalyze GTP loading, abrogated kaposin A-induced stress fiber disruption, transformation and AP-1 activity (Kliche et al. 2001).

Kaposin B is expressed in PEL and KS cells and appears to be the most abundant kaposin isoform in the PEL cell line BCBL-1 (Kliche et al. 2001; Sadler et al. 1999). The protein results from translation of the DR1 and DR2 region upstream of ORF K12 but not of ORF K12 itself (Sadler et al. 1999). Therefore, kaposin B consists of tandemly repeated copies of 23 aa peptides, which are highly conserved among different HHV-8/KSHV isolates in terms of their sequence but vary in their total number (Li et al. 2002; McCormick and Ganem 2005). Studies by McCormick and Ganem (2005) provide an interesting mechanism of how kaposin B regulates host gene expression, linking HHV-8/KSHV infection to selective mRNA turnover and cytokine biosynthesis. The protein binds and activates the kinase MK2 in the nucleus through its reiterating DR2 repeats. MK2 is a downstream target of the p38 MAPK pathway and inhibits the decay of cytokine and other mRNAs containing AU-rich elements (AREs) in their 3′-untranslated regions. It was shown that transfection of kaposin B into human foreskin fibroblasts results in a major augmentation of GM-CSF and IL-6 production, which likely results from stabilization of their ARE-containing transcripts via activated MK2. Because activated p38-MK2

has a role in cell motility (Kotlyarov et al. 2002), kaposin B might also induce the migration of HHV-8/KSHV-infected cells.

Binding to MK2 by kaposin B is mediated via its DR2 region (McCormick and Ganem 2005). Nevertheless, mRNA stabilization function requires both DR2 and DR1 (McCormick and Ganem 2006), indicating an important role of DR1 in kaposin B function. This was further supported by the identification of a single phosphorylation site for kaposin B, a specific serine residue, localized in the DR1 region (McCormick and Ganem 2006). Phosphorylation was mediated in vitro by the p38 MAP kinase (McCormick and Ganem 2006), and mutational ablation of this serine abolished phosphorylation of kaposin B by this kinase. However, this did not affect the protein's ability to extend mRNA half-life, and how DR1 contributes to kaposin B activity is therefore still unresolved.

22.2.5 vOX2

The HHV-8/KSHV vOX2 protein shows 36% identity with a cellular protein of the group of leukocyte glycoproteins, called CD200 or OX2. The protein is expressed early during lytic replication from a bicistronic mRNA containing the ORFs 74 (vGPCR) and K14 (vOX2) (Jenner et al. 2001; Kirshner et al. 1999; Talbot et al. 1999).

Interestingly, CD200-like sequences have been identified in genomes of several evolutionary diverse viruses, which include gamma- and betaherpes-viruses, yaba- and leporipoxviruses and small viruses such as duck adenovirus (Foster-Cuevas et al. 2004). This apparently independent capture of the host CD200 gene indicates a strong selective advantage conveyed by members of this family. CD200 is expressed on the surface of a wide variety of cell types including endothelial cells, B lymphocytes, T cells and neuronal cells (Wright et al. 2001) and provides immunomodulatory functions. Signal delivery of CD200 occurs through binding to a CD200 receptor (CD200R). This interaction results in inhibition of CD200R downstream signaling (including ERK, JNK and p38 MAPK activity) in monocytes and thus limiting their activation (Hoek et al. 2000; Zhang and Phillips 2006). As CD200R expression is restricted to cells of the myeloid lineage, it is suggested that CD200–CD200R interaction acts locally to modulate inflammatory cell activity at sites of infection.

However, studies on vOX2 function have so far pointed in different directions. While Chung et al. (2002) reported that vOX2 stimulates inflammatory cytokine production including IL-1β, monocyte chemoattractant protein 1 (MCP-1) and TNF-α from primary monocytes, macrophages and dendritic cells, more recent studies by two groups show the opposite, suggesting a similar function for vOX2 as for its cellular homologue CD200 (Foster-Cuevas et al. 2004; Rezaee et al. 2005). Foster-Cuevas et al. (2004) observed that vOX2 and CD200 interact with almost identical kinetics with CD200R and cells expressing vOX2 or CD200 on their surface were able to inhibit secretion of TNF-α from activated macrophages.

In HHV-8/KSHV biology and tumorigenesis the local inhibition of macrophages, which are abundant within HHV-8/KSHV-infected tissue, might be important to inhibit the host response against lytically infected cells.

Rezaee et al. (2005) confirmed the immunosuppressive function of vOX2 by demonstrating that a fusion protein of vOX2 and the Fc domain of human immunoglobulin G1 (vOX2:Fc) downregulated the activity of neutrophils directly and indirectly via inhibiting the production of the potent neutrophil chemotactic proteins IL-8 and MCP-1 from monocytes and macrophages in vitro. In vivo studies in a carrageenan-mouse model of acute inflammation demonstrated that vOX2:Fc reduced cell infiltration (Rezaee et al. 2005), suggesting that vOX2 may modulate chemotaxis.

22.3 HHV-8/KSHV Cytokines

22.3.1 vCCLs

The viral CC chemokines of HHV-8/KSHV, vCCL-1, vCCL-2 and vCCL-3, are encoded by ORFs K6, K4 and K4.1, respectively (Neipel et al. 1997; Nicholas et al. 1997; Russo et al. 1996). While vCCL-1 and vCCL-2 are structurally most closely related to CCL3 (vMIP-1α) and CCL4 (vMIP-1β), vCCL3 has no clear cellular counterpart but is related to CCL2 (MCP-1). Functionally, these viral chemokines are distinct from their cellular homologues. vCCL-1 and vCCL-2 are agonists for CCR8, the receptor for CCL1 (I-309), and vCCL-2 has been reported also to interact productively with CCR3 (Boshoff et al. 1997; Dairaghi et al. 1999; Endres et al. 1999); vCCL-3 signals through CCR4 and XCR1 (Luttichau et al. 2007; Stine et al. 2000). Additionally, vCCL-2 binds as a neutral (non-signaling) ligand to a variety of CC and CXC receptors, in addition to XCR1, thereby acting as an antagonist for cognate ligand signaling (Kledal et al. 1997; Luttichau et al. 2001; Shan et al. 2000). It has more recently been reported that CCL-2 is an agonist for CCR5 (Nakano et al. 2003), in contradiction with previous findings that vCCL-2 is a neutral ligand for CCR5; the suggestion that this discrepancy might be due to the use of eukaryotically expressed rather than chemically synthesized or bacterially expressed protein seems unlikely because another recent report using eukaryotically produced vCCL-2 also finds that it inhibits RANTES chemotactic recruitment of ($CCR1^+/CCR5^+$) Th1 cells (Rubant et al. 2006). Notwithstanding, the finding of Nakano et al. (2003) that vCCL-1 and vCCL-2 are chemotactically active on monocytic (THP-1) cells suggests that one function of these v-chemokines may be to recruit HHV-8/KSHV permissive cells into sites of ongoing lytic replication and thus promote virus dissemination. Other likely biological functions of the v-chemokines include immune evasion, either positively through recruitment of Th2 cells leading to polarization away from Th1 anti-viral responses or, in the case of vCCL-2, by interfering via neutral (antagonist) binding to

chemokine receptors with the normal function of cellular chemokines involved in promoting immune attack of infected cells.

For the purposes of this review, the main considerations are the possible roles of the v-chemokines in neoplasia. In this regard, one very important property shared by the three HHV-8/KSHV chemokines is their ability to induce angiogenesis (Boshoff et al. 1997; Stine et al. 2000). The mechanisms that operate to achieve this have not been elucidated completely, but it is known that vCCL-1 can induce VEGF production in HHV-8/KSHV latently infected PEL cell lines (Liu et al. 2001), and presumably, induction of such angiogenic factors can occur in other cell types also. Such factors are of obvious relevance to KS, but VEGF appears to be important also for the growth and dissemination of PEL cells, as observed in PEL-inoculated mice (Aoki and Tosato 1999). Another key property shared by vCCL-1 and vCCL-2 is anti-apoptotic activity, demonstrated in dexamethasone-treated PEL cells (Liu et al. 2001) and more recently in serum-deprived primary and telomerase-immortalized human microvascular endothelial cells (Choi and Nicholas 2008). CCR8-mediated anti-apoptotic signaling has been noted in murine thymic lymphoma cells, BW5147 and Ras/MAPK signaling implicated in this function (Louahed et al. 2003; Spinetti et al. 2003). In PEL, endothelial and BW5147 cells, pro-survival signaling can be conferred also by the cellular CCR8 ligand CCL1 (I-309). For endothelial cells, CCL1 desensitization experiments and shRNA knockdown of CCR8 have identified CCR8 as the receptor mediating the effects of the v-chemokines (Choi and Nicholas 2008). A key point is that the v-chemokines, unlike other known anti-apoptotic viral proteins (Feng et al. 2004; Tschopp et al. 1998), are able to mediate pro-survival signaling in a paracrine manner. Thus, their expression in a minority of lytically infected cells within a predominantly latent cell population would allow for their contribution to viral pathogenesis via promotion of the survival of latently infected cells, thereby cooperating with latency functions. Also of central relevance to KS pathogenesis is the recently proposed role of CCR8 in mediating vascular smooth muscle cell reorganization and induction of metalloproteinase-2 (Haque et al. 2004). Clearly, there is the potential for the viral CCR8 agonists vCCL-1 and vCCL-2 to contribute to angiogenesis via such effects.

22.3.2 vIL-6

IL-6 had been implicated as a contributing factor in KS and MCD prior to the discovery of HHV-8/KSHV. KS lesions showed elevated IL-6 levels and KS cells in culture were reported to show increased proliferation rates in response to IL-6; levels of serum IL-6 were found to correspond with disease severity in MCD patients and to decrease following excision of diseased lymph nodes (Burger et al. 1994; Ishiyama et al. 1994; Miles et al. 1990; Yoshizaki et al. 1989). Furthermore, IL-6 is a known mitogen and survival factor for B cells, and STAT3 (induced by IL-6) is found at elevated levels in many cancers. These

properties suggest that IL-6 activity may be important for the development and progression of HHV-8/KSHV-associated neoplasia.

The IL-6 homologue encoded by ORF K2 of HHV-8/KSHV shares only 25% amino acid identity with its human counterpart (hIL-6) but is structurally highly conserved and mediates signaling via the same signal transducer, gp130 (Molden et al. 1997). Contrary to many published reports and reviews, the specific activities of vIL-6 and hIL-6 are very similar, the reported 1000-fold reduced activity for vIL-6 being the case only for bacterially produced (and signal sequence-deleted) recombinant protein, not for vIL-6 produced in eukaryotic cells (Hu and Nicholas 2006; Wan et al. 1999). The reason for this dramatic difference in specific activity between prokaryotically and eukaryotically expressed proteins is not clear, but the signal sequence of the eukaryotically expressed protein appears to be uncleaved and glycosylation augments vIL-6 activity (Dela Cruz et al. 2004; Meads and Medveczky 2004). Protein conformation may also be key; vIL-6, unlike hIL-6, is difficult to purify in soluble form from bacteria (Boulanger et al. 2004) and tertiary protein structure appears to be crucially important for gp80-independent signaling (Chen and Nicholas 2006). Another persistent misapprehension is that vIL-6 does not utilize the non-signaling IL-6 receptor α-subunit, gp80, required by cellular IL-6 proteins. While vIL-6 can indeed signal independently of gp80 (Burger et al. 1998), there is a wealth of both physical and functional data to show that gp80 can be incorporated into vIL-6-induced signaling complexes. Furthermore, gp80 can contribute to complex stability and influence vIL-6 signal transduction (Boulanger et al. 2004; Chen and Nicholas 2006; Hu and Nicholas 2006; Wan et al. 1999). Other than gp80 independence of vIL-6, another notable difference between vIL-6 and hIL-6 is that the viral cytokine is inefficiently secreted, being expressed at high levels and able to signal intracellularly (Kovaleva et al. 2006; Meads and Medveczky 2004). This could be relevant to the function of vIL-6 during both normal virus biology and virus-induced neoplasia, where it could signal in an autocrine manner to influence the infected cell.

In consideration of the potential role of vIL-6 in HHV-8/KSHV-associated neoplasia, it is important to note that while vIL-6 is expressed most abundantly during lytic replication (as determined in lytic reactivation studies in PEL cells), it also can be expressed independently of other lytic genes (Chang et al. 2005; Chatterjee et al. 2002; Chiou et al. 2002; Nicholas et al. 1997). Indeed, vIL-6 has been found to be expressed more frequently (in more cells) than other lytic proteins in KS and PEL tissues and together with latency-associated nuclear antigen (LANA) in individual cells of KS and MCD (Brousset et al. 2001; Cannon et al. 1999; Chiou et al. 2002; Moore et al. 1996). Recent studies have identified vIL-6 as one of several lytic genes that are specifically induced via Notch signaling, independently of RTA (ORF50-encoded immediate-early transcriptional activator), raising the possibility of variability and complexity in viral gene expression programs, defined by cell type, cell signaling and so on and allowing for the possibility of autocrine actions of classically defined “lytic” proteins (Chang et al. 2005). If vIL-6 is expressed in cells independently of

productive replication, the viral protein could function as an autocrine proliferation and survival factor and, in view of concentration effects of intracellular retention, be effective even when vIL-6 gene transcription occurs at low levels. Whether or not vIL-6 can be expressed in the absence of productive infection, as a (partially) secreted viral protein vIL-6 could potentially have a role in neoplasia via paracrine signaling. It is clear that vIL-6 gene transcription and protein expression is induced upon lytic induction in PEL cells (Jenner et al. 2001; Paulose-Murphy et al. 2001) and this may represent the primary setting of vIL-6-induced pathogenesis. Effects could be exerted directly via mitogenic and survival signaling in surrounding latently infected and uninfected cells and also indirectly via the induction of cellular cytokines or other proteins, such as receptors. Examples might include hIL-6, VEGFs and other angiogenic factors, which have been demonstrated to be induced by vIL-6 in some cell types (Aoki et al. 1999; Liu et al. 2001; Mori et al. 2000), and VEGF and cytokine receptors. Inflammatory and angiogenic cytokines are known to play a role in KS, and VEGF and IL-6 appear to be important for PEL and MCD (Aoki and Tosato 1999; Burger et al. 1994; Ishiyama et al. 1994; Yoshizaki et al. 1989). In vitro, both vIL-6 and hIL-6 support PEL cell growth (Foussat et al. 1999; Jones et al. 1999).

Additional information on these proteins can be found in Chapter 24 (Kaposi's Sarcoma-Associated Herpesvirus Immune Evasion).

22.4 HHV-8/KSHV Protein-Induced Paracrine Signaling in Oncogenesis

Potential mechanisms by which the HHV-8/KSHV cytokines and chemokine receptor as well as kaposin A, B and the membrane proteins encoded by K1 and K15 might contribute to virus-associated cancers have been outlined above. All of these viral proteins are able to induce angiogenesis and/or can induce the production of cellular mitogenic and survival factors. But, how might the viral cytokines and induced secreted cellular signaling proteins contribute to cellular transformation?

In the case of vIL-6, STAT3 is likely to be a principal player. IL-6 and STAT3 are found at elevated levels in tumor tissues of a high proportion of cancers (Bowman et al. 2000; Hodge et al. 2005a). STAT3 serves primarily as a survival factor. Anti-apoptotic proteins, such as Mcl-1 and Bcl-X_L, are induced by IL-6, their genes being the targets of STAT3 transcriptional activation (Grad et al. 2000; Isomoto et al. 2005; Puthier et al. 1999; Spets et al. 2002). Furthermore, STAT3 can repress p53; it has been reported that there is an inverse relationship between levels of active STAT3 and p53 expression in 3T3 fibroblasts (Niu et al. 2005) and also that IL-6 is involved in methylation-mediated p53 gene repression via induction of methyltransferase DNMT1 in human myeloma cells (Hodge et al. 2005b). In addition, STAT3 can activate expression of cell cycle-promoting transcription factors, including c-fos and c-myc, and

also cyclin D1 (Kiuchi et al. 1999; Leslie et al. 2006; Yang et al. 2003). The overall effects of these activities could be the sustained survival and proliferation of cells containing elevated levels of STAT3. HHV-8/KSHV-encoded vIL-6 has been demonstrated to superactivate STAT3 in experimental systems via hexameric (gp80-containing) signaling complexes rather than through tetrameric complexes containing the gp130 signal transducer alone (Hu and Nicholas 2006), and this could be relevant to virus-induced neoplasia.

For the v-chemokines vCCL-1 and vCCL-2, agonists for CCR8 and able to induce signal transduction in primary endothelial, KS and PEL cells (Haque et al. 2004; Liu et al. 2001), the role in pathogenesis may be mediated primarily via protective signaling through PI3K/Akt and MAPK pathways. Ras/MAPK activation has been reported to be necessary for vCCL-1- and CCL1 (I-309)-supported survival of murine BW5147 cells (Louahed et al. 2003). Our own data have established that in endothelial cells both Akt and MAPK signaling are induced by vCCL-1, vCCL-2 and CCL1 and that chemokine treatment of endothelial cells promotes survival following serum withdrawal and leads to the suppression, via Akt-mediated FOXO transcription factor inactivation, of pro-apoptotic Bim and to the induction, likely via NF-κB, of anti-apoptotic Bcl-2 (Choi and Nicholas 2008). Clearly, there are other, well-known mechanisms of pro-survival signaling that could be targeted via the Akt pathway, for example, NF-κB-mediated induction of other anti-apoptotic Bcl-2 family members such as Bcl-X_L and IAPs, activation of mTOR and inactivation of Bad, caspase 9 and GSK3 (Nicholson and Anderson 2002). Whilst these effects would be expected to benefit virus-productive replication, and indeed we have demonstrated this in endothelial cultures, they also could have a significant impact on HHV-8/KSHV-associated neoplasia. Activation of the MAPK pathway could promote both cell survival and cell proliferation.

vGPCR is known to induce a variety of angiogenic, pro-inflammatory and growth factors of relevance to virus-induced neoplasia, especially KS (Pati et al. 2001; Schwarz and Murphy 2001). The mechanisms involved in cellular gene induction are not fully elucidated, but it is evident that many of the pro-inflammatory and angiogenic cytokines are induced via vGPCR activation of NF-κB signaling. It has been reported that VEGF is induced, at least in part, via p38-mediated phosphorylation and activation of hypoxia-inducible factor-1α (HIF-1α) that is a key regulator of VEGF gene transcription (Sodhi et al. 2000). NF-κB-regulated pro-inflammatory cytokines demonstrated to be induced by vGPCR in culture systems included vIL-6, IL-1β, IL-2, CXCL8 (IL-8), IL-14, CCL5 (RANTES), CCL2 (MCP-1) and GM-CSF, and TNFα- and AP-1-induced angiogenic factor FGF2 was also found to be positively regulated by vGPCRs (Pati et al. 2001; Schwarz and Murphy 2001). Activation of PI3K/Akt signaling by vGPCR also has the potential to contribute to cell survival, as outlined above, and activation of the MAPK cascade, via Ras/Raf and other pathways, would be expected to promote cell cycle progression. However, unless expression of vGPCR can occur independently of full lytic cycle progression (and at present there is no evidence of this), autocrine vGPCR signaling

could not contribute to HHV-8/KSHV neoplasia. Rather, the paracrine effects of vGPCR-induced secreted pro-inflammatory, angiogenic, growth and survival factors would be key players in the development of virus-induced pathogenesis. Cytokine involvement in KS and the role of VEGF in PEL have been outlined previously (Aoki and Tosato 1999; Ensoli and Sturzl 1998).

In the case of the K1- and K15-encoded terminal membrane proteins, the activation of the MAPK pathways MEK/Erk and JNK and the ensuing induction of angiogenic cytokines such as VEGF and/or IL-8 are likely to contribute to paracrine signaling in oncogenesis, as discussed above and illustrated in Fig. 22.1. In addition, both proteins that trigger pathways with survival functions, such as Akt and NF-κB, may induce the expression of cellular genes with anti-apoptotic functions and could thereby contribute to the survival of

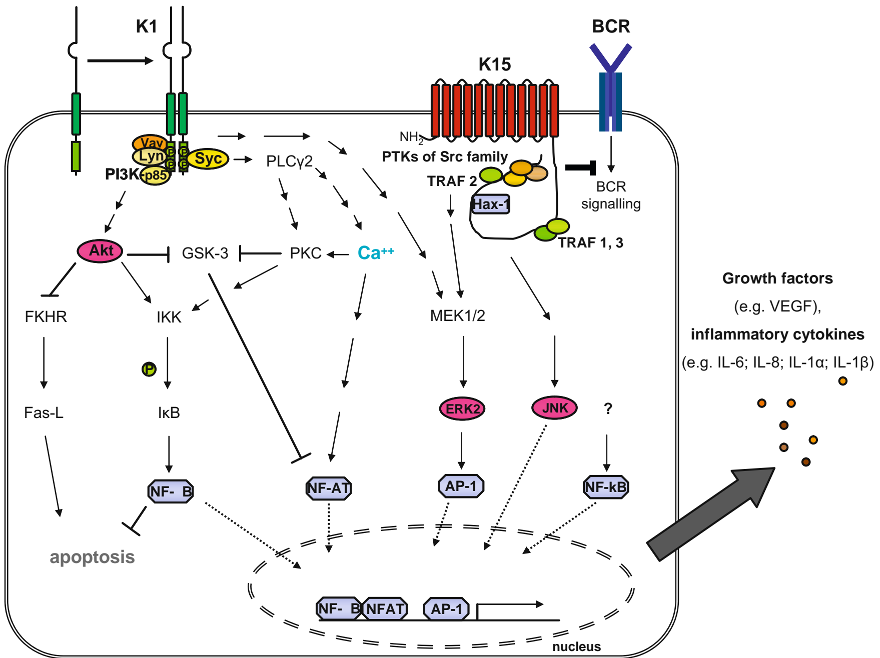

Fig. 22.1 Schematic representation of the intracellular signaling pathways engaged by the two HHV-8/KSHV terminal membrane proteins (i.e., viral proteins encoded at either end of the viral genome), K1 and K15. Both K1 and K15 proteins activate the MEK/Erk cascade and NF-κB; in addition, K1 activates Akt, while K15 activates the JNK cascade. Both recruit members of the src kinase family to either an ITAM motif (K1) or a combination of an SH2- and SH3-binding site (K15) and, in B cells, are thereby thought to interfere with Lyn (a Src kinase)-mediated signals emerging from the B-cell receptor. Their downstream effects include the activation of a range of inflammatory and angiogenic cellular cytokines, particularly VEGF (K1), IL-8 (K15), IL-1α,β and IL-6 (both K1 and K15). Signaling activities, most notably via Akt and MAPK pathways, of the HHV-8/KSHV chemokines and chemokine receptor would be envisioned to mediate similar biological effects (not illustrated, but discussed in the text)

virus-infected cells. While a direct transforming potential for K1 has been reported (see above), no evidence currently exists that would suggest a direct transforming role for K15-encoded proteins.

In contrast to all the other viral proteins discussed in this review, the mRNAs for kaposin A and B are clearly expressed in latently infected cells, although their expression markedly increases upon activation of the productive (lytic) replication cycle (see above). It is therefore conceivable that kaposin A and B might act directly on the infected cells in an autocrine manner. In the case of kaposin A this is likely to be the result of the activation of intracellular signaling pathways such as the Erk1/2 cascade. In contrast, what is currently known about kaposin B suggests that it acts by means of stabilizing ARE-containing cytokine mRNAs (see above), thereby potentially acting in both autocrine and paracrine manners.

22.5 Outlook

Of the recognized six human tumor viruses (EBV, HPV, HBV, HCV, HTLV-I, HHV-8/KSHV), HHV-8/KSHV appears to be the one in which the possibility that paracrine signaling could play an important role in viral oncogenesis has first received widespread attention and experimental support. This is in contrast to the "classic" concept of viral oncogenesis which focuses on a transforming role of viral proteins expressed during latency and in the tumor cells. It is very likely that several of the latent HHV-8/KSHV proteins [LANA-1, the D-type cyclin homologue vCyc, the FLIP homologue vFLIP, the kaposins and perhaps vIRF-3/K10.5 (LANA2)] are indispensable for virus-mediated oncogenesis and that the development of HHV-8/KSHV-associated neoplasia requires the complex interaction of proteins acting in a direct as well as paracrine manner. However, by illustrating the potential role of paracrine mechanisms in viral oncogenesis, HHV-8/KSHV has broadened our thinking. As illustrated also by, for example, *Helicobacter pylori*-mediated gastric cancer, it may not be necessary to postulate the persistence of an oncogenic agent inside the tumor cell for it to be considered oncogenic, when looking for new viral etiologies of human cancer. However, in contrast to the once popular concept of "hit and run" oncogenesis, the continuous presence of an oncogenic agent in the tumor tissue (if not necessarily in the malignant cell population) would still seem to be a prerequisite for invoking a paracrine mechanism of oncogenesis.

Acknowledgments JN is funded by grants CA76445, CA136356, CA119887 and CA113239 from the National Institutes of Health, U.S.A, and is grateful for the general support of his colleagues in the Viral Oncology Program of the Department of Oncology, Johns Hopkins School of Medicine.

SH is funded by grants Graduate School 745, and SFB 566 from the Deutsche Forschungsgemeinschaft (DFG), Germany, and by the European Union Integrated Project INCA LSHC-CT-2005-018704. She's grateful for the general support and helpful discussions at the Institute of Virology, Hanover Medical School.

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Chapter 23
KSHV Entry and Infection of Target Cells

Bala Chandran and Neelam Sharma-Walia

23.1 Introduction

To initiate and establish a successful infection in the target cells, KSHV must cross the plasma membrane and target its genome and accessory proteins to the infected cell nuclei, where gene transcription, nucleic acid replication, and viral maturation take place. Detailed knowledge about the initial stages of KSHV interactions with the host cells is crucial not only to understand the tropism and pathogenesis of KSHV but also for the development of strategies to block infection. Compared to the advances in other areas of KSHV research, knowledge regarding KSHV entry and infection is somewhat limited due to the complexity of the process which includes multiple KSHV envelope glycoproteins and a wide range of target cells, as well as the inherent difficulties in studying virus–receptor interactions. In this chapter, we discuss the available information regarding KSHV in vivo and in vitro target cells, viral envelope glycoproteins, host cell molecules (receptors) involved in KSHV binding and entry, pathways utilized for infection, different stages of infection, viral and host gene expression, signaling events triggered by infection, and the potential role of signaling events in the different stages of KSHV infection.

23.2 In Vivo Tropism of KSHV

Though the in vivo host cell range of KSHV is not yet fully characterized, detection of viral DNA and transcripts in B cells from peripheral blood, B cells in primary effusion lymphomas (PEL) or body-cavity-based B cell lymphomas (BCBL), multicentric Castleman's disease (MCD), endothelial cells of Kaposi's Sarcoma (KS) lesions, CD45+/CD68+ monocytes in KS lesions, keratinocytes,

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_23, © Springer Science+Business Media, LLC 2009

and epithelial cells demonstrates that KSHV has a broad tropism (Antman and Chang 2000; Dourmishev et al. 2003; Ganem 1998; Sarid et al. 1999; Schulz et al. 2002). Demonstration of KSHV DNA in the CD19+ peripheral blood B cells of KSHV seropositive individuals suggests that CD19+ B cells may be a primary reservoir for persistent infection. Cell lines with B cell characteristics, such as BC-1, HBL-6, JSC, BCBL-1, and BC-3, have been established from BCBL tumors (Dourmishev et al. 2003). Among these, BC-1, HBL-6, and JSC cells carry both the KSHV and the EBV genomes, while BCBL-1 and BC-3 cells carry only the KSHV genome. In BCBL cells, KSHV exists in a latent state and expresses the latency-associated ORF73, ORF72, K13, K12, and ORF 10.5 genes. Similar to B cell lines carrying EBV, about 1–3% of the BCBL cells spontaneously enter lytic cycle. KS, PEL and MCD have not been observed in veterinary pathology and attempts to infect rhesus macaques and SCID/hu mice have not been shown to produce pathological lesions (Ganem 2007).

23.3 In Vitro Tropism of KSHV

Similar to in vivo tropism, KSHV has a broader in vitro tropism. It has been shown that KSHV from the BCBL cells can infect human B cells, lymphocytes, endothelial, epithelial and fibroblast cells, and CD34+ stem cell precursors of dendritic cells. KSHV also infects a variety of animal cells such as owl monkey kidney cells, baby hamster kidney (BHK-21) cells, Chinese hamster ovary (CHO) cells, and mouse fibroblasts cells (Akula et al. 2001b; Ganem 2007; Ciufo et al. 2001; Dezube et al. 2002; Moses et al. 1999 ; Naranatt et al. 2003; Renne et al. 1998; Vieira et al. 2001; Gasperini et al. 2005). Tropism and properties of the wild-type KSHV from the saliva of infected individuals, KSHV isolates from Africa and other endemic areas for KS, are not known at present.

In contrast to EBV, infection of primary B cells by KSHV does not result in immortalization. Moreover, in contrast to alpha and beta herpesviruses, in vitro infection by KSHV is characterized by the expression of latency-associated genes and the absence of productive lytic replication. Lytic replication could be induced from these cells by phorbol esters or ORF50 (RTA) protein. Lytic replication is supported by many cells of human (primary human microvascular dermal endothelial cells [HMVEC-d], human umbilical vein endothelial cells [HUVEC], human foreskin fibroblasts [HFF] and human endothelial cells immortalized by telomerase [TIME]), monkey (monkey kidney cells), and mouse (fibroblasts) origin (Ganem 2007). Detection of KSHV LANA-1 encoded by ORF 73 after 2 days post-infection has led to the notion that the establishment of latency is the default pathway of infection (Ganem 2007; Schulz et al. 2002; Tomescu et al. 2003). However, recent studies showed that a subset of the lytic transcripts including ORF50, K8, K5, and v-IRF2 were expressed in the primary endothelial and fibroblast cells soon after infection and declined at later

time points (Krishnan et al. 2004). In vitro KSHV latent infection in primary fibroblasts, endothelial cells, or non-adherent B cells is unstable, and viral DNA is not efficiently maintained (Tomescu et al. 2003), and the proportion of KSHV-infected cells decreases over time (Grundhoff and Ganem 2004).

23.4 Mediators of KSHV in Target Cell Binding and Entry

Like other herpesviruses, KSHV virions have an icosahedral capsid surrounded by a proteinaceous tegument and an envelope composed of multiple unique glycoprotein species embedded in a lipid bilayer. The majority of KSHV envelope proteins are transmembrane glycoproteins and are expected to be critically involved in mediating attachment, entry, assembly, and egress of virus. The biochemical and the functional analyses of KSHV envelope proteins are just beginning, and much better understanding is required to reveal their specific interactions with the host plasma membrane. As in other herpesviruses, KSHV encodes the five conserved gB, gH, gL, gM, and gN glycoproteins which are encoded by ORFs 8, 22, 47, 39, and 53, respectively (Table 23.1A) (Akula et al. 2001a; Baghian et al. 2000; Naranatt et al. 2002; Neipel et al. 1997; Russo et al. 1996). KSHV also encodes unique glycoproteins, gpK8.1A, gpK8.1B, K1, K14, and K15 (Ganem 2007; Chandran et al. 1998; Neipel et al. 1997; Russo et al. 1996) that are expressed during lytic replication. Studies have shown that gB, gH/ gL, gM/gN, and gpK8.1A are associated with the KSHV virion-envelopes (Akula et al. 2001a; Baghian et al. 2000; Koyano et al. 2003; Naranatt et al. 2002; Neipel et al. 1997; Russo et al. 1996; Zhu et al. 1999a). The presence of unique complements of glycoproteins as well as subtle differences among those that are conserved with other herpesviruses could potentially be

Table 23.1A KSHV glycoproteins and their functions

KSHV Protein/Gene	Function
gB/ORF8	Attachment of cells via heparan sulfate; interacts with integrins;virus entry;egress; Signal induction
gH/ORF22	Virus entry, complexes with gL
gL/ORF47	Chaperone for gH
gM/ORF39	Codependent on gN for processing; Inhibition of cell fusion
gN/ORF53	Complexes with gM
gpK8.1A/K8.1	Attachment to cells via heparan sulfate;virus entry; Signal induction
gpK8.1B/K8.1	Unknown
OX-2/K14	Adhesion of infected cells
K1/K1	Signal induction
K15/K15	Signal induction

Table 23.1B KSHV binding, entry receptors and mode of entry in target cells

Cell type	Binding receptor(s)	Entry receptor(s)	Mode of Entry
HFF	Heparan Sulfate	$\alpha3\beta1$, $\alpha v\beta3$, $\alpha v\beta5$, xCT/CD98	Clathrin mediated endocytosis
HMVEC-d	Heparan Sulfate	$\alpha3\beta1$, $\alpha v\beta3$, $\alpha v\beta5$, xCT/CD98	?
HEK-293	Heparan Sulfate	?	?
Monocytes	?	DC-SIGN (activated macrophages)	?
B cells	Heparan Sulfate	DC-SIGN (LCL, K562 cells)	?
Keratinocytes	?	?	?

contributing to the differential tropisms of KSHV. The following gives a brief snap shot of the properties and functions of KSHV envelope glycoproteins (Table 23.1A).

23.4.1 KSHV gB

Experimentally, gB has been shown to be expressed on the surface of infected cells and in the virion envelopes (Akula et al. 2001a; Baghian et al. 2000). The 845 amino acids long gB ORF has a predicted signal sequence between residues 1 and 23, a predicted transmembrane domain between amino acids 710 and 729, and 13 potential N-glycosylation sites. There is a potential proteolytic cleavage site (RKRR/S) at amino acid position 440–441, and cleavage at this site results in two proteins with predicted masses of about 48 and 45 kDa (Akula et al. 2001a; Baghian et al. 2000). KSHV gB is synthesized as a 110 kDa precursor protein, undergoes cleavage and processing, and the envelope-associated form consists of 75 and 54 kDa polypeptides with high mannose and complex sugars that form disulfide-linked heterodimers and multimers (Akula et al. 2001a).

KSHV gB possesses the heparan sulfate-binding motifs and the integrin-binding "RGD" motif (Akula et al. 2001a), and studies have confirmed the interaction of gB with cell surface heparan sulfate and $\alpha3\beta1$ integrin molecules (Akula et al. 2002b). Anti-gB antibodies neutralize KSHV infectivity without having any effect on virus binding to target cells (Akula et al. 2002b). The ability of soluble gB to induce the integrin-associated focal adhesion kinase (FAK), Src, PI-3 K, and RhoGTPase activities (Sharma-Walia et al. 2004) clearly demonstrates that besides interaction with the host cell receptors and thus aiding in infection, KSHV gB also plays an important role in the modulation of host cell signaling pathways. Studies with gB-deleted BAC-KSHV demonstrate that besides its role in virus binding and entry into the target cells, KSHV gB also plays a role in the maturation and egress of virus from the infected cells (Krishnan et al. 2005).

23.4.2 KSHV gH/gL

gH and gL have been shown to form complexes in several members of alpha, beta, and gamma viruses and have been recognised as vital entities playing an essential role in penetration and cell-to-cell spread (Lomonte et al. 1997). The KSHV gH/ gL complex consists of a 120 kDa protein (gH) and a 41–42 kDa protein (gL) linked by non-covalent interactions and found both on the surface of the cell and in the virion envelopes (Naranatt et al. 2002). As in other herpesviruses, KSHV-gL is required for gH processing and intracellular transport, and the complex is required for entry (Naranatt et al. 2002). Anti-gH and anti-gL antibodies neutralized KSHV infectivity individually and more efficiently together, without having any effect on virus binding to the target cells (Naranatt et al. 2002).

23.4.3 KSHV gM and gN

Both these proteins have been shown to be involved in virus penetration and egress. Both KSHV-gM and gN are N-glycosylated and form heterodimers as shown by immunoprecipitation experiments, which is consistent with heteroduplex formation observed in other herpesviruses as well (Lake et al. 1998; Mach et al. 2000; Tischer et al. 2002). gN has been shown to be required for proper post-translational modification and transport of gM to the cell surface (Koyano et al. 2003). The KSHV-gM and KSHV-gN heterocomplex were shown to inhibit cell fusion in an in vitro HSV-1 and Mo-MuLV (Molony murine leukemia virus) cell fusion assay (Koyano et al. 2003).

23.4.4 KSHV gpK8.1

The KSHV K8.1 gene expressed during lytic cycle is positionally colinear to glycoprotein genes in other members of the gammaherpesvirus subfamily including the EBV gene encoding gp350/gp220, the gp150 gene of murine gamma herpesvirus 68, the ORF 51 gene of herpesvirus simiriae (HVS), and the BORFD1 gene of bovine herpesvirus 4 (Neipel et al. 1997; Russo et al. 1996). Analysis of cDNA showed that the gpK8.1 gene encodes two ORFs derived from spliced messages designated gpK8.1A and gpK8.1B. The 228 aa long gpK8.1.A contains a signal sequence, transmembrane domain, and four N-glycosylation sites. The smaller gpK8.1B is generated by an in-frame deletion of 61 amino acids from gpK8.1A, and the resulting 167 aa long ORF is a typical class I glycoprotein with a cleavable signal sequence, a transmembrane domain, and three putative N-glycosylation sites. Except for an amino acid change near the splice site (S to R), gpK8.1B shares identical amino acid sequences with gpK8.1A. Both gpK8.1A and gpK8.1B contain N- and O-linked sugars, and gpK8.1A is the predominant form detected within the infected cells and in the

virion envelopes (Chandran et al. 1998; Neipel et al. 1997; Zhu et al. 1999a; Zhu et al. 1999b). Both are immunogenic proteins (Zhu et al. 1999a), and neutralization of KSHV by anti-gpK8.1A antibodies without affecting virus binding suggests a role for gpK8.1A in the post-binding event of KSHV infection. Similar to gB, gpK8.1A also possess the heparan sulfate-binding motifs and interacts with cell surface heparan sulfate molecules (Wang et al. 2001). Soluble gpK8.1A induces a robust extracellular-regulated kinase 1/2 phosphorylation in HMVEC-d and HFF cells (Sharma-walia et al. 2005). In addition, gpK8.1 has also been shown to induce the cellular interferon response (Perry and Compton 2006).

Studies demonstrated a role for KSHV gpK8.1A-induced ERK1/2 in viral gene expression and, hence, in KSHV infection. This is in contrast to a work by Luna et al. 2004 in which a K8.1-null recombinant virus (BAC36ΔK8.1) was constructed and the authors concluded that gpK8.1A is not required for KSHV entry into 293 cells. However, for infection in these studies, infectious viral inoculum mixed with polybrene (5 μg/ml) was placed on 293 cells for 5 h, and infectivity was determined at 2 days p.i. by counting the number of GFP-expressing cells. Since polybrene is used to enhance the infectivity of various viruses by eliminating the need for receptors, the observed infection with BAC36ΔK8.1 virus does not rule out the role of gpK8.1A in KSHV in virus infectivity. When cells transiently transfected with wild-type BAC36 KSHV and BAC36ΔK8.1 were induced with TPA, estimation of genome copy numbers shows that, compared to wild-type BAC36 virus, three times more BAC36ΔK8.1 virus was present. However, during infection of 293 cells, virus preparations were not normalized to copy number. Hence the observed infection level that was twice greater with BAC36ΔK8.1 virus than WT BAC36KSHV could actually be lower if the copy numbers were adjusted to WT BAC36 KSHV. Further studies are essential to define the role of KSHV gpK8.1A in virus egress and entry.

23.5 Stages of KSHV Binding, Entry, and Infection of Target Cells

Viruses have evolved to utilize host cell surface molecules for gaining access into its interior. Entry of viruses into cells is a complex, multistep process, and for several viruses, cell attachment and internalization are distinct steps (Naranatt et al. 2005). Herpesviruses possess a large number of proteins on their envelope which they use to complete the entry process. Certain important concepts that have emerged from the study of herpesvirus–receptor interactions need to be mentioned here. The first concept is that interactions of herpesviruses with receptor are very complex processes involving multiple receptors and multiple viral proteins, and receptors vary according to cell type. For example, the γ-1

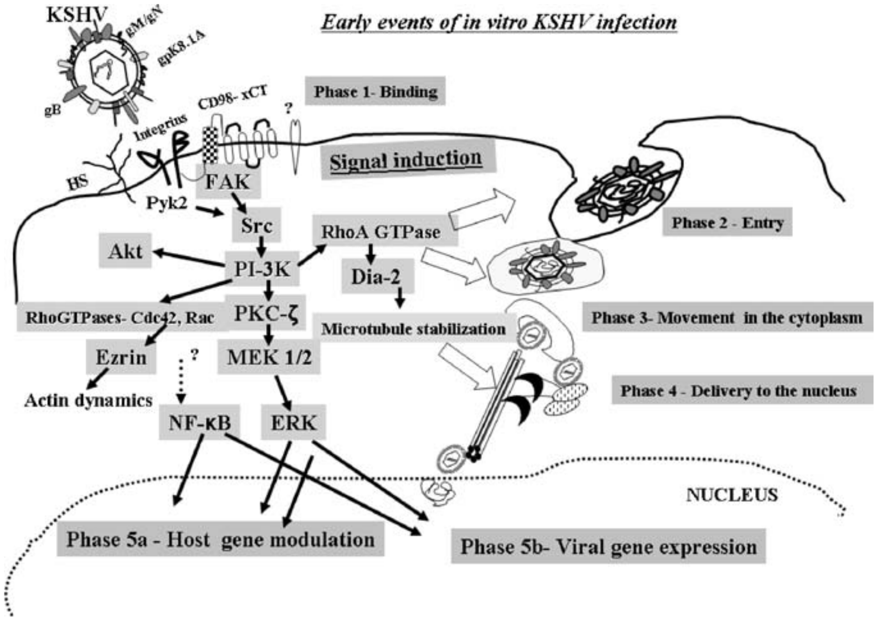


Fig. 23.1 Model depicting the overlapping dynamic phases of early events of KSHV infection in adherent target cells, KSHV induced signal pathways and their role in infection. Early events of KSHV infection are depicted in overlapping dynamic phases. In **phase 1**, KSHV infection is initiated by binding to the cell surface via its interactions with heparan sulfate proteoglycans (HSPGs) molecules via its envelope glycoproteins gpK8.1A and gB (Akula et al. 2001a, Akula et al. 2001b, Wang et al. 2001). This is followed by interaction with $\alpha3\beta1$ integrin via gB (Akula et al., 2002) binding to CD98-xCT molecules (Kaleeba and Berger., 2006) and possibly to other currently unidentified molecule(s). In **phase 2**, virus enters into the target cells, overlapping with the induction of host cell signal pathways beneficial for its entry and its gene expression. KSHV-gB interaction with cell surface integrins leads to the autophosphorylation of FAK at Tyrosine 397, which creates a binding site for the SH2 domain of Src family kinases and subsequent phosphorylation of PI3-K and RhoGTPases (Akula et al., 2002, Naranatt et al., 2003, Sharma-walia et al., 2004). FAK, Src and PI-3 K are essential for KSHV entry. In the absence of FAK, Pyk2 is induced to compensate for the function (Krishnan et al., 2006) of FAK. In **phase 3**, viral capsid/tegument moves in the cytoplasm, probably facilitated by the induced signal pathways. RhoGTPases are essential for the formation of endocytic vesicles and their movement in the cytoplasm, and the capsid is released. RhoA activates Dia-2, which in turn augments Src activation (Veettil et al., 2006), all of which are probably essential for the formation of endocytic vesicles and their movement in the cytoplasm. These events are probably mediated by the PKC-ζ pathway (Naranatt et al., 2003). In addition RhoA-GTPase facilitates the transport of capsid toward the nucleus by inducing MT stabilization and regulating MT dynamics via Dia-2 (Naranatt et al., 2005). In **phase 4**, the endocytic vesicles with virus or released capsid/tegument complexes bind to dynein motor components, transported along the MT to reach the nuclear vicinity, and deliver the viral DNA into the nucleus (Naranatt et al., 2005). RhoA-GTPases facilitate the transport of the capsid toward the nucleus by inducing microtubule stabilization and regulating microtubule dynamics (Naranatt et al., 2005). KSHV-induced ERK (Sharma-walia., 2005) and NF-κB have been shown to modulate both viral gene expression and host gene expression. Nuclear

EBV closely related to the γ-2 KSHV initiates the infection of human primary B cells via its envelope glycoprotein gp350/220 interaction with C3d complement receptor (CR2; CD28). Subsequently, EBV binds to the HLA class II molecule via its gp42 in the gH/gL complex. In contrast, infection of epithelial cells occurs via gH/gL complex interaction with an unknown receptor and integrin $\alpha 5 \beta 1$ interaction via the BMRF2 glycoprotein. Nectin-1 is the primary receptor for herpes simplex virus type 1 (HSV-1) entry into human fetal neurons. In contrast, fibroblasts have multiple receptors and mechanisms for HSV-1 entry (Simpson et al. 2005).

The second concept is that entry mechanisms vary according to cell type. Even in well-studied HSV-1, the notion that of HSV enters target cells by fusion of its envelope at the cell membrane is changing and emerging evidences suggest that HSV-1 enters different target cells by endocytosis (Nicola et al. 2005). It has been shown that HSV-1 enters human epidermal keratinocytes, but not neurons, via a pH-dependent endocytic pathway, while EBV enters primary B cells via endocytosis and human epithelial cells via fusion at the cell membrane. Similarly, clinical isolate of wild-type human cytomegalovirus (HCMV), a β-herpesvirus, enters epithelial and endothelial cells by endocytosis followed by low-pH-dependent fusion, and in contrast, enters human fibroblasts via a pH-independent fusion with the plasma membrane (Ryckman et al. 2006). Since there are a number of similarities between these concepts and KSHV infection, these need to be kept in mind by investigators entering the field of KSHV– receptor interactions for the rational analysis of their results.

Several important events occur during the early stages of target cell infection by KSHV. For better conceptual purposes, we have divided these early events into five overlapping dynamic phases (Fig 23.1) and (Fig 23.2). Phase 1 involves the binding of virus to cell surface via the interactions of viral envelope glycoproteins to the cell surface molecules (receptors). This is followed by virus entry into the target cells (phase 2), overlapping with the induction of host cell signal pathways during phase 1. In phase 3, the viral capsid/tegument moves in the cytoplasm. In phase 4, viral DNA enters into the nucleus. This is followed by expression of viral genes (latent and/or lytic) in phase 5b. Overlapping with these events are the expression of host cell genes (phase 5a) and these events are discussed in the following section.

Fig. 23.1 (continued) delivery of the KSHV genome in the infected cell nucleus is followed by simultaneous induction of viral gene expression (**Phase 5b**) and regulation of host (target cell) gene expression (**Phase 5a)**. The overlapping viral gene-induced host cell gene expression may exert an influence on subsequent viral and host gene expression. Solid arrows depict the KSHV-induced host cell signaling events that have been characterized so far and the dotted lines depict the pathways yet to be characterized. The large arrows indicate the stage of KSHV infection in which the induced signal pathways are shown to play a role

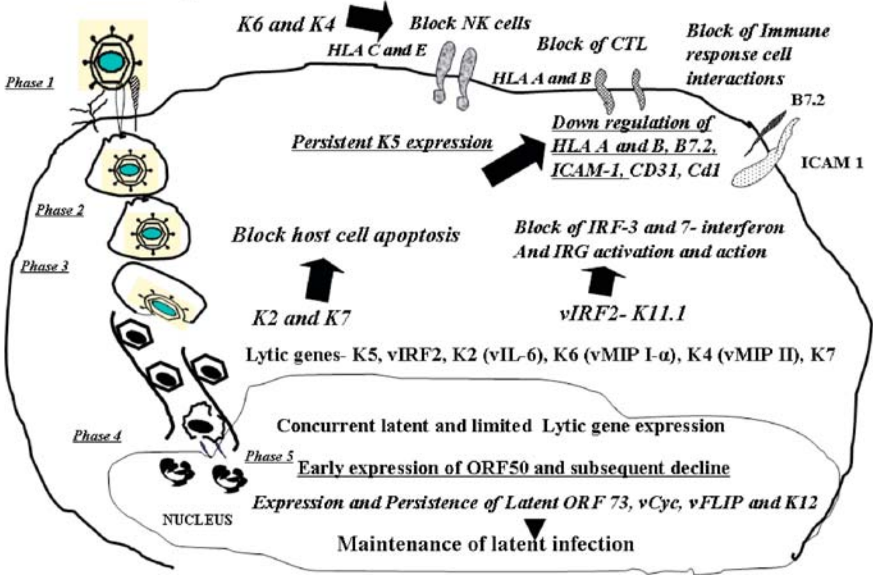


Fig. 23.2

23.6 KSHV Binding to the Target Cells (Phase 1): Binding and Entry Receptors

23.6.1 Heparan Sulfate

The study of cellular receptor(s) recognized by KSHV in the different target cells and viral glycoproteins involved is in infancy at the present time. The ability of KSHV to infect a variety of target cells in vivo and in vitro clearly suggests that KSHV recognizes cell surface molecules that are ubiquitous and/or recognize unique molecules that are specific for the specific target cells. Studies with labelled virus demonstrated that KSHV binds to a variety of target cells such as human B, endothelial and epithelial cells, monocytes (but not T and NK cells), and a variety of animal cells (Akula et al. 2001b; Dezube et al. 2002). This broad cellular tropism may be in part due to KSHV's interaction with the ubiquitous cell surface heparan sulfate (HS) proteoglycan (Akula et al. 2001b). HS has been shown to be involved in the initial virus–cell interactions of many other herpesviruses such as HSV-1 and 2, pseudorabies virus, bovine herpesvirus 1, HCMV, human herpesvirus 7 (HHV-7), and bovine herpesvirus 4. KSHV infection of primary HMVEC-d cells was difficult in the presence of the heparin, a glycosaminoglycan closely related to HS used in the growth medium of these cells. Further analyses

showed that KSHV infection can be inhibited in a dose-dependent manner by soluble heparin but not by chondroitin sulfates A and C (Akula et al. 2001b). Enzymatic removal of HFF cell surface HS with heparinase I and III reduced KSHV infectivity. Soluble heparin blocked or displaced KSHV binding to target cells and this binding was drastically reduced on mutant CHO cells deficient in HS (Akula et al. 2001b). These observations clearly suggest that KSHV interaction with HS may be the first set of ligand–receptor interactions that concentrates virus on the human B, endothelial, epithelial, and fibroblast cells (Table 23.1B), where it can subsequently bind to one or more additional host cell molecules that are essential to the entry process.

Heparin-binding proteins often contain more than one of the two consensus motifs, or heparin-binding domain (HBD), containing the XBBXBX and XBBBXXBX sequences, where B is a positively charged basic amino acid (lysine, arginine, or histidine) flanked by an additional positively charged residue separated by hydrophobic amino acids "X" (Akula et al. 2001a). Analysis of HBD of several proteins suggests that the negatively charged sulfate or carboxylate groups on heparin may interact via electrostatic interactions with positively charged cationic residues in a protein or peptide (Akula et al. 2001a). Predictive analysis of KSHV-gB revealed that its extracellular domain contains the BXXBXBBXBB (^{108}HIFKVRRYRK^{117}) type HBD, which is conserved throughout the $\gamma 2$ herpesviruses (Akula et al. 2001a). gpK8.1A also possesses two possible, although atypical heparin-binding motifs, gpK8.1A-H1 (^{150}SRTTRIRV^{157}, XBXXBXBX) and gpK8.1A-H2 (^{182}TRGRDAHY^{189}, XBXBXXBX) (Wang et al. 2001). KSHV gH lacks the typical heparin-binding domains (Naranatt et al. 2002).

Studies demonstrated that KSHV gB and gpK8.1A bind to cell surface HS molecules (Akula et al. 2001a; Ganem 2007; Wang et al. 2001), and binding of soluble forms of these proteins generated in baculovirus is saturable and can be blocked by soluble heparin (Wang et al. 2001; Wang et al. 2003). Virion envelope full length gB and gpK8.1A specifically bind heparin-agarose and can be eluted by high concentrations of soluble heparin, but not by chondroitin sulfates (Akula et al. 2001a; Wang et al. 2001). KSHV-gpK8.1A binds to heparin more strongly than gB (Wang et al. 2003), and it binds with an affinity comparable to that of glycoproteins B and C of herpes simplex virus (Ganem 2007). Even though the involvement of KSHV gB residues 108–117 and gpK8.1A residues 150–157 in binding to HS-like moieties has been convincingly demonstrated, it is also possible that other weak and/or high-affinity HBDs may appear in KSHV gB and gpK8.1A in their native quaternary structures if basic amino acids separated linearly are juxtaposed, forming a typical HBD. The presence of two or more heparin-binding glycoproteins within a single virus is not unprecedented, since the well-studied human α- and β-herpesviruses contain at least two HS-binding glycoproteins, for example: gB and gC for herpes simplex 1 and 2, gB and gCII for HCMV, and gB and gp65 for HHV-7. The presence of two HS-binding proteins in KSHV reemphasizes the importance of cell surface HS for attachment of many, although not all herpesviruses.

23.6.2 DC-SIGN

A recently published study reported that KSHV also utilizes the dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3) grabbing non-integrin (DC-SIGN; CD209) as a receptor for infection of myeloid dendritic cells (DCs) and macrophages (Rappocciolo et al. 2006) (Table 23.1B). DC-SIGN is a type II C-type lectin expressed on myeloid DCs in the dermis, mucosa, lymph nodes, lung and thymus, (Rappocciolo et al. 2006) and acts as pathogen recognition receptor that alerts macrophages and DCs to take up and process pathogens for Ag presentation to T cells. DC-SIGN was required for virus attachment to these cells and DC-SIGN-expressing cell lines. KSHV binding and infection were blocked by anti-DC-SIGN monoclonal antibody, by a natural ligand for DC-SIGN (mannan), and by soluble DC-SIGN. Pretreatment of cells with anti-DC-SIGN antibodies could not completely block KSHV binding and infection, and this residual level of binding and infection can be attributed to additional receptors such as HS and other molecules for KSHV on these cells. The nature of the KSHV envelope glycoprotein interacting with DC-SIGN needs to be evaluated further.

23.6.3 Integrins

Although KSHV gpK8.1 and gB proteins are involved in the interaction with the cell surface HS molecules, even high concentrations of rabbit polyclonal and monoclonal anti-gB and anti-gpK8.1A antibodies neutralizing infection do not block the binding of KSHV to target cells (Akula et al. 2001a; Zhu et al. 1999a). This implies a role for the proteins (cellular receptors) after attachment has occurred possibly as a result of interaction with additional cell surface molecules. Among all the gB homologs of herpesviruses sequenced to date, only KSHV-gB possesses an integrin-binding RGD (Arg-Gly-Asp) motif at amino acids 27 to 29, which is predicted to be immediately adjacent to the putative signal sequence of the protein (Akula et al. 2002b). The RGD motif is the minimal peptide region of many extracellular matrix (ECM) proteins known to interact with subsets of host cell surface integrins. KSHV infectivity of fibroblasts and endothelial cells is neutralized by RGD peptides, by antibodies to $\alpha3$ and $\beta1$ integrins, and by soluble $\alpha3\beta1$ integrin (Akula et al. 2002b) and anti-gB antibodies immunoprecipitate a complex of virus and $\alpha3\beta1$. At the same time, RGD peptides, anti-integrin antibodies and soluble integrins fail to block virus binding to target cells suggesting that KSHV uses the $\alpha3\beta1$ integrin as one of the cell receptors or coreceptor for entry (Akula et al. 2002b). Expression of human $\alpha3$ integrin also increased the infectivity of virus for CHO cells (Akula et al. 2002b). However, this increase did not reach the level of infection as seen in endothelial and fibroblast cells, suggesting that there are other receptors that are critical for entry (Table 23.1B).

Virus binding and viral DNA internalization studies suggest that $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins also play roles in KSHV entry and infection of fibroblasts or endothelial cells. Infection of fibroblasts or endothelial cells can also be neutralized by soluble $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins with higher levels of neutralization as compared to soluble $\alpha 3\beta 1$ integrin (Table 23.1B). These studies suggest the expansion of the in vivo target cells for KSHV. Using an RTA-dependent reporter 293-T cell line, Inoue et al. 2003 reported the inability of soluble $\alpha 3\beta 1$ integrin and RGD peptides to block the infectivity of KSHV. However, in this study virus was centrifuged with cell in the presence of the charged polymer polybrene, which may account for the apparent discrepancy. Polybrene is a positively charged cation which can complex with the virus envelope and may bypass the need for receptors. This property of polybrene is the basis for its use to increase the infectivity of many viruses and to deliver nucleic acid for gene therapy.

Even though anti-$\alpha 3$ or $\beta 1$ antibodies or soluble $\alpha 3\beta 1$ integrin have been shown to block KSHV infection of HMVEC-d and HFF cells, only a 50% block in infection as measured by GFP expression was observed. Role of integrin in KSHV infection of B and other cell has not been demonstrated. Integrin could be one of the receptors but only in some cells and not in all cells. This is not surprising as similar diversity in the recognized receptor has been demonstrated in EBV. Expression of DC-SIGN on B-lymphoblastoid cell lines (LCL) and K562 cells which are normally resistant to KSHV rendered them susceptible to KSHV infection (Rappocciolo et al. 2006). Since neither of these cells expressed $\alpha 3\beta 1$ on their surface, this suggested that other molecules such as DC-SIGN may be involved in infection of these target cells.

23.6.4 xCT

Another study recently reported that the 12-transmembrane cystine transporter protein xCT as the fusion-entry receptor for KSHV in adherent cells (Kaleeba and Berger 2006). The xCT molecule is part of the cell surface 125 kDa disulfide-linked hetrodimeric membrane glycoprotein CD98 (4F2 antigen) complex containing a common glycosylated heavy chain (80 kDa) and a group of 45 kDa light chains. The xCT molecule involved in glutamate/cysteine exchange is one of the light chains (Kaleeba and Berger 2006). Expression of recombinant xCT rendered otherwise not susceptible target cells permissive for both KSHV cell fusion and virion entry. Antibodies against xCT blocked KSHV fusion and entry with naturally permissive target cells such as the adherent target cells of human and nonhuman cell types. Surprisingly, xCT mRNA was not detected in human CD19 primary B cells isolated from fresh peripheral blood mononuclear cells (Kaleeba and Berger 2006), which are known target cells for KSHV. These studies further suggest that like EBV, KSHV may possess alternative receptor(s) in adherent cells and other molecules besides xCT may be involved in infection of B cells. The

nature of the KSHV envelope glycoprotein interacting with xCT molecule is not known and needs to be studied (Table 23.1B).

It is interesting to note that the CD98 complex usually associates with $\alpha 3\beta 1$ integrin and has been shown to be involved in membrane clustering and integrin-mediated signal cascades (Fenczik et al. 2001; Feral et al. 2005). This suggests that KSHV must be binding to a family of functionally related proteins such as $\alpha 3\beta 1$ integrin and CD98-xCT molecules in the endothelial, epithelial, and fibroblast cells (Table 23.1B). This is an attractive suggestion since, as discussed in the following sections, evidences suggest that KSHV interaction with these cellular receptors and co-receptors is essential for the establishment of a successful infection.

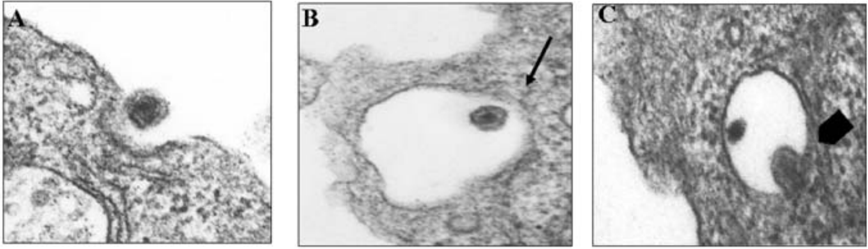
Further studies are required to define the nature of receptors in the different target cells of KSHV.

23.7 KSHV Entry into the Target Cells (Phase 2)

KSHV binds and enters a variety of human (BCBL-1, BJAB, Raji, 293, HFF, HeLa, endothelial), monkey (Vero, CV-1), hamster (BHK-21, CHO), and mouse (Du17) cells, as shown by the detection of viral DNA, limited KSHV gene expression, and GFP expression (Akula et al. 2001a; Akula et al. 2001b; Akula et al. 2002a; Naranatt et al. 2003; Renne et al. 1998; Vieira et al. 2001). Examination of internalized KSHV DNA by real-time DNA PCR demonstrates a rapid internalization of viral DNA in the infected endothelial and HFF cells (Krishnan et al. 2004). After binding to the target cell, like other viruses, KSHV faces two obstacles: the plasma membrane and the actin cytoskeleton (Medalia et al. 2002). Penetration of any enveloped virus into a cell involves fusion of the virion envelope with the membrane of the cell and can occur either at the cell surface or after endocytosis. Endocytic route of entry offers several advantages as it bypasses many barriers associated with the membrane cortex and cytosolic crowding thus affording a convenient and often rapid system of transit across the plasma membrane and through the cytoplasm for delivery of viral cargo to the vicinity of the nucleus (Sieczkarski and Whitaker 2002).

KSHV enters the human B-cell line BJAB (Akula et al. 2001b), HFF (Akula et al. 2003), human epithelial line 293 (Inoue et al, 2003; Liao et al. 2003), and endothelial cells by endocytosis. Within 5 min of infection, presence of KSHV virions in large endocytic vesicles and fusion of virion envelope with the endocytic vesicles were detected in the infected BJAB and HFF cells by electron microscopy (Akula et al. 2003; Akula et al. 2001b). Viral capsids were detected in the vicinity of nuclear membranes by 15 min after infection (Fig. 23.3). Anti-KSHV-gB antibodies colocalize with virus-containing endocytic vesicles. In HFF cells, KSHV infection is significantly inhibited by the preincubation of cells with chlorpromazine HCl, which blocks endocytosis via clathrin-coated

KSHV entry in BJAB cells



KSHV entry in HMVEC-d cells

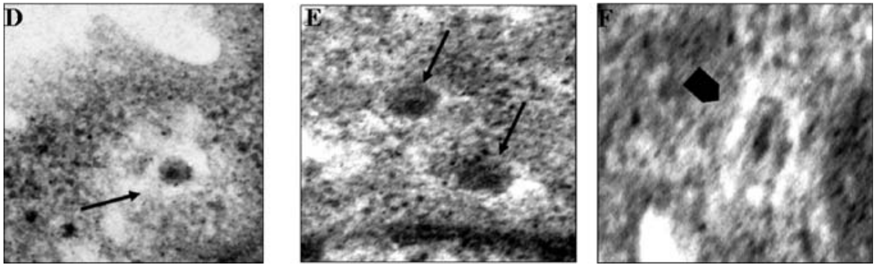


Fig. 23.3 Electron microscopic observation of KSHV entry into BJAB and HMVEC-d cells via endocytosis. BJAB cells were incubated with GFP-KSHV for 60 min at 4°C. Infection was initiated by shifting the temperature to 37°C. After 5 min at 37°C (**A**), (**B**), (**C**), cells were washed in PBS and fixed in 2% glutaraldehyde. Similarly, HMVEC-d cells were incubated with GFP-KSHV for 60 min at 4°C. Infection was initiated by shifting the temperature to 37°C. After 5 min at 37°C (**D**), (**E**), (**F**), cells were washed in PBS and fixed in 2% glutaraldehyde. Thin sections were made for ultrastructural analysis by transmission EM. Virion particles at various stages of binding and entry and in endocytic vesicles are indicated by *thin arrows* and fusion of the virion envelope with the endocytic vesicles is marked by the *large arrow*

pits, but not by nystatin and cholera toxin B which block endocytosis via caveolae and induce the dissociation of lipid rafts, respectively. Infection is also inhibited by blocking the acidification of endosomes by NH_4Cl and bafilomycin A in HFF and 293 (human embryonic kidney) cells (Akula et al. 2003; Liao et al. 2003). These findings suggest that penetration of KSHV occurs in and requires a low pH intracellular environment. Neutralization of KSHV infectivity by rabbit anti-gB, gH, and gL antibodies without affecting virus binding to HFF cells suggested that these glycoproteins play a critical role in the entry process, and it seems likely that the minimal fusion machinery of KSHV comprises gB, gH, and gL.

Further work is required to determine the mode of entry in the different target cells, the nature of the endocytic vesicles, association of cell surface molecules in these vesicles, and the role of KSHV glycoproteins in entry and fusion with the target cells.

23.8 KSHV Movement in the Cytoplasm and Delivery to the Nucleus (Phase 3 and 4)

KSHV capsid/tegument, after release into the cytoplasm must traffic through the cell for the delivery of viral DNA to the nucleus. Kinetics experiments demonstrate a rapid accumulation of KSHV viral DNA in the infected cell nuclei (Naranatt et al. 2005). Similar to HSV-1, KSHV also utilizes the dynein motors and MTs to reach the vicinity of the nucleus. KSHV infection also induced a transient increased acetylation and thickening of microtubules. Microtubules play important roles in KSHV infection since depolymerisation of microtubules, even though it did not affect KSHV binding and internalization, it inhibited the nuclear delivery of viral DNA and infection (Naranatt et al. 2005). KSHV proteins involved in the interaction of dynein motors need to be evaluated further.

23.9 Host Gene Expression in the Infected Target Cells (Phase 5a)

Establishment of latent infection by KSHV thus provides a good in vitro model for studying viral and host factors involved in the establishment and maintenance of latent infection.

As an initial step toward understanding how KSHV establishes an in vitro latent infection, modulation of host cell gene expression at 2 and 4 h p.i. of primary HMVEC-d and HFF was performed using oligonucleotide arrays (Naranatt et al. 2004). Reprogramming of host transcription regulating apoptosis, cell cycle regulation, signaling, inflammatory response, and angiogenesis (Naranatt et al. 2004) was observed. Notable among these was the strong induction of several proinflammatory cytokines, growth factors, and cyclooxygenase-2 (COX-2). Studies suggested that KSHV modulates the COX-2 gene to induce PGE2 for the maintenance of viral latent gene expression (Sharma-Walia et al. 2006). Further work is essential in defining the role of host genes in the maintenance of KSHV latency and pathogenesis.

23.10 KSHV gene expression in the infected target cells (Phase 5b)

By infecting a variety of human and animal cells, including primary endothelial cells as well as endothelial cells immortalized by telomerase (TIME) or E6/E7 proteins of human papillomavirus, several studies examined the expression of limited KSHV genes and tested the ability of target cells to support a lytic infection, as measured by the production of serially passable infectious virus (Moses et al. 1999; Poole et al. 2002). These studies were conducted at a later time point of infection, which ranged from 48 h to several days, and included 48 h p.i. in primary human umbilical vein endothelial cells with recombinant BAC36 KSHV (Gao et al. 2003), 48 h p.i. in telomerase-transformed endothelial

(TIME) cells with the BCBL-1 virus (Lagunoff et al. 2002), 24 to 48 h p.i. in a variety of human and animal target cells including TIME cells with the BCBL-1 virus (Lagunoff et al. 2002), 48 h p.i. in primary DMVEC (identical to the HMVEC-d cells used here) and TIME-DMVEC cells with BCBL-1 virus (Tomescu et al. 2003), 6 days p.i. in primary human keratinocytes with BCBL-1 virus (Dourmishev et al. 2003), 25 days p.i. in primary DMVEC with virus from JSC cells (Ciufo et al. 2001), and 7 to 14 days p.i. in human papillomavirus E6/E7-transformed human dermal endothelial cells with BCBL-1 virus (Moses et al. 1999). The detection of LANA-1 coupled with the absence of infectious virus and lytic cycle ORF 59 and gpK8.1 in these studies lead to the notion that the primary KSHV infection led to the establishment of latency without any apparent lytic cycle initiation. However, several studies also reported the detection of lytic cycle markers such as ORF 59 and gpK8.1A in <5% of cells after 48 h p.i. (Ciufo et al. 2001, Lagunoff et al. 2002, Tomescu et al. 2003), and these were considered cells entering spontaneous activation of the lytic cycle. Using recombinant BAC36 KSHV, Gao et al. 2003 detected LANA-1 in >90% of the infected primary human umbilical vein endothelial cells and KSHV ORF 65 (minor capsid protein) in <5% of cells after 2 days p.i. The reason for only the small percentage of cells entering the lytic cycle is not known at present.

Although the expression of KSHV lytic cycle switch protein-ORF 50 (RTA) following a primary infection was not measured systematically, it was presumed to be absent, and establishment of latent infection was considered as the default KSHV infection. When the kinetics of latent and lytic KSHV gene expression immediately following infection of primary HMVEC-d and HFF cells was examined by quantitative real-time RT-PCR and whole genome array, within 2 h p.i., high levels of ORF50 transcripts were detected in both HMVEC-d and HFF cells, which declined sharply by 24 h p.i. (Krishnan et al. 2004). In contrast, comparatively low levels of ORF73 expression were detected within 2 h p.i., increased subsequently, maintained at a steady state and declined slowly by 5 days p.i. Expression of ORF 50 and ORF73 proteins was observed in >90% of infected cells. Similar kinetics were also observed at an moi of 5 and 10 DNA copies/cell.

What is more interesting is that subsets of lytic transcripts with anti-apoptotic and immune-modulation functions were also expressed soon after infection, and many of these transcripts could not be detected at later time points. The detection of lytic viral transcripts by gene array in our study could also represent messages carried in the virion particles. A study by Bechtel et al. 2005 showed that 10 of the 29 RNAs detected in our system encoding ORFs such as K8.1, K12, ORF58/59, and ORF54 were present in the purified virion particles. However, other transcripts detected by us were absent suggesting de novo transcription of the remaining lytic genes during the first hours of infection. Further examination revealed a steady quantitative increase in early lytic K5, K8, and v-IRF2 gene expression in the infected HMVEC-d and HFF cells (Krishnan et al. 2004).

These results are subsequently well supported by other studies (Bechtel et al. 2005). Very early ORF50 expression and subsequent decline were also seen during primary infection of human 293 cells by KSHV (Bechtel et al. 2005). During latency, the three latency associated ORFs 73, 72, and K13 genes are transcribed from a common promoter (LTc) as part of a multicistronic mRNA. Subsequent alternative mRNA splicing and internal ribosome entry allow for the expression of each protein. Matsumura et al.(2005) have shown that transcription of LT cassette mRNA can be induced by RTA through the activation of a second promoter (LT_i) immediately downstream of the LTc promoter. Interestingly, LT_i is unresponsive to sodium butyrate, a potent inducer of lytic replication and thus demonstrating a previously unrecognized class of RTA-responsive promoters that respond to direct, but not indirect, induction of RTA. These studies suggested a mechanism by which RTA contributes to establishment of latency during de novo infections, and the initial burst of ORF50 mRNA expression shown by our studies probably facilitates the initial colonization and ultimately latency by helping to raise intracellular levels of LANA-1, v-Cyc, and v-FLIP, which are implicated in preventing apoptosis and activating the cell proliferation machinery (Matsumura et al. 2005).

How KSHV achieves selective activation of these RTA-responsive genes without initiating the full-blown lytic cascade represents an important challenge to our understanding of the regulatory network. The functions of ORF 50 can also potentially be influenced by the newly synthesized ORF 73 protein (Matsumura et al. 2005). There is evidence that LANA itself counters transactivation of certain lytic promoters by RTA, but is probably not effective at early times when RTA is more abundant (Matsumura et al. 2005). Alternatively, as shown in the following section, factors unique to the target cell during primary infection, such as host gene products, could be influencing the outcome of infection.

23.11 Potential Avenues of Immune Evasion by KSHV Early During Infection of Target Cells

Based on the above studies, we present the following summary of potential avenues of immune evasion by KSHV early during infection of target cells (Fig. 23.2).

(a) The K5 gene product has been shown to be involved in the downregulation of MHC class I A and B, ICAM-1, B7-2, PE-CAM, and Cd1d molecules, which are the most important elements necessary for the elimination of virus-infected cells by cytotoxic T and NK cells and for T-cell stimulation (Tomescu et al. 2003). Studies by Tomescu et al. 2003 demonstrated the downregulation of cell surface MHC class I, ICAM-1 (CD54), and PE-CAM (CD31) in dermal endothelial cells after infection. Our demonstration

of a high level of K5 expression in the primary infection is exciting, since together with the findings by Tomescu et al. 2003 these results suggest that expression of K5 protein probably provides an important avenue for the in vivo-infected cells to escape immune surveillance. K5 is continuously expressed in the latently infected endothelial cells (Okuno et al. 2002). It is interesting to note that K5 is also detected in KS lesion endothelial cells (Krishnan et al. 2004).

K5 selectively down regulates MHC class I-A and C molecules from the surface but not during their synthesis. The cleaved signal peptides from MHC class I-A and C molecules are loaded on the antigen-presenting grooves in MHC class I-C and E molecules before expression on the cell surface. Hence, when synthesis of class I-A and B molecules is down regulated as shown in many in vitro viral infections, this will result in the absence of signal peptides which will be recognized by NK cells. However, KSHV-infected cells will be considered by NK cells as normal as the MHC class I-C and E molecules will be still presenting the signal peptides of class I-A and C. Hence, infected cells will be in a stealth state and could escape from the host CTL and NK cells as long as K5 expression continues (Fig 23.2).

(b) KSHV lytic gene vIRF-2 (K11.1) was expressed in HMVEC-d cells for more than 24 h p.i. The vIRF-2 protein has profound inhibitory effects on IFN-α and β gene transcription, probably by its ability to bind specifically to several transcription factors, such as IRF1, IRF2, IRF3, IRF7, and IRF8 (Krishnan et al. 2004).

(c) KSHV lytic genes K4 (vMIP-II), K6 (vMIPI), and K2 (v-IL-6) were expressed briefly and these proteins have been shown to possess anti-apoptotic and immunomodulatory functions and to protect cells from IFN and NK cell action (Krishnan et al. 2004) (Fig 23.2).

(d) KSHV lytic genes K7 and K6 proteins were also expressed briefly and these proteins possess anti-apoptotic functions (Krishnan et al. 2004) (Fig 23.2).

Taken together, these studies suggest that expression of limited lytic proteins must be providing a survival advantage for KSHV-infected cells, and the necessary factors and time to establish and/or maintain latency during the initial phases of infection (Fig 23.2). For additional details on KSHV immune evasion strategies please see Chapter 24.

23.12 Induction of Pre-existing Host Cell Signal Induction by KSHV

Eukaryotic cells interact with their extracellular environments largely by utilizing ligand-induced signaling molecules exposed at their surface. Ligand mimicry is an opportunistic mechanism by which microbes, including viruses, manipulate

host-signaling molecules for their benefit (Virji 1996). Viruses take advantage of the events triggered by signaling to induce changes in the cell that promote viral entry, to facilitate intracellular transport, to manipulate cell defense mechanisms, or to induce the pattern of cellular gene expression that is most favorable to establishment of latent or productive infection. Signals can be generated in ways such as activation of cellular signaling molecules directly by using them as receptors, by clustering specific cell surface proteins, or by associating with lipid rafts to initiate highly inter-linked networks of signaling pathways. Understanding virus-induced signaling and its consequence is emerging as an important area of virology. KSHV is among the group of viruses which "knock at the door" of target cells by activating cellular signaling cascades, which in turn result in the local ligand-triggered processes required for virus entry. Available evidence suggests that this aspect of the KSHV entry and infection process is complex and a better understanding of this process is essential to fully comprehend the infectious process of KSHV.

KSHV gB interacts with integrins, which are part of a large family of heterodimeric receptors containing noncovalently associated transmembrane α and β glycoprotein subunits (Giancotti 2000; Giancotti and Ruoslahti 1999; Sastry and Burridge 2000). 19α and 9β subunits generate more than 24 known combinations of $\alpha\beta$ cell surface receptors and each $\alpha\beta$ combination has its own binding specificity and signaling properties (Giancotti 2000; Giancotti and Ruoslahti 1999; Sastry and Burridge 2000). Integrin–ligand interactions activate numerous signaling molecules, such as FAK, c-Src, p130^{CAS}, phospholipase C-γ, phosphatidylinositol 3-kinase (PI3-K), PKC and cytosolic proteins, like talin, vinculin, paxillin and α-actin, and assemble into focal adhesions which link integrins to ECM proteins on the outside and the cytoplasmic actin cytoskeleton on the inside (Giancotti and Ruoslahti 1999). These signaling events mediate a variety of cell functions such as activation of cytoskeleton elements, endocytosis, regulation of gene expression, cell motility, attachment, cell cycle progression, cell growth, apoptosis, and differentiation (Giancotti 2000; Giancotti and Ruoslahti 1999; Sastry and Burridge 2000). Integrin–ligand interactions led to a rapid increase in the tyrosine phosphorylation of pp125 focal adhesion kinase (FAK) (Giancotti and Ruoslahti 1999), which is a nonreceptor protein-tyrosine kinase that localizes in focal adhesions with vinculin, and its activation is the first step necessary for the outside-in signaling of integrins (Calderwood et al. 2000; Giancotti 2000; Sastry and Burridge 2000). Within 5 min of infection, KSHV induces the integrin-mediated activation of FAK in endothelial and fibroblast cells, and colocalizes with the focal adhesion component vinculin (Akula et al. 2002b). Soluble gB, but not soluble gpK8.1A, induces FAK autophosphorylation at tyrosine 397, which also colocalizes with paxillin (Wang et al. 2003, Sharma-walia et al. 2004). Soluble gB, independently of other viral glycoproteins, induces the FAK-Src-PI-3K-Rho GTPase-signaling pathway and extensive cytoskeletal rearrangement in target cells (Sharma-walia et al. 2004). KSHV infection also leads to cytoskeletal rearrangements and the formation of structures such as filopodia, lamellipodia, and

stress fibers in the target cells (Naranatt et al. 2003). Preincubation of virus or gB with soluble $\alpha3\beta1$ integrin or anti-gB antibodies-inhibited FAK activation and was not activated by a soluble form of gB in which the RGD sequence had been mutated (Akula et al. 2002b; Wang et al. 2003, Sharma-walia et al. 2004). The ability of anti-integrin antibodies and soluble integrin to neutralize virus infection without affecting virus entry suggests that integrin and the associated signaling pathways have a role to play in KSHV entry and infection of target cells.

23.13 Role of KSHV-Induced Signal Pathways in Entry and Infection

Studies with human $\alpha3$ integrin-transfected CHO cells, FAK knockout mouse fibroblasts Du3 (FAK−/−) and parental Du17 (FAK+/+) cells confirm that FAK plays a key role in KSHV infection (Naranatt et al. 2003, Krishnan et al. 2006). KSHV binding to both the cell types was equal and could be inhibited by soluble heparin but efficiency of infection was much lower in Du3 cells as compared to Du17 cells (Naranatt et al. 2003). Additional studies indicated that KSHV could induce the phosphorylation of FAK in FAK-positive Du17 cells early during infection and resulted in increased KSHV entry in these cells (Fig. 23.1). Du3 (FAK−/−) cells showed about 70% reduction in the internalization of KSHV DNA and expression of FAK in these cells via an adenovirus vector augmented the internalization of viral DNA thus suggesting that FAK plays critical role in KSHV entry. FAK dominant-negative mutant FAK-related non-kinase (FRNK) expression in Du17 cells significantly reduced the entry of virus. Reduced quantity of virus entry in Du3 cells, delivery of viral DNA to the infected cell nuclei, and expression of KSHV genes suggested that in the absence of FAK, another molecule(s) may be partially compensating for FAK function. KSHV infection in Du3 cells induced the phosphorylation of the FAK-related proline-rich tyrosine kinase (Pyk2) molecule, which could complement some of the functions of FAK (Krishnan et al. 2006). Expression of an autophosphorylation mutant of Pyk2 with tyrosine 402 mutated to phenylalanine 402 (Y402 to F402) reduced viral entry in Du3 cells, suggesting that Pyk2 facilitates viral entry moderately in the absence of FAK (Krishnan et al. 2006). These results suggest a critical role for KSHV infection-induced FAK in the internalization of viral DNA into target cells. Since activation of FAK is central to many paradigms of outside-in signaling by integrins, actin assembly, and endocytosis, KSHV may be taking advantage of these signaling pathways both to promote entry and to produce a cellular state that facilitates infection. Autophosphorylation of the FAK Tyrosine 397 leads to the binding of SH-2 domain of Src family kinases, activated Src kinases then phosphorylate a number of FA components. KSHV gB-induced FAK dependent Src phosphorylation in adherent target cells (Sharma-walia et al. 2004) and a recent study by Veettil et al. 2006 showed that RhoA-GTPase facilitates KSHV entry into

adherent target cells in a Src-dependent manner suggesting the involvement of Src phosphorylation in KSHV entry process (Fig 23.1).

PI 3-K, one of the important downstream effectors of FAK, is a member of a family of lipid kinases (Giancotti and Ruoslahti 1999; Sastry and Burridge 2000) that acts as second messenger for a number of cell functions including the activation of Rho-GTPases and anti-apoptotic pathway Akt molecule (Giancotti and Ruoslahti 1999; Sastry and Burridge 2000). KSHV induces PI 3-K within 5 min of infection which decreased after 15 min (Naranatt et al. 2003) and this response can be inhibited either by pre-incubating virus with integrin or by the PI 3-K inhibitors wortmannin and LY294002. Treatment of cells with genistein (general tyrosine kinase inhibitor) or LY294002 could inhibit viral DNA internalization in a dose-dependent manner suggesting the role of tyrosine kinase and PI-3 K activation in the entry of KSHV in target cells (Sharma-Walia et al. 2004). Induction of PI-3 K involved in AKT-mediated anti-apoptosis very early during infection suggests that this probably benefits KSHV by blocking apoptosis induced by virus binding and entry stages (Fig 23.1).

Reorganization and remodeling of actin cytoskeleton is another hallmark of integrin interaction with ligands, which is broadly controlled by the Rho family of small GTPases, such as Rho, Rac, and Cdc42 (Hall and Nobes 2000). The morphological changes induced by Rho, Rac, and Cdc42 activation are downstream effects of PI 3-K activation (Hall and Nobes 2000). Immediately following KSHV infection, target cells exhibit morphological changes and cytoskeletal rearrangements such as filopodia, lamellipodia, and stress fibers (Naranatt et al. 2003). This together with the phosphorylation of PI 3-K by KSHV at an early time point of infection suggests the induction of Rho-GTPases and the associated signal pathways (Naranatt et al. 2003) (Fig 23.1).

Cross talk between ECM and integrins activates FAK, which initiates a cascade of intracellular signals that eventually activate the mitogen-activated protein kinase (MAPK) pathways (Giancotti and Ruoslahti 1999; Sastry and Burridge 2000). These pathways have the core module consisting of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which eventually activates a MAPK. MAPKs are potentially involved in controlling fundamental cellular processes, such as proliferation, differentiation, survival, and apoptosis (Giancotti and Ruoslahti 1999; Sastry and Burridge 2000), in all eukaryotes. As early as 5 min post-infection, KSHV activates the MEK (MAPK/ERK kinase) and extracellular-signal-regulated kinase (ERK) (Naranatt et al. 2003) (Fig. 23.1).

Soluble KSHV gpK8.1A, but not gB, induced MEK-mediated ERK1/2 phosphorylation as early as 5 min post-treatment, and ERK1/2 phosphorylation facilitated the establishment of KSHV infection in HFF and HMVEC-d cells (Sharma-Walia et al. 2005). PI 3-K and protein kinase C-ζ (PKC-ζ) are recruited as upstream mediators of the KSHV-induced ERK pathway (Naranatt et al. 2003) and inhibitors specific for PI 3-K, PKC-ζ, MEK, and ERK significantly reduce virus infectivity without affecting virus binding to the target cells. Examination of entry of viral DNA supports a role for PI 3-K in

KSHV entry and a role for PKC-ζ, MEK, and ERK at a stage after entry (Naranatt et al. 2003). PI 3-K is also involved in the activation of Rho-GTPases that are critical for the activation of Rac, Rho, Cdc42 and Rab5 which are involved in the modulation of actin dynamics, formation of endocytic vesicles, and the fission of endocytic vesicles (Fig 23.1).

KSHV capsid/tegument, after release into the cytoplasm must traffic through the cell in order for viral DNA to be delivered into the nucleus. KSHV capsid movement in the cytoplasm depends upon microtubules (MTs), which are controlled by the RhoGTPases. KSHV induces the RhoGTPases and interaction of KSHV with cells induces the polymerization of cortical actin filaments (Naranatt et al. 2003). In KSHV-infected HFF cells, RhoA promoted the actin-stress fibers, whereas Rac1 and Cdc42 mediated the lamellipodia and filopodial extensions, respectively (Sharma-Walia et al. 2004). HSV-1 utilizes dynein motors and MTs for this purpose and the activation of RhoGTPases, which are important to control microtubules, by KSHV is consistent with a similar mechanism of transport for this virus. After internalization, KSHV exploits dynein and dynactin cytoplasmic trafficking, it moves along the stable microtubules (Sharma-Walia et al. 2004; Naranatt et al. 2005) and reaches microtubule-organizing centre (MTOC) near the nucleus. Dyneins are large protein complexes that function as MT-based molecular motors generating the driving force toward the minus end of microtubules, with the intermediate and light chains presumably involved in dynein attachment to the appropriate cargo (King 2000). KSHV induced the acetylation of microtubules, which is an essential step for microtubule aggregation and is mediated by the host cell's pre-existing signals induced by the KSHV binding and entry steps. The inactivation of RhoGTPases by Clostridium difficle toxin B (CdTxB), a specific inhibitor of Rho-GTPases, significantly reduced microtubular acetylation and subsequently the delivery of viral DNA to the nucleus (Naranatt et al. 2005). After internalization and trafficking through the cytoplasm, KSHV proceeds to nucleus to deliver its genome. Nuclear delivery of viral DNA was increased in cells expressing a constitutively active RhoA mutant and decreased in cells expressing a dominant negative mutant of RhoA (Naranatt et al. 2005; Veettil et al. 2006) (Fig 23.1).

Like in HSV-1, KSHV capsids colocalized with the microtubules, and the colocalization was abolished by the destabilization of microtubules with nocodazole and PI-3 K inhibitor affecting the RhoGTPases. These results suggest that KSHV induces RhoGTPases, modulates stabilization of microtubules and promotes the trafficking of viral capsids and the establishment of infection (Naranatt et al. 2005). *Escherichia coli* cytotoxic necrotizing factor activated RhoGTPase and significantly augmented nuclear delivery of viral DNA. Rho-GTPases activate diaphanous-related formin family molecules Dia1 and Dia2 (Ishizaki et al. 2001, Palazzo et al. 2001). Formins are one of the four major classes of poly-L-proline-containing proteins that form part of the signal transduction cascade that leads to rearrangement of the cytoskeleton. Activation of RhoA-GTP-dependent Diaphanous-2 was observed with no significant activation in the Rac- and

Cdc42-dependent PAK1/2 and stathmin molecules (Naranatt et al. 2005). These studies demonstrated for the first time modulation of the microtubule dynamics by virus-induced host cell signaling pathways to aid in the trafficking of viral DNA to the infected cell nucleus suggesting that KSHV manipulates the host cell signaling pathway to create an appropriate intracellular environment that is favorable to the establishment of successful infection.

23.14 Summary and Perspectives

Recent progress in understanding the early events of KSHV entry and infection has been promising, but many important gaps remain to be filled. Little is known about the molecular mechanism of entry of KSHV in a variety of cells types, which would be interesting to study. The determinants of tropism remain incompletely defined for KSHV. Finding answers to these questions represent exciting areas of experimentation. With newer precise tools such as single particle tracking of KSHV containing glycoproteins labeled with fluorescent proteins and live cell imaging potentially can provide insight into entry and infection mechanisms. The effect of entry on cell signaling pathways is proving to be an extremely fertile area of study, not just for understanding how KSHV establishes a suitable intracellular milieu for gene expression and DNA replication, but also for understanding how KSHV is transported to the nucleus after the cell membrane has been crossed. Studies discussed here suggest that KSHV interactions with host cell receptors has not evolved to act as a mere conduit for viral DNA entry but has evolved to manipulate the host cell signaling pathway to create an appropriate intracellular environment that is conducive to the establishment of a successful infection. Although the progress with KSHV has been particularly interesting in this regard several avenues are now open for investigation and much needs to be known about the events between entry and early transcription from the incoming viral genome for KSHV. Answering all these questions will provide useful information in generating an arsenal of drugs that can successfully block the potential entry routes hijacked by KSHV.

Acknowledgment This study was supported in part by Public Health Service Grants AI057349 and CA 075911, and Rosalind Franklin University of Medicine and Science – H.M. Bligh Cancer Research Fund to BC. We thank Keith Philibert for critically reading this manuscript.

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Chapter 24
KSHV Immune Evasion

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Abstract Kaposi's sarcoma-associated herpesvirus (KSHV) has been consistently implicated in the pathogenesis of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL), and multicentric Castleman's disease (MCD). The hallmark of KSHV infection is to establish life-long persistency that has imposed enormous pressure on this virus to escape host immune recognition. Although many of the mechanistic details require further elucidation, KSHV devotes a significant portion of its genome to gene products that together sabotage almost every aspect of host's immune system. A more thorough understanding of these strategies helps to unravel the complexities of viral pathogenesis and may reveal targets for novel therapeutic strategies. This review provides an overview of the current understanding of how KSHV evades or mitigates the host immune response.

24.1 Introduction

Co-existence of viruses with their hosts requires a balance between host immune responses and their evasion by viruses. To deal with the sophisticated host immune system, viruses have evolved a myriad of immune evasion mechanisms to avoid elimination by the host's immune response. Gifted with large genetic capacity, DNA viruses encode a wider array of viral immune modulators. Adding to the complexity of viral immune evasion, herpesviruses have the additional lifestyle to hide from the host's immune recognition by establishing latency. One sophisticated case in this respect is KSHV, a most recent addition to the DNA tumor viruses.

KSHV is classified as a γ2-herpesvirus, closely related to herpesvirus saimiri (HVS), rhesus monkey rhadinovirus (RRV), and murine gammaherpesvirus 68 (γHV-68 or MHV68) [3, 26, 89, 141, 165, 171]. Like other members of the

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DOI 10.1007/978-0-387-68945-6_24, © Springer Science+Business Media, LLC 2009

γ-herpesviruses, KSHV establishes a life-long persistent infection in lymphocytes and drives the proliferation of infected lymphocytes. Crucial to the ability of the virus to establish and maintain persistency is an evasion of host immune responses that would otherwise eliminate the virus. Through conjoined evolution with their host, herpesviruses have developed elaborate mechanisms for either outwitting or adopting host's immune responses. Almost 50% of KSHV genome is dedicated to the tasks of modulating host immune response. Intriguingly, most of these viral immune modulators seem to be pirated from the host and expressed as natives or homologs of host proteins to avoid being targeted by a particular arm of host immune system.

The immune evasion strategies of KSHV in the context of host innate and adaptive immune responses include alteration of the host chemokine network through mimics of both cellular chemokines and chemokine receptors; interference with a number of soluble innate immune effectors such as interferon (IFN) and complement, and blockage of innate apoptotic and autophagic effects; as well as evasion of MHC class I-mediated and coordinated immune responses through downregulation of major immunomodulatory proteins. Notably, these viral innate and adaptive immune evasions are intimately linked. A case in point is that by encoding mimicry of cytokines KSHV polarizes the adaptive immune response and upsets the homeostatic balance of humoral and cell-mediated immunity. Overall, these evasion strategies ensure persistent infection and spread of KSHV, and contribute to the pathogenesis of KSHV-associated diseases.

24.2 Innate Immune Evasion

The first barrier to overcome for a successful viral infection is the rapid innate immune response of the host. This includes the complement cascade, IFN production, inflammatory cytokine, macrophage and NK cell activity, apoptosis and autophagy. Blocking any of these responses can also skew the adaptive immune response to infection. So, it is not surprising that a battery of KSHV genes are dedicated to downregulate components of the host innate immunity at various levels.

24.2.1 The Complement System

The complement system is long considered to be a key arm of the innate immunity response that limits viral infection. Complement cascade can be activated by specific antibody–pathogen binding (classical pathway), surfaces of pathogens (alternative pathway), or through recognition of microbial surface carbohydrate by lectins (lectin pathway). All these three activation pathways result in the cleavage of proenzymes to enable the formation of the C3 and C5

convertase complexes, then end in assembly of the membrane attack complex (MAC) which punches a hole in infected cells or pathogens (for review see [129]). Beside the direct lysis of invading pathogens, deposition of the complement component C3b and C4b onto the pathogen surfaces, so-called opsonization, also enhances phagocytosis and increases the humoral response to those pathogens [18, 207].

To avoid autologous complement attack and/or inappropriate activation on host cell, the complement cascade is tightly controlled by regulatory proteins, referred to as regulators of complement activation (RCA). All these proteins, including membrane-bound regulators such as complement receptor 1 (CR1; CD35), membrane cofactor protein (MCP; CD46), decay accelerating factor (DAF; CD55) and CD59, and soluble regulators such as factor H (FH), and C4b-binding protein (C4BP), contain 4–35 short consensus repeat (SCR) and share significant homology. Each SCR is a tightly packed structure of 60–70 amino acids held together by four invariant disulphide-linked cysteines, and by a hydrophobic core consisting of several hydrophobic/aromatic residues [145]. Two mechanisms are mainly attributed to the RCA-mediated complement inhibition: (i) accelerating decay of C3/C5 convertase (enzymatic complexes that cleave C3/C5 leading to deposition of C3b/C5b on the activating surface) by dissociation of enzyme subunits; (ii) cofactor for the cleavage of C3a or C4b to inactive fragments by Factor I (reviewed in [109]). Some viruses including human cytomegalovirus (HCMV), HIV, human T-cell lymphotropic virus (HTLV-1), and SIV have subjugated the host RCAs to their own ends for complement evasion by either upregulating RCA expression on host cell surface, recruiting protective RCA to viral surface, and/or incorporating host RCAs into their envelopes during egress process, to protect the virion from complement-mediated attack [78, 190].

All γ2-herpesviruses, including KSHV, HVS, RRV, and MHV68, are equipped to interfere with the complement by encoding host complement control protein homologs that disrupt the progression of the complement cascade [57, 92, 183]. The *orf4* gene of KSHV encodes a lytic complement control protein (KCP) of 550 amino acids, which consists of four N-terminal tandem SCRs repeats. Three protein isoforms are identified for KCP that are thought to be produced by alternative splicing of the primary transcript, all of which retain the four SCRs motif, and all have equivalent complement inhibition function [8, 9, 32, 47, 56, 62]. KCP has been shown in several studies to enhance the decay of the classical C3 convertase, prevent C3b deposition on sensitized cells, and acts as a cofactor for factor I-mediated inactivation of C3b and/or C4b [136, 137, 182, 183]. Mapping of the functional domains of KCP using SCRs deletion/swapping or antibody blocking showed that SCRs 1 and 2 are required to decay the classical C3 convertase, while all the four SCRs are essential for the alternative C3 convertase decay accelerating activity [32, 39, 40]. Moreover, the minimum region necessary for the factor I cofactor activity was confined to SCRs 2–3 [137]. Notably, KCP has been recently shown to be expressed on the surface of KSHV virions and bind heparin through SCR1–2. Pre-incubation of KSHV with

a monoclonal anti-KCP antibody targeting heparin-binding site of KCP as well as with soluble KCP reduces KSHV infection. This result suggests that KCP expression at the viral surface not only mediates immune evasion, but also facilitates viral entry by binding to heparin sulfate at the cell surface [121, 137].

The importance of complement evasion in γ2-herpesviruses infection was recently underscored in a mouse model, in that deleting the viral RCA of MHV68 leads to decreased virulence in acute and chronic infection and that the attenuated phenotype of the mutant could be reversed by deletion of host C3 [91]. Unlike other herpesviruses, HVS encodes two distinct complement regulatory proteins. The HVS complement control protein homolog (CCPH) shows similar functional activity to those described for KCP, in that it inhibits C3b deposition and C3 convertase activity [57]. A second, encoded by HVS *orf15,* is a functional homolog of CD59, which tightly binds to C5b-8, blocks the formation of the terminal MAC, and protects cells from the complement-mediated lysis [1].

Instead of encoding RCA protein homologs, some herpesviruses, for example herpes simplex viruses (HSV), express complement control proteins lacking any structural homology to host RCAs [99]. The glycoprotein C (gC) of HSV-1 interacts with native C3 and its cleaved fragments, blocks binding of properdin (a positive regulator of C3b and Bb) and C5 to C3b, and accelerates the decay of alternative pathway C3 convertase. Thus, viruses have devised multiple strategies to manipulate the complement system highlighting the importance of complement cascade to their lifecycle.

24.2.2 Interferon Signaling

The interferons (IFNs) are a large family of cytokines dedicated to coordinate immunity to viruses and other pathogens, and also involved in cell growth, differentiation, and immuno-regulatory activities (reviewed in [67, 80, 81, 155]). At least three distinct types of IFNs are classified. Type I IFNs (IFN-α/β) are produced in direct response to virus infection by nearly all cell types, while type II IFN, referred to as IFN-γ, is typically released by cells of the immune system such as activated T lymphocytes or natural killer (NK) cells (reviewed in [204]). Recently, another member of the IFN family including IFN-λ1, -λ2, and -λ3 (also known as interleukin-28A [IL-28A], IL-28B, and IL-29) has been identified and referred to as type III IFN, which are structurally distinct from Type I, but share similar antiviral activity by inducing IFN-stimulated genes (ISGs) [155, 203]. The production of IFN-α/β upon viral infection is primarily controlled at the transcription level, wherein IFN regulatory factors (IRFs) play central roles (reviewed in [80–82]). The IRF family is characterized by a highly conserved amino-terminal DNA-binding domain (DBD) with five tryptophan repeats. Among nine hitherto identified members (IRF1 to IRF9) of the IRF family, IRF3, and IRF7 are the key regulators of the *IFN*-α/β gene expression

elicited by viruses. Upon viral infection, cytoplasmic IRF3 and IRF7 undergo serine phosphorylation of its C terminus, resulting in the homodimerization or heterodimerization of IRF3 and IRF7. These dimers then translocate to the nucleus, where they form a complex with the coactivators and directly induce the expression of type I IFN [208]. Nascent type I IFNs act in both autocrine and paracrine fashion to alert the presence of viral infection. By signaling through the type I IFN receptor, IFNs stimulate the expression of a large set of genes that together establish the "antiviral state" in host cells to suppress the viral replication and spread [185, 186, 202].

The efficacy of the IFN response has led to many viruses evolving various strategies against IFN system [23, 64, 67, 93]. Nearly all aspects of the IFN regulatory pathway have been targeted by viruses including perturbation of IFN expression, neutralization of secreted IFN molecules, disruption of IFN signaling, or inhibition of antiviral actions of the IFN-induced products (for review see [93]). For example, E1A of adenovirus disrupts IFN-induced signaling by decreasing the level of STAT-1 and p48 [76, 102]; HSV uses at least two known functions to circumvent PKR action, an IFN-response pathway, by encoding US11 and ICP34.5 [76, 157].

A unique feature of KSHV-modulated IFN evasion is directly tied to their encoding of viral homologs of IRF. The viral IRFs are proposed to act as the negative regulators of IFN responses, in this case, the inhibition does not appear to act directly at the level of IFN signaling, but rather inhibits the function of the IFN-inducible product IRF1, IRF3, and/or IRF7, thus IFN synthesis and transcriptional responses to IFN cannot be sustained. KSHV encodes a cluster of four viral homologs of IRFs (vIRF1-4), one unspliced (vIRF1 from *k9*) and three spliced (vIRF2 from *k11*, vIRF3, also called LANA-2 from *k10.5* and vIRF4 from *k10*) [141, 165]. The identification of four KSHV vIRFs is intriguing, which might reflect evolutionary adaptation to select for vIRFs that function more effectively in a particular host [41]. The KSHV vIRFs display differential expression profiles in KS lesion, KSHV-associated B-cell lymphoma, and MCD. vIRF1 mRNA is present in every KS sample, yet vIRF3 transcript can only be detected in KSHV-associated lymphoma but not in endothelial-derived KS tumors [44]. Except for vIRF3 transcript, the other three vIRFs were clearly inducible and display lytic kinetics [44]. In this context, vIRFs may not readily be functionally redundant, but activated differentially depending on the nature of the cell type and the stage of viral infection, thereby eliciting distinct IFN evasion.

vIRF1. vIRF1 was the first viral IRF family member that was discovered to effectively repress cellular IFN response [63, 216]. It is expressed low but can be induced in response to TPA in PEL cell lines [30]. vIRF1 possesses a partially conserved N-terminal tryptophan-rich DNA-binding domain but lack significant DNA-binding activity; instead, it interacts with the p300/CBP transcriptional coactivator as demonstrated in several studies [21, 104, 108]. The interaction of vIRF1 with p300/CBP inhibits p300 histone acetyltransferase (HAT) activity, resulting in global chromatin condensation and

therefore restricting transcriptional activity of cellular genes, including those encoding cytokines [104]. Moreover, vIRF1 binds with several cellular IRFs, including IRF1, IRF3, and IRF7, but selectively inhibits IRF3-mediated transcriptional activation by sequestration of p300/CBP, which blocks IRF3 recruitment of the CBP/p300 [104]. Nevertheless, the efficacy of vIRF1 to block IFN response was recently challenged by the short half-life and duration of vIRF1 in KSHV-infected cells [158], raising the concern that vIRF1 might function at vulnerable stages of viral replication or other vIRFs and viral immunomodulators are required to confer complete inhibition.

Similar to the cellular IRF2 proto-oncogene, vIRF1 also prevents IFN induction of the cell cycle inhibitor $p21^{waf}$ and induces cell-growth transformation [139, 173]. NIH 3T3 cells that stably express vIRF1 undergo transformation and consequently display features of malignant fibrosarcoma in nude mice, this is presumably mediated in part by interfering with the tumor suppressor p53 function [104]. The putative DNA-binding region of vIRF1 directly associates with p53, suppresses the level of phosphorylation and acetylation of p53, and inhibits the transcription and pro-apoptotic activities of p53 [139, 173]. Recently, Shin et al. [175] have extended these results demonstrating that vIRF1 further deregulates p53 stability by interacting with and inhibiting upstream ATM-kinase activity, which may circumvent host growth surveillance and facilitate viral replication in infected cells. Further evidence in support of the oncogenic property of vIRF1 is the fact that vIRF1 binds to and inhibits the activities of cell death regulator GRIM19 and Smad3/Smad4 transcriptional proteins in TGF- β signaling, as well as downregulation of CD95L expression [95, 172]. In spite of its many repressive activities as described, vIRF1 acts as a transcriptional activator to induce the vIL-6 and c-myc gene [104].

Other vIRFs. The functions of vIRF2–4 are incompletely characterized and in some respects contentious. vIRF2 gene is now known to encode a spliced transcript of 2.2 kbp from two exons *K11.1* and *K11*. Fuld et al. [60] have shown that full length of vIRF2 inhibits IRF1 and IRF3 transactivation as well as IFNα- and IFNλ-induced ISRE signaling. The underlining mechanism, however, is yet to be defined, but is less likely through p300 sequestration or IRF3 binding as shown on vIRF1 [60]. Moreover, vIRF2 does not share with vIRF1 activity in cell-growth transformation [60], whereas the first exon of vIRF2 was shown to inhibit apoptosis through transcriptional suppression of CD95L expression in activated T cell [95]. Unlike other vIRFs, vIRF3 is a B-cell-specific latently expressed nuclear protein that affects p53-mediated apoptosis [162], PKR-mediated translational control [50], and NF-κB activation [174]. Lubyova et al. [115] have shown that vIRF3 directly associates with both IRF3 and IRF7, but the biological function of vIRF3 as a transcription activator or repressor is puzzled due to the contradicting results from the same group [115, 116]. Nonetheless, our group recently observed that vIRF3 specifically interacts with IRF7 and that this interaction clearly results in a significant inhibition of IRF7 DNA-binding activity, thereof abrogation of

IFNα production and IFN-mediated immunity [88]. The apparent importance of KSHV to modulate IRF7 activity is evident from the observation that the two immediate-early lytic proteins of KSHV, ORF45 and RTA, also negatively regulate IRF7 by preventing its phosphorylation and nuclear translocation, or by promoting its ubiquitination for proteasome-mediated degradation, respectively [213–215].

Deregulation of IRF3- and IRF7-mediated transactivation by viruses is not unprecedented. Similar to vIRF1, the E1A interacts with p300 and blocks IFN signaling [117], whilst the E6 protein of human papillomavirus [163] and the NSP1 of the rotavirus [68, 75] directly bind and prevent the transactivation ability of IRF3. Recently, Hahn et al. [75] have shown that BZLF-1 of EBV, an immediate-early gene product, subverts the antiviral IFN response through the negative regulation of IRF7. The various mechanisms employed by viruses to interfere with IRFs activities reflect the importance of IRF functions in antiviral defense.

Besides vIRF-mediated regulation, KSHV also modulates the IFN response at different levels. Recent study by Perry et al. [154] indicates that the envelope glycoprotein gpK8.1 of KSHV stimulates the production of IFNβ and expression of ISGs, perhaps through IRF3 activation, and is independent of viral replication. A similar situation exists in other enveloped viruses, where viral glycoprotein interactions might signal rapid IFN response; this includes but not limits to the gB of CMV [15], gD of HSV-1[7], gM of transmissible gastroenteritis virus [101], and g120 of human immunodeficiency virus (HIV) [6]. Intriguingly, the gpK8.1-mediated IFN induction, but not upregulation of IL-6 expression, can be abrogated by the intact KSHV virions. This study suggests that other structural components are present in the virion to not only repress an effective antiviral response but also selectively modulate cellular response to their own ends.

24.2.3 Chemokine Network

Viral infection stimulates the production of cytokines and chemokines that initiate the activation and migration of immune cells to sites of infection, with a role to play in lymphocyte development and differentiation, in angiogenesis, and in antiviral defense [11, 65]. The large chemokine family can be divided into four subfamilies (CC-, CXC-, C-, and CX_3C-) on the basis of the relative positions of two N-terminal cysteine residues. Their activities are mainly mediated through 7-transmembrane, G-protein-coupled receptors (GPCRs) to which specific chemokines bind [217]. In addition, chemokines interact with glycosaminoglycans (GAGs), which are also important for effective chemokine-mediated cell migration [159].

Many viruses, particularly herpesviruses and poxviruses, utilize a variety of methods to modulate the chemokine machinery by encoding their own version of chemokines (virokines) or chemokine receptors (viroceptors), or by secreting

chemokine-binding proteins (vCKBPs), that are presumed to assist in evading the immune response [125, 138]. Virokines might function as either agonists that are secreted by infected cells for the attraction of lymphocyte subsets or antagonists by competing with endogenous ligands for binding and blocking signal transduction. These viral-encoded homologs generally have broader spectrums than their host counterpart, and also have altered receptor activation [209]. Some viroceptors are constitutively active and may induce proliferation and migration of the infected cells. On the other hand, they may function as a sink to sequester chemokines hence influence the microenvironment of the infected cells. vCKBPs can act as decoy protein to sequester chemokines from receptor binding and activation. As immune evasion strategies, viral mimicry of chemokine and their receptors has a preference for specific inhibitory mechanism or it can re-direct the outcome of the immune response for the advantage of viral replication. Alternatively, viruses can hijack chemokine pathways to induce cell proliferation or migration for viral pathogenesis. Furthermore, certain chemokine receptors can be subverted for facilitating viral entry, for example, HIV utilize chemokine receptors (CXCR4 and CCR5) for its entry into susceptible cells [51]. Additional descriptions of KSHV-encoded cytokines, chemokines, and chemokine receptors can be found in Chapter 22 (HHV-8/ KSHV proteins involved in signaling and transformation).

vCCL-1, vCCL-2, vCCL-3. KSHV encodes three viral CC- class chemokines termed vCCL1 *(ORF K6)*, vCCL2 *(ORF K4)*, and vCCL3 *(ORF K4.1)*, formerly known as vMIP-I, vMIP-II, and vMIP-III, respectively. vCCL1 and vCCL2 display higher amino acid identity (~41%) to the CC chemokine, macrophage inflammatory protein (MIP-1α), which is thought to be a potent activator and chemoattractant for monocytes, T lymphocytes, NK cells, granulocytes, and dendritic cells [132, 141].

Unlike cellular chemokines counterpart, vCCL2 (vMIP-II) appears to be a promiscuous antagonist for at least 10 known human CC-, CXC-, and CX_3C-chemokine receptors [16, 42, 96, 119]. In competition and chemotaxis assays in vitro, vCCL2 inhibits binding, signaling, and trafficking of responsive leukocytes to multiple pro-inflammatory chemokine receptors [96, 118, 119]. In particular, vCCL2 binds and antagonizes CXCR4 and CCR5, the two major coreceptors of HIV, thereof prevents macrophage-tropic strains of HIV from infecting $CD4^+$ cells in vitro by competing with the virus for the coreceptors binding [16]. Consistent with these in vitro findings, vCCL2 effectively blocks the RANTES-induced firm arrest of monocytes and Th1-type T cells under flow condition [211]. More recently, Rubant et al. [164] demonstrate that vMIP-II not only abrogates Th1 lymphocytes recruitment into the target tissue but also inhibits generation of immunity by adoptive transfer of immunity experiment. Together, these findings indicate that vCCL2 is a broad anti-inflammatory molecule thereof can act as a promising agent to interfere with acute and chronic inflammatory diseases.

In contrast to vCCL2, vCCL1, and vCCL3 are not chemokine antagonists, instead, vCCL1 acts as agonist of CCR8, whilst vCCL3 selectively agonizes CCR4 [16, 42, 48]. Interestingly, vCCL2 has also been shown to be a unique

agonist toward CCR3 [16]. Each of these chemokine receptors is preferentially expressed on Th2 lineage T lymphocytes, and their associated cellular ligands are potent Th2 lymphocyte chemoattractant [166]. The selective chemotactic activity of vCCLs correlates with the presence of predominant $CCR3^{+}$ Th2 lymphocytes infiltrate in KS lesions [181]. Th2 cells mainly secrete cytokines such as IL-4 and IL-5 to promote humoral immunity, while Th1 cells mainly release cytokines such as lymphotoxin and IFN-γ to promote cellular immune responses [135]. Therefore, by chemoattracting Th2 rather than Th1 lymphocytes preferentially to the site of infection, the vCCLs deviate host immune response away from an undesirable cell-mediated immune responses to a more favorable antibody-predominant Th2 microenvironment, thereby facilitating evasion from cytotoxic reactions [181, 211]. The Th2 polarization has also been observed in EBV, CMV, murine CMV, and poxviruses, which suggests that this may represent a generalized viral strategy for dampening host immune response [100, 184, 200]

Apart from their respective immunomodulatory roles, vCCLs all display angiogenic properties when applied to the chorioalantonic membrane of chicken eggs even though their cellular CC chemokines have not been reported to have such activities [16, 189]. These findings suggest that the expression of vCCLs might account for the formation of new blood vessels, the hallmark of KS lesions, in cooperation with other KSHV angiogenic gene products such as vGPCR and vIL-6 (discussed below).

vGPCR. ORF74 of KSHV, a "pirated" human chemokine receptor, is a member of the GPCR superfamily [169]. vGPCR is transcribed as an early gene during lytic infection and shares ~70% similarity to the human angiogenic interleukin-8 chemokine receptor (IL-8R) [25]. The binding studies demonstrate that compared to other viral or cellular chemokine receptors, the vGPCR binds with high affinity to a much broader array of both CXC and CC chemokines [10, 24]. Unlike most cellular chemokine receptors, however, the vGPCR is ligand independent and constitutively active. Along with this finding, the N-terminal extracellular region of vGPCR is shown to be necessary for chemokine binding, but dispensable for its signaling activity [79], whereas the last five amino acids of the cytoplasmic tail of vGPCR is major determinant for its constitutive activation [170].

Expression of vGPCR is sufficient to induce cellular transformation and VEGF-mediated angiogenesis in fibroblasts [12]. Transgenic mice expressing vGPCR in cells of the T-cell lineage develop VEGF-driven angioproliferative lesions in multiple organs similar to the KS [73]. Mechanistic studies indicate that vGPCR can activate PI3K/AKT/mTOR cell survival pathway, which may directly contribute to cell transformation in endothelial cells, also protection of cells from apoptosis to favor virus replication [178, 180]. Furthermore, KSHV-GPCR has been shown to constitutively activate the transcription factors NF-κB, AP-1, and hypoxia inducible factor-1α (HIF-1α), which, in turn, leads to the expression of NF-κB-dependent cytokines (IL-1β, IL-8, GRO-α, IL-6, and TNF-α), the AP-1-dependent basic fibroblast growth factor (bFGF),

as well as HIF-1α-regulated VEGF expression and release, all of which are known to be expressed in KS [12, 69, 170]. Also, vGPCR upregulates the expression of the VEGF receptors, which is associated with the release of VEGF. VEGF and other factors may act in autocrine or paracrine fashion to promote KS pathogenesis [12]. The direct contribution of vGPCR to KS pathogenesis was recently dissected by Grisotto et al. [69] in the context of a conditional transgenic system with simultaneous expression of vGPCR and β-gal (LacZ), which showed that vGPCR directly triggers angioproliferation in endothelial cells by activating intracellular pathways, autocrine factors, or both. Nevertheless, a major conundrum is that how a lytic gene of KSHV could be so important in deregulating cell growth. The deregulated expression of vGPCR during the latent phase of KSHV infection perhaps helps explain this paradox [179].

Due to the lack of efficient cell culture system and appropriate animal models of infection, the role of vGPCR in the context of viral infection is limited. vGPCR is conserved in all members of γ-2-herpesviruses such as MHV68, HVS, and RRV [10, 205]. Analysis of the vGPCR deletion mutants of MHV-68 reveals that vGPCR of γ-2-herpesviruses is required for chemokine-stimulated viral replication as well as reactivation from latency, which may represent a novel mechanism by which viruses subvert immune system [133].

vCKBP. The first viral chemokine-binding protein of herpesvirus was identified in MHV68 (M3 protein). The M3 has broad spectrum of chemokine-binding activity, interacting with CC-, CXC-, C-, and CX_3C-chemokines. M3 sequesters chemokines from binding to their receptor as well as GAG and blocks chemokine-mediated signal transduction [152, 210]. Target disruption of M3 does not affect lytic replication but M3 is necessary for establishment of a normal latent load and protection from acute viral meningitis [19, 201]. Unlike MHV68, no vCKBP has been identified in KSHV.

24.2.4 Other Cytokine-Modulating Factors

vIL6. Originally identified as a B-cell differentiation factor, interleukin (IL)-6 is a pleiotropic inflammatory cytokine that has important roles in the regulation of the immune response, inflammation, cell proliferation, and angiogenesis [144]. Of note, KSHV *orf k2* encodes a homolog of human IL-6 (hIL-6), termed vIL-6, that exhibits nearly 25% amino acid identity with hIL-6 and is unique to KSHV [140]. vIL-6 is primarily expressed as a lytic gene, can be induced specifically by IFNα in PEL cells, and protects cells from IFNα-mediated cell cycle arrest and apoptosis [27]. vIL-6 activates the same downstream JAK/STAT-signaling pathway as their cellular counterpart [131, 148]. Expression of vIL-6 plays key role in immune evasion and also contributes to KSHV-associated malignant disease [132, 140, 143]. Despite their similarities in sequence and function, vIL-6 differs from hIL-6 in its receptor engagement

and utilization [27]. While hIL-6 requires both IL-6R (gp80) and gp130 for intracellular signaling, vIL-6 directly binds and activates gp130 independent of gp80. The lack of gp80 requirement for vIL-6 signaling is thought to subvert the host immune response mediated by IFNα, which downregulates surface expression of gp80, thus prevents hIL-6 signal transduction. Moreover, the presence of ISRE element in the promoter of vIL-6 makes it inducible by IFNα, which in turn further amplifies the vIL-6-signaling pathway by an autocrine or paracrine loop [27]. Of relevance to this finding, the infected PEL cells exhibit reliance on vIL-6, but not on hIL-6-mediated signaling.

In spite of gp80 independency, more recently the role of gp80 in vIL-6-signaling complex formation and activation is getting appreciated. In addition to the formation of a stable tetramer vIL-6/gp130 complex, Boulanger et al. found that the vIL-6 also can signal via hexameric vIL-6/gp80/gp130 complex with enhanced vIL-6-signaling potency [17]. In line with this finding, gp80 has been shown to stabilize gp130 dimerization induced by vIL-6 [29]. In a recent report, Hu et al. have further illustrated the positive role of gp80 in modulating vIL-6-mediated signaling [83], which in fact is not equivalent to the biological response of hIL-6. Combining these, it is conceivable that vIL-6 may utilize gp80-independent tetrameric and/or gp80-dependent hexameric complex to elicit distinct-signaling activity to adapt to host stress responses against infection.

Kaposin B. The molecular link between KSHV infection and cytokine release recently was demonstrated to be a latent protein of KSHV, kaposin B, which increases the production of cytokines by blocking the degradation of their messenger RNAs (mRNAs) [124]. Kaposin B is transcribed from the *Kaposi* locus, which contains *orf12* preceded by two direct repeats (DR2 and DR1). The complex translational program of Kaposi locus generates at least three protein species, of which kaposin B is the predominant product and consists of DRs alone. McCormick et al. [125] have shown that kaposin B directly binds and activates cellular MAPK-associated protein kinase 2 (MK2), a key inhibitor of mRNA stability, through its DR2 repeat. This binding leads to the blockage of AU-rich element (ARE) containing cytokine mRNAs decay and ultimately increase cytokine release such as IL-6, which drives proliferation and tumor formation. Further study of the same group demonstrates that both DR2 and DR1 are functionally required, and that a specific serine residue in DR1 can be directly phosphorylated by p38MAPK [123], but the molecular mechanism of Kaposin B-mediated MK2 activation has yet to be elucidated [123]. Nevertheless, this study represents a striking example of how viral protein regulates host gene expression.

vOX2. The *orf k14* of KSHV encodes a lytic protein vOX2, also termed vCD200, which is a viral homolog of CD200. Cellular CD200 belongs to a group of leukocyte glycoprotein and is expressed on a variety of cells. The signal delivery of CD200, however, is mainly restricted to myeloid cells that express CD200 receptor (CD200R) [212]. The CD200/CD200R interaction is thought to deliver a negative control to myeloid cells and, thus, limits their function. vCD200 exhibits limited homology to the cellular counterpart yet appears to

bind the same receptor CD200R with identical affinity and kinetics [58]. While the exact role of CD200 in the context of viral infection is unclear, several studies using recombinant K14 protein indicate that KSHV might modulate macrophage activation by pirating CD200 on the surface of infected cell to provide a favorable environment for viral replication, dissemination, or pathogenesis [34, 58, 161].

24.2.5 Modulation of Apoptosis

Apoptosis is a conserved cell death program that contributes to restriction of viral replication and elimination of infected cells. Whether triggered via internal inducers such as DNA damage, viruses (intrinsic pathway, mitochondria-dependent), or via external stimuli such as engagement of the TNF receptor (extrinsic pathway, death receptor-mediated), apoptosis proceeds through a cascade of regulated internal proteolytic digestion, resulting in a collapse of cellular infrastructure, mitochondrial potential, genomic fidelity, and cell membrane integrity (for review see [54]). Indeed, apoptosis represents a predominant form of virally infected cell demise. In response, viruses have evolved numerous ways of circumventing this host cell apoptosis. Most of the DNA viruses including oncogenic KSHV are genetically equipped with antiapoptotic ability to ensure viral replication and propagation, which at certain stage of viral life cycle may also influence viral pathogenesis.

Several critical steps in the cell death pathway have been shown to be targeted by KSHV, including: (i) inhibition of death receptor signaling by expression of viral FLICE/caspase 8 inhibitory protein (FLIP); (ii) modulation of mitochondria stability by expression of viral homologs of Bcl-2; (iii) interference with caspases by viral inhibitor of apoptosis (IAP); and (iv) deregulation of internal sensor activity of p53. The complementary functions of these antiapoptotic viral proteins ensure persistent infection and contribute to the oncogenicity of KSHV.

vFLIP. KSHV-encoded viral FLIP homolog vFLIP, also called K13, is one of the few viral proteins to be expressed in latently infected KS and PEL cells, therefore likely contributes to the latent signatures of KS. Like its cellular counterpart, vFLIP contains two death effector domains (DEDs), and is well documented in vitro to protect virally infected cells against death receptor-mediated apoptosis [45], which is initiated by the ligation of cell surface Fas, TNF, or TRAIL death receptor and the formation of the death-inducing signaling complex (DISC). In particular, vFLIP interacts with Fas-associated death domain protein (FADD) or pro-caspase 8 through DEDs and interferes with the recruitment and activation of caspase 8, resulting in the disruption of DISC and blockade of extrinsic apoptotic signaling. Indeed, the expression of vFLIP strongly blocks Fas-mediated apoptosis, correlating with decreased activity of downstream effector caspases [45]. Similar activities are shared by

the vFLIPs of other viruses including E8 from equine herpesvirus 2, MC159L from the molluscum contagiousum virus (MCV) as well as vFLIP from HVS and RRV [14, 66, 84, 196]. Subsequent studies by several groups, however, reveal that the antiapoptotic property of vFLIP cannot be solely explained by its direct caspase 8 inhibition, instead, it is primarily related to the constitutive activation of NF-κB pathway, which induces the transcriptional activation of several cell survival factors such as cIAP-1, cIAP-2, and cFLIP thus preventing virally infected cells from spontaneous apoptosis [28, 71, 194]. Consistent with these findings, vFLIP has been reported to physically associate with and activate the IκB complex, which in turn leads to IκBα phosphorylation and degradation, followed by activation of NF-κB activity [55, 110]. More recently, Guasparri et al. [72] pointed out that the interaction of vFLIP with IκB complex is mediated by the adaptor molecule TRAF2 and that both TRAF2 and TRAF3 are necessary for vFLIP signaling to NF-κB. Thus, it is conceivable that vFLIP blocks host cell apoptosis either directly through caspase inactivation or indirectly by activating transcription factors important in inhibiting apoptosis, the latter of which is in fact essential for the virally infected cells survival [71].

The constitutive activation of NF-κB is also mechanistically associated with the cell transformation potential of vFLIP, which is not shared by other identified vFLIPs [33, 122, 194]. vFLIP transgenic mice display constitutive activation of NF-κB pathways, enhanced lymphocyte proliferation, and increased incidence of lymphoma [122], which of note is independent of inhibition of Fas-induced apoptosis [33, 191, 192, 194]. Furthermore, vFLIP expression was found to induce NF-κB-regulated cytokines expression and secretion [70, 193] and likely contributes to the pro-inflammatory microenvironment of KS. More intriguingly, a recent study by Grossmann et al. [70] demonstrates that vFLIP of KSHV is sufficient to induce endothelial cell spindling, a hallmark of KS lesion, which is unexpectedly linked to NF-κB activation. Collectively, as a result of its strong activation of the NF-κB pathway, vFLIP plays a plethora of roles in viral cell survival, transformation, morphological changes, and inflammatory activation, thereof directly contributes to KSHV pathogenesis. Other than vFLIP, KSHV proteins such as vGPCR, K1, K15, vIL-6 have been implicated in regulating NF-κB activity, suggesting a critical role of NF-κB signaling in viral pathogenesis.

vBcl-2. Multiple apoptotic-signaling (both extrinsic and intrinsic) pathways converge on mitochondria to induce mitochondrial outer membrane permeabilization (MOMP), causing the release of cytochrome *c* and other apoptogenic factors. This mitochondrial cell death is regulated tightly by the interplay of pro- and antiapoptotic molecules of the B-cell lymphoma 2 (Bcl-2) family [98]. The defining characteristic of Bcl-2 family is the presence of multiple conserved Bcl-2 homology (BH) domains. The pro-survival cellular Bcl-2 proteins (e.g., Bcl-2, Bcl-xL) are thought to function to block MOMP by heterodimerization with pro-death Bcl-2 such as BAX, BAK, and BAD, which disrupt the outer mitochondrial membrane integrity and promote cytochrome *c* release. Although

generally recognized as apoptosis modulators, Bcl-2 family members are critically involved in the regulation of cellular homeostasis, development, and immunity as well as in major pathologies such as hematological malignancies, with far-reaching therapeutic implications.

Many viruses have acquired the capacity to intercept the mitochondrial-signaling pathway leading to cell death. Death-inhibitory Bcl-2 analogs have been identified in all members of γ-herpesviruses, including orf16 of KSHV, HVS, and RRV, BHRF1 and BALF-1 of Epstein–Barr virus (EBV), and M11 of MHV68 (for review see [156]). KSHV Bcl-2 is expressed as a 175-amino acid product with a modest identity (15–20%) to cellular Bcl-2 and the homology is largely restricted to the BH1 and BH2 domains [31]. As indicated, the KSHV Bcl-2 RNA is detected in KS lesions and PEL cell lines and is upregulated in PEL cells upon activation of lytic cycle. The solution structure of KSHV Bcl-2 is overall similar to the cellular Bcl-2, Bcl-xL, and the MHV68 vBcl-2 in that it shares the same BH3-binding groove [85, 113]. Like its EBV counterpart BHRF1, KSHV Bcl-2 is expressed in early lytic phase and inhibits apoptosis in vitro induced by several stimuli, including Bax, viral cyclin, and sindbis virus infection as tested [147, 168]. Of interest, recent finding by Altmann et al. shows that both two Bcl-2 s of EBV are not only essential for initial apoptosis evasion in lytic viral replication but also necessary for establishing a latent infection and cell transformation [4]. This suggests that vBcl-2 may contribute directly to the pathogenicity of the γ-herpesviruses. However, the in vivo role of KSHV vBcl-2 in pathogenesis needs further exploration.

In spite of structural and functional similarity, viral Bcl-2 s differ in important ways from their cellular counterpart. They display different binding affinity to the BH3 peptides from cellular Bcl-2, which, in the case of vBcl-2 of KSHV and MHV is BAK>BAX>BAD, whereas that of cellular Bcl-2 is BAD>BAX>BAK ([85]; our unpublished results). In addition, the long functionally inhibitory loop between $\alpha 1$ and $\alpha 2$ regions is diminished in the most case of vBcl-2 s including KSHV Bcl-2, resulting in the viral evasion of host caspase cleavage and regulatory phosphorylation, all of which would otherwise inhibit their antiapoptotic function [13]. Therefore, vBcl-2 s have evolved to have advantageous activity compared to their host counterpart to effectively evade apoptosis.

vIAP (K7): Caspase activity can be directly inhibited by a conserved family of inhibitor of apoptosis proteins (IAP). KSHV K7, a mitochondrial protein expressed in lytic replication, is originally identified as a viral ortholog of the human survivin (surviving-ΔEx2), a cellular IAP [206]. K7 has been shown to be able to protect cells from apoptosis induced by various stimuli in vitro [52, 206], and this activity is partially attributed to its inhibition of caspase-3 activity and apoptosis by forming a bridge between cellular Bcl-2 and active effector caspase-3. In addition, K7 is found to target cellular calcium-modulating cyclophilin ligand (CAML) to modulate intracellular calcium effluxes, thereof suppresses ER-stress-induced apoptosis [52]. Further characterization by Feng et al. [53] extends the role of K7 beyond apoptosis modulation, in that it binds

and inhibits a regulator of the host ubiquitin/proteasome pathway, thereof interferes with the right turnover of crucial host defense molecules such as p53 and IκB, which may facilitate the avoidance of host immune surveillance.

p53 deregulation. The p53 protein serves as an internal sensor of apoptosis and induces the transcription of genes involved in both external and internal apoptotic signaling [90, 130, 146]. Several KSHV proteins have been reported to inhibit p53-mediated transcriptional activation as well as p53-dependent apoptosis including LANA-1 [59], vIRF1, LANA-2 (vIRF3), RTA [74],, and K-bZIP [150] highlighting the significance of the p53-mediated apoptosis pathway in host surveillance mechanisms.

24.2.6 Modulation of Autophagy

Autophagy has been recently confirmed as an important effector of host immunity and also plays indispensable roles in cell homeostasis, development, and disease [43, 97, 103]. In contrast to the self-destruct apoptotic program, cellular autophagy (Greek for *self-eating*) involves the self-recycling, whereby the cytoplasmic materials are sequestered into a characteristic double-membrane-coated vesicle, termed autophagosome, and is delivered to lysosomes for degradation and turnover. Although originally recognized as a cell survival mechanism, under certain conditions, excessive autophagy can promote cell death termed class II-programmed cell death (PCD) [103]. Apoptosis and autophagy, characterized by distinctive morphological and biochemical changes, are not mutually exclusive [8, 128, 197]. Several apoptotic signals such as TNF-α, TRAIL, and FADD mediate autophagy; inhibition of class I PI3K/AKT/mTOR-signaling pathway suppresses both apoptosis and autophagy. In keeping with its primary function in cell homeostasis, autophagy serves as a mechanism for the removal of intracellular bacteria and viruses. A case in point is that a crucial autophagy gene, Beclin-1, protects mice from lethal sindbis virus encephalitis [107]. Also, herpes simplex virus type I (HSV-1) virions are found to be engulfed in autophagosomes for degradation [195]. Inevitably, viruses have in turn selectively evolved mechanisms to avoid or subvert autophagy; for example, poliovirus hijacks autophagosome membrane as a replicative niche for viral replication [87], and HIV envelope glycoprotein triggers the autophagic death of $CD4^+$ T cells as part of pathogenesis [49].

The involvement of autophagy in γ2-herpesviruses infection has been recently implicated [106, 153], in that the antiapoptotic Bcl-2 homologs of γ2-herpesviruses directly target the cellular autophagy pathway through their interaction with and inhibition of autophagy protein Beclin-1. The advantageous autophagy suppression of vBcl-2 over their cellular counterpart suggests that autophagy might in part account for the biological effects of vBcl-2. On the other hand, it can be postulated that vBcl-2, by coordinately blocking both apoptosis and autophagy, might circumvent host immune response for persistent viral infections. Nevertheless, given the potential significance of autophagy

in host response to pathogens, it would be expected that this pathway is also manipulated by KSHV via vBcl-2 or other unidentified molecules, which remains to be understood completely.

24.2.7 Natural Killer (NK) Cell Immunity

NK cells are a subpopulation of lymphoid cells that function as an interface between the host innate and the adaptive immune responses (for review see [111, 112]). Unlike B or T cells, NK cells lack specific antigen receptors, instead display both activating and inhibitory receptors. Integrated signaling from both receptors determines the outcome of NK cell activity. Activation of NK cell triggers the direct lysis of target cells through the release of cytotoxic granules (that contains perforin and granzymes), also secretes inflammatory cytokines, such as IFN-γ, to enhance the adaptive cellular responses against the cell.

NK cell activity is fine-tuned with the signaling from both activating and inhibitory receptors through their engagements with positive or negative molecules on target cell surface. The most prominent negative molecule is MHC class I which is recognized by certain inhibitory receptors of NK cells such as the killer cell immunoglobulin-like receptors (KIR) or CD94/NKG2. Downregulation of MHC class I molecules from the cell surface through any of a variety of mechanisms renders the cells sensitive to recognition and killing by NK cells, provided that an activating receptor is engaged. These activating NK receptors include the natural cytotoxicity receptors (NCR), DNAX accessory molecule-1 (DNAM-1) and coreceptors such as 2B4, NTBA, CD59, NKp80, and CD86. Integrins represent a distinct category of NK cell-activating receptors. The engagement of integrin, for example, LFA-1 by intracellular adhesion molecule 1 (ICAM-1) or ICAM-2 is sufficient to induce adhesion and stabilizes the interaction between NK cell and target cell, a prerequisite for NK cell effector functions [20]. Engagements of these activating receptors by appropriate ligands initiate NK response for both cytolysis and cytokine production (for review see [134]).

Many viruses, especially herpesviruses, in turn, countermeasure NK cell-mediated immunity at several levels including avoidance of NK-receptor-mediated recognition of the virus-infected cell, blockade of NK-activating cytokines such as IFNγ, or inhibition of NK-effector pathways.

K3 and K5. KSHV expresses two viral proteins, K3 and K5, which, in concert, act to block both the cytotoxic T-cell response and the NK cell killing. K3 and K5 share 40% amino acid identity with one another and their expression is part of the lytic replication cycle [142]. Both contain PHD/LAP family zinc-finger motifs at the amino terminus and two transmembrane domains in the central region but are of varying size in the carboxy terminal tail [142]. They are predominantly localized to the endoplasmic reticulum (ER), but act distally in the secretory pathway, promoting endocytosis and targeted degradation of

surface MHV-I molecules (as discussed below), a major inhibitory ligand for NK cells. Of note, despite their similarity in sequence and function, K3 drastically downregulates HLA-A, -B, -C, and -E, whereas K5 exclusively downregulates HLA-A and -B [36, 86]. One would expect that the indiscriminate downregulation of HLA allotypes by K3 would enhance the visibility of KSHV-infected B cells to NK cells. However, K5-expressing or K3- and K5-coexpressing cells are more resistant to NK cell-mediated cytolysis than naïve cells. Further mechanistic studies reveal that, in addition to lowered levels of MHC class I, KSHV K5 (but not K3) protein significantly downregulates ICAM-1 (CD54) and B7-2 (CD86) from the B-cell surface, two co-activating molecules for NK cell, by inducing their endocytosis and degradation [35, 86]. As outlined above, ICAM plays an important role in the initial adherence between NK cells and potential targets. By downregulating ICAM from the cell surface, K5 reduces the ability of NK cells to maintain contact with target cells and diminishes the likelihood of a positive signal being transmitted to NK cells. Hence, K5 abrogates NK cell-mediated lysis, not by transmitting a negative signal, instead by preventing a positive one. Although K3 and K5 are not expressed during latency, recent studies by Sirianni et al. on PEL cells indicate that KSHV latently infected PEL cells also exhibit decreased MHC-I expression and impaired NK cell activity, and are therefore less susceptible to CTL and NK killing [176, 177]. This may be explained by the abortive lytic expression of K3 or K5, or KSHV may use other ways to ensure comprehensive protection from host immune effectors during different phase of viral infection. The exact mechanism, however, awaits clarification.

Besides eluding NK cell recognition and cytolysis, K3 and K5 can inhibit the action of NK-activating cytokines IFNγ (discussed below). The effect of many other cytokines released by NK cells such as TNF-α and chemokines can also be blocked by KSHV-encoded immunomodulators as described in the previous sections.

Due to our inability to test mutants of KSHV for pathogenicity in vivo, the importance of this interference with NK responses is still unclear. From other viral systems, however, it is apparent that the NK response is critical to control viral spread [150, 151, 160].

24.3 Adaptive Immune Evasion

When an infectious agent breaches the first lines of host defense, an adaptive immune response will ensue. The adaptive immune system consists of two branches, the cellular, cytotoxic CD8$^+$ T-cell arm and the humoral, antibody-producing B-cell arm. Both typically require antigen presentation in conjunction with MHC and a costimulatory signal for full activation. Accordingly, the antigen-processing machinery, particularly, the MHC class I pathway has been targeted by many viruses including KSHV [126].

24.3.1 Evasion of Cytotoxic T-Cell (CTL) Responses

CTL evasion is a prerequisite for persistent viral replication; this is particularly true of herpesviruses, which establish persistent infection. Indeed, virtually every herpesvirus implements at least one gene product to deal with the MHC class I-restricted CTL responses by interfering with MHC protein synthesis, assembly, peptide loading, or cell surface transport (for review see [2, 5]). For example HCMV encodes four separate proteins, US3, US2, US11, and US6, to block MHC class I presentation at three fundamentally different steps [199]. As outlined in Section 3.7, KSHV encodes two gene products, K3 and K5, which are involved in the downregulation of MHC class I and the prevention of antiviral CTL responses [35, 36, 86]. Although predominantly located in the ER, K3, and K5 do not affect the assembly or transport of MHC class I molecules through the secretory pathway. Instead, they mediate ubiquitination of cell surface MHC class I molecules; once ubiquitinated, MHC class I molecules are rapidly internalized and degraded by the lysosome [38, 77].

The structural requirements for the MHC-I downregulation by K3 were analyzed in detail. K3 and K5 belong to a large family of E3 ubiquitin ligases [38, 61, 77, 127]. They contain a plant homeodomain (PHD) motif, a specialized form of zinc finger, at their amino termini that carries E3 ubiquitin ligase activity. It has been shown that an intact PHD is required for MHC class I ubiquitination, and that mutational ablation of the lysine residues in the MHC class I cytosolic tail abolishes both ubiquitin tagging and MHC class I downregulation [37, 38, 77]. However, the E3 ligase activity of K3, but not K5, can be lysine independent; K3 can alternatively ubiquitinate cysteine residues of target molecules [22]. The substrate specificity of K3 and K5, however, is determined by their transmembrane domains [38, 167]. On association with its target protein, the PHD domain of K3 recruits E2 ubiquitin-conjugating enzymes and promotes transfer of the ubiquitin from the E2 onto the target protein. Two E2 enzymes, UbcH5b/c and Ubc13, are recently identified by Duncan et al. [46] that contribute to the Lys-63-polyubiquitination of class I molecules, a prerequisite for efficient endocytosis and endolysosomal degradation of class I molecules. The C-terminal cytoplasmic region contains a number of motifs including a YXXΦ endocytosis motif, four amino acids (NTRV) conserved in both K3 and K5, a potential SH3-binding domain, and two acidic clusters (DE1 and DE2). Detailed mutational analysis indicates that the N-terminal PHD motif, the central endocytosis motif, and conserved NRTV residues of K3 contribute to endocytosis of MHC class I molecules, whereas the C-terminal diacidic cluster region is engaged in lysosomal recruitment of MHC class I molecules [127]. These results demonstrate that K3 exerts control at both the initial internalization step and the downstream lysosomal degradation step, notably, the actions of which are functionally and genetically separable. In addition, the endocytic pathway of the MHC class I molecule depends on a late endosome-sorting protein, TSG101, in that depletion of TSG101 does not

affect internalization but block K3-mediated MHC class I degradation [77]. Moreover, Lorenzo et al. report that the ubiquitin-proteasome system is somehow also involved in modulating endocytic trafficking of MHC class I molecules [114].

Similar to MHC class I, ICAM-1 and B7-2 (see above), platelet-endothelia cell adhesion molecule (PECAM) [120] and IFN-γR1 [105] undergo ubiquitination and endocytosis upon K3 or K5 expression with overall similar schemes. Notably, diminishing cell surface ICAM-1 and B7-2 not only evades NK cell recognition (see above) but also inhibits T-cell stimulation [38]. By downregulating the IFN-γ receptor, KSHV blocks the ability of the cell to transmit an IFN-γ signal as exemplified by the decreased phosphorylation level of STAT1 and significant repression of IFN-γ-mediated transcriptional activation, thereby, inhibiting IFN-γ action. In particular, K3 is more pronounced in MHC-I downregulation, while K5 is more evolved to downregulate ICAM-1 and B7-2, PECAM, and IFN-γR1. Orchestration of K3 and K5, as a result, suppresses both cytokine-mediated and cell-mediated immunity, which ensures a comprehensive avoidance of host immune controls.

The in vivo importance of K3/K5-mediated CTL evasion via MHC-I downregulation can be inferred from the K3 protein of MHV68, which is an ortholog of KSHV-K3 possessing an N-terminal PHD motif and two transmembrane domains. Stevenson et al. have shown that cells expressing MHV68-K3 are decreased in their ability to present peptides to T-cell hybridomas in a MHC-I-restricted manner, indicating both potential evasion of CTL lysis and activation [187]. MHV68-K3 directly associates with and ubiquitinates the MHC-I molecules, however, in striking contrast to KSHV-K3, MHV68-K3 targets them for proteasomal degradation. K3 deletion of MHV68 has minimal effect on the viral clearance from the lung, but leads to attenuated viral latency amplification, a defect that can be reversed by CTL depletion [188]. Whether K3 and K5 have similar potent effect in KSHV infection is as yet unknown. Nonetheless, downregulation of class I molecules and co-stimulators is also observed in latent KSHV-infected cells [198], suggesting CTL evasion is a constitutive demand for KSHV.

24.3.2 Evasion of B-Cell Response

Although cell-mediated immune responses are often necessary to clear viral infection, humoral response is highly effective in reducing viral load and preventing revisit of viruses. Protective antibodies sequester viruses from accessing host cell receptors and mark them for phagocytosis. Likewise, antibody-coated infected cells can be lysed by NK lymphocytes. As may be expected, viruses are able to subvert antibody actions through diverse strategies [199]. For example, HSV encodes two glycoproteins, gL and gE that together bind to the Fc of antibodies, limiting the antibody-mediated neutralization of virus. However, no specific viral genes have been identified to directly interfere with antibody-mediated

neutralization of KSHV. Kimball et al. have shown that the titers of neutralizing Abs in KS-positive group are significantly lower than that in KS-negative group, suggesting a potential role of antibody in the control of KSHV infection and progression of KSHV infection to KS [94]. Given the significance of antibody in antiviral immunity, it is expected that KSHV would have a strategy for evading the neutralizing antibody response awaiting identification.

24.4 Concluding Remarks

Life-long KSHV infection is maintained by a delicate balance between host immune system and viral immune evasion mechanisms. In this review, we have discussed the potential immune escape strategies employed by KSHV as schematized in Fig. 24.1 and summarized in Table 24.1. By probing the molecular details of each virus-designed or pirated immunomodulatory molecules, researchers have gained important insight not only into diverse aspects of virus–host interactions but also to the inner workings of host immunity. Nonetheless, due to the absence of tractable tissue culture or in vivo models for KSHV, the function of many viral immune evasion proteins in the context of viral infection is not fully understood. Greater use of closely related animal model systems such as RRV, HVS, and MHV68 along with improved culture systems may provide new understandings of

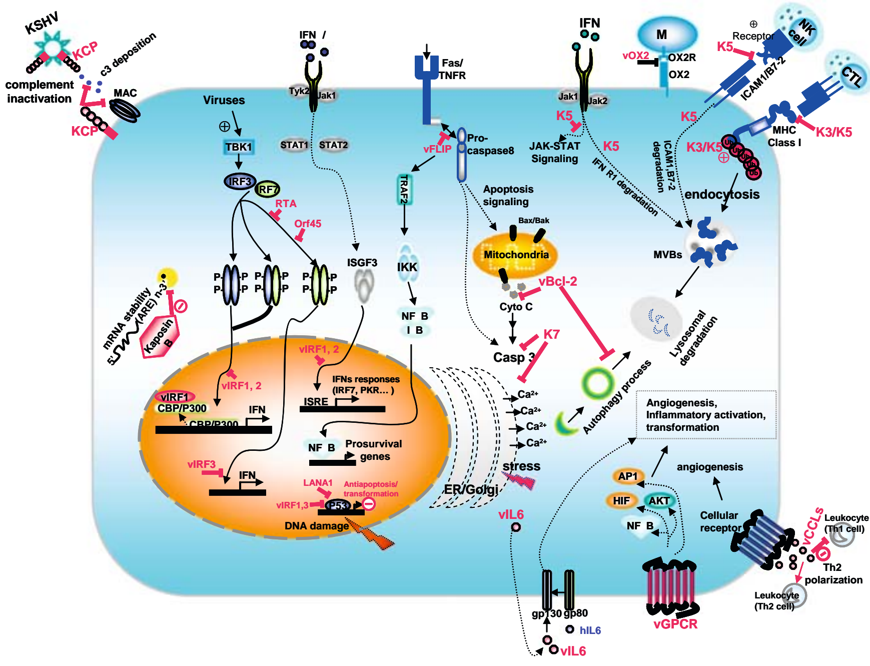

Fig. 24.1. Schematic representation of KSHV immune evasion strategies

Table 24. 1 Potential immune evasion mechanisms of KSHV gene/gene products

Responses	ORF	Gene Product	Functions	Mechanisms
Complement cascade	ORF4	KCP	Blocks complement activation	Enhances C3 convertase decay; prevents C3b deposition; factor I cofactor activity
IFN signaling	K9	vIRF1	Inhibits IRF3-mediated transcription; cell transformation	P300/CBP sequestration; antagonizes p53, ATM,GRIM19 activity and TGFβ signaling, activates vIL-6 and c-myc
	K11.1-K11	vIRF2	Inhibits IRF1, IRF3-mediated transcription	
	K10.5-	K10.6	vIRF3	Inhibits IRF7-mediated transcription
		Inhibits IRF7 DNA	binding	
	ORF50	RTA	Prevents IRF7 activation;	IRF7 ubiquitination and degradation
ORF45		Prevents IRF7	activation;	Prevents IRF7 phosphorylation/ nuclear translocation
Cytokine Network	K4	vCCL2	Th2 chemotaxis, angiogenesis	CCR3 agonist; C-, cc-, cxc-, CX_3C-chemokine antagonist
	K6	vCCL1	Th2 chemotaxis, angiogenesis	CCR8 agonist
	K4.1	vCCL3	Th2 chemotaxis, angiogenesis	CCR4 agonist
	ORF74	vGPCR	Inflammatory activation; angiogenesis; Cell transformation	Constitutive active GPCR, activates P13K/AKT pathway; activates $NF_{\kappa}B$, AP-1, HIF-1α trascriptional activities
	K2	vIL6	Antagonizes IFNα activity in PEL Inflammatory activation; angiogenesis	IL-6 homologue; gp80-independent signaling
	K12	Kaposin B	Increases cytokines release	Activates MK2, blocks cytokine mRNA decay
	K14	vOX2	Modulates macrophage activity	Cellular CD200 homologue, binds to CD200R
Apoptosis/			Autophagy	K13
	vFLIP	Anti-	apoptosis, cell transformation, cell	Block recruitment/ activation of caspase 8;

Table 24. 1 (continued)

Responses	ORF	Gene Product	Functions	Mechanisms
			spindling, inflammatory activation	$NF_{\kappa}B$ pathway activation
	ORF16	vBcl-2	Anti-apoptosis, anti-autophagy	Binds and neutralizes pro-apoptotic Bcl-2 family proteins activity; blocks Beclin-1-mediated autophagy
	K7	vIAP	Anti-apoptosis, interferes with ubiquitin-proteasome pathway	Inhibits effector caspase 3 activity, CAML activity; inhibits PLIC-regulated ubiquitin-proteasome pathway
	ORF73	LANA	Anti-apoptosis	Inhibits p53 transcriptional activity
	K9	vIRF1	Anti-apoptosis	Inhibits p53 transcriptional activity
	K10.5-10.6	vIRF3	Anti-apoptosis	Inhibits p53 transcriptional activity
ORF50	RTA	Anti-	apoptosis	Inhibits p53 transcriptional activity
CTL and NK cell responses	K3/K5	vMIR1	Inhibit CTL and NK activations and their effectors pathway	Downregulation of MHC-1 molecules
		vMIR2		Downregulation of ICAM-1, B7-2, PECAM
				Downregulation of $IFN_{\gamma}R1$

viral immune evasion and pathogenesis, which will aid the development of immuno-therapeutics for KSHV-associated disorders.

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Chapter 25
Non-coding Regulatory RNAs of the DNA Tumor Viruses

Christopher S. Sullivan and Bryan R. Cullen

Abstract Viral non-coding RNAs (ncRNAs) exist in various forms, many of which appear unique to a particular DNA tumor virus family. In contrast, virally encoded microRNAs (miRNAs) represent a strategy used by several DNA tumor virus families to tap into the same processing and effector machinery utilized by host-derived miRNAs. Whether encoded during latent or lytic infection, ncRNAs are often among the most highly expressed viral transcripts, implying that they have important functions in the viral life cycle. Viral ncRNAs have been shown to contribute to viral gene autoregulation, modification of the host cell apoptotic response, and enhance the translation of viral proteins. While our knowledge of the various viral ncRNAs continues to grow, there remain surprising gaps in our understanding of the functions of some viral ncRNAs, especially given their abundance and the fact that they were discovered decades ago. Here, we provide an overview of the current understanding of the regulatory ncRNAs encoded by the DNA tumor viruses, including the VA RNAs, EBERs, HSURs, PAN, and the recently discovered viral miRNAs.

25.1 Introduction

Despite obvious differences in their genomic capacity, the replication cycles of the large (Herpesviridae) and small (Adenoviridae, Papillomaviridae, Polyomaviridae) DNA tumor viruses share much in common. Upon entry into the cell, the challenges faced for effective lytic replication are similar, and therefore their strategies for accomplishing successful replication often overlap

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_25, © Springer Science+Business Media, LLC 2009

mechanistically. For example, during lytic replication both large and small DNA viruses have to create a cellular environment that is conducive to viral DNA replication and that avoids the induction of apoptosis and host immune responses. Lytic replication of both kinds of viruses requires driving the cells into a state of "replicative preparedness", ensuring a sufficient supply of metabolites and enzymes to support robust DNA replication and virion production. As such, most DNA tumor viruses have evolved various strategies to interfere with the RB and p53 tumor suppressor families and their associated pathways (albeit with differing outcomes: the small DNA tumor viruses elicit an S phase-like state in infected cells, whereas some members of the herpesviral families elicit a G0-like state) (Ahuja et al., 2005; An et al., 2005; Berk, 2005; Doniger et al., 1999; Flemington, 2001; Gwack et al., 2001; Moore and Chang, 2003). In addition, some members of both classes of DNA tumor viruses (and many RNA viruses as well) have evolved mechanisms to reduce host gene expression, preferentially allowing for viral gene expression ("host shutoff") and resulting in greater viral access to the host gene expression machinery (Glaunsinger and Ganem, 2006). An additional benefit to the virus is that such shutoff mechanisms often limit the host's innate immune response to infection as well. Both the large and the small DNA viruses must deal with host adaptive and innate immune responses. The larger viruses encode specialized proteins (in some viruses several proteins) to combat various aspects of the host response, whereas the mechanisms of avoidance of the host immune response in the smaller viruses necessarily rely on fewer proteins that are often multifunctional and composed of several domains. Nonetheless, the ability to form lifelong, persistent, or latent infections (common to most DNA tumor viruses – the exception being adenoviruses) necessarily means that both types of DNA tumor viruses have evolved strategies to combat host interferon responses as well as the other innate (NK) and adaptive (B and T cell) responses (Finlay and McFadden, 2006; Langland et al., 2006; Lodoen and Lanier, 2006). While both the large and the small DNA tumor viruses have evolved proteins to accomplish these tasks, some have also evolved non-coding RNAs that are able to regulate these processes.

In recent years, a paradigm shift in the way we view the genomes of mammals has taken place (Mattick and Makunin, 2006). Previous conceptions of the genome containing mostly insignificant, non-protein-coding "junk" sequences are changing as it has become evident that a majority of these sequences are transcribed into RNA (Carninci et al., 2005; Maeda et al., 2006). The significance of this observation is still a matter of some debate (Huttenhofer et al., 2005; Werner and Berdal, 2005), however this notion, combined with the understanding of the profound role miRNAs and other RNAi-related mechanisms play in regulating gene expression, demonstrates the importance of regulatory ncRNAs in several critical biological processes. As our appreciation of the "transcriptome" of the mammalian genome deepens, it is interesting to note that the viral genomes of the DNA tumor viruses are more similar to the host genomes they infect than previously appreciated. In both viruses and hosts, the majority of the genomes are transcribed and many of these transcripts are at least partially overlapping, in

either the sense or the antisense (AS) orientation, with other transcripts. In addition, it has been known for over 25 years that some viral ncRNAs play an essential role in the virus life cycle as mutants in these ncRNA are defective for growth in cultured cells (Thimmappaya et al., 1982) .Therefore, it is likely that the study of viral ncRNAs will not only illuminate the field of virology but also provide valuable insights into host cell biology.

The repertoire of functions attributed to mammalian non-RNAi-associated ncRNAs spans a vast landscape including transcription initiation, telomerase function, regulation of splicing, regulation of kinase activity, and many more (Butcher and Brow, 2005; Hirose et al., 2006; Kwek et al., 2002; Matera and Shpargel, 2006; Yang et al., 2001). Although the mechanisms remain elusive, some antisense transcripts have been demonstrated to play essential roles in the control of gene expression, particularly noteworthy examples of which include regulation of Hif-1alpha and myosin heavy chain expression (Luther, 2005; Luther et al., 1997; Mattick and Makunin, 2006; Rossignol et al., 2004; Rossignol et al., 2002; Thrash-Bingham and Tartof, 1999; Zezza and Heywood, 1986). Antisense transcripts have the potential to form dsRNA, and as such, some antisense transcripts trigger RNA editing, a process whereby dsRNA is deaminated converting adenosine nucleotides to inosines. This can result in the trapping of mRNAs within the nucleus and the consequent robust silencing of specific gene expression (Bass, 2002; DeCerbo and Carmichael, 2005; Zhang and Carmichael, 2001). In addition, long ncRNAs may play a role in directing chromatin silencing, as has been observed with the non-coding RNA Xist in X chromosome inactivation (Brown and Chow, 2003; Plath et al., 2002). Thus, as our understanding of the functionality of host-derived long ncRNAs grows, it seems likely that more examples of viral utilization of similar strategies will be uncovered.

There are examples of viruses that express ncRNAs with some of the same functions described above, for example, in Marek's disease virus (MDV), a herpesvirus that causes neurological disease and tumors in chickens. Recently, it was established that MDV expresses two genes encoding viral versions of the RNA component of telomerase. Deletion of both of these genes results in reduced induction of lymphomagenesis by MDV infection (Fragnet et al., 2003; Trapp et al., 2006). Additionally, polyomaviruses likely utilize extensive editing caused by overlapping antisense transcripts to downregulate early gene expression at late time points during infection (Kumar and Carmichael, 1997, 1998). Furthermore, viruses encode ncRNAs with functions that are apparently viral specific, with no known host counterpart. For example, the pol III-derived $\sim$165 nt small RNAs that inhibit interferon-induced translational repression and apoptosis (see VA RNA and EBER RNA sections below).

In addition to the long, i.e., $\geq$50 nt ncRNAs described above, it has now also become clear that several DNA tumor viruses, including polyoma and herpesviruses, also encode miRNAs. As described in more detail below, miRNAs are short, generally 20–23 nt long non-coding RNAs that function as post-transcriptional regulators of gene expression. There are reports documenting the regulation of both viral and host cell genes by viral miRNAs, and knowledge of

the function of viral miRNAs appears likely to expand rapidly over the next few years. Current evidence suggests that virally encoded miRNAs likely function by the same general mechanisms seen with cellular miRNAs, that is, they function as sequence-specific, post-transcriptional inhibitors of mRNAs bearing regions of homology to the miRNA. This is to be expected, given that viral miRNAs appear to be entirely dependent on the host cell RNAi machinery for their function.

The fact that viruses utilize ncRNAs is not surprising given the limited genomic space ncRNAs require (from a few kilobases to less than 100 nucleotides) and their presumed ability to rapidly evolve (for example, as inverted hairpins during genome replication). ncRNAs may represent a particularly favorable strategy for viruses to affect host cell targets since they do not elicit the adaptive (T and B cell) immune response that is precipitated by proteins. However, it should be noted that viral utilization of ncRNAs is not restricted to viruses that infect multicellular organisms with sophisticated immune responses, but rather is an ancient strategy that is conserved in evolutionary distant viruses, including bacteriophages (Briani et al., 2000; Heinrich et al., 1995; Wu et al., 1987; Zhang et al., 1998). As with their host cell counterparts, the functions of most viral ncRNAs remain unknown. So far, viruses have been shown to encode antisense RNAs (including those that trigger editing), miRNAs, and RNAs with as yet no known host counterpart such as EBERS and VA RNAs, which presumably act as competitive, stoichiometric binding sinks for host cell proteins. For the purpose of this review, the viral ncRNAs will be divided into two classes: the non-RNAi-associated ncRNAs (Table 25.1) and miRNAs.

Table 25.1 Viral regulatory ncRNAs (other than microRNAs)

Virus	Family	Notes	Refs.
Herpes simplex virus 1	Herpesviridae	LAT, abundant latent transcript, anti-apoptotic possibly because it serves as a precursor to generate a miRNA	(Bloom, 2004; Gupta et al., 2006; Kent et al., 2003)
Marek's disease virus	Herpesviridae	vTR, functions as RNA subunit of telomerase, may be oncogenic	(Fragnet et al., 2003; Trapp et al., 2006)
Cytomegalovirus	Herpesviridae	beta2.7, ~2,700 nt, polyadenylated, binds to mitochondrial enzyme complex I and prevents apoptosis and promotes stable ATP production during viral life cycle	(Kulesza and Shenk, 2004, 2006; Reeves et al., 2007)
		5 kb RNA, a stable intron of unknown	

Table 25.1 (continued)

Virus	Family	Notes	Refs.
		function in HCMV, MCMV encodes an ~7 kb homolog that enhances persistent infection in vivo, mechanism unknown	
Epstein–Barr virus	Herpesviridae	EBER RNAs, ~170 nt Pol III- derived, inhibits interferon-induced apoptosis.	(Clemens, 2006; Prang et al., 1999)
		AS transcript, possible regulation of immediate-early genes	
Herpes saimiri virus	Herpesviridae	HSURs, 70–140 nt, Pol II-derived, functions in activation of T cells?	(Albrecht and Fleckenstein, 1992; Cook et al., 2005; Lee et al., 1988; Lee and Steitz, 1990; Murthy et al., 1986; Wassarman et al., 1989)
Kaposi's sarcoma-associated herpesvirus	Herpesviridae	PAN (Nut-1), ~1.1 kb, pol II-derived polyadenylated nuclear transcript, function unknown.	(Lukac et al., 1999; Saveliev et al., 2002; Sun et al., 1996; Zhong et al., 1996)
		AS transcripts to master lytic switch protein RTA, function unknown	
Murine polyomavirus	Polyomaviridae	AS transcript, encoded late during infection, promotes extensive RNA editing of transcripts and nuclear retention of early transcripts	(DeCerbo and Carmichael, 2005; Kumar and Carmichael, 1997)
Adenovirus	Adenoviridae	VA RNAs I & II, ~160 nt, Pol III derived, inhibits PKR-mediated translation inhibition	(Mathews and Shenk, 1991)
Papillomavirus	Papillomaviridae	AS transcript, expressed in some tumors, antisense to portions of early region, function unknown	(Belaguli et al., 1997; Higgins et al., 1991; Vormwald-Dogan et al., 1992)

25.2 DNA Tumor Virus ncRNAs

25.2.1 Non-RNAi-Associated ncRNAs

Unlike proteins, for which an established set of partner binding experiments can provide clues to their function (such as the yeast two-hybrid assay and the TAP tag affinity co-purification assay), ncRNAs present a challenge to identifying their function in the absence of other knowledge. Additionally, many viral ncRNAs are found in human viruses for which good animal models are lacking. Therefore, mutant analysis in a relevant host is not possible. Thus, our understanding of the functions of most viral ncRNAs is either partial or entirely lacking. With that said, there is no doubt that the functions of at least some of these ncRNAs are important, given the amount of replicative energy expended on transcribing them. In many cases, such as EBV EBERs, adenovirus VA RNAs, saimiri HSUR RNAs, and KSHV PAN, these RNAs are among the most abundant transcripts made during viral infection. The extraordinary abundance of these viral ncRNAs suggests that their role is likely non-catalytic and may in fact require stoichiometric binding to cellular partners.

25.2.2 EBV EBER and Adenoviral VA RNAs: Pol III-Derived Inhibitors of the Interferon Response

25.2.2.1 VA RNAs

Perhaps the best-characterized viral ncRNAs are the adenovirus virus-associated (VA) RNAs (Ma and Mathews, 1996; Mathews and Shenk, 1991). These are highly structured, ~160 nt RNAs (Fig. 25.1B) transcribed by RNA polymerase III that are abundantly expressed late during infection. A majority of the human adenoviruses have two genes that code for products of similar size and predicted secondary structure (VAI and VAII), however some have only one copy – VAI (Ma and Mathews, 1996). Mutants defective for expression of both VA RNAs are severely defective for growth in cultured cells, implying an important cellular function (Thimmappaya et al., 1982).

After transcription, VA RNAs are exported from the nucleus via the karyopherin Exportin 5 (Exp5) – the same mode of export utilized by pre-miRNAs (see below) (Gwizdek et al., 2001), which consequently localizes the VA RNAs predominantly to the cytoplasm (Schneider et al., 1984). The VA RNAs are abundantly expressed, reaching up to 10^8 copies per infected cell, roughly equivalent to the number of ribosomes (Mathews and Shenk, 1991). Their abundance and subcellular localization imply a cytoplasmic binding target. Indeed, VA RNAs have been found to directly associate with PKR (Katze et al., 1987; Mellits et al., 1990), a cytoplasmic, interferon-induced kinase that is activated by dsRNA. Direct binding of PKR to long dsRNA occurs concurrent with PKR dimerization and autophosphorylation, suggesting that dsRNA may

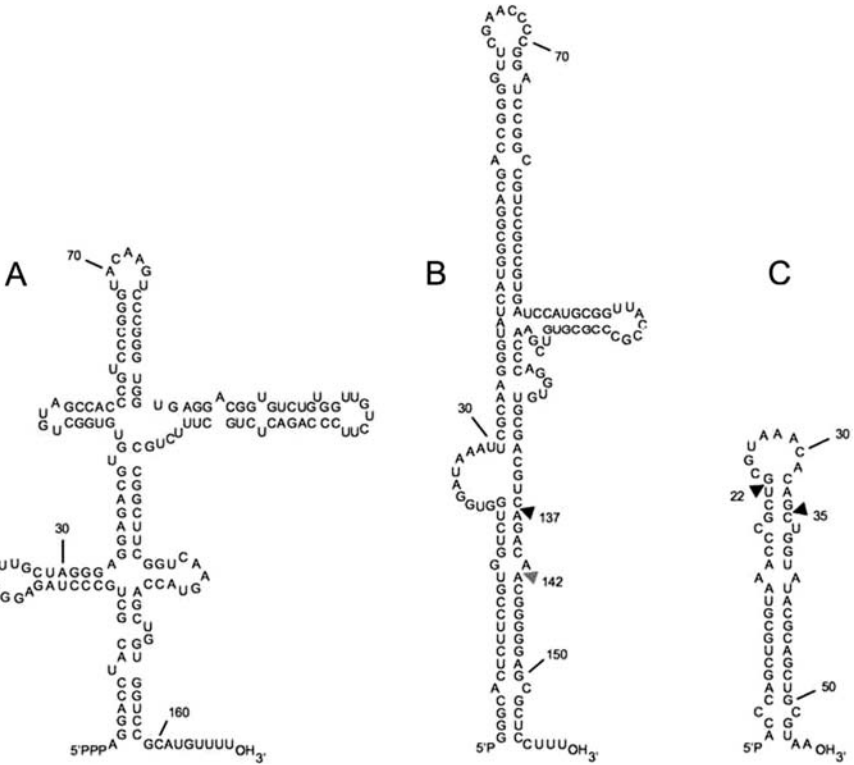

Fig. 25.1 Predicted RNA secondary structure of (**A**) an EBER ncRNA encoded by EBV (Glickman et al. 1988), (**B**) the VAI ncRNA encoded by adenovirus type 2 (Ma and Mathews, 1996) and (**C**) the pre-miRNA for miR-K9 encoded by KSHV. A Dicer cleavage site in VAI proposed by Aparicio et al. (2006) is shown by a gray arrow, while a Dicer cleavage site in VAI proposed by Sano et al. (2006) is shown by a black arrow. The known Dicer cleavage sites in KSHV miR-K9 are also indicated

serve as a scaffold to promote the homodimerization of PKR (reviewed in Garcia et al., 2006). PKR binding to the shorter, structured VA RNAs may serve as a competitive interaction that inhibits PKR homodimerization and autophosphorylation, and thus its kinase activity. Activated PKR phosphorylates the translation initiation elongation factor eIF2α, which results in a general inhibition of protein expression. The model of VA RNA prevention of PKR homodimerization and eIF2α phosphorylation is supported by the observation that expression of the VA RNAs enhances protein translation. However, it should be noted that there are other substrates for PKR, such as NF kappa B and IRF-1, whose phosphorylation by PKR is pro-apoptotic (Gil et al., 1999; Gil et al., 2004; Kirchhoff et al., 1995; Kumar et al., 1997). Therefore, it is likely that VA-mediated inhibition of the activation of these pathways is also advantageous to viral replication and could conceivably contribute to its

effect of increasing protein expression in infected cells. Recently, the VA RNAs have also been shown to interact with components of the cellular RNAi machinery. This will be discussed in more detail in the viral miRNA section of this review (see below).

25.2.2.2 EBERs

Another pair of regulatory small ncRNAs, called EBERs, is encoded by Epstein–Barr virus (EBV) (reviewed in Clemens, 2006). EBV encodes two EBERs, each an abundantly expressed ($\sim 10^7$/cell) (Lerner et al., 1981), ~ 170 nt RNA with highly structured features reminiscent of the adenoviral VA RNAs (Fig. 25.1A and B) (Glickman et al., 1988). The EBERs are found in many tumors latently infected with EBV, including nasopharyngeal carcinoma and Burkitt's lymphoma; due to their abundance, they are often used as a marker for EBV infection in these tumors (Khan et al., 1992). EBERs are transcribed by Pol III (Arrand et al., 1989; Rosa et al., 1981) and have been shown in several studies to prevent apoptosis induced by interferon treatment or triggers of the interferon response (Clemens, 2006; Nanbo et al., 2005; Ruf et al., 2005; Wong et al., 2005; Yamamoto et al., 2000). EBERs are similar in size and structure to the adeno VA RNAs and early reports demonstrated that the EBERs could interact with and inhibit the activation of PKR in vitro, leading to the hypothesis that the EBER RNAs function via the same mechanism as the adeno VA RNAs (Clarke et al., 1991; Sharp et al., 1993). In fact, a mutant adenovirus in which the region of the genome encoding the VA RNAs was replaced with the region of EBV encoding the two EBERs showed partial rescue of the growth defect associated with adenoviral VA null mutants (Bhat and Thimmappaya, 1983). In addition, there is at least one report arguing that expression of EBERs in nasopharyngeal epithelial cell lines results in decreased apoptosis and phosphorylation of PKR when cells are treated with the dsRNA analogue poly I:C (Wong et al., 2005). However, there are some notable differences between EBERs and VA RNAs. The EBER RNAs are expressed almost exclusively in latently infected cells, while adenovirus expresses the VA RNAs late during lytic infection. In addition, the EBERs are almost exclusively nuclear while the VA RNAs are found predominantly in the cytoplasm (Barletta et al., 1993; Fok et al., 2006; Howe and Steitz, 1986). As PKR is also found predominantly in the cytoplasm, and the accepted model for how VA RNAs inhibit PKR autophosphorylation involves stoichiometric association with PKR monomer to prevent homodimerization (Mathews and Shenk, 1991), it seems unlikely that the EBERs function in a manner identical to the VA RNAs. Indeed, in at least some cell types, expression of EBERs inhibits interferon-induced apoptosis but clearly does not robustly inhibit phosphorylation of PKR or its downstream substrate eIF2α (Ruf et al., 2005). In addition, recent studies demonstrate a PKR-independent role for the EBERs in preventing translational repression (Laing et al., 2002; Wang et al., 2005). Combined, these data raise the possibility of a nuclear localized, stoichiometric target for

the EBER RNAs, binding to which inhibits interferon-triggered translation repression and/or apoptosis. Perhaps, binding of this hypothetical nuclear target triggers a pathway parallel to or epistatic to PKR, thus bypassing phospho-PKR-mediated apoptosis and translational repression.

The EBERs were originally described as RNAs that co-precipitate with antibody that recognizes the autoantigen La (Lerner et al., 1981), an abundant protein involved in RNA processing (Wolin and Cedervall, 2002), and EBER1 has also been shown to interact with the ribosomal protein L22 (Toczyski and Steitz, 1991). However, the virological relevance of these associations remains obscure. Given the diversity of binding partners reported for the EBERs, it is possible that other functions exist in addition to their established role in the prevention of IFN-mediated apoptosis or that EBER association with La and/or L22 somehow prevents IFN-induced apoptosis by an as yet unappreciated mechanism. Thus, while it is clear that EBER-mediated inhibition of apoptosis may contribute to the tumorigenesis associated with EBV infection, the definition of the mechanisms by which the EBERs prevent apoptosis, and whether or not the EBERs encode additional functions, awaits future studies. For further information on EBERs, please see Chapter 14 (Epstein–Barr Virus Latent Infection Nuclear Proteins; Genome Maintenance and Regulation of Lymphocyte Cell Growth and Survival).

25.2.3 Herpesvirus Saimiri HSURs, KSHV PAN, and the Antisense Transcripts

25.2.3.1 HSURs

Herpesvirus saimiri (HVS) is a potent transforming virus that induces T-cell malignancies in New World monkeys. Infection with HVS is sufficient to transform primary T cells in culture (Desrosiers et al., 1986; Szomolanyi et al., 1987). HVS encodes seven small ~70–150 nt, pol II-derived ncRNAs, called herpes saimiri virus U RNAs (HSURs), that are the most abundantly expressed viral transcripts in latently infected cells (~ 2,000–20,000 copies/cell) (Albrecht and Fleckenstein, 1992; Lee et al., 1988; Lee and Steitz, 1990; Murthy et al., 1986; Wassarman et al., 1989). HSURs were originally identified by Northern blot analysis using probes spanning a subsection of the genome essential for the transformation activity associated with the virus (Murthy et al., 1986). In spite of this, the HSURs are dispensable for transformation of cultured T cells in vitro as well as for viral replication (Ensser et al., 1999; Murthy et al., 1986; Murthy et al., 1989). It was later found that some of the HSURs share similarities with a subset of mRNAs that contain the nucleotide sequence AUUA in the 3′ UTR – a so-called AU-rich element (Lee et al., 1988). The presence of AU-rich elements conveys instability by inducing a rapid but regulated turnover of mRNAs that contain them (Chen and Shyu, 1995). AU-rich elements are particularly enriched in transcripts that encode cytokines, growth factors,

and oncogenes (Bakheet et al., 2001). Thus, this observation led to a model whereby the abundant HSURs could serve as a “sink” to soak up host AU-rich element-binding proteins and thereby contribute to HVS-induced transformation by increasing the expression of AU-rich element-containing transcripts (Myer et al., 1992). In fact, it was demonstrated that some HSURs do associate with AU-rich element-binding proteins, however these associations had no effect on the half-life of host-derived transcripts containing AU-rich elements (Cook et al., 2004). Therefore, the significance of the ability of the HSURs to associate with AU-rich element-binding proteins remains unclear. Recently, Cook et al. compared cells infected with wild-type saimiri virus to those infected with a mutant that does not encode HSURs 1 and 2 using microarray expression profiling (Cook et al., 2005). Cells infected with the mutant virus expressed lower levels of genes associated with T- and NK-cell activation. These results suggest that at least some HSURs could be involved in promoting an activated state in T cells, perhaps leading to a growth advantage for the infected cells that could be advantageous to the virus. Whether this is the primary function of HSURs 1 and 2, the mechanism of how these small RNAs alter host gene expression and whether the other HSURs have similar functions remain unanswered questions. Nonetheless, with the first phenotypic differences for an HSUR mutant of HVS in hand, it is possible that a molecular function could soon follow.

25.2.3.2 PAN

KSHV PAN (polyadenylated nuclear RNA, Nut1, T1.1) is a pol II-derived, $\sim$1,080 nt polyadenylated transcript that is localized to the nucleus and is the most abundant viral transcript expressed during lytic infection (estimated $\sim 1–5 \times 10^5$ copies/cell). Consequently, it was one of the first KSHV-specific transcripts discovered (Sun et al., 1996; Zhong et al., 1996). It is an early lytic gene product that makes up as much as 80% of total polyadenylated transcripts in infected cells, yet it contains no intron and its longest predicted open reading frame is only $\sim$60 codons, suggesting it functions as an ncRNA (Song et al., 2001; Zhong and Ganem, 1997). In spite of its abundance, its biological function is still something of an enigma; although a small fraction ($<$10%) of PAN has been found to associate with the spliceosome-associated Sm proteins, no function with obvious relevance to the viral life cycle has been described (Sun et al., 1996; Zhong and Ganem, 1997). Conrad and Steitz demonstrated that PAN contains a 79-nt region just upstream of its polyadenylated $3'$ end that accounts for its high levels of nuclear expression (Conrad and Steitz, 2005). Recent studies have shown that the PAN element functions by preventing the action of a previously unappreciated mammalian RNA decay process that rapidly eliminates intronless polyadenylated transcripts (Conrad et al., 2006). This element is also sufficient to increase the steady-state levels of several intronless reporter RNAs in the nucleus. These observations, combined with previous reports that a small percentage of PAN is associated with host nuclear

Sm proteins, suggest that PAN may have a function in altering mRNA processing or export, perhaps to aid in expression of viral transcripts (a majority of lytic KSHV transcripts are intronless). In addition, Bechtel and colleagues have demonstrated that PAN as well as several other abundant KSHV transcripts are packaged into KSHV virions and are detectable in de novo infected cells (Bechtel et al., 2005). This raises the possibility that PAN could have a function soon after infection or even possibly within the virion. In spite of these observations, several key questions regarding the enigmatic PAN remain, not the least of which being its virological function. For more information on KSHV PAN, please see Chapter 19 (Organization and Expression of the Kaposi's Sarcoma-Associated Herpesvirus Genome).

25.2.3.3 AS-RNAs

Non-coding antisense transcripts have been described for several DNA tumor viruses including papilloma, polyoma, KSHV, and EBV. Although the function of these antisense transcripts is, for the most part, unknown, it is likely that at least some of these transcripts will serve a function similar to host-derived antisense transcripts, including silencing of gene expression in an RNA editing-dependent or -independent manner and/or directing chromatin modification. Therefore, these transcripts could play an important role in the biology of these viruses. dsRNAs have been described from cells latently infected with EBV (Prang et al., 1999). This dsRNA is found in latently infected cells as a hybrid between intronic portions of the latent EBNA-1 transcript and the unspliced (and therefore non-expressed) immediate-early lytic transcript BZLF-1. Formation of dsRNA between latent and lytic transcripts could represent a viral strategy that reinforces the inhibition of lytic activation and the maintenance of latent replication. Indeed, the other human gamma herpesvirus, KSHV, encodes multiple, presumably non-coding transcripts that are antisense to the gene encoding the master lytic switch activator protein RTA (Lukac et al., 1999; Saveliev et al., 2002). RTA expression is necessary and sufficient to trigger the lytic cascade of gene expression in latently infected cells (Lukac et al., 1998; Sun et al., 1998). Deciphering the role of these antisense transcripts could lead to a better understanding of the regulation of the life cycle of KSHV, given the central importance of RTA in regulating the lytic/latent switch. It is interesting to note that AS-RNAs have been reported that could contribute to the regulation of the virus life cycle in several families of small DNA tumor viruses. Tumors positive for high-risk papillomavirus type 16 encode RNAs that are antisense to portions of the early region of the genome (Higgins et al., 1991; Vormwald-Dogan et al., 1992) and transfection studies have demonstrated that similar antisense RNAs are detectable, and form dsRNA, in cells (Belaguli et al., 1997). It has been proposed that these AS-RNAs could regulate early papillomavirus gene expression (Belaguli et al., 1997), however this has not yet been verified during the course of infection. There is precedence for antisense-mediated viral gene autoregulation – murine polyoma virus has been shown to

encode transcripts late during infection that are antisense to the early transcripts (Acheson, 1978). These antisense transcripts induce editing of the early transcripts and their retention in the nucleus, resulting in a decrease in early gene expression (Kumar and Carmichael, 1997). Since its discovery in polyomavirus-infected cells, nuclear retention and inhibition of the expression of hyperedited RNAs has been well characterized (DeCerbo and Carmichael, 2005). Given the power of viral genetics, it is possible that antisense transcripts in other viruses will also serve to elucidate new gene regulation strategies relevant to the host.

25.2.4 microRNAs Encoded by DNA Tumor Viruses

25.2.4.1 miRNA Biogenesis and Function

miRNAs are short, ~22 nt long regulatory RNAs encoded by all known metazoan eukaryotes (Bartel, 2004). Human cells encode >400 distinct miRNAs that are found either singly or in clusters in the human genome. Most cellular miRNAs are initially transcribed by RNA polymerase II (pol II) as part of a capped, polyadenylated primary miRNA transcript (Cai et al., 2004; Lee et al., 2004). Within the primary miRNA, the mature miRNA sequence forms part of one arm of the stem of an ~80 nt long imperfect RNA hairpin. The first step in miRNA processing involves the recognition of the primary miRNA hairpin by the RNase III enzyme Drosha acting in concert with the dsRNA-binding protein DGCR8 (Cullen, 2004) (Fig. 25.2).

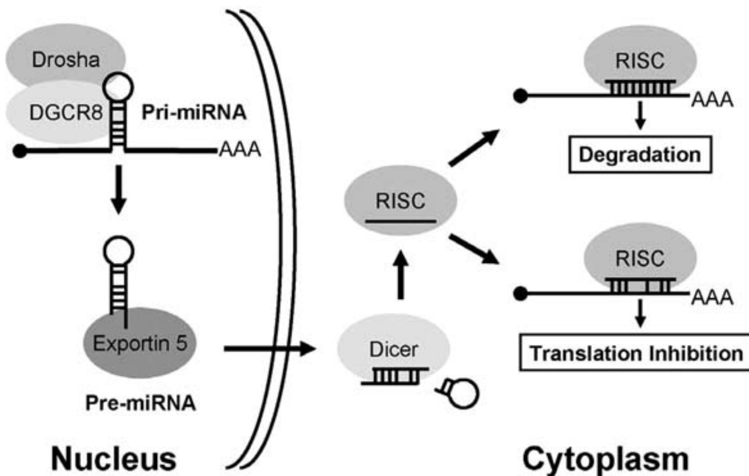

Fig. 25.2 Transcription and processing of microRNAs in vertebrate cells. The various steps in the biogenesis of mature viral or cellular microRNAs are indicated. See text for detailed discussion

This results in the specific cleavage of the primary miRNA hairpin, at a distance of ~22 bp from the junction of the stem and terminal loop, to give the ~60 nt long pre-miRNA intermediate. The pre-miRNA hairpin contains an ~2 nt 3′ overhang – a characteristic feature of RNase III processing events – and one end of the mature miRNA is defined by Drosha cleavage (Figs. 25.1C, 25.2). Drosha cleaves the primary miRNA transcript into at least three distinct RNA fragments, i.e., the pre-miRNA and the two long flanking regions that are retained in the nucleus and degraded (Cai et al., 2004). Therefore, function as a primary miRNA is incompatible with simultaneous function as an mRNA (Note, however, that Drosha cleavage is rarely 100% efficient and some primary miRNAs may therefore exit the nucleus and undergo translation.). As a result, the large majority of cellular and viral miRNAs are found either in exons in non-coding pol II transcripts or in the introns of coding or non-coding pol II transcripts (Cullen, 2004).

Once the ~60 nt pre-miRNA is cleaved out of the long primary miRNA, it is recognized by the Ran-dependent nuclear RNA export factor Exp5 (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Exp5 recognizes short 3′ overhangs at the base of short RNA stems and is responsible for the nuclear export of not only pre-miRNAs but also other viral and cellular ncRNAs, such as adenovirus VAI and cellular "Y" RNAs (Gwizdek et al., 2001; Zeng and Cullen, 2004). Once the pre-miRNA reaches the cytoplasm, it is recognized by the RNase III enzyme Dicer (Fig. 25.2). Dicer specifically binds adjacent to the 2 nt 3′ overhang found at the base of the pre-miRNA stem and then cleaves ~22 bp away, thus precisely removing the terminal loop (Cullen, 2004). Dicer, like Drosha, also leaves a ~2 nt long 3′ overhang. One strand of the resultant ~20 bp long dsRNA duplex intermediate, the mature miRNA strand, is then loaded into the RNA-induced silencing complex (RISC), while the passenger strand is degraded (Fig. 25.2). The mature miRNA now serves as a guide RNA to target RISC to cellular mRNAs bearing regions of homology (Bartel, 2004). If the homology is essentially complete, RISC will cleave the mRNA, leading to its degradation. However, if the homology is less extensive, then RISC will remain associated with the mRNA and inhibit its translation (Hutvagner and Zamore, 2002; Zeng et al., 2003). The mechanism of translational inhibition by RISC remains poorly understood but effective inhibition appears to require the binding of several RISCs acting in concert (Doench et al., 2003).

25.2.4.2 Virally Encoded miRNAs

Consideration of the life cycle of viruses suggests that DNA tumor viruses would be the most likely viruses to encode miRNAs. As noted above, the first step in miRNA processing occurs in the nucleus. Therefore, RNA or DNA viruses that replicate entirely in the cytoplasm (e.g., poxviruses, flaviviruses, picornaviruses, etc.) would have difficulty accessing the nuclear miRNA processing machinery. Moreover, because Drosha cleavage destroys the primary miRNA precursor (Cai et al., 2004; Lee et al., 2004), RNA viruses that encode

miRNAs as part of their genome or anti-genome would be subject to Drosha cleavage. This would appear likely to inhibit RNA virus replication and it is therefore not surprising that analyses of RNA viruses such as HIV-1, human T-cell leukemia virus type I, yellow fever virus, and hepatitis C virus have so far failed to identify any virally encoded miRNAs (Lin and Cullen, 2007; Pfeffer et al., 2005). We note that one laboratory has reported that HIV-1 does encode a miRNA (Omoto and Fujii, 2005; Omoto et al., 2004), but this stands in contradiction to two reports arguing that HIV-1 does not encode any miRNAs (Lin and Cullen, 2007; Pfeffer et al., 2005).

On the other hand, as DNA tumor viruses are, of course, DNA viruses that replicate in the nucleus, they would appear to be well positioned to express miRNAs in infected cells. Moreover, miRNA genes occupy little space and miRNAs are not antigenic and offer a highly specific mechanism by which to regulate host and/or viral gene expression. It is therefore not surprising that several DNA tumor viruses have indeed been found to encode miRNAs, as listed in Table 25.2.

While only a few viral-encoded miRNAs have well-defined functions, several mRNA targets have been identified. Some viral miRNAs target viral transcripts by directing their cleavage or translational repression, while others direct translational repression of host transcripts. Examples of each class are discussed in detail in Table 25.3.

Table 25.2 DNA tumor virus miRNAs

DNA virus family	Viral species	Number of known miRNAs	Refs.
α-Herpesviruses	Herpes simplex virus type 1	2	(Burnside et al., 2006; Cui et al., 2006; Gupta et al., 2006; Yao et al., 2007)
	Marek's disease virus type I	8	
	Marek's disease virus type II	17	
β-Herpesviruses	Human cytomegalovirus	11	(Buck et al., 2007; Dolken et al., 2007; Dunn et al., 2005; Grey et al., 2005; Pfeffer et al., 2005)
	Mouse cytomegalovirus	18	
γ-Herpesviruses	Kaposi's sarcoma-associated herpesvirus	12	(Cai et al., 2005; Cai et al., 2006b; Grundhoff et al., 2006; Pfeffer et al., 2005; Pfeffer et al., 2004; Samols et al., 2005; Schafer et al., 2007)
	Epstein–Barr virus	23	
	Rhesus lymphocryptovirus	16	
	Rhesus monkey rhadinovirus	7	
	Mouse γ-herpesvirus 68	9	
Polyomaviruses	SV40	1	(Sullivan et al., 2005)
	SA12	1	(Cantalupo et al., 2005; Sullivan et al., 2005)
Adenoviruses	Human adenovirus	1	(Andersson et al., 2005; Aparicio et al., 2006; Sano et al., 2006)
Papillomaviruses	Human papillomavirus 31	0	(Cai et al., 2006a)

Table 25.3 DNA tumor virus miRNA targets and functions

Virus	MiRNA	mRNA target/ function	Refs.
Herpes simplex Virus 1	miR-LAT	Prevents apoptosis by downregulating translation of both host TGF-β and SMAD3	(Gupta et al., 2006)
Human cytomegalovirus	hCMV-miR-UL1112	Downregulates translation of host MICB, resulting in reduced killing by natural killer cells	(Stern-Ginossar et al., 2007)
	hCMV-miR-UL1112	Downregulates translation of HCMV transcript IE72	(Grey et al., 2007)
Epstein–Barr virus	miR-BART2	Likely cleaves EBV BALF5 transcript that encodes viral DNA polymerase	(Pfeffer et al., 2004)
	miR-BARTs 16, 17–5p, 1–5p	Downregulates translation of EBV LMP1	(Lo et al., 2007)
Kaposi's sarcoma-associated herpesvirus	miR-K1, K3-3p, K6, K11	Downregulates expression of host thrombospondin resulting in decreased TGF-β activity	(Samols et al., 2007)
	miR-K11	Downregulates numerous mRNAs that are also targeted by host mir-155, a cellular miRNA associated with lymphomagenesis	(Gottwein et al., 2007; Skalsky et al., 2007)
	miR-K5	Downregulates BCLAF-1/Btf and may regulate apoptosis	(Ziegelbauer et al.)
SV40	SVmiRNA	Cleaves viral SV40 early mRNA transcripts late during infection, reduces early protein levels	(Sullivan et al., 2005)

25.2.4.3 The γ-Herpesviruses

The first report of virally encoded miRNAs identified five viral miRNAs in EBV (Pfeffer et al., 2004). Subsequent work identified an additional 18 miRNAs

in EBV (Cai et al., 2006b; Grundhoff et al., 2006) as well as 12 miRNAs in Kaposi's sarcoma-associated herpesvirus (KSHV) (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005), 16 in rhesus lymphocryptovirus (rLCV) (Cai et al., 2006b) and 9 in mouse γ-herpesvirus 68 (MHV68) (Pfeffer et al., 2005). It is therefore likely that γ-herpesviruses, which establish lifelong latent infections in susceptible hosts, rely extensively on viral miRNAs to help establish and maintain latent viral infections in vivo.

25.2.4.4 miRNAs Encoded by KSHV

As discussed in detail elsewhere in this volume, KSHV, the prototype of the rhadinovirus subgroup of the γ-herpesviruses, is an opportunistic human pathogen that infects both lymphoid and epithelial cells and readily establishes latent infections, particularly in human B cells. During latent infection, KSHV expresses only a small segment of its genome primarily encompassing the viral *kaposin (K12)*, *ORF71*, *ORF72*, and *ORF73* genes (Dittmer et al., 1998; Sarid et al., 1998). The cloning and computational identification of small RNAs from latently KSHV-infected cells by several groups identified 12 viral miRNAs, all of which are expressed in both latently and lytically KSHV-infected human B cells (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005).

An interesting feature of the KSHV miRNAs is that they are all encoded within the latency-associated region of the KSHV genome. Ten of the viral miRNAs (miR-K1 to miR-K9 as well as miR-K11) are found between the viral *kaposin* and *ORF71* genes while the other two are found within the kaposin K12 open reading frame (miR-K10) or within the 3′ UTR of kaposin (miR-K12) (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005) (Fig. 25.3). Analysis of gene expression from this region of the KSHV genome in latently or lytically infected cells has shed light on the transcriptional origin of these viral miRNAs (Cai and Cullen, 2006). In latently infected cells, transcription of the four latency-associated protein-coding genes, as well as all 12 viral miRNAs, is dependent on two fairly weak latent promoters located immediately 5′ to either the *ORF73* or the *ORF72* gene (Fig. 25.3). Immediately 3′ to *ORF71*, there is a leaky mRNA polyadenylation site. If this site is used, then the resultant mRNAs are used for translation of the ORF71, ORF72, and/or ORF73 proteins, depending on splicing (Cai and Cullen, 2006). However, if this polyadenylation site is not used, then the resultant mRNA is instead polyadenylated 3′ to the *kaposin* gene. The ORF71, ORF72, and ORF73 coding sequences, as well as the miR-K1 to miR-K9 and miR-K11 viral miRNAs, are excised by mRNA splicing and the above 10 viral miRNAs are then processed out of the intron by Drosha (Fig. 25.3). The resultant mature ~1.6 kb KSHV mRNA encodes the kaposin gene products and also acts as a primary miRNA for miR-K10 and miR-K12. Apparently, processing of miR-K10 by Drosha is quite inefficient as this viral miRNA appears to be expressed at significantly lower levels in latently KSHV-infected cells than are the intronic viral miRNAs

miR-K1 to miR-K9 plus miR-K11 (Cai et al., 2005). Moreover, as kaposin protein expression is readily detectable in latently KSHV-infected cells (Sadler et al., 1999), it is apparent that a significant proportion of the kaposin mRNA must have reached the cytoplasm in an intact form.

Upon activation of lytic KSHV replication, expression of the latency-associated viral proteins ORF71, ORF72, and ORF73, and of the miR-K1 to miR-K9 plus miR-K11 viral miRNAs, increases at most modestly (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005), consistent with data arguing that the viral latent promoters are not significantly activated during lytic replication (Dittmer et al., 1998). However, kaposin protein expression, as well as miR-K10 and miR-K12 expression, is strongly enhanced. This results from the presence of a powerful lytic viral promoter immediately 5′ to the kaposin ORF (McCormick and Ganem, 2005) (Fig. 25.3). Therefore, while all 12 KSHV miRNAs are detectable in latently

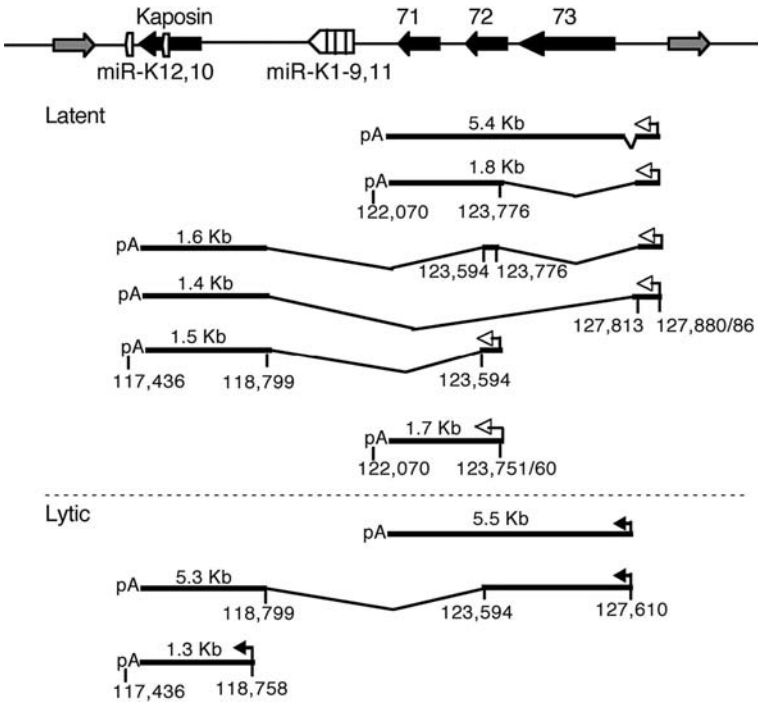

Fig. 25.3 Transcriptional origin and expression of the miRNAs encoded by KSHV. This schematic shows the genes and miRNAs found in the KSHV latency-associated region as well as the pre-mRNAs and mature mRNAs that have been identified. Numbers refer to the sequence coordinates of transcription start sites, splice sites and polyadenylation sites within the KSHV genome. Latent transcripts are shown above the dashed line and lytic transcripts below the line. Sizes of mature mRNAs are given in kilobases and their promoters are indicated by *arrows*. pA, Poly(A)-addition site. (Modified, with permission, from Cai and Cullen 2006.)

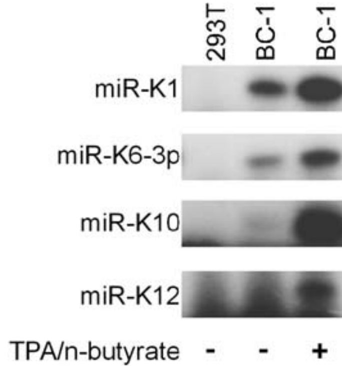

Fig 25.4 Effect of induction of lytic KSHV replication on viral miRNA expression. Latently KSHV-infected BC-1 cells, a human B-cell line, were either cultured normally or treated with TPA and n-butyrate for 48 h prior to RNA isolation, as described previously (Cai and Cullen 2006). This drug treatment induces lytic KSHV replication. The level of expression of the indicated KSHV miRNAs was then determined by primer extension analysis. Uninfected 293 T cells were used as the negative control. Reproduced from Gottwein et al., Cold Spring Harbor Symposia in Quantitative Biology, Vol. LXXI, 2007

infected cells only 2 of these, i.e., miR-K10 and miR-K12, are strongly induced during lytic replication (Cai and Cullen, 2006; Pfeffer et al., 2005) (Fig. 25.4). The significance of this activation will not be understood until the mRNA targets for miR-K10 and miR-K12 have been defined.

There are no computer-predicted mRNA targets showing extensive homology to any of the KSHV miRNAs found in any cellular or KSHV mRNAs and so it appears likely that the viral miRNAs act by regulating mRNAs at the translational level. Indeed, several recent studies have identified host mRNAs that are translationally repressed by KSHV miRNAs. miRs-K1, K3-3p, K6, and K11 were shown to downregulate thrombospondin 1 (THBS1) expression when exogenously expressed in human embryonic kidney (HEK) 293 cells (Samols et al., 2007). Although it has not yet been demonstrated that endogenous expression of these miRNA during KSHV infection has similar effects, this finding could be of enormous importance to understanding KSHV-induced tumorigenesis since THBS1 is a known tumor suppressor and anti-angiogenic factor. miR-K11 shares seed identity with miR-155, a cellular miRNA associated with lymphomagenesis (Nair and Zavolan, 2006). This shared seed identity is sufficient to allow both miR-K11 and miR-155 to downregulate an overlapping set of mRNA targets with seed complementary binding sites in their 3′ UTRs (Gottwein et al., 2007; Skalsky et al., 2007). At least one of these targets, FOS (part of the AP-1 transcriptional activator complex), is targeted for translational repression by endogenous miR-K11 in PEL cells (Gottwein et al., 2007). Thus, miR-K11 represents the first example of a viral miRNA that binds to and regulates host mRNA through some of the same binding sites that are utilized by a host miRNA. Finally, endogenous miR-K5 has been shown to target the pro-apoptotic

transcriptional repressor BCLAF/Btf in multiple different screens involving exogenous transient and stable expression of miR-K5 in multiple cell types (Ziegelbauer et al.). Furthermore, antisense inhibition of endogenous miR-K5 in PEL cells induces expression of BCLAF/Btf. Reduced levels of BCLAF/Btf results in less sensitivity to etoposide-induced Caspase activation, implying a protective effect against apoptosis. Combined, these results suggest that a major function of at least some KSHV miRNAs is to regulate host transcripts to promote cell survival during infection. As more targets are identified, it will be interesting to see if this trend holds or whether some KSHV miRNA will target viral transcripts similar to the other DNA viruses (SV40, EBV, and hCMV, see below). Efforts to fully define the mRNA targets for the various KSHV miRNAs are currently ongoing in several laboratories.

25.2.4.5 miRNAs Encoded by EBV

EBV is the prototype of the lymphocryptovirus subgroup of the γ-herpesviruses and is only very distantly related to KSHV, a rhadinovirus. EBV is a human pathogen that can cause both acute and chronic diseases, including several types of cancer. However, in immunocompetent individuals, EBV generally establishes a long-term latent infection that is both lifelong and symptomless after an initial flu-like disease termed infectious mononucleosis in the USA and glandular fever in the UK. While EBV infects both epithelial and lymphoid cells, the most common targets for latent EBV infection are B cells. EBV can establish three distinct forms of latent infection termed type I, type II, and type III (Kieff and Rickinson, 2001). Type I latency is the most common form in vivo in both normal and virus-transformed B cells, such as Burkitt's lymphoma (BL) cells. This form of latency involves the expression of only the viral EBNA1 protein and the EBER non-coding RNAs.

After EBV-infected primary B cells or BL tumor cells are removed from a patient and cultured in vitro, a significant proportion eventually switches to a second form of latency, termed type III latency. This form of viral latency is also seen in lymphoblastoid cell lines established by infection of B cells with EBV ex vivo. In type III latency, the cells express a series of viral gene products including the nuclear EBNA1, EBNA2, and EBNA3 proteins, the viral latent membrane proteins LMP1 and LMP2 as well as the EBER non-coding RNAs. A third form of EBV latency, termed type II latency, is in some ways intermediate between types I and III and is often seen in EBV-induced epithelial cell tumors, such as nasopharyngeal carcinomas (NPCs). This form of latency involves the expression of EBNA1 and LMP1, but not of the EBNA-2 and EBNA-3 gene products, and is also characterized by high-level expression of a family of alternatively spliced mRNAs that derive from the viral *BART* gene, a possibly non-coding gene of unclear function (Chen et al., 1992; Gilligan et al., 1990).

Work from several laboratories has now identified no less than 23 miRNAs encoded by EBV that exists in two distinct clusters (Cai et al., 2006b; Grundhoff et al., 2006; Pfeffer et al., 2005). The BHRF1 miRNAs are found in a cluster of

three miRNAs that flank the EBV BHRF1 ORF. It was initially suggested that the BHRF1 miRNAs might derive from mRNAs that encode BHRF1, an early lytic *EBV* gene (Pfeffer et al., 2005). However, it is now clear that these miRNAs are actually processed out of a large intron that forms part of the EBNA1 mRNAs that are selectively expressed in Type III latency due to activation of two viral promoter elements referred to as Cp and Wp (Cai et al., 2006b). In contrast, in Type I and Type II EBV latency, the Qp promoter, which is located between the BHRF1 miRNAs and the EBNA1 ORF, is used for EBNA1 expression. Therefore, in Type I and Type II latency, the EBV BHRF1 miRNAs are not detectably expressed (Cai et al., 2006b).

Surprisingly, the second EBV miRNA cluster, consisting of the 20 BART miRNAs, is also only expressed in some forms of viral latency. Specifically, because all the BART miRNAs are located within the introns of the EBV *BART* gene, the BART miRNAs are only expressed in EBV-infected cells that express BART (Cai et al., 2006b). BART is not expressed during Type I latency and is generally expressed at only very low, albeit detectable, levels during Type III latent infection of B cells (Chen et al., 1992; Gilligan et al., 1990). However, the *BART* gene is expressed at high levels in Type II EBV latency, particularly in NPC cells. Why the EBV miRNAs are differentially expressed in different cell types undergoing different types of latent viral infection is currently unclear. Again, this will not be understood until the identity of the mRNA targets for the EBV miRNAs is established. At present it is unclear whether these are viral or cellular mRNAs or a mixture of both.

While the mRNA targets for most of the EBV miRNAs remain unknown, two viral mRNA targets may have been identified (Lo et al., 2007; Pfeffer et al., 2004). One EBV miRNA, i.e., miR-BART2, is encoded within the EBV genome at a location that lies exactly opposite the viral open reading frame that encodes the EBV DNA polymerase. Therefore, miR-BART2 is fully complementary to this viral mRNA, and a transcript that likely results as a cleavage product from miR-BART2-guided RISC cleavage of this mRNA has been identified (Furnari et al., 1993). The purpose of this cleavage is not clear, although it has been suggested that it could perhaps help to maintain latent EBV infections by inhibiting EBV DNA polymerase expression (Pfeffer et al., 2004). A second report has shown the potential for several EBV-encoded miRNAs, miR-BARTs 16, 17–5p, and 1–p, to prevent translation of the viral transcript encoding the LMP1 protein (Lo et al., 2007). Exogenous expression of a cluster of miRNAs containing miR-BARTs 16, 17–5p, and 1–p reduces toxicity associated with expression of LMP1 in the presence of genotoxic stress. Combined, these results suggest that a major role for at least some EBV miRNAs will be to autoregulate viral gene expression.

25.2.4.6 miRNAs Encoded by rLCV

The primate lymphocryptoviruses have co-evolved with their primate hosts and it is therefore believed that the evolutionary distance between any two primate

lymphocryptoviruses is very similar to the evolutionary distance between their primate host species (Gerner et al., 2004). As humans and rhesus macaques last shared a common ancestor ~16 million years ago, it is likely that this same evolutionary distance applies to EBV and to the rhesus form of EBV, termed rLCV. Analysis of the miRNA-coding capacity of rLCV therefore has the potential to shed light on the evolutionary conservation of herpesvirus miRNAs and, hence, their importance in the EBV life cycle. Moreover, rLCV represents an important animal model for the study of EBV pathogenesis that is likely to become genetically tractable in the near future (Rivailler et al., 2004).

Analysis of the miRNAs encoded by rLCV identified 16 distinct viral miRNAs, of which 8 are closely related to 8 of the 23 miRNAs encoded by EBV (Cai et al., 2006b; Grundhoff et al., 2006). The other eight rLCV miRNAs are entirely novel. Because evolution of a very short ncRNA, such as a miRNA, should occur very easily, the fact that 50% of the rLCV miRNAs have been conserved over 16 million years of evolution strongly suggests that these miRNAs do play key roles in the EBV life cycle. What these roles are remains to be determined.

25.2.4.7 miRNAs Encoded by MHV68

MHV68 is a rhadinovirus that is only very distantly related to KSHV. However, MHV68 nevertheless represents an interesting, and genetically tractable, animal model for the study of rhadinovirus pathogenesis (Speck and Virgin, 1999). Analysis of MHV68-infected cells showed that this virus expresses at least nine miRNAs that are encoded in a cluster located very close to one end of the linear viral genome (Pfeffer et al., 2005).

There are two features of note about the MHV68 miRNAs. Firstly, these nine miRNAs were the first described that are transcribed by RNA polymerase III. All nine miRNAs are transcribed as fusions to a short tRNA-like sequence that contains the "box A" and "box B" internal promoter elements for pol III transcription initiation. The resultant tRNA/miRNA fusions are initially processed into pre-miRNAs and tRNA-like molecules by cellular proteins that remain to be identified. While the tRNAs are non-functional, i.e., are not aminoacylated, and are eventually degraded, the MHV68 pre-miRNAs are further processed, presumably by Dicer, to give functional viral miRNAs (Pfeffer et al., 2005).

While the function of the nine MHV68 miRNAs remains unknown, a clue comes from a study of naturally occurring deletion mutant of MHV68, termed MHV76 (Macrae et al., 2003). The MHV76 variant lacks all nine MHV68 miRNAs, as well as four adjacent ORFs, but nevertheless grows indistinguishably from MHV68 in tissue culture. However, the MHV76 variant is highly attenuated in vivo, with lower levels of virus replication, a greater host inflammatory response, and more rapid clearing by the immune system of infected mice (Macrae et al., 2003). Moreover, MHV76 differs from MHV68 in being unable to effectively establish long-term, latent infections in mice. While this interesting phenotype could reflect the loss of one or more of the four viral

protein-coding genes, it does clearly argue that the nine MHV68 miRNAs do not have a role in viral replication per se and suggests that these miRNAs may instead play a role in modulating host innate and/or adaptive immune responses in vivo. It is, of course, unclear whether these MHV68 data can be extrapolated to any human herpesviruses.

Although not DNA tumor viruses, we have also discussed the miRNAs of human cytomegalovirus (hCMV) and herpes simplex virus Type 1 below.

25.2.5 The β-Herpesviruses

25.2.5.1 miRNAs Encoded by Human Cytomegalovirus

Analysis of cells infected by the prototypic β-herpesvirus, hCMV, has resulted in the cDNA cloning and identification of 11 hCMV-encoded miRNAs (Dunn et al., 2005; Grey et al., 2005; Pfeffer et al., 2005). For all but one of these viral mRNAs, neither the mRNA targets nor the role played by these miRNAs in the viral life cycle has been determined. However, several of the hCMV miRNAs are evolutionarily conserved in chimpanzee CMV, thus suggesting that these miRNAs do play a significant role (Grey et al., 2005).

Two properties of the hCMV miRNAs distinguish these viral miRNAs from the miRNAs encoded by the γ-herpesviruses described above. Firstly, the 11 hCMV miRNAs are not clustered and are instead scattered over the hCMV genome. Also, unlike the γ-herpesvirus miRNAs, which are all found encoded by one strand of the linear viral DNA genome, the hCMV miRNAs derive from both strands (Dunn et al., 2005; Grey et al., 2005; Pfeffer et al., 2005). While the transcriptional origin of the hCMV miRNAs remains to be determined, it therefore seems likely that these are expressed from several different promoters.

A second interesting property of the hCMV miRNAs is that 5 of the 11 miRNAs are found within viral coding sequences, 3 in an antisense orientation, and 2 in the sense orientation. In the cases where the viral miRNA and coding sequence are in the same orientation, nuclear excision of the pre-miRNA would clearly disrupt the expression of the underlying ORF. This situation is similar to that seen with miR-K10 in KSHV, which is located within the kaposin/K12 ORF (Cai et al., 2005; Grundhoff et al., 2006; Pfeffer et al., 2005; Samols et al., 2005) (Fig. 25.3). In the case of miR-K10, Drosha cleavage of the primary miRNA precursor, i.e., the kaposin/K12 mRNA, appears to be inefficient and it is possible that this is also true in hCMV. On the other hand, it is possible that hCMV somehow regulates viral (and cellular?) miRNA processing in infected cells, although no evidence in support of this hypothesis currently exists.

As noted above, three of the hCMV miRNAs are encoded antisense to an underlying hCMV ORF. As the miRNA is therefore fully complementary to any mRNAs encoding the relevant viral protein, one would predict that these viral miRNAs would cleave the viral mRNAs, leading to their eventual destruction. As noted above, this is indeed seen in EBV, where the miR-BART2

miRNA is encoded antisense to the EBV DNA polymerase ORF and induces the cleavage of at least a portion of the cognate mRNA (Pfeffer et al., 2005). Whether this occurs in hCMV remains unknown. It is worth noting, however, that RNA structure can readily block access of a miRNA to even a fully complementary mRNA target (Cullen, 2006), so that autoregulation of the expression of these three hCMV mRNAs by viral miRNAs present in an antisense orientation is not inevitable.

Only one hCMV miRNA has any reported targets. Exogenous expression of miR-UL112 repressed translation of luciferase reporters containing 3′ UTR segments of the immediate-early gene IE72 (Grey et al., 2007). Furthermore, overexpression of a miR-UL112 oligonucleotide mimic reduces the efficiency of viral replication as measured by quantification of viral genome copy number by real-time PCR. Amazingly, miR-UL112 has also been shown to downregulate a seemingly completely unrelated host target – major histocompatibility complex-related chain B (MICB). miR-UL112 reduces translation of MICB, a stress-induced ligand of natural killer (NK) cells, and this results in reduced killing of infected HFF cells by co-cultured NK cells (Stern-Ginossar et al., 2007). Thus, miR-UL112 may represent a new class of viral miRNAs, those that can target transcripts of *both* host and viral origin.

25.2.6 The α-Herpesviruses

25.2.6.1 miRNAs Encoded by Marek's Disease Virus

Marek's disease virus (MDV) type I is an avian pathogen that induces T-cell lymphomas in susceptible chickens. Analysis of MDV I-infected cells has identified eight miRNAs encoded by MDV I, five in a cluster surrounding the viral *meq* oncogene and three more located in an intron in the MDV latency-associated transcript (LAT) (Burnside et al., 2006). All eight MDV I miRNAs are expressed in latently infected cells and in tumors induced by MDV. Recently, MDV II, a non-oncogenic antigenically similar virus has been shown to encode 17 pre-miRNAs. Estimates suggest that MDV I and MDV II have been separated by ~26 million years of evolution, a number comparable to EBV and LcV. Interestingly, unlike EBV/LcV, miRNAs from MDV Types I and II do not share any sequence identity. However, the relative genomic positions of miRNAs from both types are conserved (Yao et al., 2007). The role of these miRNAs in the viral life cycle or in viral tumorigenesis is not currently understood, however, the relatively similar genomic location of expression may imply that some of these miRNAs are enriched for viral targets.

25.2.6.2 miRNAs Encoded by Herpes Simplex Virus Type 1

Herpes simplex virus Type 1 (HSV1) is an important human pathogen that establishes long-term, latent infections in human neuronal cells, particularly in

the trigeminal ganglia (Whitley, 2001). These latently infected neurons are subject to spontaneous activation of lytic HSV1 replication, generally resulting in a localized infection. In infants or in immunocompromised or aged patients, HSV1 can sometimes spread to the brain causing a lethal encephalitis.

Although HSV1 readily establishes latent infections in vivo, it does so only in terminally differentiated, non-dividing neurons and no good tissue culture model of HSV1 latency currently exist. However, HSV1 will replicate lytically in culture in a wide range of cell types. HSV1 lytic replication induces the large-scale degradation of host cell mRNA and non-coding RNA species. Therefore, it has not proven possible to cDNA clone HSV1 miRNAs from lytically infected cells based on their characteristic size of 20–24 nt, as there are huge levels of intracellular RNA breakdown products of the same size. Conversely, obtaining sufficient RNA for cDNA cloning from latently HSV1-infected neurons has also proven difficult.

Because of these practical problems, efforts to identify HSV1-encoded miRNAs have largely relied on the identification of candidate viral pri-miRNA hairpin structures by computer analysis followed by attempts to validate the existence of the mature form of these miRNAs using analytical techniques, such as Northern blots. An initial report hypothesized that HSV1 encoded seven miRNAs, several of which were proposed to be encoded within the HSV1 LAT RNA (Pfeffer et al., 2005). LAT is a highly unstable, $\sim$8 kb capped polyadenylated non-coding transcript that represents the only viral RNA expressed in latently HSV1-infected neurons (Jones, 2003). The fact that LAT is both non-coding and unstable has historically made it difficult to ascribe a function to the LAT RNA, although it had been reported that LAT expression inhibits apoptosis and enhances the ability of HSV1 to enter a lytic replication cycle (Jones, 2003). However, if LAT was shown to be a primary miRNA precursor, then this would explain why LAT was non-coding, why it was unstable, and, most importantly, how it could exert a phenotypic effect. As noted above, analysis of MDV had previously identified three miRNAs encoded within the LAT region of this distantly related α-herpesvirus (Burnside et al., 2006).

Subsequent analyses have identified two HSV-1 encoded miRNAs. One group (Cui et al., 2006) has identified an HSV miRNA encoded $\sim$450 bp upstream of the LAT transcription start site. No function for this miRNA has been proposed. Of more interest, Gupta et al. (2006) identified a second HSV1 miRNA, termed miR-LAT, derived from the HSV1 LAT transcript itself. Moreover, these workers presented evidence arguing that miR-LAT inhibited the expression of both TGFβ and SMAD3 in infected cells by targeting RISC to the 3$'$ UTR regions of their cognate mRNAs (Gupta et al., 2006). Both these proteins exert a pro-apoptotic effect and it has been proposed (Gupta et al., 2006) that inhibition of the expression of these two cellular proteins by HSV1 miR-LAT is both necessary and sufficient to explain the anti-apoptotic phenotype exerted by the full-length LAT transcript. It currently remains unclear whether HSV1 encodes additional miRNAs, although this appears likely given the precedent of MDV (Burnside et al., 2006) and given the fact that computer

analysis predicts the existence of additional HSV1 miRNAs (Cui et al., 2006; Pfeffer et al., 2005).

25.2.7 The Polyomaviruses

25.2.7.1 miRNAs Encoded by SV40 and SA12

The polyomavirus family is composed of small viruses with dsDNA genomes that have so far been identified to have mostly been identified in avian, rodent, or primate hosts (Pipas, 1992). Most polyomaviruses have a similar genomic architecture and life cycle consisting of a circular DNA genome of $\sim$5 kb that contains a bidirectional promoter that transcribes the early gene products (T antigens) in one direction and the late gene products (encoding the structural proteins) in the other (Cole, 1996). Similar to the herpesviruses, all of the polyomaviruses that have been studied in detail, including the murine polyomavirus muPyV, the macaque polyomavirus SV40, and the human polyomaviruses JCV and BKV, can form lifelong infections in their respective hosts. For the most part, exposure to polyomaviruses eventually results in a persistent infection with periodic shedding.

SV40, the prototypic primate polyomavirus, encodes a pre-miRNA late in infection that is processed into a miRNA and a miRNA* that are both active in RISC (for some pre-miRNAs both strands are capable of entering RISC, albeit one strand preferentially enters [miRNA] and the less abundant small RNA from the other strand that enters less efficiently is designated as miRNA*) (Sullivan et al., 2005). Since both SVmiRNA and SVmiRNA* are encoded on the late strand in a region of the genome that is completely complimentary to the early mRNAs, both direct the siRNA-like cleavage of the early mRNAs late during infection. So far, this is the only example known where a single pre-miRNA generates different miRNAs from both arms of the hairpin precursor that are active on the same mRNA target(s). This miRNA-mediated autoregulation of early mRNA levels results in a reduction in T antigen protein levels, since an increase in the steady-state levels of the early proteins is detected in a mutant that is unable to generate the miRNAs. However, this autoregulatory function of the SVmiRNAs is not essential in cultured cells as the miRNA mutant replicates as robustly as wild type (Sullivan et al., 2005).

Despite the non-essential nature of the SVmiRNAs for infection of cultured cells, their function(s) is presumably important as the pre-miRNAs are predicted to be conserved in the related human polyomaviruses JCV and BKV (Sullivan et al., 2005). Furthermore, SA12, a baboon polyomavirus that is more similar at the genomic level to JCV and BKV than it is to SV40, encodes homologous miRNAs that are readily detectable by Northern blot (Cantalupo et al., 2005; Sullivan et al., 2005). Interestingly, the SV40 and SA12 pre-miRNAs share features that are atypical; both generate robust, approximately equal amounts of the miRNA and miRNA* (as judged by Northern blot analysis),

and both are inefficiently processed by Dicer. The functional relevance of these atypical features remains unknown. However, it is likely that they have evolved to optimize the amount of early protein expressed late during infection. In support of this notion, muPyV encodes a pre-miRNA that, despite being unrelated in sequence to the SV40-like pre-miRNAs of the primate polyomaviruses, shares much in common at the level of processing and molecular function (CS Sullivan, AT Grundhoff, and D Ganem, unpublished).

Since the polyomavirus miRNAs serve no replicative purpose in cultured cells, it is likely that their function is important during natural infection of their animal hosts. Thus, it is conceivable that the polyomavirus miRNAs play a role in viral animal to animal transmission, spread throughout the host or in avoidance of host immune defenses. In support of the later hypothesis it is well established that the polyomaviral T antigen proteins elicit a strong cytotoxic T-cell response (Moser and Lukacher, 2001) and thus, decreasing the levels of T antigen late during infection could allay the adaptive immune response. SV40 infection of monkey cells (that have been engineered to express murine MHC class I) in the presence of cytotoxic T lymphocyte (CTL) clones that recognize T antigen epitopes results in CTL-mediated lysis of infected cells (Bates et al., 1988). CTL-mediated lysis is more robust when cells are infected with a mutant virus that does not make the SVmiRNAs and consequently encodes increased antigen levels (Sullivan et al., 2005). This suggests that controlling early protein antigen levels is likely of central importance to the viral life cycle in vivo. It seems polyomaviruses have adopted several strategies to downregulate early protein levels late during infection; SV40 also represses transcription of the early genes late during infection (Cole, 1996), and murine polyomavirus reduces early protein levels by utilizing extensive editing induced by overlapping AS transcripts (Kumar and Carmichael, 1997). Thus, it is clear that in the proper context, polyomavirus miRNA-mediated autoregulation of gene expression can play a role in avoiding the host CTL killing of infected cells. Whether or not this occurs in vivo and the possibility that these miRNAs may encode additional functions are the subjects of future studies.

25.2.8 The Papillomaviruses

Papillomaviruses are small DNA tumor viruses that establish long-term, persistent infections that lead to the development of warts or other superficial hyperplastic lesions. Particularly in the case of cervical infections, these can eventually lead to the development of malignant tumors. As such, one might predict that human papillomaviruses (HPVs) would be likely to have evolved miRNAs as one tool to regulate the cellular environment of infected cells. However, initial computer analyses of the genome of the oncogenic HPV18 genotype predicted that HPV18 did not encode any viral miRNAs (Pfeffer et al., 2005). A more recent cDNA cloning analysis of the miRNAs present in

cells undergoing latent or lytic infection by a second oncogenic HPV, HPV31, failed to detect any viral miRNAs although numerous cellular miRNAs were recovered (Cai et al., 2006a). It therefore seems likely that at least the major oncogenic HPVs do not encode any viral miRNA. However, there are numerous HPV genotypes, many of which are quite divergent from HPV16 and HPV31, as well as even more divergent papillomavirus species that infect bovines, rabbits, and so on. Therefore, it remains possible that other members of this extensive virus family do encode miRNAs that are expressed during persistent infection of their cognate host species.

25.2.9 The Adenoviruses

As noted above, human adenoviruses generally express two ~160 nt long ncRNAs in infected cells, called VAI and VAII (Ma and Mathews, 1996; Mathews and Shenk, 1991). VAI is a potent inhibitor of PKR activation and therefore allows adenoviruses to avoid inhibition by the cellular interferon response (Katze et al., 1987; Mellits et al., 1990). However, recent data show that VAI also interacts with the host cell RNAi machinery (Andersson et al., 2005; Aparicio et al., 2006; Lu and Cullen, 2004; Sano et al., 2006).

VAI is transcribed in the nucleus by pol III and then is exported to the cytoplasm by Exp5, the same nuclear export factor utilized by pre-miRNAs (Gwizdek et al., 2001). However, while cellular miRNAs are expressed at a total level of $\sim 10^5$ copies/cell (Bartel, 2004), VAI is expressed at a far higher $\sim 10^8$ copies/cell during lytic adenoviral replication (Mathews and Shenk, 1991). Also, mutational definition of the structural requirements for nuclear export of either VAI or pre-miRNAs by Exp5 have shown that, for both RNA substrates, Exp5 recognizes a short terminal stem of $\geq$16 bp ending with a short, ~2 nt 3′ overhang (Gwizdek et al., 2001; Zeng and Cullen, 2004) (Figs. 25.1B and 1C). It is important to note that the requirements for RNA substrate recognition by Exp5 are closely similar to the RNA structure requirements for recognition by Dicer, which binds to and cleaves structured RNAs containing a terminal stem of $\geq$20 bp with a short, ~2 nt 3′ overhang (Zhang et al., 2004).

Initial analyses of the effect of VAI expression on miRNA biogenesis and function showed that VAI acted as a potent competitive inhibitor of pre-miRNA nuclear export, apparently by simply competing for a limiting supply of Exp5 (Lu and Cullen, 2004). In addition, it was demonstrated that VAI was able to bind Dicer and thereby inhibit Dicer cleavage of pre-miRNAs to form the miRNA duplex intermediate (Andersson et al., 2005; Lu and Cullen, 2004). The overall effect of VAI was therefore to strongly inhibit the de novo biogenesis and function of cellular miRNAs (Lu and Cullen, 2004).

Although VAI is a very poor substrate for Dicer cleavage, and primarily acts as a competitive inhibitor of Dicer function, some cleavage of VAI by Dicer does occur, resulting in the processing of ~0.5–1% of VAI in infected cells

(Andersson et al., 2005; Aparicio et al., 2006; Sano et al., 2006) (Fig. 25.1B). While this is clearly a very inefficient reaction, the high-level expression of VAI ($\sim 10^8$ copies/cell) means that a substantial number of VAI molecules are processed by Dicer, leading to the production of an adenovirus-derived miRNA that is loaded into RISC and that is functional in infected cells (Andersson et al., 2005; Aparicio et al., 2006; Sano et al., 2006). More recently, it has been argued that inhibition of the function of this VAI-derived adenovirus miRNA, using 2′ O-methyl antisense oligonucleotides, results in a modest, ~10-fold drop in the efficiency of adenovirus replication in culture (Aparicio et al., 2006). No mechanism underlying this reported inhibition has been reported, but clearly this adenovirus-derived miRNA could be targeting viral and/or cellular mRNAs.

At this point, we are left with three possibilities. On the one hand, adenovirus VAI may have evolved to not only block PKR activation but also to block cellular miRNA biogenesis and function (Andersson et al., 2005). How this would be of benefit to the virus is not entirely clear. Secondly, VAI (and perhaps VAII) may also have evolved to give rise to an adenovirus miRNA that, by targeting one or more unidentified mRNAs, somehow facilitates adenovirus lytic replication (Aparicio et al., 2006). Finally, these phenomena may all be physiologically irrelevant. It is possible that VAI has simply evolved to exit the nucleus by using Exp5 (no other factor that can export small RNAs that are not tRNAs from the nucleus is known). Because of the very high level of expression of VAI, this inadvertently blocks Exp5 function and hence, pre-miRNA export and processing. Because Dicer has essentially the same RNA substrate recognition characteristic as Exp5, VAI, like pre-miRNAs, is bound by Dicer after export resulting in an inadvertent inhibition in Dicer function. VAI has, however, evolved to be very ineffectively cleaved by Dicer, in order to maintain its ability to bind and inhibit PKR. Nevertheless, a small percentage of VAI – too small to affect VAI function – is cleaved by Dicer to give a viral miRNA that is loaded into RISC. This again may be inadvertent, i.e., this viral miRNA may have no role in the adenoviral life cycle and the drop in adenovirus replication noted by Aparicio et al. (2006), when this adenovirus miRNA was inhibited, could be non-specific. At present, it is not possible to distinguish between these mutually exclusive scenarios, and the functional significance of the interaction of adenovirus VAI with the cellular RNAi machinery therefore remains uncertain. Nevertheless, adenovirus clearly does produce at least one functional miRNA in infected cells (Table 25.2).

25.3 Conclusions

The study of viral ncRNA has not only been relevant to understanding viral replication cycles but also has been illuminative to host processes, in particular in the areas of miRNA biogenesis and editing. With that said, some viral

ncRNAs have been the subject of study for over a decade and their functions still remain unknown. So, while progress has been made, there is still much work to be done. As with their host counterparts there are many questions that remain regarding viral-derived regulatory ncRNAs. How have the viral ncRNAs evolved, were they pirated from the host, or do they arise as fortuitous accidents? Is the fact that some ncRNAs are packaged within virions simply a neutral phenomenon – a result of their sheer abundance – or does this packaging have biological significance? Do other ncRNAs, such as miRNAs, also enter with the virus upon infection? Most importantly, what are the functions of these ncRNAs, and how can we develop new technologies to uncover these functions (this latter point is particularly relevant to the non-miRNA viral ncRNAs).

While much remains unknown, it seems likely that we are about to witness rapid progress in this field. New mutant phenotypes are being reported for the larger viral ncRNAs and, because of the rapid progress in understanding the molecular biology of cellular miRNAs, we stand poised for major progress in identifying the molecular targets (both host and viral) of viral miRNAs. Because viruses expend a significant amount of metabolic energy generating these ncRNAs at such abundant levels, it is reasonable to assume that most of them will have important functions. Therefore, the study of viral ncRNAs should continue to be fruitful, contributing much to our understanding of the life cycles of the DNA tumor viruses and potentially identifying new targets for pharmacological intervention in the viral life cycle.

Acknowledgments Work in CSS lab is funded by UT Austin start up funds & a Fellowship from the UT Austin Institute for Cellular and Molecular Biology. Research into viral miRNA function in the Cullen laboratory is supported by NIH Grant AI067968. We thank Dr. Joan Steitz and Latham Fink for helpful comments regarding this chapter, and Jennifer Lin, Xuezhong Cai, Eva Gottwein, and Latham Fink for help with the figures. The manuscript proposing the existence of a miRNA called miR-LAT within the LAT transcripts of HSV-1 (Gupta et al., 2006) has now been retracted. A recent manuscript (Umbach, J. L., Kramer, M. F., Jurak, I., Coen, D. M., & Cullen, B. R. 2008, MicroRNAs expressed by Herpes Simplex Virus 1 during latent neuronal infection regulate viral gene expression. Nature, 454, 780–783) has now reported that HSV-1 LAT is processed to give rise to four viral miRNAs, miR-H2 to miR-H5, while an additional HSV-1 miRNA, miR-H6, was found to be encoded outside LAT. All five HSV-1 miRNA were found to be expressed in latently infected neurons *in vivo*. Moreover, miR-H2 and miR-H6 were found to inhibit expression of the HSV-1 immediate early transcription factors ICP-0 and ICP-4 respectively. This suggests that these viral miRNA may stabilize HSV-1 latency in infected neurons *in vivo*.

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Chapter 26
AIDS and Associated Malignancies

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Abstract Malignancies are a major complication of AIDS. The most frequently diagnosed HIV-associated cancer is Kaposi's sarcoma (KS) followed by AIDS-related non-Hodgkin lymphoma (ARL). The development of highly active antiretroviral therapy (HAART) has resulted in a marked decrease in the number of cases of KS and to a lesser degree ARL in countries where these drugs are widely available; however, these tumors are increasing in incidence in developing nations and still pose a major threat in severely immunocompromised patients. AIDS-related KS and ARL are distinct from their counterparts seen in HIV-1 seronegative patients. About half of all cases of ARLS are associated with a gamma herpesvirus, Epstein–Barr virus (EBV) or human herpesvirus-8 (HHV-8) and AIDS-related KS is far more aggressive than the classical variety of the disease. Several other types of tumors occur with increased frequency in AIDS patients including leiomyosarcoma in children, squamous cell carcinoma of the conjunctiva and multi-centric Castleman's disease. It is likely that as the epidemic expands and AIDS becomes more of a chronic condition the spectrum of HIV-associated cancer will also evolve.

26.1 AIDS-Related Lymphomas (ARL)

Chronically immunocompromised patients including organ transplant recipients, those with inherited immunodeficiencies or AIDS have a markedly increased risk of developing lymphoproliferative disease. The incidence of non-Hodgkin lymphomas (NHL) is increased nearly 200-fold in HIV-positive patients and about half of all

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_26, © Springer Science+Business Media, LLC 2009

cases of ARL are associated with a gamma herpesvirus, EBV or HHV-8 (Lim and Levine 2005; Scadden 2003; Rabkin et al. 1999). ARLs are often diagnosed at a very advanced stage in debilitated patients and frequently involve unusual anatomical sites (oral cavity, GI tract, central nervous system) (Carbone 2002; Ambinder 2001; Boshoff and Weiss 2002). The development of ARL is likely quite complex. Progressive immune suppression, chronic antigen stimulation and resultant B-cell proliferation, initially polyclonal and proceeding to oligo and monoclonal lymphoid expansion are probably critical to the evolution of these tumors. Elevated expression of activation-induced cytidine deaminase (AID) preceding the development of ARL has recently been reported. Associated immune activation and dysregulation of cytokine modulatory pathways (especially interleukin-6 and interleukin-10); altered bcl-6, p53 and c-myc expression and coexisting viral infection(s) have also been implicated in the pathogenesis of ARL (Nador et al. 2003; Carbone 2002; Epeldegui et al. 2007).

26.1.1 Subtypes of AIDS NHLs

Almost all ARLs are derived from B lymphocytes. Three varieties of ARL have been categorized by the World Health Organization and each of these into several subtypes. The most common types include those that also occur in HIV-negative patients such as Burkitt's (BL) and diffuse large cell (immunoblastic, anaplastic and diffuse) lymphomas (DLCL). These two varieties account for about 90% of ARLs (Carbone and Gloghini 2005; Bellan et al. 2003).

DLCL generally develops in the setting of moderate to severe immunosuppression (CD4+ lymphocyte counts below 100 mm^3/ml). These tumors, particularly the immunoblastic variant, are often associated with EBV and if so express the viral oncoprotein latent membrane protein-1 (LMP-1) (Carbone and Gloghini 2005; Knowles 2001). LMP-1 functions in a similar manner to tumor necrosis factor receptors by activating cellular anti-apoptotic transcription factors such as nuclear factor kappa B (NF-κB) (Soni et al. 2006; Eliopoulos et al. 1996). DLCLs frequently contain genetic alterations in Bcl-6. These events are thought to occur during the process of somatic hypermutation. Although the consequences of these mutations have not been fully defined recent reports indicate that Bcl-6 suppresses the expression of the p53 tumor suppressor (Phan and Dalla-Favera 2004).

AIDS-related Burkitt's lymphoma (BL), for reasons that remain unclear, generally occurs in more immunocompetent patients (Levine 2002). AIDS BLs share features with endemic-African BL in that both overexpress c-myc due to one of three reciprocal translocations that bring the transactivator under the influence of potent promoter sequences within immunoglobulin (Ig) gene loci. Inactivating mutations and deletions of the p53-ARF pathway is also common in all types of BL (Carbone 2003). A distinguishing feature between AIDS related and endemic BL is that the former is associated with EBV far less frequently than

the latter (30–35% versus nearly 100%) (Navarro and Kaplan 2006; Bociek 2005). Chronic stimulation of B cells whether driven by HIV antigenemia or malaria probably results in an increased likelihood of myc translocations. A hallmark of all variants of BL is their phenotypic similarity to germinal centroblasts (CD10+, CD77+ and Bcl-6+). These tumors are incredibly aggressive with brief doubling times. Flow cytometric analysis typically reveals that over 90% are in S phase (Knowles 2001; Carbone 2003; Carbone and Gloghini 2005). Ongoing tumor lysis syndrome even in the absence of concomitant chemotherapy is often noted. AIDS-related BLs carry a poor prognosis even when compared to AIDS-related DLCL (Levine 2002; Lim et al. 2005).

A recently described variant of ARL, plasmablastic lymphoma, occurs in a small percentage of HIV+ patients. These tumors are generally associated with EBV or HHV-8 (Cioc et al. 2004; Cattaneo et al. 2005). As opposed to BL or DLCL plasmablastic lymphomas are typically CD20 and bcl-6 negative. Response to conventional chemotherapy is poor and some investigators have suggested that viral-targeted approaches may be beneficial (Oksenhendler et al. 2002).

A rapidly fatal subtype of AIDS NHL is primary central nervous system lymphoma (PCNSL). These tumors are most frequently classified as large cell immunoblastic and occur in the most immunosuppressed patients. In contrast to PCNSL in HIV-negative patients they are virtually always associated with EBV (Newell et al. 2004). Post-transplant PCNSL is also an EBV+ tumor. Recent published data suggest that the lack of EBV-specific CD4+ T cells puts HIV+ patients at risk for the development of PCNSL regardless of the overall CD4 count (Gasser et al. 2007). These tumors are generally LMP-1+ and have recently been shown to express IRF-7 (Zhang et al. 2004). Detection of EBV sequences in the cerebrospinal fluid by polymerase chain reaction (PCR) coupled with positive thallium spectroscopy has proven to be a helpful diagnostic tool in these patients who are generally poor candidates for risky diagnostic procedures (Cingolani et al. 1998). These patients are often afflicted by many complications of HIV infection. Standard therapy with conventional chemotherapy combined with radiation therapy results in only about a 4-month survival although long-term remission has been reported in patients treated with HAART alone (Hoffmann et al. 2001) and others (including post-transplant PCNSL) with high-dose zidovudine and ganciclovir (Raez et al. 1999; Roychowdhury et al. 2003) .

The most commonly identified virus associated with AIDS-related lymphomas is EBV and there is a large body of published work on the oncogenic mechanisms of this agent. B lymphocytes transformed by EBV (lymphoblastoid cell lines) in vitro express an array of virus-encoded proteins including six EBV nuclear antigens (EBNAs) and three LMPs. EBNAs are generated from differential splicing of a transcript that arises from one of two promoters (Cp or Wp). This form of latency is termed latency III, the type observed in immunoblastic lymphomas (Carter et al. 2002; Young and Rickinson 2004). A type II form of latency where EBNA 1, LMP-1 and LMP-2a are expressed has been identified in some EBV-associated lymphomas, most notably Hodgkin disease. In latency I (typical of Burkitt's lymphomas) only EBNA-1 (generated from the Qp

promoter) and EBV-encoded RNAs (EBERs) are expressed (Young et al. 2004; Kelly et al. 2005). Recent studies have indicated that some heterogeneity in EBV gene expression and EBNA promoter usage exists among endemic BL and may correlate with a variable response to therapy (Kelly et al. 2002).

26.1.2 Primary Effusion Lymphomas

In addition to Kaposi sarcoma, HHV-8 has also been associated with two other AIDS-associated neoplasms, primary effusion lymphoma (PEL) and multi-centric Castleman's disease (MCD). PELs were first identified as a unique subset of body-cavity-based lymphomas, which were subsequently called PELs (Cesarman et al. 1995; Cesarman et al. 1996). In reality neither term is totally accurate since solid tumor variants of HHV-8-associated lymphomas have been identified (Carbone and Gloghini 2007). PELs are unique as they carry HHV-8, are most frequently diagnosed in men (often with a previous history of KS) and occur almost exclusively in immunocompromised patients. This type of lymphoma is distinguished from others by its distinctive morphology that bridges large cell immunoblastic lymphoma and anaplastic large cell lymphoma. PELs most often present as lymphomatous effusions in the pleural, peritoneal and/or pericardial cavity. PELs are usually CD20 negative and often express CD45 and CD30. These lymphomas are usually of B-cell origin with clonal immunoglobulin gene rearrangements. Most are co-infected with EBV but lack e-myc gene rearrangements and LMP-1 is expressed at very low levels if at all (Horenstein et al. 1997; Carbone and Gloghini 2007). Diagnosis and association with HHV-8 is usually confirmed by immunohistochemical analysis with the monoclonal antibody to the viral latency-associated nuclear antigen (LANA). PELs are relatively rare tumors, and estimated to be about 0.13% of all AIDS-related malignancies in AIDS patients in the United States (Engels 2007). Thus, HHV-8-associated lymphomas represent a rare, distinct pathobiologic category which is often, but not always, associated with an effusion in AIDS patients. The role of HHV-8 in the development of these lymphomas is not clear since this type of tumor is still rare even in populations with high seroprevalence rates. Other events must be needed for the development of PELs which may include EBV infection and/or immunosuppression. Unlike BL these tumors are most often p53 wild type (Petre et al. 2007). Solid tumor variants with plasmablastic features tend to be rapidly fatal although recent data suggest that PEL lines are quite sensitive to inhibition of NF-κB and may be responsive to antiviral therapy (Keller et al. 2006; Ghosh et al. 2003).

26.1.3 Prognostic Factors in ARL

Prior to the widespread implementation of HAART, clinical prognostic indicators (prior opportunistic infections, poor performance status and low-CD4

lymphocyte count) or lymphoma-specific factors (stage, elevated lactate dehydrogenase and extranodal involvement) were shown to be predictive of the outcome of HIV-positive DLBCL patients. With the introduction of HAART, the outcome of ARL has improved, with the currently reported median duration of survival of 15–34 months (Navarro and Kaplan 2006; Barclay et al. 2007). Identification of molecular prognostic factors that more accurately predict DLBCL outcomes in HIV-positive patients is therefore of great importance and may form the basis for risk-adapted treatments. Beyond outcome prediction, molecular prognostic models may provide the rationale for new therapeutic initiatives. These studies have not been done although they should be feasible given the substantial number of patients that have been enrolled on clinical trials.

26.1.4 Diagnosis and Therapy of AIDS NHL

Diagnosis of ARL is established by pathological confirmation of malignant lymphoma on biopsy material of involved lymph node(s), bone marrow or other extranodal site(s). Tissue diagnosis of AIDS-related CNS lymphoma is often quite difficult due to the invasive nature of the procedure and the debilitated state of most patients. In the absence of tissue confirmation the detection of EBV DNA in cerebrospinal fluid by polymerase chain reaction coupled with imaging studies is strongly suggestive of the diagnosis. Treatment of the ARL remains challenging. Polychemotherapy regimens have produced similar results although regimens that combine potent antiretrovirals with conventional chemotherapy may prove superior (Navarro and Kaplan 2006; Yarchoan et al. 2005). HIV-positive patients often have poor bone marrow reserve which compromises the ability to deliver full dose chemotherapy. Concomitant opportunistic infections may also lead to a decrease in chemotherapy delivery (Lim et al. 2005). In general, response and survival rates for common NHL regimens are lower than for the HIV-negative population. Complete responses occur but tend to be of shorter duration with frequent relapses. Our experience has been that patients concomitantly diagnosed with HIV infection and lymphomas do better with antiretroviral and anti-lymphoma therapy than do those who develop lymphoma after becoming refractory to antiretrovirals. A recently completed study performed by the NCI-sponsored AIDS malignancy consortium (AMC) demonstrated the feasibility of concomitant chemotherapy with HAART (Ratner et al. 2001). Probably the best reported results for chemotherapy in ARL were from the group at the National Cancer Institute (Little et al. 2003). Using the EPOCH regimen, they achieved remission in 22 of 24 patients with a progression-free survival of 23 months. These patients had favorable prognostic factors (median CD4+ lymphocyte count of 233 mm^3/ml). HIV+ patients are prone to develop complications that are not seen in the general population. Enhanced toxicity of rituximab (an anti-CD20 antibody) and

CHOP chemotherapy was recently noted in a large multi-center trial conducted by the AMC (Kaplan et al. 2005). The addition of rituximab as compared to CHOP alone led to increased infectious complications and deaths attributable to sepsis. It is possible that delayed recovery of humoral immunity could contribute to this increased risk of life-threatening bacterial infections in HIV-infected patients. There have been several reports on the feasibility and efficacy of high-dose chemotherapy and autologous stem-cell transplant for ARL (Behler and Kaplan 2006; Re et al. 2003). It seems reasonable that patients with well-controlled HIV and good performance status should be considered candidates for this therapy. Newer approaches that may benefit patients with ARL include EBV-specific cytotoxic T cells, inhibitors of NF-κB and agents that activate the lytic program of gamma herpesviruses, thereby sensitizing the tumors to antivirals (Rooney et al. 1998; Feng et al. 2004; Kurokowa et al. 2005).

26.2 Kaposi's Sarcoma

Kaposi's sarcoma was first described by Moritz Kaposi in 1872 in several cases of multi-focal pigmented sarcoma in elderly Mediterranean men (Kaposi 1872). There are four forms of KS. The first is known as classic KS or sporadic KS. These tumors are mainly found in elderly men from Mediterranean countries, such as Italy (Iscovich et al. 2000). The lesions tend to be found in the lower extremities and are generally indolent. The second type, known as endemic-African KS, is more aggressive than the classic KS and can also involve the lymph nodes. This form of KS was seen in the African continent prior to the HIV epidemic and found in adults (both men and women) and in children (Franceschi and Geddes 1995). The third is the iatrogenic form of KS, which normally occurs after transplantation in patients treated with immunosuppressives. This form of KS seems to vary in geographical prevalence, and is more common in individuals of Mediterranean ancestry (Zmonarski et al. 2005). The fourth form of KS is AIDS related. This is a very aggressive variant first described in early 1980 s in homosexual men (Friedman-Kien 1981). AIDS-KS not only involves skin, but may also involve lymph nodes and visceral organs such as the lungs, gastrointestinal tract, liver and spleen.

KS is composed of a mixture of irregular shaped, round capillaries, and slit-like endothelium-lined vascular spaces and spindle-shape cells with infiltrating mononuclear cells. It is not clear whether KS represents a clonal neoplastic process or a polyclonal inflammatory lesion. Studies have shown monoclonal, oligoclonal and polyclonal lesions in different patients (Dezube 2000; Gill et al. 1998). The origin of KS spindle cells is also not clear; it has been suggested that KS cells represent a heterogeneous population of cells, arising from pluripotent mesenchymal precursor cells and may be of lymphatic endothelial cell origin (Wang et al. 2004).

26.2.1 Human Herpesvirus and KS

An infectious agent has long been suspected in the development of KS. In 1994, a novel human herpesvirus was identified by Chang and Moore (Chang et al. 1994). For more on the discovery of KSHV the readers are referred to Chapter 18 (Introduction to Diseases Associated with Kaposi's Sarcoma-Associated Herpesvirus). This virus is now known as KSHV or HHV-8 and is necessary, but not sufficient for the development of all types of KS. It is clear that other co-factors, such as immunosuppression, are required for KS development. HHV-8 is found in all KS lesions, and is mainly located in the vascular endothelial cells and perivascular spindle-shaped cells (Wang et al. 2004; Li et al. 1996).

HHV-8 belongs to the γ-herpesvirus family, which can further be divided into two subgroups, γ-1 or lymphocrytovirus and γ-2 or rhadinovirus. EBV is the prototype of γ-1 virus and the simian herpesvirus saimini is the prototype of γ-2 herpesvirus. HHV-8 is classified as a γ-2 rhadinovirus and is the first human virus of this subfamily identified. Like other herpesviruses, HHV-8 is a double-stranded deoxyribonucleic acid (DNA) virus (Edelman 2005; Roizmann et al. 1992). Its genome is linear, is about 165 kbp in length and contains at least 87 viral genes. A feature of some DNA viruses, particularly of herpesviruses and HHV-8, is the ability of these viruses to incorporate or pirate host genes into their genome: these genes can then play a role in the replication, survival and transformation functions of the virus. HHV-8 encodes human homologous genes that regulate cell cycling like cyclin D, growth factors like interleukin 6 or genes that may prevent programmed cell death such as bcl-2 (Schulz 2000). Deciphering the functions of these viral genes will lead to a better understanding of viral oncogenesis and potential therapy.

Unlike most other herpesviruses, HHV-8 infection does not seem to be widely distributed in most populations. Its detection relies on the presence of antibodies against either lytic and/or latent antigens and varies among the different tests that were used in different seroprevalence studies. In general, the frequency of infection appears to be low in North America, certain Asian countries and in Northern European nations such as the United Kingdom and Germany, with most studies reporting a seroprevalence rate in normal blood donors of less than 5% (Pellet et al. 2003). In these countries the seroprevalence of KSHV in different risk groups mirrors the incidence of AIDS-KS, with a seroprevalence rate of between 25 and 50% among homosexual men. In other countries such as Italy, Greece and Israel, especially Southern Italy, the infection rate seems to be much higher in the general population, and is more variable, ranging between 5 and 35%. In contrast to North America and Europe, HHV-8 infection is widespread in the African continent. High seroprevalence rates between 40 and 50% have been found in Central, West as well as South Africa (Gao et al. 1996). Therefore, HHV-8 seroprevalence tracks very closely with KS, with the highest infection rate in geographic areas where classic or endemic forms of KS are more common. KS has a particularly high incidence

in Central African countries like the Republic of Congo, Uganda and Zambia; these countries also have the highest HHV-8 infection rates in the world (He et al. 1998; Olsen et al. 1998).

26.2.2 Impact of HAART on KS

Since the beginning of the AIDS epidemic in the early 1980 s, AIDS-KS has become one of the most common AIDS-associated malignancies with HIV-infected homosexual males at the highest risk, and those with AIDS had a 50% lifetime rate of developing KS early in the HIV epidemic (Katz et al.1994). However, the rate of AIDS-KS has since steadily declined both in the United States and the Europe (Beral 1991). It has been suggested that the disease may have shifted from an early disease to a late manifestation during the course of HIV infection. Several studies have shown that there was a marked decrease in KS incidence since HAART was introduced, a decline of nearly 80-fold. Interestingly, the reduced KS risk was only observed with HAART, but not with double or single anti-HIV drugs (Jones et al. 2000). Even though the incidence of KS in the treated HIV-infected individuals in the Western world has decreased dramatically, in the setting where HAART is still not widely available, such as sub-Saharan Africa, AIDS-KS still remains a major problem.

26.2.3 Therapy for AIDS-Related KS

Therapy for AIDS-related KS is chronic and unlike ARL, chemotherapy by itself rarely, if ever, produces complete remissions. Prior to the institution of HAART a variety of agents were employed such as interferon and Velban and were used with some success (Krown 1998). One of the most interesting and as yet poorly understood clinical scenarios is the disappearance of KS in some patients treated with HAART alone (Tam et al. 2002; Jones et al. 2000). This occurs with sufficient frequency that most experienced practitioners will try this approach in patients with non-life-threatening disease. Despite this, it is not uncommon to see patients with aggressive KS despite well-controlled HIV infection. The mainstays of therapy for KS now are the liposomal anthracyclines doxil and daunosome (Dezube 2000). These drugs are highly active in the disease and have a generally favorable toxicity profile. Another commonly used agent is taxol and combinations (doxil/taxol) have been used successfully in some refractory patients. Recently more targeted approaches have been employed for KS. These have been made possible by the evolution in the understanding of the disease. HHV-8 infection of dermal microvasculature endothelial cells is associated with the upregulation of the c-kit receptor, and expression of c-kit in HIV-related KS in vivo has been demonstrated by immunohistochemistry (Asou et al. 1998; Moses et al. 2002). These observations have led to small clinical trials in KS with the c-kit inhibitor imatinib mesylate (Gleevec). In some patients responses have been

observed. An important observation was made in 2005 when clinicians noticed that renal transplant patients with KS on cyclosporine experienced regression of their disease when switched to an alternative immunosuppressant, rapamycin (sirolimus) (Campistol et al. 2004; Stallone et al. 2005) . This is thought to occur due to the effect of the drugs on the mammalian target of rapamycin (mTOR) and has led to novel clinical trials which are now underway. Interestingly there is evidence that this agent may also be active in PEL (Sin et al. 2007).

26.2.4 Multi-centric Castleman's Disease

Multi-centric Castleman's disease (MCD) has also been associated with HHV-8 infection. MCD is a rare and poorly understood B-cell lymphoproliferative disorder with vascular proliferation in the germinal centers, and is thought to be related to immune dysregulation (Corbellino et al. 1996). HHV-8 is found in almost all cases of MCD in AIDS patients and in about 50% of cases of MCD in HIV-negative individuals (Sarid et al. 2002). However, the role of KSHV in the pathobiology of MCD is not well understood, and it is not clear whether there are any clinical differences between those with and without HHV-8. The diagnosis of Castleman's disease is made by histological analysis of the lesion. There are several variants of the tumor. The hyaline vascular variant is characterized by extensive capillary proliferation and a lymphocyte-predominant infiltrate surrounding small germinal centers. The plasma cell variant has prominent sheets of plasma cells within the interfollicular tissues surrounding germinal centers (Corbellino et al. 1996). MCD is almost exclusively derived from the plasma cell variant. Patients with AIDS-related MCD are often acutely ill. The clinical manifestations are fever, cytopenias, organomegaly and polyclonal hypergammaglobulinemia. Interestingly, the levels of detectable HHV-8 have been found to correlate with the symptoms associated with the disease. In addition there appears to be a substantial amount of HHV-8 lytic activity associated with this disease (as opposed to KS and PEL). There is also strong evidence of a role played by vascular endothelial growth factor (VEGF) in MCD as well as human and viral (HHV-8)-associated IL-6 (Oksenhendler et al. 2000; Aoki et al. 2001). A variety of cytoreductive therapies have been employed in HIV-related MCD although not within the context of a clinical trial. Newer approaches have included rituxan, interferons and antiviral agents (Corbellino et al. 2001; Ocio et al. 2005; Marcelin et al. 2003; Casper et al. 2004)
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26.3 Human Papilloma Virus (HPV)-Associated Malignancies

It is now widely accepted that 95% of invasive cervical cancers worldwide are linked to human papilloma virus (HPV) infection (Walboomers et al. 1999; zur Hausen et al. 1999). Genital cancers have been a worldwide public health problem,

especially in the context of HIV infection and immunosuppression. HIV infection has been shown to substantially enhance the development of cervical cancer precursor lesions (Bosch et al. 2002; Massad et al. 2001). Since 1993, the CDC has included invasive cervical cancer as one of the AIDS-defining illnesses. In the US adult population, about 20 million are infected with genital HPVs and there are 5.5 million new infections each year (Cates and Dallabetta 1999). It is also now the most common malignancy in women in many developing countries, especially in sub-Saharan Africa where HIV infection is epidemic (Waggoner 2003). The rates of cervical cancer in Africa are fourfold higher in than in North America and Europe.

HPV infection of genitals can either lead to asymptomatic infection or a wide range of genital lesions, ranging from genital warts to mild dysplasian to invasive carcinomas. Genital lesions are often referred to as cervical intraepithelial neoplasia or CIN, which is graded from I to III depending on the degree of epithelial atypia. CIN1 encompasses mild dysplasia or low-grade squamous intraepithelial lesions (SIL). CIN II represents high-grade SIL with moderate dysplasia, while severe dysplasia is referred to as CIN III (Massad et al. 1999).

HIV and HPV infections have a number of common features, both are sexually transmitted diseases (STD) and there is a high prevalence of HPV infection among HIV-seropositive women, especially those with low-CD4+ T-cell counts (Ahdieh et al. 2001; Sun et al. 1997). A number of studies have shown a strong and consistent association between HIV and HPV co-infection and the development of CIN and genital cancer (Chiasson et al. 1997, Heard et al. 2000; Palefsky and Barrasso 1996; Sirera et al. 2006), and HIV-positive women have a significantly higher rate of CIN than their counterparts and are more likely to progress to invasive carcinoma than HIV-negative women (Durante et al. 2003; Williams et al. 1994). Up to 20% of the HIV and HPV co-infected individuals developed HPV-associated pre-malignant lesions of the uterine cervix within 3 years of HIV infection (Ellerbrock et al. 2000). A study by Silverberg et al. (Silverberg et al. 2002) found that HIV-seropositive women were 3.2-fold more likely to present with genital warts than HIV-seronegative women. The prevalence and severity of genital tract infection in these women are also more pronounced (Duerr et al. 1997; Minkoff et al. 1999). The rates of invasive cervical carcinoma are 15–18 times higher in women with AIDS compared to the general population (Franceschi et al. 1998; Serraino 1999). Several studies have also shown that HIV-positive women have 2–3 times more HPV DNA in cervicovaginal washings and 15 times more in anal swabs as compared to HIV-negative individuals (Hillemanns et al. 1996, Sun et al. 1997).

Even though HPVs are most commonly associated with cervical cancer, it is now known that a number of other cancers are also caused by HPV. They include penile, anal, oral and conjunctival malignancies (Koutsky 1997; Newton et al. 2002; Syrjanen 2003; Waddell et al. 1996). About 80% of the anal cancers are found in HPV-infected individuals (Hankey et al. 1999; Zippin and Lum 1993). Even though these cancers are relatively rare it appears that the

incidence has been rising in the past 10 years, despite the implementation of HAART (Gloeckler Ries et al. 2003). The incidence of anal cancer in men with history of anal intercourse is at 35 per 100,000 individuals (Piketty et al. 2003; Sun et al. 1995). The highest level of risk for anal cancer caused by HPV is associated with men who have sex with men (MSM) (Piketty et al. 2004). Among MSM who are HIV positive, the rate of anal cancer is twofold higher than HIV-negative MSM (Fakhry and Gillison 2006). High-risk HPVs have also been implicated recently in approximately 30% of oral cancers (Gillison et al. 2000). In fact HPVs are responsible for cancers in the tonsils, the palate, gums, tongue and the larynx (Aaltonen et al. 2005; de Villiers et al. 1986; El-Mofty and Lu 2003; Lopez Amado et al. 1996; Syrjanen 2005). High-risk HPVs have been further implicated in upper respiratory tract and lung cancers (Cheah and Looi 1998; Clarke et al. 1991). Furthermore, evidence suggests that some digestive cancers are also HPV positive (Milde-Langosch et al. 1989).

26.3.1 Effects of HAART on HPV-Associated Cancers

The effects of HAART on HIV and the immune status of HIV-infected individuals are well known, but its effects on the course of HPV-related cervical lesions in HIV-infected women are still not well established. HAART has not been shown to affect HPV detection and its effects on the natural history of cervical intraepithelial neoplasia are unclear (Palefsky 2003). There were a number of studies examining the effects of HAART on the course of cervical lesions (Heard et al. 1998). A study by the Women's Interagency HIV Study (WIHS) group has reported on the regression of cervical lesions with the use of HAART (Minkoff et al. 2001) while others did not (Lillo et al. 2001). In addition, a 5-year multi-center study of the effects of HAART on cervical lesions in over 700 women followed also did not show any correlation (Schuman et al. 2003). However, these studies primarily focused on evaluating the effects of HAART on existing cervical lesions and the date of onset of those lesions is not known; thus it is not surprising that their outcome was controversial. To address these concerns, a study by Ahdieh-Grant et al. (Ahdieh-Grant et al. 2004) determined whether HAART alters the natural history of CIN among HIV-infected women that were regularly followed every 6 months for 7 years. This study provided evidence that HAART has a modest benefit for HIV-infected women that were at risk for cervical neoplasia. With the success of the HPV vaccine, the potential exists to prevent HPV infection and hence the development of cancers associated with this virus. The vaccine has the long-term potential to reduce HPV-induced cancers by 70%. Even though the availability of the vaccine is increasing the cost of the vaccine is still too high for many developing countries, and population-based studies indicate that, until all girls are immunized prior to the onset of sexual activity, the vaccine will prevent only 30–50% of cervical malignancies. Thus it is expected most countries, especially those that are afflicted

by the HIV epidemic will continue to face a great deal of cervical cancer morbidity and mortality in the years to come.

26.4 Future Outlook

Since the introduction of HAART, there has been a dramatic improvement on the prognosis for patients with AIDS-associated cancers. There has also been a substantial decrease in the number of cases of KS and NHL. In spite of the improvement in prognosis of cancers in HIV-infected patients, there are still concerns. Given the prolonged survival rate of HIV-infected individuals with HAART, it is likely that AIDS-associated malignancies will continue to pose a challenge in the AIDS epidemic. HAART treatment, even though effective, still only leads to partial immune reconstitution. Prolonged immunosuppression is likely to lead to a resurgence of AIDS-associated cancers and perhaps different varieties than those noted previously. Moreover, even though HAART has been introduced in parts of the world where AIDS has the greatest impact, such as the African continent, it is still not widely available. It is expected that AIDS-associated cancers will continue to pose a major challenge in these populations. Since many of the cancers associated with HIV-infected individuals were found to have viral etiology, the development of effective strategies for preventing or controlling the infection by these viral agents will be of paramount importance. In addition, as the population living with HIV increases and ages we may expect that cancers seen in older patients (lung, breast, digestive) will become more common.

Acknowledgment This publication was made possible, in part, by support from the following NIH grants: PHS awards CA70058 and CA82274 to WH and CA76958, NCRR COBRE grant RR15635 and INBRE grant P20 RR016469 to CW.

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Chapter 27
Primate Models for Gammaherpesvirus-Associated Malignancies

Ilhem Messaoudi, Blossom Damania, and Scott W. Wong

27.1 Introduction

Non-human primates (NHP) represent an invaluable resource for elucidating and understanding disease processes in humans, as humans and NHP share close developmental, physiological and evolutionary relationships (Hendrickx and Binkerd 1990). For infectious disease research, NHP have historically played an important role as they are either susceptible to infectious agents that cause disease in humans (Kirschstein et al. 1960) or harbor infectious agents that are closely related to those that infect and cause disease in humans (Wenner et al. 1975). For example, NHP harbor herpesviruses that have co-evolved with their hosts and are genetically more closely related to human herpesviruses than other mammalian herpesviruses. More importantly, these simian herpesvirus homologues, which include alpha (α), beta (β) and gamma(γ)herpesviruses, are capable of causing similar, if not identical, disease manifestations in their natural host, which makes them excellent models to dissect the complicated host–pathogen interactions that lead to disease.

NHP can be divided into two groups, Old World and New World, both of which harbor γ-herpesviruses that can be divided into two classes: lymphocryptovirus (γ-1) and rhadinovirus (γ-2). The phylogenetic relationship of the simian γ-herpesviruses with human γ-herpesviruses is shown in Fig. 27.1, and demonstrates their close evolutionarily relationships. The remainder of this chapter will discuss representative simian γ-herpesviruses from Old World and New World monkeys and their utility as models of human disease. Table 27.1 lists the γ-herpesviruses identified to date. Some of these viruses have not been isolated and cultured, but have been so named on the basis of limited DNA sequence analysis.

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_27, © Springer Science+Business Media, LLC 2009

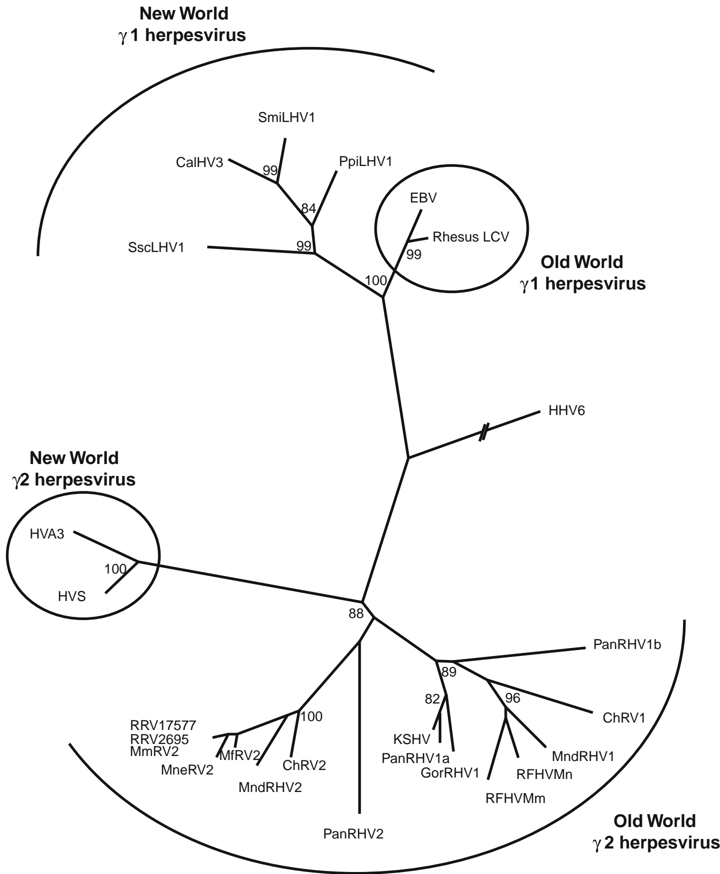

Fig. 27.1 Phylogenetic analysis of the DNA polymerase genes of representative primate γ-herpesviruses (adapted from de Thoisy et al. 2003). Protein distance tree for the 142 amino acid residues of primate γ-herpesvirus DNA polymerase identified by degenerate PCR analysis (Rose et al. 1997). Sequences were aligned by ClustalW and were analyzed with the PROTDIST and NEIGHBOR programs in PHYLIP

27.2 Rhadinovirus of New World NHP: *Herpesvirus saimiri*

Herpesvirus saimiri (HVS, saimirine herpesvirus 2) is the prototypical *rhadinovirus*, whose natural host is the squirrel monkey (*Saimiri sciureus*). It was originally isolated from a kidney cell culture of a healthy squirrel monkey (Melendez et al. 1968). The closely related *Herpesvirus ateles* (HVA) can be isolated from spider monkeys (Melendez et al. 1972). In the wild, squirrel monkeys are infected via saliva within the first 2 years of life and remain asymptomatically and persistently infected for the remainder of their lives (Melendez et al. 1968). In fact, HVS can be isolated from peripheral blood

cells of persistently infected squirrel monkeys by co-culture with permissive cell lines such as owl monkey kidney cell line (OMK, reviewed in (Desrosiers and Falk 1982)). Although HVS infects squirrel monkeys without any apparent development of disease, the virus can cause fatal T-cell lymphomas in other New World primates such as tamarins, marmosets and owl monkeys within 2 months following experimental inoculation (reviewed in (Fickenscher and Fleckenstein 2001)). Interestingly, HVS can also cause T-cell lymphomas in Old World monkeys such as rhesus macaques and cynomologus macaques. Furthermore, HVS can transform human T cells in vitro to continuous growth, which can provide useful tools for T-cell immunology studies. A series of transformed T-cell lines that were derived from tumors of virus-infected tamarins could be cultivated for several years. However, virus production was lost after prolonged culture and episomal viral DNA was found heavily methylated and some of the cell lines carried several mutations including large deletions (Desrosiers et al. 1979; Kaschka-Dierich et al. 1982). Future studies of HVS biology will be facilitated by the availability of a bacmid clone of HVS strain C488 (Ensser and Fleckenstein 2005).

HVS strains can be classified into three subtypes – A, B and C – based on their pathogenic potential and sequence divergence in the left-terminal non-repetitive genomic region. Subgroup C strains have the most oncogenic properties, whereas subgroup B contains the least oncogenic strains. HVS strains A and C immortalize common marmoset T lymphocytes to IL-2-independent growth, and the most highly oncogenic HVS strain C can also immortalize human, rabbit and rhesus monkey lymphocytes and cause fulminant lymphomas in Old World as well as New World primates. The most studied representative strains are A11 (Falk et al. 1972) for subgroup A, B-S295C and B-SMHI (Daniel et al. 1975) for subgroup B and C488 (Biesinger et al. 1990) for subgroup C.

The genomes of two HVS strains have been sequenced: A11 (Albrecht et al. 1992) and C488 (Ensser et al. 2003). The HVS genomes contained 76–77 open-reading frames (ORFs) and 5–7 URNAs. The closely related HVA strain #73 contains 73 ORFs and 2 URNA genes (Albrecht 2000). While most genes are well conserved amongst different HVS strains, there is extensive sequence divergence at the left end of the HVS L-DNA and in the region of the transactivator gene ORF50 and the glycoprotein ORF51 discussed later in this section. The classification of HVS genes into immediate early, early and late has been severely hampered by the fact that HVS infection occurs in an asynchronous manner (Randall et al. 1985). Therefore, current classification was based on studies where cyclohexamide was used to inhibit viral protein synthesis.

Three genes were classified as IE genes: ORF14, ORF57 and ORF50. ORF14 encodes a gene product with homology to murine superantigens. Later experiments indicated that it was dispensable for virus replication (Duboise et al. 1998), which renders a regulatory role for ORF14 rather unlikely. ORF50 encodes a larger protein, ORF50A, and a smaller C-terminal variant, ORF50B, due to differential promoter usage and splicing. Viral transactivator function analogous to that of the R transactivator of EBV (BRLF1) was mapped to the C-terminus of

both proteins (Nicholas et al. 1991). ORF50A transactivated transcription from early virus gene promoters such as ORF6, a major DNA-binding protein. The genomic region of ORF50 was found to be highly variable between different HVS strains. In fact, the most pronounced diversity was observed for ORF50B and adjacent ORF51. The whole region occurs in two alleles: one specific for subgroups A and B and a second allele specific for subgroup C strains. Therefore, it seems that the different alleles of ORF50B segregate with the transforming ability of the subgroup (Ensser et al. 2003).

Last, but not least, ORF57 encodes a nuclear phosphoprotein with sequence homology to IE63/ICP27 of herpes simplex and BMLF1/EB2 of EBV. Correspondingly, IE57 stimulates the expression of unspliced and suppresses that of spliced viral RNA transcripts at a post-transcriptional level (Whitehouse et al. 1998). In addition, IE57 regulates nuclear RNA export (Goodwin et al. 2000; Goodwin et al. 1999), and participates in the redistribution of the nuclear components of the splicing machinery. Therefore, IE57 appears to be a transcriptional regulator. ORF57 was found to regulate the expression of ORF50 as well as its own (Goodwin et al. 2000).

HVS ORF73 was classified as a latency-associated nuclear antigen. ORF73 protein co-localizes to the host cell nucleus and, like the latency-associated antigen LANA of KSHV, it can associate with chromosomal DNA (Schafer et al. 2003). It can also form dimers and associate with mitotic chromosomes via carboxyterminal domains (Calderwood et al. 2004). Furthermore, ORF73 was shown to interact with ORF50 and prevent entry into lytic phase by down-regulating the activity of ORF50A and ORF50B promoters (Schafer et al. 2003). However, unlike LANA, ORF73 expression in HVS-transformed human T cells is not detectable by northern blot (Hall et al. 2000).

27.3 HVS Homologues of Cellular Genes

Like other γ2-herpesviruses, HVS encodes a battery of viral genes encoding homologues of cellular proteins. The acquisition of these genes remains a controversial topic to this day since they are intron-less in the viral genome. These genes can be divided into several subclasses (described below) and can encode proteins that function similarly to their cellular counterpart or modulate the activity of their cellular counterpart. The deletion of these genes was shown in several studies not to interfere with viral replication and transformation in different in vitro models. The lack of specific pathogen-free squirrel monkeys has, however, prevented the rigorous testing of the function on these different proteins in their natural host. These observations led to the current belief that viral homologues are more important in the establishment of latency and evading host detection rather than mediating pathogenesis. As mentioned above, formal proof for the importance of these different proteins in pathogenesis will await the availability of HVS-free squirrel monkeys.

27.3.1 Viral Homologues of Cell Cycle-Associated Proteins

ORF2 of HVS encodes a homologue of dihydrofolate reductase (DHFR). ORF70 encodes a homologue of thymidylate synthase (TS). In addition, ORF3 and ORF75 encode large tegument proteins which share local homology to formylglycineamide ribotide amidotransferase (FAGART) (summarized in (Ensser et al. 2003)). The function of these enzymes may be to increase the pool of free nucleotides thereby facilitating or enhancing viral DNA replication.

ORF72 encodes a homologue of cyclin-D which, unlike its cellular counterpart, is resistant to inhibition by cyclin-dependent kinase inhibitors (Jung et al. 1994). Therefore v-cyclin has the ability to push the cell cycle through S phase. Interestingly the deletion of ORF72 has no impact on viral replication in vitro.

27.3.2 Viral Homologues of Complement Cascade

Two viral homologues of complement cascade regulators are encoded by HVS. ORF4 encodes a complement control protein that inhibits C3 convertase, a critical enzyme to the initiation of the complement cascade (Fodor et al. 1995). ORF15 is a viral variant of CD59, a protein that prevents the insertion of the membrane attack complex formed by C8 and C9. ORF15 therefore has the potential to block the terminal complement cascade (Rother et al. 1994).

27.3.3 Viral Homologues of Anti-apoptotic Molecules

ORF16 protein is a viral Bcl-2 that can stabilize mitochondrial membranes. Furthermore, studies have shown that this protein can inhibit apoptosis induced by dexamethasone, menadione or by irradiation (Bellows et al. 2000;Derfuss et al. 1998;Nava et al. 1997). These data suggest that this viral Bcl-2 can interfere with both cell autonomous or receptor-mediated cell death. ORF71 encodes a viral FLICE (FADD-like interleukin 1-converting enzyme-like protease) inhibitory protein, referred to as vFLIP (Thome et al. 1997). This protein can inhibit the formation of the death signal-induced signaling complex and consequently prevent caspase 8 activation.

27.3.4 Viral Homologues of Growth Factors

ORF13 is a viral homologue of IL-17 (Rouvier et al. 1993), a cytokine normally secreted by a specific subset of CD4 T cells referred to as TH17. IL-17 has pleiotropic effects, and induces the secretion of several other cytokines such as IL-6, IL-8, G-CSF by fibroblasts, endothelial and epithelial cells (Stockinger

et al. 2007). IL-17 can also induce the maturation of hematopoeitic progenitor cells into neutrophils and supports the proliferation of T cells.

ORF74 is viral G-protein-coupled receptor (vGPCR), and more specifically IL-8 receptor (Nicholas et al. 1992). A synergistic interaction between these two proteins has been proposed whereby HVS-infected cells produce IL-17 which can induce IL-8 secretion by adjacent stromal cells. The secreted IL-8 can then interact with the viral IL-8R on the surface of infected cells, resulting in a paracrine loop that promotes further T-cell activation. This scenario remains speculative and has not been shown to occur (Ensser and Fleckenstein 2005).

The protein product of ORF14 bears homology to murine superantigens MMTV and mls (Knappe et al. 1997). Superantigens crosslink specific T-cell receptor β chains based on the variable region family they express to MHC class II molecules on the surface of antigen-presenting cells (Bueno et al. 2007). This interaction leads to an efficient antigen-independent T-cell stimulation that results in T-cell proliferation and cytokine secretion. In some events this stimulation is so massive that it can lead to severe disease or death as seen in toxic shock syndrome. Recombinant ORF14 protein can bind MHC class II molecules and cause T-cell proliferation, however, no Vβ specificity has yet been detected (Duboise et al. 1998).

ORF5 protein has structural similarities to the adaptor molecule linker for activated T cells (LAT), including an amino terminal myristoylation site and several SH2-binding sites. Indeed, overexpression of ORF5 in 293T and Jurkat cells leads to the interaction with SH2 domain-containing proteins and augments T-cell receptor signaling (Lee et al. 2004).

27.4 HVS-Encoded Oncogenes

The variable region at the left end of the HVS L-DNA harbors genes responsible for the T-cell transforming potential of HVS strains ((Desrosiers et al. 1985; Koomey et al. 1984)). In subgroups A and B there is only one gene at this position termed saimiri transformation-associated protein (Stp) A and StpB, respectively (Hor et al. 2001). Members of subgroup C, on the other hand, have two different genes: StpC and tyrosine kinase-interacting protein (Tip) (Biesinger et al. 1990). Both genes are expressed from a common bicistronic mRNA which is transcriptionally induced by T-cell activation (Ensser et al. 2003). Interestingly, the closely related virus HVA encodes one protein that bears sequence homology to Stp and Tip called Tio (two in one, (Albrecht et al. 1999)).

27.4.1 Oncogenic Potential of the STPs

Despite the fact that StpA, B and C are only weakly homologous (on average 25% sequence homology), they show structural similarities. StpA and StpC have a highly acidic N-terminal end followed by a series of collagen-like repeats,

and finally a hydrophobic membrane anchor (Choi et al. 2000). StpB, on the other hand, lacks the collagen-like repeat clusters and is incapable of transforming rodent fibroblasts. If collagen repeats are introduced into StpB, it gains the ability to transform rodent fibroblasts (Choi et al. 2000). This structural difference could explain why subgroup B is the least pathogenic.

All three Stp proteins have tumor necrosis factor receptor-associated factors (TRAF) binding motifs. However, only StpC can lead to NF-kB activation through interaction with TRAFs (Choi et al. 2000). Moreover, StpC was also shown to interact with Ras to activate mitogen-activated protein kinase (MAPK) (Jung and Desrosiers 1995).

StpA and B are phosphorylated by Src kinase (Hor et al. 2001). Furthermore, StpA can interact with signal transduction and activator of transcription (STAT-3) and mediate its phosphorylation by Src, which results in the upregulation of the transcriptional activity of STAT-3. StpB was also shown to bind STAT-3, but weakly activates it (Park et al. 2004).

27.4.2 Oncogenic Potential of Tip

Tip, which is only expressed in strains of subgroup C, has been the subject of several studies. StpC and Tip are derived from a bicistronic transcript, and are together responsible for the oncogenic potential of HVS strain C (reviewed in (Brinkmann and Schulz 2006)). Recombinant HVS lacking either StpC or Tip is unable to immortalize T lymphocytes in vitro or produce fatal lymphomas in susceptible marmosets. In C488-transformed human T cells, StpC and Tip are the only viral proteins constitutively expressed (Biesinger et al. 1995). The transgenic expression of Tip can induce T-cell lymphomas in mice (Wehner et al. 2001). Tip is believed to exert its transforming potential through interaction with the tyrosine kinase Lck (Biesinger et al. 1995; Jung et al. 1995a). This interaction is believed to be direct and results in the activation of Lck. However, the expression of recombinant Tip in Jurkat cells resulted in a decrease in T-cell signaling (Jung et al. 1995b). This result shed doubt on the exact role of Tip; however, later studies showed that the transforming potential of Tip can be separated from its interaction with Lck (Duboise et al. 1998). Besides its interaction with Lck, Tip can also interact with STAT-1 and 3 (Lund et al. 1999), and a third cellular protein called Tap (Tip-associated protein), which was identified by a yeast two-hybrid system (Yoon et al. 1997).

Since Stp and Tip genes are necessary for transformation and pathogenicity, they are the major determinants of the oncogenic potential of the HVS strains. However, experiments with retroviral vectors expressing Stp and Tip in the absence of the rest of the HVS genome strongly suggest that these two proteins are not sufficient for transformation (Jung et al. 1991). Therefore, additional viral factors are required. A better understanding of what these factors might be and thus how they might contribute to the pathogenicity of the different HVS strains is still required.

27.5 HVS as a Tool

27.5.1 Transformation of Human T-Cell Lines and Clones

The derivation of primary T-cell lines is a laborious process that requires continuous availability of antigen-presenting cells with the appropriate restricting MHC element and antigen. Furthermore, primary T-cell lines have a finite life span. On the other hand, efficient immortalization of human B cells using EBV has been available for decades and has contributed to the advancement of our understanding of B-cell function. HVS offers the possibility of a similar methodology to immortalize T cells, thereby offering scientists the possibility to generate a sufficient amount of material to achieve similar understanding of T-cell function.

HVS strain C488 can efficiently transform human T cells to stable growth in the absence of antigen and antigen-presenting cells (Biesinger et al. 1992). More importantly, transformed human T cells maintain their antigen specificity and relatively normal function, and can remain stable for several months in culture (Berend et al. 1993). These observations have opened up novel research endeavors in T-cell signal transduction pathways. However, a few abnormalities were observed in HVS-transformed human T cells. The cells develop a hypersensitivity to CD2 ligation, acquire de novo expression of the protein tyrosine kinase lyn, which is normally expressed in B cells (Fickenscher et al. 1997), and shift their cytokine pattern toward higher levels of IL-2 and IFNγ (De Carli et al. 1993). Moreover, HVS-transformed cells can show an inducible natural killer cell-like cytotoxic activity against K562 targets (Biesinger et al. 1992).

27.5.2 Utility of HVS Transformants

HVS transformation has enabled the study of T cells from patients with different immunodeficiencies. The amplification of these primary cells has facilitated the understanding of the mutations associated with CD3γ chain, IL-2Rγ chain, IL12-R, MHC class II, Wiskott–Aldrich syndrome and CD18 molecule (reviewed in (Ensser and Fleckenstein 2005)). Immortalization of rhesus macaque T cells has also been achieved. Studies have shown that infusion of autologous-transformed T cells can protect the animals from challenge with HVS-C-488 (Knappe et al. 2000).

HVS-transformed human CD4 T cells can be used to propagate poorly growing HIV isolates that have a narrow host range (Nick et al. 1993) as well as macrophage-tropic HIV strains without selecting for variants or altering cell tropism (Vella et al. 1999a;Vella et al. 1999b). HVS-transformed T cells can also be persistently infected with HIV.

Lastly, gene transfer into human T cells for adoptive immune therapy has remained very difficult. Given that HVS transformation maintains T-cell

phenotype and HLA restriction, this method provides an alternative to gene therapy using traditional retroviral vectors. There are several hurdles and safety issues that would need to be resolved before; notably the presence of episomal HVS DNA and the hyper-response to CD2 ligation.

27.6 Rhadinoviruses of Old World Monkeys

The discovery of Kaposi's sarcoma-associated herpesvirus as the etiological agent of Kaposi's sarcoma (KS) prompted a search for a related herpesvirus in retroperitoneal fibromatosis (RF), a malignancy reported in Old World monkeys that possess cellular features that resemble KS (Giddens et al. 1985). Specifically, RF is characterized by ill-formed bundles of spindle-like cells that frequently contain lymphocytic and plasmacytic aggregates.

To search for a related γ-herpesvirus, Rose et al. (1997) utilized degenerate PCR primers to a highly conserved region of herpesvirus DNA polymerase gene and DNA purified from archived RF tissue obtained from a *Macaca nemestrina*. By this approach the investigators amplified DNA sequences that were highly homologous to KSHV and HVS. Moreover, they further demonstrated that the primers could amplify DNA sequences from RF tissue derived from rhesus macaques. From this information they hypothesized that a herpesvirus closely related to KSHV, referred to as retroperitoneal fibromatosis-associated herpesvirus (RFHV), is the etiological agent (Rose et al. 1997). The investigators have reported DNA sequences for RFHV from *Macaca nemestrina* (RFHVmne) and *Macaca mulatta*(RFHVmmu), but these putative viruses have yet to be isolated and cultured.

Shortly after the report of RFHVmmu, Desrosiers et al., from the New England National Primate Research Center (NENPRC), reported the isolation of a rhadinovirus from peripheral blood mononuclear cells obtained from a normal rhesus macaque (Desrosiers et al. 1997). This particular rhadinovirus isolate grew well in cell culture and short DNA sequence analysis of this isolate revealed that it was closely related to KSHV, as the virus encoded an IL-6-like ORF similar to KSHV. This virus was subsequently referred to as rhesus macaque rhadinovirus (RRV2695) and represents rhadinovirus group 2 (RV2). These investigators also reported that greater than 90% of the adult rhesus macaques at their colony were serologically positive for RRV infection, suggesting that the virus is a natural infectious agent of rhesus macaques.

A second RRV isolate was independently isolated at the Oregon National Primate Research Center (ONPRC). This isolate was obtained from an SIV-infected rhesus macaque that developed widespread lymphoproliferative disease (LPD) (Wong et al. 1999). Short DNA sequence analyses of this isolate, referred to as RRV17577, also revealed that it was closely related to KSHV and RRV2695, yet distinct from RFHVmmu. Screening of rhesus macaques at the ONPRC for prior exposure to RRV confirmed the NENPRC results that RRV

is a natural pathogen of macaques. Both groups also found that RRV, like KSHV, was capable of establishing a persistent/latent infection in B lymphocytes, which further supported RRV as a closely related rhadinovirus to KSHV (Bergquam et al. 1999).

Based upon limited DNA sequence analysis and not whole genome analysis, rhadinoviruses of Old World NHP can be classified into two groups, RV1 and RV2 (Greensill et al. 2000b; Schultz et al. 2000;Strand et al. 2000). RFHVmne and RFHVmmu represent RV1 and there exists evidence for additional RV1 isolates from African green monkeys, chimpanzees and gorillas (Greensill et al. 2000a; Lacoste et al. 2000). Like the pigtail and rhesus macaques viruses, these isolates have yet to be cultured as well. RV2 is represented by RRV2695 and RRV17577. Other RV2 isolates have been reported from pigtail and cynomologus macaques. The primary difference between RV2 and RV1 isolates appears to be the ability to grow in cell culture.

The genomes of RRV2695 and RRV17577 have both been determined, whereas limited sequence information is available for RFHVmne or RFHVmmu. As such, the remainder of this discussion of Old World monkey rhadinoviruses will be devoted to RRV, which has been further characterized.

The genome of RRV17577 was the first to be described and found to be essentially colinear with KSHV. The RRV17577 genome encodes 84 ORFs, with 80 of these having homologues in KSHV (Searles et al. 1999). The genome of RRV2695 was subsequently determined and reported to be nearly identical to RRV17577 and closely related to KSHV (Alexander et al. 2000). Both strains of RRV lack homologues of immune modulators K3 and K5, and homologues of K7 and K12 (Russo et al. 1996). Additionally, differences between RRV and KSHV include position of the dihydrofolate reductase (DHFR) gene, copies of the macrophage inflammatory protein (MIP) homologues and viral interferon regulatory factors (vIRFs). In RRV the DHFR gene is located adjacent to the left terminus, whereas in KSHV, DHFR is 16 kb to the right near the viral interleukin-6 homologue. RRV also encodes only a single MIP homologue compared to three for KSHV. Finally, RRV encodes eight vIRFs (R6-R13) compared to four for KSHV. The close homology between R6, R7, R8 and R9 to R10, R11, R12 and R13 suggests that this locus was duplicated in RRV. The RRV vIRFs are discussed in greater detail below.

The RRV genome also shares structural features with KSHV. Specifically, the RRV genome contains three highly repetitive regions, which correspond to three repetitive regions found in KSHV. Two are tandem repeats and are located in the divergent loci DL-B 1, 2 and DL-E 1, 2, and correspond to KSHV tandem repeats *frnk* and *zppa*, respectively. The third, DL-F is a short repeat located near the right terminal repeat and is much shorter than the *mdsk* repeat of KSHV.

The functions of the repeat regions were not known at the time the genomes were determined, but it was presumed that they served as origins of DNA replication. Subsequent studies by Pari et al. (2001) confirmed that repeat region DL-E1, 2 contains a lytic origin of DNA replication, as does the *zppa* region of KSHV (AuCoin et al. 2002).

27.7 Genes Encoded by Rhesus Macaque Rhadinovirus

27.7.1 Viral Homologues of Cellular Genes

Like HVS and KSHV, RRV encodes several homologues of cellular genes that most likely function to assist the virus in replication by providing sufficient dTMP pools in resting infected cells. These include DHFR (ORF 2) and TS (ORF 70), where only the DHFR gene product has been shown to express an active protein as of yet.

RRV like KSHV and HVS also encodes proteins that help prevent infected cells from entering into apoptosis, which would limit virus production and spread. These include a Bcl-2 homologue (ORF 16) and a cyclin-D homologue (ORF 72). The close homology these RRV ORFs share with KSHV and HVS would suggest that they encode functional proteins, but no reports have been published on these two homologues yet.

The ability to persist in an immunocompetent host is an important characteristic of herpesviruses. The γ2-herpesviruses accomplish this by evading the host immune system utilizing ORFs that encode proteins to squelch the host response. One ORF that is conserved amongst RRV, HVS and KSHV is ORF 4, which encodes the complement control protein (CCP). The complement control proteins from RRV2695 and RRV17577, referred to as RCP-H and RCP-1, respectively, have been characterized in vitro and it is interesting to note that they are quite different (Mark et al. 2007). RCP-1 is larger than RCP-H, in that RCP-1 encodes eight CCP domains, compared to the four that RCP-H or KCP encodes. Despite the difference in size both RCP-1 and RCP-H are capable of suppressing complement activation. CCP appears to play a role in γ-herpesvirus pathogenesis, as recombinant MHV-68 lacking a CCP homologue was found to be attenuated in mice (Kapadia et al. 2002).

Interestingly, KSHV and RRV both differ from HVS by the fact that they lack ORF 15, the viral variant of CD59 which is thought to protect HVS-infected cells from complement-mediated lysis. This could indicate that KSHV and RRV possess different mechanism(s) to evade the host immune response. For example, KSHV and RRV both encode vCD200 molecules (RRV ORF R15) that have been shown to be expressed on the surface of infected cells undergoing lytic replication and are capable of downmodulating macrophage activation through binding to CD200R on activated macrophages and thereby reducing the level of TNF production (Foster-Cuevas et al. 2004; Langlais et al. 2006).

ORF R2 is the viral homologue of IL-6, a multifunctional cytokine that plays a vital role in host defense by way of its broad range of immune and hematopoietic activities. RRV vIL-6 is a 207-amino acid polypeptide with overall amino acid identity of 12.7 and 17.8% (27.4 and 35.6% similarity) with KSHV vIL-6 and rhesus macaque IL-6, respectively (Kaleeba et al. 1999). More importantly, RRV vIL-6 is functionally equivalent to KSHV vIL-6 with regards to its ability to support proliferation of an IL-6-dependent cell line (Kaleeba et al. 1999).

RRV encodes a single homologue of macrophage inflammatory protein-1α (MIP-1α), ORF R3, whereas KSHV encodes three MIP homologues, K4, K4.1 and K6 (Russo et al. 1996). RRV vMIP has yet to be characterized for function; however, our laboratory has a manuscript in preparation that describes RRV vMIP function in vitro and in vivo.

Like HVS and KSHV, RRV encodes an IL-8-like receptor from ORF 74. This seven-transmembrane-spanning G-protein-coupled receptor (GPCR) shares 40.8% amino acid sequence identity with KSHV ORF 74 and, more importantly, possesses similar properties in vitro. Specifically, RRV ORF 74 expression results in increased vascular endothelial growth factor secretion and activation of the ERK1/2 mitogen-activated protein kinase-signaling pathway (Estep et al. 2003).

As mentioned above, the RRV genome encodes eight vIRFs: R6 through R13 in the RRV17577 genome and R9.1 through R9.8 in the RRV2695 genome (Alexander et al. 2000; Searles et al. 1999). The nomenclature of RRV17577 will be utilized as these eight vIRFs are in fact related yet distinct. RRV encodes eight vIRFs (R6–R13), with the first four showing significant homology to the last four, suggestive of a duplication event. Likewise, four of the vIRFs encoded in RRV share between 26 and 30% similarity with KSHV vIRF1, and RRV R10 shows the greatest similarity to both KSHV vIRF1 and cellular IRFs (Searles et al. 1999). This suggests possible overlap in function of vIRFs within RRV as compared to KSHV; however, functional studies have yet to be performed on any of the RRV vIRFs.

27.7.2 Other Viral Homologues

Other important homologues include RRV R1, which is the positional homologue of K1 and shares 28% identity and 33% similarity with K1. K1 and R1 are structurally similar to the B-cell receptor (BCR) and the cytoplasmic tail of both molecules contains an immunoreceptor tyrosine-based activation motif (ITAM) which is capable of activating signaling pathways similar to those activated by the BCR complex in B lymphocytes (Damania et al. 1999). However, unlike the BCR, RRV ORF R1 is thought to be constitutively active through an oligomerization process that leads to phosphorylation of the ITAM and recruitment of the major B-cell kinase, Syk, which is consistent with B-cell survival. In vitro experiments further reveal that R1, like K1, possesses oncogenic potential, as R1-expressing cells formed tumors in mice and a recombinant HVS-expressing R1 was capable of transforming T cells (Damania et al. 2000).

RRV encodes other homologues of KSHV that are essential for the virus. Specifically, RRV encodes two viral transcription factors, R-Rta and R-bZIP, which are postulated to be required for lytic viral replication, based on homology to KSHV Rta and bZIP (DeWire et al. 2002; Lin et al. 2002). ORF 50 was

initially predicted to encode Rta, but R-bZIP was not identified as the product is derived from a highly spliced message (DeWire et al. 2002; Lin et al. 2002). ORF 73 encodes the homologue of KSHV ORF 73, also referred to as the latency-associated nuclear antigen (LANA). In vitro studies indicated that RRV LANA expression during lytic replication results in an inhibition of RRV replication, much like what has been reported for KSHV LANA (DeWire and Damania 2005). RRV also encodes a homologue of KSHV K8.1, a glycoprotein that is well conserved amongst the γ-herpesviruses (DeWire et al. 2002).

Like KSHV and HVS, RRV encodes a viral FLIP from ORF 71. RRV vFLIP shares overall amino acid sequence identity 30.8 and 15.1% (38.8 and 25.3% similarity) with KSHV and HVS, respectively (Searles et al. 1999). Functional activity has yet to be established for RRV vFLIP.

The relative importance of miRNAs to virus replication and pathogenesis is emerging as miRNAs have been found to be conserved amongst related herpesviruses (Pfeffer et al. 2005). Not surprisingly, RRV, like KSHV, which encodes 12 viral miRNA, encodes 7 distinct miRNAs that are located in a single cluster and in an analogous position in the RRV genome (Schafer et al. 2007). Interestingly, these miRNAs do not share sequence homology with the KSHV miRNAs, suggesting they either target different sequences on the same target or different targets all together that result in similar outcomes for the virus in vitro and in vivo.

27.8 Infection of Old World Monkeys with Rhadinoviruses as Models of KSHV

Developing an animal model of KSHV infection that recapitulates KSHV infection in humans would greatly facilitate studies on KSHV. Unfortunately, attempts to infect non-human primates with KSHV were not met with success. Specifically, Renne et al. reported that experimental transmission of KSHV into rhesus macaques, neonatal and SIV-infected, did not lead to KSHV-related pathology (Renne et al. 2004). The investigators did observe that animals exposed to KSHV showed evidence of persistent infection, but failed to seroconvert to KSHV, implying that the virus was under tight control of the innate immune system, which would be consistent with no related pathology in highly immunocompromised animals. As such, Renne et al. concluded that this animal model will not allow for the study of viral determinants involved in KS or other KSHV-related pathologies (Renne et al. 2004).

Several laboratories have initiated studies to create an Old World monkey model of rhadinovirus disease. One involved the direct injection of RF tissue homogenate in the peritoneal cavity of rhesus macaques that were infected 2 weeks earlier with simian retrovirus type-2 (SRV-2) (Bosch et al. 1999). Initial reports of RF were observed in macaques with simian-acquired immune deficiency syndrome (SAIDS) caused by SRV, so the investigators were postulating

that this approach would lead to recovery of RFHVmmu and development of disease. Unfortunately, these animals did not develop RFHVmmu-related pathologies, but they did show evidence of persistent infection, as evidenced by PCR, which the authors subsequently concluded was present before initiation of their infections as the DNA sequences detected were different from the input. The investigator did, however, report that SRV infection led to reactivation of RFHVmmu, even though no virus was isolated or cultured (Bosch et al. 1999).

Two groups have tried to experimentally infect rhesus macaques with RRV to determine if RRV is associated with rhadinovirus-related pathologies. The investigators from Oregon experimentally inoculated rhesus macaques with RRV17577 that had been previously infected with SIV_{mac239} to induce immunodeficiency (Wong et al. 1999). These investigators rationalized that since RRV is naturally present in the colony and is not associated with any identifiable pathology, experimental inoculation into an immunodeficient animal would be a suitable host for viral-mediated disease. Additionally, the original RRV17577 isolate was derived from an SIV-infected rhesus macaque that had died from widespread lymphoproliferative disease (LPD). Hence, this approach tests aspects of Koch's postulates that the agent was indeed the causative agent of the LPD.

By taking this infection protocol, these investigators found that SIV-infected animals subsequently inoculated with RRV1777 developed persistent lymphadenopathy and splenomegaly, and hypergammaglobulinemia (Wong et al. 1999). Interestingly, autoimmune hemolytic anemia was found in a subset of animals. Microscopic examination of the peripheral lymph nodes of the dually infected animals revealed marked angiofollicular lymphoid hyperplasia characterized by giant, secondary reactive germinal centers that were irregularly shaped and lacked distinct mantel zones. Both the medullary cords and the paracortical areas were infiltrated with vascularity and sheets of plasma cells. The hyperplastic LPD resembled the plasma cell variant of multi-centric Castleman's disease B-cell hyperplasia and was found to be widespread at necropsy. RRV/SIV-infected animals also exhibited persistent viremia with little or no RRV-specific antibody response, whereas animals infected with RRV alone displayed transient viremia followed by a vigorous anti-RRV response and lacked evidence of LPD. More recently, the Oregon investigators have found evidence that RRV is associated with lymphomagenesis in SIV-infected rhesus macaques (Orzechowska et al. 2008).

The results described above with RRV17577 differ somewhat from those performed at the New England Primate Research Center. These investigators reported that experimental co-inoculation of RRV2695 and SIV_{mac251} yielded lymphadenopathy characterized initially as paracortical hyperplasia and vascular hypertrophy/hyperplasia that was subsequently replaced with follicular hyperplasia, which eventually resolved (Mansfield et al. 1999).

Exactly what is responsible for these clinical differences is not known. Potentially, the two RRV strains, although similar, possess sufficient genetic

differences that contribute to different clinical outcomes. Additionally, the strain of SIV utilized to induce immune deficiency and the time following SIV infection may have contributed to the differential outcomes.

Further studies into the RRV/SIV-infected rhesus macaque are warranted and can be expanded into studies with recombinant RRV isolates, now that two recombinant strains have been generated utilizing different systems (Bilello et al. 2006; Estep et al. 2007). Both systems enable the creations of recombinants to investigate the role specific RRV genes have in viral-mediated pathogenesis, as well as the ability to create chimeric RRV/KSHV viruses. These types of recombinants provide an invaluable tool for the evaluation of the function of KSHV ORFs in the context of a relevant in vivo animal model.

27.9 Lymphocryptoviruses of Old World Monkeys

There are several lymphocryptoviruses in both Old and New World monkeys (Table 27.1) that were originally identified from studies demonstrating that the serum of several Old World primates exhibited antibody cross-reactivity against human EBV (Dunkel et al. 1972; Kalter et al. 1972; Landon and Malan 1971; Levy et al. 1971; Naito et al. 1971). In the human population, there are two types of EBV isolates, EBV-1 and EBV-2, that can be distinguished by genetic polymorphisms in the EBNA genes (Dambaugh et al. 1980; Dambaugh et al. 1984; Rowe and Clarke 1989; Rowe et al. 1989; Sample and Kieff 1990; Zimber et al. 1986). Similarly, rhesus macaques contain two lymphocryptoviruses (type 1 and type 2) (Cho et al. 1999). The two types of rhesus LCVs have similar genetic polymorphisms, as seen in the human isolates, and share the same biological properties (Cho et al. 1999). These data suggest that the human and rhesus lymphocryptoviruses arose from the same ancestral lymphocryptovirus and that there exists a similar selection pressure for the evolution of two different strains in both humans and macaques

The complete rhesus LCV (type 1) genome has been cloned and sequenced (Rivailler et al. 2002b). Rhesus LCV encodes 80 open-reading frames (ORFs) and each ORF shares homology to a corresponding gene in human EBV. The average gene homology between EBV and rhesus LCV is 75.6% (Rivailler et al. 2002b) with each ORF being located at an equivalent position in the viral genome (Rivailler et al. 2002b). Many of the latent and lytic genes of rhesus LCV are conserved with those of human EBV. In addition, both the rhesus LCV and the human EBV genomes have four homologues of cellular genes: CSF1R (BARF1), two bcl-2 homologues (BHRF1 and BALF1) and an IL-10 homologue (BCRF1) (Rivailler et al. 2002b). Interestingly, although the bcl-2 homologues are conserved between the Old and the New World lymphocryptoviruses, the IL-10 homologue and the colony-stimulating factor 1 receptor were recently acquired genes, and are not present in an LCV genome isolated from a common marmoset (Rivailler et al. 2002a).

Table 27.1 Nomenclature of Primate Gammaherpesviruses Lymphocrytoviruses

Species	Full Virus Name	Other Name	ICTV	Abbreviation	Reference
Old World Primates					
Chimpanzee	*Pan troglody*tes LCV1	Herpesvirus pan	*Pongine herpesvirus 1*	PtroLCV1	Greensill et al., 2000
Bonobo	*Pan paniscus* LCV1			PpanLCV1	Ehlers et al., 2003
Gorilla	*Gorilla gorilla* LCV1	Gorilla herpesvirus	*Pongine herpesvirus 3*	GgorLCV1	Ehlers et al., 2003
Gorilla	*Gorilla gorilla* LCV2	Gorilla herpesvirus	*Pongine herpesvirus 3*	GgorLCV2	Ehlers et al., 2003
Orangutan	*Pongo pygmaeus* LCV1	Orangutan herpesvirus	*Pongine herpesvirus 2*	PpygLCV1	Ehlers et al., 2003
White-cheeked gibbon	*Hylobates leucogenys* LCV1			HleuLCV1	Ehlers et al., 2003
White-handed gibbon	*Hylobates lar* LCV1			HlarLCV1	Ehlers et al., 2003
Hanuman langur	*Semnopithecus entellus* LCV1			SentLCV1	Ehlers et al., 2003
Hamadryas baboon	*Papio hamadryas* LCV1	Herpesvirus papio	*Cercopithecine herpesvirus 12*	PhamLCV1	Ehlers et al., 2003
Hamadryas baboon	*Papio hamadryas* LCV2	Herpesvirus papio	*Cercopithecine herpesvirus 12*	PhamLCV2	Ehlers et al., 2003
Mandrill	*Mandrillus sphinx* LCV1			MsphLCV1	Ehlers et al., 2003
Mandrill	*Mandrillus sphinx* LCV2			MsphLCV2	Ehlers et al., 2003
Black and White colobus	*Colobus guereza* LCV1			CgueLCV1	Ehlers et al., 2003
Western red colobus	*Piliocolobus badius* LCV1			PbadLCV1	Ehlers et al., 2003
Black mangabey	*Cercocebus aterrimus* LCV1			CateLCV1	Ehlers et al., 2003
Rhesus macaque	*Macaca mulatta* LCV1	Rhesus EBV	*Cercopithecine herpesvirus 15*	MmuLCV1	Franken et al., 1996
Cynomolgus macaque	*Macaca fascicularis* LCV1			MfasLCV1	Ehlers et al., 2003
Japanese macaque	*Macaca fuscata* LCV1			MfusLCV1	Ehlers et al., 2003
Japanese macaque	*Macaca fuscata* LCV2			MfusLCV2	Ehlers et al., 2003
Wanderoo	*Macaca silenus* LCV1			MsilLCV1	Ehlers et al., 2003
Magot	*Macaca sylvanus* LCV1			MsylLCV1	Ehlers et al., 2003
Tibet macaque	*Macaca tibetana* LCV1			MtibLCV1	Ehlers et al., 2003
Patas monkey	*Erythrocebus patas* LCV1			EpatLCV1	Ehlers et al., 2003
African green monkey	*Chlorocebus aethiops* LCV	African green monkey EBV-like virus	*Cercopithecine herpesvirus 14*	CaetLCV1	Bocker et al., 1980
New World Primates					
Common squirrel monkey	*Saimiri sciureus* LCV1			SsciLCV1	Ehlers et al., 2003
Common squirrel monkey	*Saimiri sciureus* LCV2			SsciLCV2	Ehlers et al., 2003
Saki	*Pithecia pithecia* LCV1			PpitLCV1	Ehlers et al., 2003
White-fronted capuchin	*Cebus albifrons* LCV1			CalbLCV1	Ehlers et al., 2003
Black spider monkey	*Ateles paniscus* LCV1			ApanLCV1	Ehlers et al., 2003
Black-penciled marmoset	*Callithrix penicillata* LCV1			CpenLCV1	Ehlers et al., 2003
Common marmoset	*Callithrix jacchus* LCV1	Marmoset LCV		CjacLCV1/ CalHV3	Cho et al., 2001
Red-handed tamarin	*Saguinus midas* LCV1			SmidLCV1	Ehlers et al., 2003

27.10 Genes Encoded by Rhesus LCV

27.10.1 Homologues of Epstein–Barr Virus

The latency gene EBV nuclear antigen -1 (EBNA-1) is a critical gene required for establishment and maintenance of the viral genome in the latent state. Both baboon LCV (*cercopithecine herpesvirus 12* or herpesvirus papio) and rhesus LCV (*cercopithecine herpesvirus 15*) encode homologues of EBV EBNA-1 that are highly conserved (Blake et al. 1999; Yates et al. 1996). These simian viral EBNA-1 proteins are slightly smaller than the human viral protein due to differences in the glycine–alanine (GAR) repeat domain. The baboon and rhesus LCV EBNA-1 proteins can both function to support EBV ori-P-dependent plasmid replication and maintenance, similar to EBV EBNA-1 (Marechal et al. 1999; Ruf et al. 1999).

The EBNA-2 protein in the rhesus and baboon LCVs shows only limited homology to each other and to human EBV. However, it appears that their functional characteristics such as their interactions with the transcription factor RBP-Jk have been retained (Cho et al. 1999; Ling and Hayward 1995; Ling et al. 1993; Peng et al. 2000). Genetic polymorphisms in the EBNA-2 genes have suggested the presence of two types of LCVs in rhesus macaques, similar to the situation in humans.

The rhesus LCV latency-associated genes, EBNA-3A, 3B and 3C, show limited homology to human EBV EBNA-3A, 3B and 3C genes. Despite the limited conservation in sequence among these proteins, the interaction with RBP-Jk has been retained (Dalbies-Tran et al. 2001; Jiang et al. 2000; Zhao et al. 2003). Further, the transactivation function of EBNA-3C has also been conserved between the rhesus, baboon and human viral proteins, and all three proteins can interact with the Spi proteins (Zhao et al. 2003). Both non-human primate 3C proteins can support transcriptional activation mediated by the Spi proteins in the presence of EBNA-2 (Zhao et al. 2003). Despite these similarities, gene replacement studies demonstrated that the rhesus LCV EBNA-3 genes were unable to functionally substitute for the EBV EBNA-3 genes in the EBV genome, for immortalization of human B cells (Jiang et al. 2000). However, the ability of this recombinant virus to immortalize rhesus B cells was not tested.

The EBV-encoded latent membrane protein 1 (LMP1) structurally and functionally resembles a constitutively active TNF family receptor. LMP1 aggregates in the plasma membrane and can activate a multitude of signaling pathways in the cell resulting in cell proliferation and transformation. Sequence analysis of the LMP-1 proteins from baboons (*cercopithicine herpesvirus 12*or herpesvirus papio) and rhesus monkeys (*cercopithicine herpesvirus 15*) showed that although the transmembrane domains of these proteins are conserved with that of EBV LMP-1, there is great divergence within the carboxyterminal cytoplasmic domains of these proteins (Franken et al. 1996). The C-terminal

domain of EBV LMP1 has been shown to be essential for B-cell immortalization and interaction with members of the tumor necrosis factor receptor family. A comparative study of the simian LMP-1 proteins with EBV LMP-1 showed that the simian LCV LMP-1 proteins could induce NF-kB activity, bind tumor necrosis factor associated factor-3 (TRAF3) and induce ICAM1 expression (Franken et al. 1996). This is likely through the multiple TRAF-binding sites (PXQXT/S) that are contained within the simian LCV LMP1 C-terminal domain (Franken et al. 1996). A similar study performed with an LCV LMP1 protein from cynomolgus monkeys (*Macaca fascicularis*) shows that this simian LMP1 protein also contains two PXQXT/S motifs and retains its ability to activate the NF-kB pathway (Faucher et al. 2002). Further, Eliopoulos and Young (1998) have reported that LMP1 homologues from the baboon and rhesus EBV are also capable of activating the c-Jun N-terminal kinase (JNK) pathway (Eliopoulos and Young 1998). Thus, activation of the NF-kB and JNK pathways by the primate LMP1 proteins is an important function that has been conserved throughout evolution.

The LMP2A homologues in the baboon and rhesus LCV genomes show homology to EBV LMP2 and contain an immunoreceptor tyrosine-based activation motif (ITAM) (Franken et al. 1996; Rivailler et al. 1999). All three primate LCV LMP2A proteins contain 12 transmembrane domains, and although the amino acids in the cytoplasmic domain are quite different, the ITAM and proline-rich domains are well conserved. These conserved motifs/domains in EBV LMP2A have been shown to be required for interaction with protein tyrosine kinases (Portis et al. 2002) and are essential for LMP2A function. Moreover, the rhesus LCV LMP2B gene is located at an equivalent genomic position as EBV LMP2B (Rivailler et al. 1999).

Given the close homology rhesus LCV and EBV share, it is not surprising that rhesus LCV would also share homology with the miRNAs encoded by EBV. Indeed, Cai et al. have reported that rhesus LCV encodes 16 miRNAs in the analogous position as those in EBV (Cai et al. 2006). Moreover, 8 of the 16 miRNAs identified in rhesus LCV are conserved in EBV at the primary sequence (Cai et al. 2006). Conservation of these viral miRNAs suggest that the two viruses have closely evolved with their hosts and that they likely target the same mRNAs, be it viral or host to achieve virus replication or viral persistence.

27.11 Infection of Old World Monkeys with Rhesus LCV as an Animal Model System for EBV

Historically, several investigators attempted and failed to infect Old World primates such as baboons and rhesus macaques with human EBV (Frank et al. 1976; Gerber et al. 1969; Levine et al. 1980). This failure to establish EBV infection in Old World primates may be due to pre-existing antibody

cross-reactivity due to natural LCV infection in the primates, the route of inoculation used in these studies or the utilization of non-transforming deletion mutants of EBV, namely P3HR1 (Levine et al. 1980). Additionally, there might be a species restriction imposed on EBV infection of non-human primates, since LCVs tend to be competent for immortalization of B cells from the same or closely related species, but immortalization potential varies for B cells from a more divergent species. For example, Moghaddam et al. (1998) found that rhesus LCV did not immortalize human B cells, and human EBV could not immortalize rhesus monkey B cells (Moghaddam et al. 1998), despite the fact that the rhesus LCV could infect human B cells (Moghaddam et al. 1998). Rhesus and baboon LCVs have been reported to be incapable of immortalizing human B cells (Falk et al. 1977), although other reports suggest that this is possible at a much lower frequency (Gerber et al. 1977; Rabin et al. 1977a; Rabin et al. 1977b). In a similar manner, EBV has been shown to immortalize B cells from chimpanzees, which are more closely related to humans, and chimpanzee LCV can immortalize human B cells (Gerber et al. 1977; Ishida and Yamamoto 1987).

In light of the fact that human EBV fails to establish long-term infection in rhesus macaques, rhesus LCV serves in the rhesus macaque as a valuable animal model to study EBV pathogenesis. Experimental infection of naïve rhesus macaques with rhesus LCV has been previously reported (Moghaddam et al. 1997) and showed that orally infected macaques displayed acute and persistent LCV infection, which closely resembled that seen with primary EBV infection of humans. Initial viral load peaked between 4 and 21 days and declined to almost undetectable levels over a 3-month period (Rao et al. 2000). Immune activation was evidenced 3 days after oral inoculation by the presence of T cells expressing IL-2, IL-10 and gamma interferon. Activated CTL activity against LCV-infected cells was observed in the first few weeks post-infection similar to what has been described for infectious mononucleosis patients in the human population (Wang 2001). In addition, antibody responses against EBNA-2 and viral capsid antigens develop within 2 weeks post-inoculation (Moghaddam et al. 1997; Mohle et al. 1997; Rao et al. 2000). Persistent viral infection could be detected in the oropharynx of infected animals by PCR (Cho et al. 1999; Moghaddam et al. 1997; Mohle et al. 1997; Rao et al. 2000) and in B cells, and a percentage of these infected B cells were immortalized and could be grown in vitro (Moghaddam et al. 1997; Mohle et al. 1997). Persistent infection as measured by RT-PCR of the rhesus LCV EBER genes in peripheral blood mononuclear cells (PBMCs) was detected 3-years post-infection (Rao et al. 2000). Hence, primary, acute and persistent infection of macaques with rhesus LCV appears to resemble EBV infection of humans, further validating the use of rhesus LCV as an animal model system to study EBV pathogenesis.

The potential to use the rhesus LCV animal model system to study EBV-associated tumorigenesis is the subject of current and future investigation. Feichtinger et al.(1992a,b) have shown that when cynomolgus monkeys naturally infected with LCV were infected with simian immunodeficiency virus

(SIV), malignant B-cell lymphomas containing DNA which cross-hybridized with human EBV were detected. In another study, squamous epithelial proliferative lesions in SIV-infected rhesus monkeys were also shown to contain EBV-like sequences by immunohistochemistry and in situ hybridization (Baskin et al. 1995). The current availability of the genomic sequence of rhesus LCV will facilitate the construction of recombinant LCVs that can be tested in rhesus macaques. Such studies will provide an understanding of the contribution of individual genes to the life cycle of these lymphocryptoviruses and serve to verify rhesus LCV as a tractable system to model EBV pathogenesis.

27.12 Other Lymphocryptoviruses in Old World Primates

A recent study by Ehlers et al. has identified the presence of novel lymphocryptoviruses in several species including baboons, chimpanzees and gorillas (Ehlers et al. 2003) (Table 27.1).

27.13 Lymphocryptoviruses of New World Monkeys

Early serological studies had indicated that there was no evidence for the presence of lymphocryptoviruses in New World primates since there was no antibody cross-reactivity with human EBV from sera of New World monkeys (Frank et al. 1976). However, Ramer et al. (2000) reported the identification of novel viral DNA sequences from common marmoset monkeys (*Callithrix jaccus)* afflicted with spontaneous B-cell lymphomas. These sequences were most closely related to EBV and suggested the presence of a novel lymphocryptovirus in New World primates. Subsequently, the first EBV-related herpesvirus in New World primates was cloned from common marmoset monkeys (Cho et al. 2001; Jenson et al. 2002). This virus was formally named Callitrichine herpesvirus 3 or CalHV3. Sequencing of CalHV3 revealed that the genomic organization was similar to that of EBV (Rivailler et al. 2002a). Sequence analysis of the 73 open-reading frames (ORFs) revealed that although many genes showed high homology to genes in EBV, there were some striking differences between the two genomes as well.

27.14 The Marmoset LCV (CalHV3) Genome

The marmoset LCV genome is comprised of approximately 160,000 nucleotides. Of the 73 ORFs found in the marmoset LCV genome, 59 of these share homology to genes found in all herpesviruses. Six additional genes encoded by ORFs 1, 6, 39, 43, 44 and 45 show homology to EBV BALF1, BILF1, EBNA-1, BZLF1, BZLF2 and gp350, respectively (Rivailler et al. 2002a). The eight other marmoset ORFs show no sequence relatedness to either cellular or viral genes

and were named C0 through C7. Based on their genomic position only C0, C1, C2, C3, C4, C5 and C7 are in the equivalent genomic locations as EBV EBNA-LP, LMP1, BILF-2, EBNA-3, BHLF1, EBNA-2 and LMP2, respectively. In addition to encoding unique genes, there are also 11 EBV genes that are not present in marmoset LCV. These include the EBERs, BARF0, BCRF1, BARF1 and BDLF3 (Rivailler et al. 2002a).

27.15 Genes Encoded by CalHV3

C1 is a positional homologue of EBV LMP1. Although it shares no homology with LMP1 at the amino acid level and does not contain the PXQXT/S motifs that are contained in the C-terminus of the rhesus LCV and human EBV, C1 is a functional homologue of LMP1. It can transform rodent fibroblasts in vitro and can also induce NF-kB activity to similar levels as LMP1. Hence, C1 can interact with the TRAFs through an alternative TRAF-binding motif (Wang 2001).

The CalHV3 EBNA-1 protein shows homology to EBV EBNA-1 in the C-terminal domain and the GR-rich domains, which in EBV EBNA-1 are required for episomal maintenance (Wang 2001). However, the CalHV3 EBNA-1 homologue does not contain the Gly–Ala repeat region, which has previously been shown to be involved in immune-modulation (Levitskaya et al. 1995).

CalHV3 C5 is a positional homologue of EBNA-2 and shares no relatedness at the amino acid level. However, like EBV EBNA-2, the C5 protein has a cluster of C-terminal acidic residues that may be important for transcriptional transactivation (Rivailler et al. 2002a). Unlike the EBV and rhesus LCV EBNA-2, C5 is missing the polyproline repeat, which is present in EBV EBNA-2 (Yalamanchili et al. 1996).

C7 is a positional homologue of EBV LMP2. Similar to LMP2, it contains 12 transmembrane domains but a shorter N-terminus and a longer C-terminus (Rivailler et al. 2002a). The latter contains five tyrosine residues, three of which may serve as part of five different Src-homology-2 (SH2)-binding motifs (Rivailler et al. 2002a).

27.16 New World Primates as an Animal Model System for EBV

Historically, an extensive body of work has been published on using New World primates as an animal model system to study EBV (Miller et al. 1972; Miller et al. 1977; Shope et al. 1973). EBV readily infects and immortalizes B cells from common marmosets (Desgranges et al. 1976; Rabin et al. 1977b). In fact one of the most widely used EBV-infected cell lines, B95-8, is a marmoset B-cell line infected with human EBV (Miller et al. 1972). New World monkey species, including the cotton-top tamarin (*Sanguinus oedipus*) and owl monkey (*Aotus trivirgatus*) develop B-cell lymphomas upon infection with EBV (Cleary et al.

1985; Epstein et al. 1973a; Epstein et al. 1973b; Johnson et al. 1983; Miller et al. 1977; Werner et al. 1975).

A comprehensive study on lymphocryptoviruses (LCV) that infect New and Old world primates has identified the presence of many lymphocryptoviruses from a multitude of primates (de Thoisy et al. 2003; Ehlers et al. 2003) (Table 27.1). These studies were done using degenerate PCR and revealed that some New and Old World monkeys are infected with two different lymphocryptoviruses, denoted as LCV1 and LCV2 (Table 27.1). In each primate species examined, one LCV virus was more closely related to human EBV and grouped in the same genogroup as EBV, while the viruses that were less closely related to EBV all grouped together in a second lymphocryptovirus genogroup (Ehlers et al. 2003).

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Chapter 28
Murine Gammaherpesvirus 68 Infection of Mice: A Small Animal Model for Characterizing Basic Aspects of Gammaherpesvirus Pathogenesis

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Abstract The human gammaherpesviruses, EBV and KSHV, have a narrow host range, limiting development of small animal models for characterizing aspects of EBV and KSHV pathogenesis. As such, over the past 15 years there has been increasing interest in studying murine gammaherpesvirus 68 (γHV68, MHV68) infection of inbred strains of laboratory mice. In this chapter, we review advances in this model system and how these findings may offer insights into EBV and KSHV infections in humans.

28.1 Discovery, Epidemiology and Initial Characterization of MHV68

Not surprisingly, gammaherpesviruses have readily been recovered from all mammalian species examined. MHV68 (murine herpesvirus 4) is one of several closely related gammaherpesviruses isolated from murid rodents in the late 1970s (Blaskovic et al., 1980). The others include MHV60, MHV72, which along with MHV68 were recovered from yellow-necked mice (*Apodemus flavicollis*) and bank voles (*Clethrionomys glareolus*), and MHV76 and MHV78 that were recovered from wood mice (*Apodemus flavicollis*). In addition, a MHV68-like virus, MHV-Brest, was more recently recovered from a greater white-toothed shrew (*Crocidura russula*) – a small insectivorous mammal that is not a rodent (Chastel et al., 1994). It remains unclear whether these are all strains of the same virus or represent several distinct viruses. Serological analysis of several species (five rodent species, deer, boar, sheep, and humans) has provided evidence that infection with MHV68 is widespread, although it remains undetermined whether the results of these studies reflect the presence of cross-reacting antibodies or bona fide infection with MHV68 (Mistrikova et al., 2006b; Mistrikova et al., 2000). More recently, both serologic and PCR evidence of MHV68 infection in wood

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B. Damania, J.M. Pipas (eds.), *DNA Tumor Viruses*,
DOI 10.1007/978-0-387-68945-6_28, © Springer Science+Business Media, LLC 2009

mice has been reported (Blasdell et al., 2003). A subsequent detailed analysis of the epidemiology of MHV68 in England has provided evidence that MHV68 infection in this region is significantly more prevalent in wood mice than in bank voles (Telfer et al., 2007). In addition, it appears that infected animals may only be transiently seropositive – indicating that some caution is warranted in solely relying on serology for monitoring MHV68 infection. While no study of the presence of MHV68 (or a closely related virus) in feral *Mus musculus* populations has been reported, it is notable that MHV68 does establish a chronic infection in immunocompetent-inbred laboratory mice in the absence of any overt disease manifestations. As such, MHV68 infection of laboratory mice provides a valuable and tractable small animal model for assessing the requirements for establishing and maintaining chronic gammaherpesvirus infection.

Initially, MHV68 was judged to be an alphaherpesvirus since it appeared neurotropic and caused fatal encephalitis in neonatal mice (Blaskovic et al., 1980). However, adult mice showed no neurologic disease, but instead a severe exudative pneumonia and hematogenous dissemination of MHV68 to multiple organs, including the trigeminal ganglia (Rajcani et al., 1985; Sunil-Chandra et al., 1992a). MHV68 infects numerous organs and largely persists in B cells and macrophages following clearance of acute virus replication. In addition, it has been shown that the virus exhibits tropism for vascular smooth muscle cells with infection leading in some cases to a large vessel arteritis (Weck et al., 1997). A common feature of MHV68 infection is splenomegaly that is observed during acute infection, and is associated with a two- – threefold increase in the number of $CD4^+$ T cells, $CD8^+$ T cells and B cells in the spleen (Ehtisham et al., 1993; Sunil-Chandra et al., 1992b; Usherwood et al., 1996a; Weck et al., 1996). It is important to note, however, that horizontal transmission of MHV68 among inbred mice has not generally been observed or extensively investigated, with the notable exception of a single report in which two to three dams became infected from eating experimentally infected pups (Rajcani et al., 1985).

28.2 Genome Organization and Identification of Genes Required for Virus Replication

Based on the organization of the viral genome and limited sequence analysis, MHV68 was reclassified as a γ-herpesvirus (Efstathiou et al., 1990a; Efstathiou et al., 1990b). This assignment was confirmed when the entire genome was sequenced and analyzed (Virgin et al., 1997). Comparison of candidate genes encoded by MHV68 to the known γ-herpesviruses (lymphocryptoviruses) demonstrated that MHV68 is most closely related to the $\gamma 2$ herpesviruses (rhadinoviruses) HVS and KSHV, and is more distantly related to the $\gamma 1$ herpesvirus EBV (Virgin et al., 1997) (Fig. 28.1). Notably, all the γ-herpesviruses share blocks of conserved genes, which are interspersed with genes that appear to be largely virus specific (i.e., no significant homology to genes present

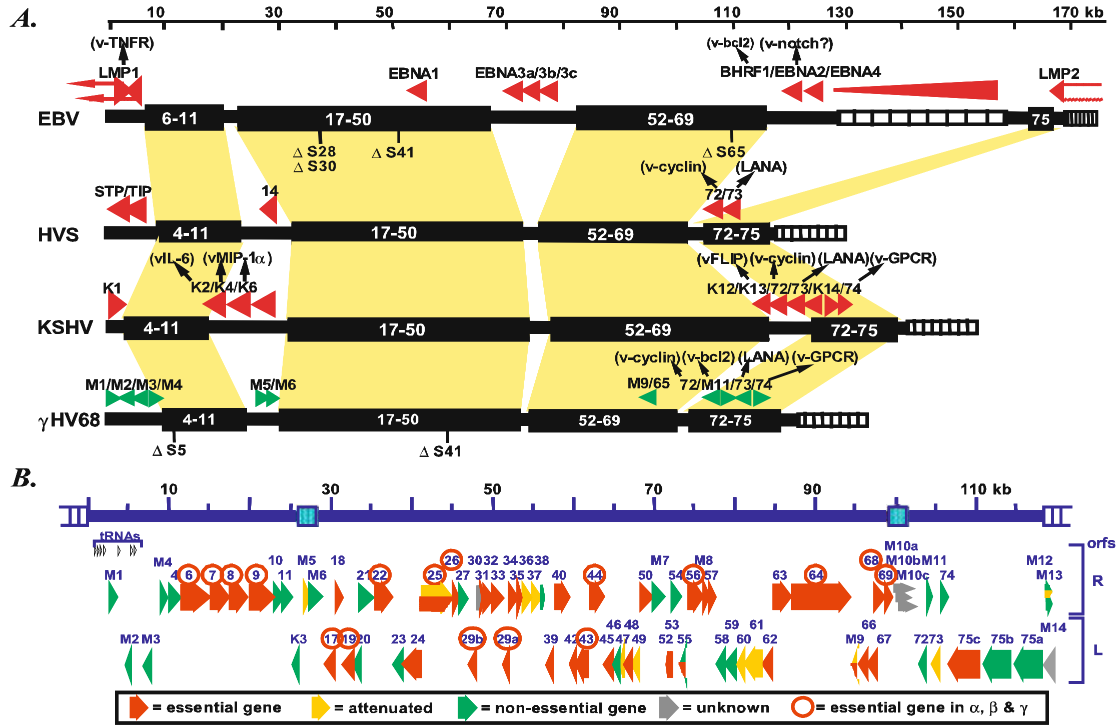

Fig. 28.1 **(A)** Alignment of sequenced gammaherpesvirus genomes. Conserved blocks of genes are indicated along with genes that are unique to each gammaherpesvirus. **(B)** Summary of mutagenesis studies identifying viral genes involved in virus replication in vitro. Data were largely compiled from two large transposon mutagenesis screens (Moorman et al., 2004; Song et al., 2005), but also include data compiled from targeted mutagenesis studies (see Table 28.1 and references therein). The locations of the 40 and 100 bp internal repeats are shown as light blue boxes on the viral genome (*See* Color Insert)

Table 28.1 Phenotypes of Targeted MHV68 Mutants

Gene	Known function	In vitro replication	Replication lungs	Latency: Spleen establish	Latency: Spleen reactiv	Latency: PECs establish	Latency: PECs reactiv
M1	Secreted; superantigen?	wt	wt	wt	wt	▲3x	▲10-15x
M2	adaptor protein	wt	wt	wt - ▼100x	wt - ▼100x	wt	▼3-fold
M3	chemokine binding protein	wt	wt	wt	wt	wt	wt
M4	secreted protein	wt	wt	▼10x	▼10x	nd	nd
orf4	RCA protein	wt	wt	wt	wt	wt	wt
orf6	ssDNABP	essential	nd	undetected	nd	wt	undetected
MK3	E3 ligase - MHC I modulation	wt	wt	wt	▼10-50x	nd	nd
orf11	?	wt	▼3-5x	wt	wt	nd	nd
orf18	?	essential	nd	nd	nd	nd	nd
orf21	tk	wt	▼1,000x	nd	▼10x	nd	nd
orf27	gp48	wt - ▼10x	▼10x	nd	▼10x	nd	nd
orf28	glycoprotein	wt	wt	nd	wt	nd	nd
orf31	?	essential	nd	▼30x	nd	nd	nd
orf39	gM	essential	nd	nd	nd	nd	nd
orf45	?	essential	nd	nd	nd	nd	nd
orf47	gL	▼10x	▼5-10x	wt	wt	nd	nd
orf50	Rta	essential	undetected	undetected	undetected	nd	nd
M7	gp150	wt	wt	nd	wt	nd	nd
orf72	v-cyclin	NE	wt - ▼10x	wt or nd	wt - ▼40x	wt	▼10-50x
M11	v-bcl2	wt	wt	wt - ▼3x	wt - ▼10x	wt	▼4-5x
orf73	LANA	wt - ▼10x	▼10x	▼>100x	undetected	nd	nd
orf74	v-GPCR	wt	wt	wt	wt	▲10x	wt

In most cases latency phenotypes determined at early times post-infection (e.g., day 16) are shown, although in cases where the maximal phenotype was observed at later time points those phenotypes are indicated (refer to individual references for experimental details). Route and inoculating dose had a substantial impact in some cases, and for those mutants the range of phenotypes observed is indicated. Establish., establishment of latency; Reactiv., ex vivo reactivation from latency; wt, like wild type virus; nd, not determined. Phenotypes were compiled for each mutant from the following references: M1, Clambey et al. (2000); Evans et al. (2008); M2, Jacoby et al (2002), Herskowitz et al. (2005); M3, van Berkel et al. (2002); M4, Evans et al. (2006); Geere et al. (2006); orf4, Kapadia et al. (2002); orf6, Tibbetts et al. (2006); MK3, Stevenson et al. (2002); orf11, Boname et al. (2005); orf18, Arumugaswami et al. (2006); orf21, Coleman et al. (2003); orf27, May et al. (2005); orf28, May et al. (2005a); orf31, Jia et al. (2004), Flano et al. (2005); orf39, May et al. (2005b); orf45, Qingmei et al. (2005); orf47, Gillet et al. (2007); orf50, Pavlova et al. (2003); M7, de Lima et al. (2004); orf72, Hoge et al. (2000), van Dyk et al. (2000), Upton et al. (2006); M11, Gangappa et al. (2002); de Lima et al. (2005); orf 73, Moorman et al. (2003); Fowler et al. (2003); orf74, Moorman et al. (2003).

in other γ-herpesviruses) (Fig. 28.1A). As discussed below, a number of these virus-specific genes are involved in regulating viral latency and/or manipulation of the host immune response. Analysis of the MHV76 genome suggests that it is a spontaneous deletion mutant of MHV68, having lost ca. 9 kb from the left-hand end of the unique region of the viral genome – resulting in a loss of the M1, M2, M3, M4 genes and the non-coding tRNA-like and miRNA genes encoded in this region (Macrae et al., 2001). Notably, a nearly identical deletion mutant of MHV68 was recovered during in vitro passage of MHV68, suggesting that this deletion may occur relatively frequently during growth of MHV68 in tissue culture (Clambey et al., 2002).

Viral genes required for virus replication in tissue culture have been identified by screening transposon mutagenesis libraries, as well as targeted mutation of specific viral genes. Moorman et al. (2004) characterized 53 distinct transposon mutants that disrupted 29 known open reading frames in the MHV68 genome. Of these, 16 were identified as essential genes while transposon insertions in 6 other open reading frames resulted in attenuated virus replication. A more extensive analysis of a library of 1,152 MHV68 transposon mutants, characterizing those transposon mutants with insertions near the predicted 5′ends of known MHV68 open reading frames (Song et al., 2005), identified 41 essential genes, 6 genes whose disruption resulted in an attenuated phenotype and 26 genes that were non-essential for replication in tissue culture. The results from the two transposon mutagenesis screens were in good agreement. However, some caution in interpreting the results obtained with transposon mutants is warranted, since large insertions in the viral genome may affect expression of adjacent genes and as such could lead to complex phenotypes that are not solely attributable to the open reading frame that has been disrupted. Finally, a number of open reading frames in the MHV68 genome have been specifically disrupted – in many cases by the introduction of small disruptions (e.g., insertion of a translation termination codon) to minimize effects on neighboring gene expression (see Table 28.1 and references therein). Notably, with respect to in vitro replication phenotypes, the results obtained with the targeted gene mutations correlate well with the data obtained from the transposon mutagenesis screens. A summary of the essential, attenuated and non-essential viral genes compiled from these analyses is summarized in Fig. 28.1B.

28.3 Sites of MHV68 Latency and Identification of Latency-Associated Genes

Detection and quantitation of MHV68 latently infected cells. Latency is the presence of the MHV68 viral genome in a reactivatible form in the absence of ongoing production of infectious virus. Thus, chronic MHV68 infection differs from chronic infection with LCMV, HCV, or HIV in which continuous productive infection occurs, and this fundamental difference very likely has a

significant impact on mechanisms of immunity involved in controlling γ-herpesvirus infection. Antigens expressed in latent cells may stimulate an immune response. Intermittent reactivation from latency, perhaps at discreet anatomical sites, likely occurs, providing a potential source of chronic stimulation with lytic-viral antigens.

To date, MHV68 latency has primarily been assessed by determining the frequency of cells that reactivate MHV68 from latently infected tissue upon ex vivo culture. A critical control for all reactivation analyses is a quantitative assessment of the level of preformed infectious virus present in the samples being analyzed, since this can dramatically alter the interpretation of the results obtained. Notably, latency and persistent productive infection can coexist in the same animal. For example, B-cell-deficient mice have demonstrable latency in spleen and peritoneal cells (Weck et al., 1996; Weck et al., 1999a; Weck et al., 1999b) while persistent productive infection has been clearly demonstrated in lung (Stewart et al., 1998) and in the aortic wall (Dal Canto et al., 2000; Weck et al., 1997). Initial studies of MHV68 latency attempted to measure viral latency by assessing plaque formation, detected by 5–7 days in an infectious center assay, using plaque assays to monitor the presence of preformed infectious virus (Cardin et al., 1996; Sarawar et al., 1997; Sunil-Chandra et al., 1992a; Sunil-Chandra et al., 1992b). However, these assays have not been standardized to demonstrate that they have equivalent sensitivity and as such the absence of detectable preformed infectious virus by plaque assay does not assure that the infectious centers observed solely reflect reactivation of latently infected cells. The limitation of plaque assays for detecting preformed infectious virus has been demonstrated for MHV68 (Stevenson et al., 1999c; Stewart et al., 1998; Weck et al., 1996) as well as MCMV (Kurz et al., 1997; Mercer et al., 1988; Pollock and Virgin, 1995). Limiting dilution analysis provides a different assay for simultaneously quantitating both preformed infectious virus and cells that reactivate latent MHV68 (Weck et al., 1996; Weck et al., 1999a). To distinguish preformed infectious virus from latent virus, cells are mechanically disrupted (latent virus cannot reactivate from dead cells) and diluted in parallel with live cells onto permissive monolayers, and the presence of infectious virus is detected by cytopathic effect on the monolayer over 2–4 weeks in culture. Importantly, controls have demonstrated that preformed infectious MHV68 is not inactivated during tissue preparation or by the mechanical disruption procedure employed (Weck et al., 1996; Weck et al., 1999a).

The frequency of cells harboring viral genome is also often determined (Weck et al., 1996; Weck et al., 1999a). However, the relationship between the frequency of cells harboring viral genome and latent infection remains unclear. For example, the frequency of splenocytes from infected normal immunocompetent mice that spontaneously reactivate virus upon explant into tissue culture is never greater than 10% of the cells harboring viral genome (Weck et al., 1996; Weck et al., 1999a). Are the 90% of viral genome-positive cells that fail to reactivate latently infected, or are some or all of these cells functionally dead-end forms of infection that are incapable of reactivating virus? To complicate matters further, while the frequency of splenocytes that spontaneously reactivate virus upon ex

vivo culture precipitously drops to nearly undetectable levels from 2 weeks post-infection to 6 weeks post-infection, the frequency of viral genome-positive cells only drops ca. 10-fold (Weck et al., 1999a). However, reactivation of virus from B cells at late times post-infection can be significantly enhanced by cross-linking surface Ig (Moser et al., 2005b). Analysis of latently infected peritoneal exudate cells (PECs) has demonstrated that nearly all viral genome-positive PECs reactivate ex vivo at 2 weeks post-infection, but that the efficiency of reactivation drops at later times post-infection (Weck et al., 1999a; Weck et al., 1999b). The factors involved in the drop in the frequency of splenocytes and PECs-reactivating virus remain to be determined, but may involve epigenetic phenomenon such as methylation of the viral genome – which occurs during EBV infection in normal seropositive individuals (Paulson and Speck, 1999). As our understanding of virus reactivation increases, it may be possible to better determine the ability of latently infected cell populations to reactivate virus at late times post-infection.

Identification of cell types latently infected by MHV68. MHV68 can establish a latent or persistent infection in the spleen, lymph nodes, lung, peritoneal exudate cells (PECs), blood and bone marrow (Stewart et al., 1998; Sunil-Chandra et al., 1992b; Weck et al., 1996; Weck et al., 1999a). Latency in the spleen has been the most intensively studied, where the pool of latently infected cells that reactivate upon ex vivo culture expands over the first ca. 2 weeks of infection and then decreases in size over the ensuing several weeks (Sarawar et al., 1997; Usherwood et al., 1996a; Weck et al., 1996; Weck et al., 1999a). The mechanism(s) responsible for this decrease is not clear. Notably, PECs are to date the reservoir with the highest frequency of MHV68 latently infected cells, where ca. 1 in 100 cells reactivate MHV68 from normal infected mice at 9–10 days post-infection (Weck et al., 1999a; Weck et al., 1999b).

What cell type(s) harbor latent MHV68? B lymphocytes are clearly a major hematopoietic reservoir of latent virus in infected mice (Sunil-Chandra et al., 1992b). However, MHV68 can efficiently establish a latent infection in B-cell-deficient mice (Weck et al., 1996), and independent analyses reported the persistence of viral DNA in the lungs of B-cell-deficient mice (Stewart et al., 1998). The latter studies provided compelling evidence that mature B cells are not required for MHV68 latency, and raised the possibility that MHV68 may be able to establish latency in another cell type(s). Subsequent analyses have demonstrated the presence of latent virus in both macrophages and dendritic cells (Flano et al., 2000; Weck et al., 1999b). However, analysis of latently infected cell populations in the spleen as a function of time post-infection have shown that non-B-cell reservoirs for latent MHV68 do not contribute significantly to long-term maintenance of MHV68 latency in the spleens of normal immunocompetent mice (Willer and Speck, 2003). Analysis of latently infected PECs from infected C57BL/6 mice has demonstrated that macrophages are the predominant cell type harboring MHV68, and that these viral genome-positive macrophages can reactivate MHV68 indicating that they represent a bona fide latency reservoir (Weck et al., 1999b). In addition, this analysis of latently infected PECs revealed that ca. 10% of the viral genome was present in B cells. Further analysis of viral

infection in the lungs has provided evidence that lung epithelial cells may harbor latent MHV68 (Stewart et al., 1998). However, additional characterization of these viral genome-positive lung epithelial cells will be required to demonstrate that (i) they do not shed low levels of virus and (ii) MHV68 can reactivate from these cells. Notwithstanding the latter issues, it is clear that MHV68 is capable of establishing a latent infection in multiple cell types; the significance of specific MHV68-latency reservoirs remains to be elucidated.

Analysis of MHV68 in different B-cell subsets. For EBV infection it has been shown that latent virus is predominantly found in circulating memory B cells (Thorley-Lawson, 2001). Furthermore, analysis of EBV-latency programs has persuasively argued in support of a model in which EBV manipulates normal B-cell differentiation to gain access to the memory B-cell reservoir. Is there any evidence that other γ-herpesviruses re-program lymphocytes differentiation to persist with the host? Analysis of MHV68 latency in mice as a function of time post-infection has shown that initially all mature B-cell populations (naïve, germinal center, and memory) harbor MHV68 (Flano et al., 2002a; Willer and Speck, 2003). As infection progresses, MHV68 infection is predominantly found in isotype-switched memory B cells. Thus, there are likely strong parallels between EBV and MHV68 chronic infection. It remains to be determined whether MHV68 actively drives the differentiation of naïve murine B cells into the memory reservoir, as has been proposed for EBV infection in humans.

28.4 MHV68 Genes Associated with Latency and Reactivation

As observed with primate gammaherpesviruses in their natural hosts, MHV68 tumorigenesis occurs rarely and is perhaps even less appreciated given the short life span of mice and the lack of potential oncogenic catalysts in experimental settings. Moreover, MHV68 does not readily transform primary cells in culture, although this may owe more to inherent difficulties in culturing murine cells than to any “deficit” in the virus. Nonetheless, MHV68 does promote the development of lymphoproliferative disease and lymphoma in immunosuppressed or immunodeficient BALB/c mice (Sunil-Chandra et al., 1994; Tarakanova et al., 2005). Such experiments facilitated the isolation of a MHV68-positive, latently infected cell line, S11 (Sunil-Chandra et al., 1994). The S11 cell line is unique in that, although other lymphoma lines have been established from MHV68-infected mice, only S11 has maintained the MHV68 genome in an episomal and reactivation competent form (Husain et al., 1999; Usherwood et al., 1996b; Wu et al., 2000). Martinez-Guzman and colleagues performed gene expression analyses on S11, thus revealing viral genes that may directly influence viral persistence and cellular transformation (Martinez-Guzman et al., 2003). These include ORF74 (v-GPCR), ORF73 (LANA homolog), M11 (v-bcl2), M2, and ORFs 75a, b and c. As further implication for potential roles in transformation, transcripts for these genes and others, especially ORF72 (v-cyclin), also are detected in vivo

during viral latency (Marques et al., 2003; Virgin et al., 1999). Since latent infection is a correlate of transformation for primate gammaherpesviruses, particularly in immunocompromised individuals, the role of genes that influence MHV68 latency and reactivation will be reviewed.

Viral cyclin. Of the putative transformation-related MHV68 genes, only the v-cyclin is directly linked to tumorigenesis. V-cyclin is a conserved rhadinovirus gene that, as its name implies, is a viral ortholog of cellular cyclins. The cellular cyclins, through temporal transcriptional regulation and associations with other proteins, perform cell-cycle regulatory functions that control progression through the growth (G0/G1/G2), DNA synthesis (S) and mitotic (M) stages of the eukaryotic cell life cycle. Aberrations in controlling appropriate expression or activation of cellular cyclins, especially cyclins D and E, can positively influence cellular transformation. Consistent with this notion, v-cyclin expression in a T-cell-specific transgenic setting mediates the development of high-grade lymphoblastic lymphomas and increased cell-cycle progression (van Dyk et al., 1999). Determination of the structure of v-cyclin suggests it is an ortholog of the cellular D-type cyclins through orientation of the defining cyclin box (Card et al., 2000). The same is also true of the KSHV cyclin ortholog (Schulze-Gahmen et al., 1999). However, v-cyclin exhibits a more promiscuous substrate range than cellular D-type cyclins, functionally interacting with CDKs 1 and 2 to promote their activity (Upton et al., 2005). Thus, v-cyclin potentially is a functional mimetic of cyclins A, D, E and B.

Consistent with a role in modulating cell-cycle related functions, v-cyclin critically regulates the capacity of MHV68 to reactivate or reinitiate the productive replication cycle from latent infections. Following experimental infections of inbred mice, v-cyclin-deficient MHV68 is severely compromised for reactivation following ex vivo plating of latently infected cells (Hoge et al., 2000; Upton and Speck, 2006; van Dyk et al., 2000). This defect can be partially ameliorated by mitogenic stimuli (Moser et al., 2005b; Upton and Speck, 2006), a finding consistent with a role for v-cyclin in promoting cell-cycle progression to facilitate reactivation from latency. Further, the v-cyclin-dependent reactivation deficit is more pronounced in PECs than splenocytes, and B-cell-deficient mice are incapable of supporting long-term latency after infection with v-cyclin-null viruses (Upton and Speck, 2006; van Dyk et al., 2000; van Dyk et al., 2003). These findings demonstrate the importance of gammaherpesviruses of being able to “drive” the cell into cycle, particularly in certain cell types, to maintain latency and promote emergence from a quiescent replicative state.

Additional experiments revealed that v-cyclin functions in both CDK-dependent and CDK-independent manners, with v-cyclin mutants from the latter category retaining their capacity to reactivate ex vivo following compromised acute replication (Upton and Speck, 2006). As cyclin activity was generally thought to depend on CDK interactions, these findings suggested previously unappreciated roles for cyclins in regulating viral, if not cellular, replication functions. This paradox was further supported by the finding that cellular cyclin E also possesses CDK-independent activity that functions to promote replication

origin loading for DNA synthesis (Geng et al., 2007). Whether v-cyclin functions in a similar fashion remains to be determined, and if so, whether it promotes replication origin loading for the virus, cell or both. Further, it will be interesting to learn if cellular or viral cyclins can functionally complement each other. Moreover, as cyclin E has been shown to promote MEF focus formation independent of its CDK-binding capacity in conjunction with p53 inactivation (Geng et al., 2007), understanding the CDK-independent functions of both cellular and viral cyclins may illuminate unknown cyclin-related functions that influence oncogenesis.

M2 gene product. Although other viral genes have not been directly linked to tumor formation, their role in transformation may be predicted based on mutant phenotypes in vivo, functional analyses or inference from other γ-herpesviruses. Perhaps the most studied of these genes is M2. M2 is a unique MHV68 gene with no known homology to other viral or cellular genes. The M2 protein is proline rich, and as such contains several SH3 protein–protein interaction motifs. These motifs, as well as a positively charged region and tyrosine-directed phospho-acceptor, are thought to guide M2's putative role as a scaffolding protein to either promote or inhibit cellular-signaling programs to benefit the virus (Liang et al., 2006b; Liang et al., 2004; Madureira et al., 2005; Rodrigues et al., 2006). Described M2 functions in isolation are diverse. M2 has been shown to limit IFN signaling and to deregulate DNA damage responses (Liang et al., 2006b; Liang et al., 2004). M2 expression in WEHI-231 B cells correlated with diminished cell-cycle arrest and apoptosis following B-cell receptor cross-linking, presumably dependent on an interaction with the guanylate exchange factor Vav via M2 SH3 domains (Madureira et al., 2005). Further, M2 enhances Vav phosphorylation by the src-family kinase Fyn and is itself a Fyn substrate in vitro (Rodrigues et al., 2006). Consistent with the results in overexpression systems and cell lines, M2 expression in primary B cells promotes proliferation and survival upon LPS stimulation, as well as substantial secretion of cytokines including IL-6 and IL-10 (Siegel et al., 2008). Moreover, the expansion of M2-expressing cells is dependent on IL-10 responsiveness (Siegel et al., 2008). Remarkably, MHV68 infection of vav1 or vav2-null mice results in accumulation of virus in germinal center B cells (Rodrigues et al., 2006), while infection of IL-10-deficient mice results in an overall reduction in latency (Peacock and Bost, 2001). Both of these findings are consistent with phenotypes of M2-null MHV68 infection of wild-type mice (see below), and further suggest the importance of M2-directed manipulation of B-cell signaling in MHV68 persistence.

The biochemical data suggesting M2-dependent host-cell manipulation are supported by several in vivo studies. M2-null MHV68 exhibits inefficient reactivation ex vivo following intraperitoneal inoculation of mice (Herskowitz et al., 2005; Jacoby et al., 2002). However, intranasal inoculation with M2-null viruses revealed a dose-dependent failure of MHV68 to spread to distal latency reservoirs and a further incapacity to reactivate ex vivo, despite normal acute virus replication in the lung (Herskowitz et al., 2005; Jacoby et al., 2002). Over time, whereas infection with wild-type MHV68 promotes lymphocyte

proliferation and splenomegaly leading to latent viral accumulation in memory B cells, M2-deficient virus fails to promote lymphocyte expansion and is preferentially found in non-class switched B cells (Herskowitz et al., 2005; Simas et al., 2004). An elegant study utilizing viruses with M2 mutations in Src-homology domains further supports a putative role for M2 in manipulation of host-cell signaling to promote latent MHV68 infection (Herskowitz et al., 2008). However, since it is not clear that mutation of the vav interaction motifs in this system yields a negative phenotype in vivo, these data highlight the complexities and question the reliability of limited biochemical analyses of viral gene functions. Finally, it is intriguing to note that latent infection in type I interferon non-responsive mice is characterized by increased M2 transcript detection in vivo (Barton et al., 2005). Given the role of M2 in promoting efficient ex vivo reactivation and the hyper-reactivation phenotype displayed by MHV68 in these mice, these data suggest a role for IFN in regulating M2 expression and further predict the capacity of M2 to drive host B-cell signaling events that facilitate viral reactivation.

LANA homolog. ORF73, the MHV68 homolog to the KSHV LANA protein, is another likely contributor to MHV68-induced tumorigenesis. Reminiscent of LANA in KSHV-associated tumors, ORF73 is the most abundant transcript in S11 cells (Martinez-Guzman et al., 2003). Following intranasal inoculation, ORF73 is required for establishment of latent infection in the spleen (Fowler et al., 2003; Moorman et al., 2003b), the absence of which would clearly impact potential viral oncogenic insults to the host. Additionally, ORF73 is capable of blocking MHC class I-restricted epitope presentation to cytotoxic T cells (Bennett et al., 2005), which could facilitate evasion by MHV68-harboring cells from the host tumor immunosurveillance machinery. Interestingly, a role in immune-evasion is consistent with a role for ORF73 in promoting viral replication, expansion of early latency or reactivation-based seeding of latency reservoirs. Indeed, Moorman and colleagues observed an acute replication defect in the lungs of C57BL/6 mice (Moorman et al., 2003b), a phenotype suggested by ORF73's immediate-early expression pattern (Coleman et al., 2005) and borne-out by very low multiplicity infections of primary fibroblasts in culture (Forrest et al., 2007). In the latter study, ORF73-deficient MHV68 activated p53 and caused increased cell death relative to a MR virus. Moreover, ORF73 expression inhibited p53 stabilization and cell death following treatment with DNA-damaging agents. A capacity to alter normal p53 function also is consistent with primate rhadinovirus ORF73/LANA functions and further predicts a role for ORF73 in cellular transformation.

Viral bcl-2. Another potentially oncogenic MHV68 protein is the viral bcl-2 homolog (v-bcl-2) encoded by the M11 gene. Ectopically expressed v-bcl-2 is capable of inhibiting apoptosis induced by a variety of stimuli, including Fas, TNF-α, and Sindbis virus infection (Bellows et al., 2000; Roy et al., 2000; Wang et al., 1999). When expressed as a transgene using a T-cell-specific promoter, v-bcl-2 provides thymocyte protection from γ-irradiation, dexamethasone treatment and anti-CD3 stimulation (Loh et al., 2005). Further, a bcl-2 homology 3 (BH3) peptide-binding groove contained within the BH1 region of v-bcl-2

facilitates interactions with pro-apoptotic bcl-2 family members Bax and Bak to protect yeast from death induced by these proteins. Transgenic v-bcl-2 expression also promotes expansion of total thymocytes in this setting, although no indication of tumorigenesis was described (Loh et al., 2005), perhaps reflecting a role for v-bcl-2 in another cell type or in conjunction with other viral gene products. A role for cellular and viral bcl-2 proteins in the regulation of autophagy through interactions with beclin 1 also was suggested (Liang et al., 2006a; Pattingre et al., 2005); however, the impact of these events in viral replication or pathogenesis has not been described.

The phenotype of v-bcl-2 mutant MHV68 is primarily one of persistent replication, particularly in IFNγ non-responsive mice. It should be noted that de Lima and colleagues report a modest reduction in total MHV68 genomes in the spleen (rather than genome-positive cells) during the early establishment and expansion of latency following intranasal inoculation (de Lima et al., 2005). In IFNγ-sufficient C57BL/6 mice, lack of v-bcl-2 results in a mild defect in ex vivo reactivation from PECs following high-dose intraperitoneal inoculation (Gangappa et al., 2002b). However, v-bcl-2-null and BH-domain mutant MHV68 exhibit decreased persistent replication (a correlate of disease in IFNγR–/– mice) in the PECs of IFNγ- R–/– mice, despite equivalent presence of viral genome-positive cells (Gangappa et al., 2002b; Loh et al., 2005). This deficit in persistent viral replication corresponds to a decrease in lethality over time (Gangappa et al., 2002b). The decreased reactivation capacity and persistent virus replication suggest a critical role for v-bcl-2 as an integrator of host-cell reactivation triggers or as a determinant of the virus' "fitness" in responding to such stimuli. Further characterization of the biochemical events leading to reactivation will likely reveal a critical role for the v-bcl-2 in promoting cell survival during the switch from latency to lytic replication.

Viral GPCR. Another conserved gammaherpesvirus gene with putative transformation potential is the viral G-protein-coupled receptor (v-GPCR) or ORF74. The v-GPCRs are homologs to cellular CXCR chemokine receptors, and both the KSHV and the MHV68 proteins were demonstrated to facilitate transformation of mouse NIH 3T3 fibroblasts (Bais et al., 1998; Flore et al., 1998; Wakeling et al., 2001). However, unlike the KSHV v-GPCR, the v-GPCR of MHV68 is not constitutively active, and requires chemokine binding (particularly by KC and MIP-2) for signaling – presumably through MAPK- and PI3K-responsive cascades (Lee et al., 2003; Verzijl et al., 2004). Furthermore, in contrast to the KSHV v-GPCR (Flore et al., 1998; Verzijl et al., 2004), the NF-kB response from the agonists tested was remarkably weak (Verzijl et al., 2004), suggesting functional differences for the two presumed oncogenes.

In vivo, MHV68 v-GPCR transcripts are detectable during the latency-establishment phase in splenocytes and become restricted to latently infected lung epithelia over time (Wakeling et al., 2001), perhaps reflecting cell-type-specific requirements for the gene product. Mutagenesis experiments indicate that ORF74 is not required for acute replication or pathogenesis, establishment of latency in PECs or splenocytes, or reactivation from PECs, but may modestly

influence ex vivo reactivation from splenocytes (Moorman et al., 2003a). Further support comes from the finding that direct infection of primary splenocytes with ORF74-mutant viruses yields decreased capacity for reactivation in culture (Lee et al., 2003). Provocatively, while wild-type MHV68 responds to KC treatment with enhanced replication in cultured fibroblasts, ORF74-mutant viruses failed to respond to KC treatment (Lee et al., 2003). Given the localization of ORF74 transcripts to lung epithelia during MHV68 latency (Wakeling et al., 2001), one must speculate that analyses of MHV68 v-GPCR function are incomplete, and further analysis should examine the role of ORF74 in promoting reactivation and/or shedding during acute host stress, as well as define signaling pathways that promote cellular transformation.

RTA. ORF50 is a conserved gammaherpesvirus gene that encodes the homolog of the EBV and KSHV lytic transactivator protein, RTA. RTA is an immediate-early gene product (Liu et al., 2000; Wu et al., 2000) capable of transactivating viral early gene promoters (Allen et al., 2007; Pavlova et al., 2005). RTA is required for MHV68 replication, as disruption of the gene or expression of dominant-negative RTA blocks the progression of viral replication (Pavlova et al., 2003; Wu et al., 2001b). Replication of ORF50-null MHV68 can be complemented by growth in a stable cell line expressing MHV68 RTA, indicating that ORF50 is both necessary and sufficient for productive replication (Pavlova et al., 2003). Further, transfection of latently infected S11 cells with RTA expression constructs yields increased viral transcription, production of lytic antigens, rolling circle DNA replication and higher output titers (Wu et al., 2000). Viruses engineered to overexpress RTA exhibit faster replication kinetics in culture and in vivo, and fail to establish latency (May et al., 2004). Conversely, ORF50-deficient MHV68 establishes latent infection at the primary site of inoculation, but fails to expand and disseminate to downstream latency reservoirs – similar to inhibiting viral replication with cidofovir treatment (Moser et al., 2006). Together these data suggest that while RTA-dependent replication is required for seeding of latent reservoirs, negative regulation of ORF50 is a requisite step for viral latency.

28.5 Immunomodulatory Viral Genes

M1 gene product – superantigen activity. As mentioned earlier for ORF73, immunomodulatory functions for viral gene products highlight underlying correlates of MHV68 disease. Several of the unique, or M, viral genes positioned at the left end of the MHV68 genome (see Fig. 28.1) function in this regard. The M1 gene encodes a secreted protein (Evans et al., 2008), whose presence correlates with negative regulation of MHV68 reactivation upon ex vivo plating (Clambey et al., 2000). The presence of M1 is also required for the expansion of CD8+ T cells bearing the Vβ4 T-cell receptor element (Evans et al., 2007). These T cells predominate other TCR elements and are maintained

for the lifetime of the host following wild-type MHV68 infection (Doherty et al., 2001), but are glaringly absent despite equivalent latent viral loads following infection by M1-mutant MHV68 (Evans et al., 2007). The Vβ4 T cells are potent secretors of IFNγ and TNF-α when stimulated with anti-CD3 or by exogenously expressed recombinant M1 protein (Evans et al., 2007). Since the M1-mutant phenotype in vivo is hyper-reactivation (Clambey et al., 2000), this finding is consistent with the described role for IFNγ in controlling MHV68 reactivation (Steed et al., 2006). Impressively, IFNγ receptor-deficient mice develop aortitis and inflammatory-related fibrotic diseases of the lung and spleen which are associated with high mortality following wild-type MHV68 infection; however, infection of these mice by M1-null MHV68 does not result in fibrosis or death, despite normal establishment of latent infection and induction of vascular disease (Clambey et al., 2000; Evans et al., 2007). These data directly implicate the Vβ4 T cells themselves as active participants in disease progression, rather than solely the lack of IFNγ responsiveness in controlling persistent virus replication.

M3 gene product – chemokine binding. The M3 protein shares substantial amino acid identity with M1 and is abundantly expressed during lytic replication in culture (van Berkel et al., 1999). M3 functions as a broad-spectrum chemokine-binding protein, binding and squelching the activity of CC, CXC, and CX3C chemokines (Alexander et al., 2002; Parry et al., 2000; van Berkel et al., 2000). Remarkably, however, M3 does not engage KC or MIP-2, the two most potent stimulators of v-GPCR activity described (Lee et al., 2003; Parry et al., 2000; van Berkel et al., 2000; Verzijl et al., 2004), suggesting that M3 and v-GPCR might work in concert to achieve a common goal. Solution of the M3 structure revealed a homodimer with the capacity to engage two independent chemokine molecules by proposed structural mimicry of both chemokine self-interaction and GPCR-binding motifs (Alexander et al., 2002). M3 is not required for replication in culture or in lungs or spleens of BALB/c or C57BL/6 adult mice in vivo (Bridgeman et al., 2001; van Berkel et al., 2002), but critically influences viral replication, inflammation and lethality following intracranial inoculation of 21-day-old CD1 mice (van Berkel et al., 2002). Although one study utilizing a lacZ inserted MHV68 to ablate M3 indicated that M3 influences the early seeding and expansion of latent infection in BALB/c mice in a CD8+ T-cell dependent manner (Bridgeman et al., 2001), analysis of a more subtle, but equally disruptive, M3 mutation in C57BL/6 mice demonstrated that M3 is not required for latent infections or ex vivo reactivation (van Berkel et al., 2002). Perhaps these conflicting data suggest the influence of additional mouse strain-dependent factors such as the Vβ4+ T-cell expansion described above in determining the importance of chemokine sequestration by M3 in vivo, or simply reveal the potentially disparate influences of large insertions in a densely packed coding region.

RCA protein. ORF4 is conserved gammaherpesvirus gene whose product encodes a regulator of complement activation (RCA). MHV68 RCA is expressed as a late antigen during lytic replication in culture in both cell-surface associated and soluble forms (Kapadia et al., 1999). RCA is capable of

inhibiting both classical and alternative pathways of C3-mediated complement activation/deposition (Kapadia et al., 1999). The importance of this function is evident from acute pathogenesis studies in vivo. While replicating efficiently in culture, RCA-null MHV68 is attenuated for acute replication in C57BL/6 mice or persistence in IFNγR –/– mice, and the absence of RCA yields a dramatic deficit in viral replication and lethality following intracranial inoculation of CD-1 weanling mice (Kapadia et al., 2002). Remarkably, wild-type MHV68 pathogenesis was restored to RCA-deficient virus in C3-null mice (Kapadia et al., 2002). These findings further demonstrate yet another mechanism by which MHV68 has evolved to evade the host immune response.

MK3 gene product – modulation of MHC I expression. The most extensively studied immunomodulatory MHV68 gene is mK3, so named due to its homology to the KSHV K3 gene. MK3 was identified in a functional screen to define MHV68 proteins that blocked CTL epitope presentation (Stevenson et al., 2000). This original study demonstrated reduced cell-surface MHC class I expression in the presence of MHV68 infection, or mK3 expression, and diminished stability of MHC I. Subsequent studies very elegantly demonstrated that not only does mK3 facilitate ubiquitin-mediated degradation of MHC class I by RING-CH domain-directed interaction with class I heavy chain (Boname and Stevenson, 2001), but that mK3 also stimulates the degradation of the TAP-dependent peptide-loading complex via direct interaction with TAP1 – especially after IFNγ treatment (Boname et al., 2004b). Perhaps controversially, another study utilizing ubiquitylation site MHC mutants demonstrated mK3-dependent degradation via a ubiquitin-independent ER-associated degradation pathway (Wang et al., 2005), a process that involves MHC translocation into the cytosol mediated by Derlin1 and p97 (Wang et al., 2006). Nonetheless, mK3-mutant MHV68 does not facilitate MHC class I downregulation on the surface of fibroblasts in culture and is characterized by decreased expansion of latently infected B cells in the spleen, more robust epitope-specific CD8+ T-cell expansion and diminished reactivation potential (Stevenson et al., 2002b). Importantly, CD8+ T-cell depletion rescues colonization of the spleen by the mK3-mutant MHV68 (Stevenson et al., 2002b).

28.6 Immune Response to MHV68 Infection

Loss of immune control during chronic infection leads to reactivation of latent infections. This in turn makes the host cell susceptible to unchecked alterations in survival and proliferation, placing the cell at risk for further tumorigenic insults. Investigations of the immune response to MHV68 have yielded a wealth of information regarding host control of lytic and latent phases of gammaherpesvirus infection. The ability to examine gammaherpesvirus infection in mice that are deficient in immune cell subsets or immune-signaling molecules by knock-out or antibody depletion experiments is an undeniable asset of the

MHV68 system. These findings have been thoroughly covered in recent reviews (Doherty et al., 2001; Flano et al., 2002b; Gasper-Smith and Bost, 2004; Nash et al., 2001; Olivadoti et al., 2007; Stevenson et al., 2002a; Stevenson and Efstathiou, 2005) . The results of these investigations as they relate to control of virus infection are summarized in Table 28.2. Here we will bring attention to more recent advances in unraveling the complexity of immune control during chronic gammaherpesvirus infection.

Adaptive immunity: B cells. B cells have multiple roles in chronic infection by MHV68. B cells are a major reservoir for latent virus and are required for efficient colonization of the spleen with MHV68 latently infected cells upon intranasal infection with MHV68 (Flano et al., 2002a; Stewart et al., 1998; Weck et al., 1999a; Willer and Speck, 2003). While only a fraction of B cells are infected with MHV68 at the peak of splenic colonization, antigen-independent polyclonal activation of B cells and the production of non-viral-specific antibodies is a hallmark of MHV68 infection (Sangster et al., 2000; Stevenson and Doherty, 1998; Stevenson and Doherty, 1999). This polyclonal B-cell activation requires CD40-mediated CD4+ T-cell help (Stevenson and Doherty, 1999; Sangster et al., 2000). However, chronic virus infection is established in B-cell-deficient mice and these animals exhibit abnormal control of virus reactivation at late times post-infection (Weck et al., 1997). The latter result was the first demonstration of alternative cell types harboring latent virus, and suggested a role for antiviral antibodies in regulating virus latency. Intriguingly, McClellan et al. (2006) recently observed that non-viral-specific B cells (anti-HEL Ig transgenic mice) restored normal control of virus latency (in comparison to normal C57Bl/6 mice and B-cell-deficient mice), indicating that the mechanism(s) involved is independent of virus-specific antibody production. The latter result suggested that B cells are functioning either as antigen-presenting cells (APC), providing critical costimulatory signals to activate T cells, or that they fill a requirement for the virus to gain access to the spleen and perhaps other lymphoid tissue, which in turn drives T-cell responses required for appropriate control of latency (McClellan et al., 2006; Stewart et al., 1998; Usherwood et al., 1996c; Weck et al., 1999a). Consistent with the lack of dependence on antiviral antibody responses for normal control of virus infection, the absence of GC formation in CD28–/– mice results in rapid loss of viral-neutralizing antibody (Kim et al., 2002). Notably, these mice appear at late times post-infection to control virus infection. However, following depletion of T cells recrudescence occurs in the lungs of CD28–/– mice, but not normal controls, and this can be controlled by passive transfer of neutralizing antibodies. Other data also demonstrate that antibody can contribute to long-term control of latency and the prevention of recrudescence in the lungs (Gangappa et al., 2002a; Tibbetts et al., 2002). Finally, infection of CD40–/–, which are known to have defective antibody responses (Kawabe et al., 1994), results in greatly increased levels of persistent virus replication in the lungs at late time post-infection (Willer and Speck, 2005). Thus, while a definitive demonstration of the importance of antiviral antibody responses in the control of chronic MHV68 infection is absent, it is very

Table 28.2 Phenotypes of MHV68 in knock-out mouse strains

Mouse genotype	Lung replication	Splenic latency	PEC latency	Disease
Wild-type mice - C57BL/6,129 Balb/c	acute replication cleared by ~12 dpi	lifelong latency, peaks ~16 dpi, reactivation nearly undetectable by 42 dpi	lifelong latency, peaks ~16 dpi, reactivation detectable 42 dpi	transient vasculitis, pneumonitis, splenomegaly
μMT-/- (B cell-deficient)	wt	None post-IN ▲ reactivation post-IP	▲latency and reactivation post-IP	>90% mortality by 200 dpi post-IP
MHCII-/-	cleared, recrudescence after 3 wks	▼ reactivation early (IC) ▲ reactivation late (IC)	nd	>80% mortality by 130 dpi
CD8-/-	▲ acute	▲ latency & reactivation	▲ latency & reactivation	nd
β2 microglobulin -/-	▲ acute delayed clearance	▲ latency & reactivation	▲ latency & reactivation	Mortality 3-31 wks
MHC Ia-/-	wt	wt	wt	nd
CD28-/-	▲ acute	▼ latency	nd	nd
Caspase3-/-	nd	nd	▲ latency	nd
Granzyme-A, -B, -B cluster	▼ acute for granzyme B, wt for A and cluster	nd	▲ latency in triple knock-out	nd
Perforin-/-	wt	▲ reactivation	▲ reactivation	nd
Fas-/-	wt	wt	nd	nd
CD80/CD86-/-	cleared, recrudescence after 41 dpi	▲ latency	nd	nd
SAP-/-	cleared, recrudescence after 45 dpi	▼ latency	nd	▲ lymphocyte infiltration
Protein Kinase Cθ-/-	wt	nd	nd	nd
4-1BBL-/-	wt	▲ latency	nd	nd
CD40L/CD40-/-	persistence	▼ latency	nd	50% mortality
LTα-/-	delayed clearance	▲ reactivation	nd	nd
IFNα/βR-/-	▲ acute	▲ reactivation	▲ reactivation	Dose-dependent lethality
STAT1-/-	nd	nd	nd	100% mortality by d21
IRF1-/-	nd	nd	nd	70% mortality
iNOS-/-	wt	nd	nd	discrepancy regarding mortality
ISG15-/-	▲ acute	nd	nd	No mortality
IFNγ-/-	▲ acute	wt	▲ reactivation	persistence, arteritis
IFNγR-/-	▲ acute	▲ reactivation	▲ reactivation	dose and age-dependent mortality, arteritis, multi-organ fibrosis
IFNα/β/γR-/-	nd	nd	nd	100% mortality by 16 dpi
IL6-/-	wt	wt spleen, ▲ reactivation in MLN	nd	nd
IL-10-/-	nd	▼ reactivation	nd	nd
IL-12-/-	▲ acute	▲ reactivation	nd	nd
CXCR3-/-	delayed clearance	▲ (slightly) reactivation	nd	nd
CCR2-/-	nd	nd	nd	▲ lung inflammation
Apolipoprotein E -/-	nd	nd	nd	accelerates atherosclerosis

Phenotypes were compiled for each mutant from the following references: Wild-type including: C57BL/6, 129, Balb/c, (Nash et al., 2001; Stevenson and Efstathiou, 2005; Virgin and Speck, 1999; Weck et al., 1997); μMT-/-, (Brooks et al., 1999; Weck et al., 1999; Gangappa et al., 2002; McClellan et al., 2006); MHCII-/-,(Cardin et al., 1996; Sarawar et al., 2001); CD8-/-, (Braaten et al., 2006; Tibbetts et al., 2002); β2 microglobulin-/-, (Stevenson et al., 1999; Weck et al., 1997; Braaten et al., 2006); MHC Ia-/-, (Braaten et al., 2006); CD28-/-, (Kim et al., 2002; Lee et al., 2002; Lyon and Sarawar, 2006); Caspase3-/-, (Loh et al., 2004); Granzyme-A, -B, -B cluster, (Loh et al., 2004); Perforin-/-, (Tibbetts et al., 2002; Topham et al., 2001; Usherwood et al., 1997); Fas-/-, (Topham et al., 2001); CD80/CD86-/-, (Fuse et al., 2006; Lyon and Sarawar, 2006); SAP-/-, (Chen et al., 2005; Yin et al., 2003), (Kim et al., 2007); Protein Kinase Cθ-/-, (Giannoni et al., 2005); 4-1BBL-/-, (Fuse et al., 2007); CD40L/CD40-/-, (Brooks et al., 1999; Kim et al., 2003; Lee et al., 2002; Willer and Speck, 2005); LTα-/-, (Lee et al., 2000); IFNα/βR-/-, (Barton et al., 2005; Dutia et al., 1999; Weck et al., 1997); STAT1-/-, (Barton et al., 2005; Weck et al., 1997); IRF1-/-, (Dutia et al., 1999); iNOS, (Dutia et al., 1999; Kulkarni et al., 1997); ISG15-/-, (Lenschow et al., 2007); IFNγ-/-, (Sarawar et al., 1997; Tibbetts et al., 2002; Weck et al., 1997); IFNγR-/-, (Dal Canto et al., 2001; Ebrahimi et al., 2001; Steed et al., 2006; Weck et al., 1997); IFNα/β/γR-/-, (Barton et al., 2005); IL6-/-, (Gasper-Smith et al., 2006; Sarawar et al., 1998); IL-10-/-, (Peacock and Bost, 2001); IL-12-/-, (Elsawa and Bost, 2004); CXCR3-/-, (Lee et al., 2005); CCR2-/-, (Cadillac et al., 2005); Apolipoprotein -/-E, (Alber et al., 2000).
wt, wild-type; nd, not determined; dpi, days post-infection; IN, intranasal inoculation; IP, intraperitoneal inoculation; IC, intracranial inoculation; MLN, mediastinal lymph node; persistence, ongoing virus replication post-clearance of acute replication.

likely that they play an important role in conjunction with other components of the host immune response.

Adaptive immunity: T cells. CD4 + T cells are critical players in the establishment of splenic latency and for immune control (Brooks et al., 1999; Cardin et al., 1996; Christensen and Doherty, 1999; Flano et al., 1999; Flano et al., 2001; McClellan et al., 2004; Sparks-Thissen et al., 2004; Usherwood et al., 1996a). CD4 + T cells provide helper functions to B cells and CD8 + T cells, yet they also have helper-independent functions during MHV68 infection. The autonomous viral-suppressive function of CD4 + T cells does not depend on their antigen specificity, but does involve IFNγ (Sparks-Thissen et al., 2005; Sparks-Thissen et al., 2004).

CD8 + T cells are important in the control of primary lytic replication and long-term chronic infection (Cardin et al., 1996; Ehtisham et al., 1993; Tibbetts et al., 2002). While preformed infectious virus is nearly undetectable in most tissues at late time post-infection, there is likely a low-level, episodic reactivation of virus in mucosal tissues such as the lungs. Viral recrudescence occurs in the lungs in the absence of CD8 + T cells (Cardin et al., 1996; Ehtisham et al., 1993). CD8 + T cells specific for lytic- or latency-associated antigens reduce lytic replication and latency, respectively (Bennett et al., 2005; Braaten et al., 2005; Usherwood et al., 2000; Usherwood et al., 2001).

Mechanisms of immune control by CD8 + T cells vary with the cell types involved at different stages of chronic infection (Tibbetts et al., 2002). Cytotoxic CD8 + T cells may help control lung infection via the release of perforin or engagement of Fas; lytic titers were substantially increased in perforin- or fas-deficient mice upon depletion of CD4 + helper T-cell depletion (Topham et al., 2001a). After intraperitoneal infection, perforin impacts latency and reactivation to a greater extent in the spleen than the PECs (Tibbetts et al., 2002). Granzymes and the granzyme B-responsive caspase 3 also have roles in the control of latency in PECs (Loh et al., 2004).

A hallmark of MHV68 infection is the unusual expansion of Vβ4 + CD8 + T cells, which occurs following clearance of acute virus replication and establishment of latency in the spleen (Stevenson et al., 1999a; Tripp et al., 1997). Based on the analysis of MHV68 M1 gene function, it appears that the Vβ4 + CD8 + T cells control virus reactivation in a cell-specific manner via IFNγ (Evans et al., 2007). The observation that the expansion and activation of this subset of CD8 + T cells occur in mice lacking MHC class Ia molecules, coupled with the ability of these mice to control chronic infection, points toward a role for this unconventional subset of CD8 + T cells in regulating chronic MHV68 infection (Braaten et al., 2006; Evans et al., 2007).

Costimulatory signals. Costimulatory signals provided through the CD80/CD86-CD28 pathway are essential for the development of humoral and cellular immune responses. CD28–/– mice lack germinal centers, resulting in impaired virus-specific antibody responses and decreased IFNγ production upon infection. These mice have slightly lower peak splenic latency, but the virus is maintained without lung recrudescence at late time points (Kim et al., 2002; Lee et al.,

2002). Mice deficient in both CD80 and CD86 signaling molecules on the surface of APCs that bind CD28 of T cells, exhibit lower peak splenic latency, with decreased neutralizing antibodies and IFNγ production as found for CD28–/– mice (Fuse et al., 2006; Lyon and Sarawar, 2006). Surprisingly, CD80/CD86–/– mice are unable to maintain long-term control of virus infection, suggesting a new CD28-/CTLA-4-independent role for CD80/CD86 in immunosurveillance (Fuse et al., 2006; Lyon and Sarawar, 2006). The absence of the 4-1BBL/4-1BB costimulatory pathway results in a significant loss of virus-specific CD8+ T-cell function, but a substantial increase in long-term viral latency in the spleens and the lungs without viral recrudescence in the lungs (Fuse et al., 2007).

Lymphocyte-signaling pathways. Now that a framework of MHV68 pathogenesis is in place, viral–host interactions in biologically relevant tissues and cell subsets can be investigated. Specific host-signaling pathways that are modulated by MHV68 are an active area of investigation as a result of the ever-expanding variety of knock-out mice, conditional knock-out mice and a new generation of recombinant viruses expressing CRE recombinase or transdominant inhibitors of specific pathways (Krug et al., 2007; Moser et al., 2005a). The activation of NF-kB by gammaherpesviruses is critical for their ability to alter B-cell biology in order to drive cell survival, proliferation, cellular gene expression and lymphomagenesis (Bowie et al., 2004; Lam and Sugden, 2003). However, since NF-kB signaling is integral to innate and adaptive immune responses, complete knock-out of signaling molecules may lead to immunodeficiencies that alter the normal biology of the virus. CD40L engagement by CD40 on B cells activates NF-kB and is critical for cell survival, germinal center formation and humoral protection (Sen, 2006). CD40 signaling, likely via CD4+ T-cell interactions with B cells, is required for initiating splenomegaly upon MHV68 infection (Brooks et al., 1999; Usherwood et al., 1996a). Interestingly, MHV68 gained access to a CD19+ IgD– memory B-cell population in CD40–/– mice, but a B-cell-intrinsic role for CD40 in viral latency is difficult to discern since virus replication persisted in the lungs and possibly served as a constant source of reseeding (Willer and Speck, 2005). This may reflect a requirement of CD40-mediated CD4+ T-cell help in long-term CD8+ T-cell immune control (Brooks et al., 1999; Cardin et al., 1996; Sarawar et al., 2001). Alternatively, similar to EBV, signaling through CD40 may provide signals that suppress virus reactivation (Adler et al., 2002; Sarawar et al., 2001).

The generation of mixed CD40–/– and CD40+/+ bone marrow chimeric mice permitted an evaluation of gammaherpesvirus latency in CD40-sufficient and CD40-deficient B cells in the context of a normal host immune response (Kim et al., 2003). Interestingly, while MHV68 established latency in both CD40-sufficient and CD40-deficient cells, long-term latency was not maintained in the CD40-deficient B cells (Kim et al., 2003). These data suggest that infected B cells require CD40 signaling for long-term maintenance of virus latency in B cells, at least in competition with CD40-sufficient B cells. Notably, however, chronic virus infection in CD40–/– mice is maintained, although these animals have significant ongoing persistent virus replication in the lungs (Willer and Speck, 2005). In the latter situation, it may be that ongoing

virus reactivation and replication in MHV68-infected CD40–/– mice serves to continually reseed the B-cell latency reservoir due to the absence of a sufficiently robust humoral immune response.

Krug et al. (2007) generated a recombinant MHV68 that expressed IkBaM, a transdominant inhibitor of NF-kB signaling. While overexpression of the NF-kB p65 was shown to inhibit MHV68 lytic replication in tissue culture (Brown et al., 2003), inhibition of NF-kB signaling by MHV68-IkBaM did not result in increased acute titers in vivo (Krug et al., 2007). However, this transgenic virus was defective in B-cell latency after intranasal infection (Krug et al., 2007). As with other latency-deficient mutants, B-cell activation was decreased, suggesting that this virus was unable to drive activation-induced proliferation to reach and/or establish peak latency in the spleen. Further investigation of NF-kB function and viral modulation of this pathway will likely provide insights critical for understanding gammaherpesvirus latency in B cells.

Antiviral activity of interferons. Innate immune mechanisms are critical for the control of MHV68 lytic replication in the lungs and inhibit reactivation from latency. The infection of mice non-responsive to type I interferons (IFN α/βR–/–) results in faster replication kinetics and higher peak titers in multiple organs (Dutia et al., 1999). This systemic spread leads to high rates of mortality in a dose-dependent manner (Barton et al., 2005; Dutia et al., 1999; Weck et al., 1997). Lethal infections are also observed with mice lacking the type I IFN-inducing factor, IRF-1 (Dutia et al., 1999) or the IFN-induced signaling molecule STAT1 (Barton et al., 2005; Weck et al., 1997). IFNα/βR–/– mice that survive infection have altered latency programs, the peritoneal cells have a hyper-reactivation phenotype and the latency-associated gene expression profile differs in splenocytes (Barton et al., 2005). Taken together, type I interferons have potent antiviral activity during early infection and later serve to modulate latency.

IFNγ is not the major antiviral molecule that mediates clearance of acute replication in the lungs, but rather appears to be critical for CD8 + T-cell-mediated control of chronic infection (Dutia et al., 1997; Sarawar et al., 1997). IFNγ blocks MHV68 reactivation from latently infected PECs and non-B-cell splenocytes upon explant, and in vivo depletion of IFNγ leads to enhanced PEC reactivation (Steed et al., 2007; Steed et al., 2006). The deletion of macrophages, but not B cells, reduced the frequency that PECs reactivated upon explant (Steed et al., 2007). Taken together, IFNγ has potent antiviral activity against macrophages, but not B cells in MHV68-infected mice.

Mice deficient in IFNγ or the INFγ receptor initially clear acute replication in the lungs, but exhibit increased reactivation and persistent replication (Dal Canto et al., 2001; Dal Canto et al., 2000; Dutia et al., 1997; Ebrahimi et al., 2001; Tibbetts et al., 2002; Weck et al., 1997). Persistent MHV68 replication in mice deficient in IFNγ activity results in the development of vasculitis in the great elastic vessels and chronic inflammation leading to fibrosis in the spleen, lymph nodes, liver, and lungs. These pathologies are associated with significant mortality (Dal Canto et al., 2001; Dal Canto et al., 2000; Dutia et al., 1997; Ebrahimi et al., 2001; Mora et al., 2005; Tibbetts et al., 2002; Weck et al., 1997). Antibody

depletion of CD8+ or CD4+ T cells prevented fibrosis (Dutia et al., 1997), indicating that host immune pathology plays a role in this disease process.

28.7 MHV68-Associated Diseases

The infection of inbred strains of mice with MHV68 drives splenomegaly, pneumonitis and vasculitis, yet mice rarely appear symptomatic even upon high-dose infection. The infection of mice deficient in particular components of their immune system leads to viral persistence that is associated with an increased incidence in lymphoproliferative disorders and inflammatory disease. Understanding the basis for MHV68 disease in immunosuppressed mice is likely to provide mechanistic insights into EBV and KSHV reactivation-associated disease and cancers in immunosuppressed patients.

Lymphoproliferative diseases. As might be expected with an infectious agent that has co-evolved with its host for millennia, infection of immune-competent mice by murine gammaherpesvirus 68 is not highly tumorigenic. Sunil-Chandra et al. (1994) reported a 9% incidence of lymphoproliferative disease (LPD) in BALB/c mice over a period of 3 years. Lymphoma development has been reported in other inbred strains of mice, but BALB/c mice have polymorphisms that lead to alterations in DNA repair and cell-cycle control (Dragani, 2003; Yu et al., 2001) that might explain a higher incidence of MHV68-associated LPD on the BALB/c background (Sunil-Chandra et al., 1994; Tarakanova et al., 2005). Notably, the majority of lymphoma cell lines derived from MHV68-infected mice do not harbor the MHV68 genome (Usherwood et al., 1996b), indicating a possible hit-and-run mechanism of oncogenesis or direct paracrine and/or inflammatory-mediated events caused by MHV68 infection that promote lymphomagenesis in the infected mice (Tarakanova et al., 2005; Usherwood et al., 1996b). Viral-associated lymphomas are B220+, often clonal, and are found in the liver, lung and kidneys (Tarakanova et al., 2005; Usherwood et al., 1996b). An increased incidence of LPD has also been reported in mice infected with MHV78 and MHV60, isolates that are closely related to MHV68 (Mistrikova et al., 2006a; Pappova et al., 2004).

The occurrence of human gammaherpesvirus-associated lymphoproliferative disease is increased in immunocompromised hosts. Cyclosporine A treatment increased the incidence of LPD in MHV68-infected BALB/c (Sunil-Chandra et al., 1994). In a careful analysis by Tarakanova et al. (2005), β2 microglobulin-deficient mice on a BALB/c, but not 129/Pas background, infected with MHV68 exhibited a higher frequency and more rapid development of LPD than naïve mice. β2-microglobulin-deficient mice are characterized by a loss of CD8+ T cells in addition to decreases in other non-classical class I molecules, and alterations in immunoglobulin homeostasis (Israel et al., 1996). Further phenotypic analyses of the cells that harbor virus in lymphoproliferative specimens, in addition to the molecular signature of viral gene expression

in the expanded B-cell population, may provide mechanistic insight into MHV68-associated lymphomagenesis.

Infectious mononucleosis-like syndrome. Infectious mononucleosis (IM) often arises during primary EBV infection of adolescents. IM is characterized by a rapid expansion of CD8+ T cells, polyclonal B-cell activation and an associated enlargement of lymph nodes and the spleen. MHV68 infection of mice leads to a striking expansion of the Vβ4 subset of CD8+ T cells that is observed subsequent to the resolution of the acute phase of replication and the contraction of splenomegaly, well after the peak of lytic antigen-specific CD8+ T cells (Tripp et al., 1997; Brooks et al., 1999; Flano et al., 1999; Stevenson et al., 1998). The latency-associated Vβ4+ CD8+ T cells remain elevated at late time points, impressively accounting for 30–60% of CD8+ T cells (Chen et al., 2005; Flano et al., 2004; Tripp et al., 1997). These unconventional CD8+ T cells are MHC Ia haplotype-independent, self-renewing, proliferative and can produce IFNγ and TNF-α, but have altered phenotypic markers for T-cell activation (Coppola et al., 1999; Evans et al., 2007; Flano et al., 2004; Tripp et al., 1997). The presence of Vβ4+ CD8+ T cells does not impact splenic latency, but may control low-level reactivation events that occur in other tissues and cell reservoirs such as the peritoneum during chronic infection (Flano et al., 2004; Steed et al., 2007; Evans et al., 2007).

Latently infected B cells, CD4+ T cells, and intact CD40 signaling are required for Vβ4+ T-cell expansion, suggesting that CD4+ T-cell and B-cell interactions precede the stimulation of Vβ4+ CD8+ T cells (Brooks et al., 1999; Flano et al., 1999). Indeed, we have recently identified the secreted MHV68 M1 product as a Vβ4+ CD8+ T-cell stimulatory factor (Evans et al., 2007). Viruses lacking M1 fail to drive Vβ4+ CD8+ T-cell expansion (Evans et al., 2007). Furthermore, in the IFNγR–/– model of persistent replication and chronic inflammation, the lack of M1-driven Vβ4+ CD8+ T-cell expansion protects these infected mice from multi-organ inflammation and fibrosis (Evans et al., 2007).

Interestingly, EBV infection activates an endogenous human retroviral superantigen and drives activation of a Vβ-specific CD4+ T-cell population (Hsiao et al., 2006; Sutkowski et al., 2001; Tai et al., 2006). It has been proposed that this T-cell response in humans provides B-cell help and promotes EBV latency, and/or plays a role in EBV-associated autoimmune diseases (Tai et al., 2006). While the impact of the Vβ4+ CD8+ T-cell expansion during MHV68 infection in mice likely involves IFNγ-mediated control of virus reactivation (Evans et al., 2007), further characterization of their derivation in the MHV68 system will likely provide important insights into how gammaherpesviruses may manipulate host T-cell responses during chronic infection.

X-linked Lymphoproliferative (XLP) Syndrome. XLP syndrome is characterized by hypogammaglobulinemia and fatal infectious mononucleosis upon EBV infection (Purtilo et al., 1982; Tatsumi and Purtilo, 1986). The gene encoding the signaling lymphocyte activation molecule-associated protein (SAP) is altered in these patients. SAP is an SH2 domain-containing adaptor

protein that alters signaling to regulate Th2 cytokines and perhaps T-cell homeostasis (Czar et al., 2001; Wu et al., 2001a). Notably, MHV68-infected SAP–/– mice exhibit hypogammaglobulinemia and a striking increase in the cytotoxic activity of virus-specific CTL and Vβ-specific CD8 + T cells (Chen et al., 2005; Kim et al., 2007; Yin et al., 2003). Thus, although there has been only limited study of MHV68 in SAP–/– mice, it appears that aspects of XLP syndrome in humans are recapitulated in this small animal model. As such, further studies using this model system offer promise for understanding the association between gammaherpesvirus infection and the development of severe immune-dysfunction in these patients.

Pulmonary Fibrosis. Idiopathic pulmonary fibrosis (IPF) is a progressive fibrotic lung disease that kills patients 3–5 years from diagnosis (Selman, 2002). Recent reports suggest an association of human gammaherpesvirus infection with IPF and pulmonary hypertension (Cool et al., 2003; Tang et al., 2003). The intranasal infection of IFNγR–/– mice results in multi-organ fibrosis (Ebrahimi et al., 2001), including lung disease with clinical manifestations similar to idiopathic pulmonary fibrosis in humans (Mora et al., 2005). Understanding the role of immune cell subsets and immune molecules during MHV68 chronic infection may yield important insight into the development of therapeutics for virus-associated lung disease (Doran and Egan, 2005). Notably, pulmonary fibrosis also occurs in MHV68-infected immunocompetent BALB/c mice treated with bleomycin, consistent with a multi-factoral induction of this disease (Lok et al., 2002).

Dysregulated host repair in response to ongoing alveolar epithelial injury is one component of IPF (Reynolds, 1987). In the MHV68 IPF model, viral proteins are detected in type II alveolar epithelial cells of infected IFNγR–/– mice (Mora et al., 2005). Depending on the time of administration, antiviral treatment can diminish fibrosis and lower TGFβ levels and other markers of macrophage activation (Mora et al., 2007). In addition, infection with a MHV68 reactivation-defective mutant also resulted in a reduction in fibrosis, further supporting the hypothesis that virus persistence drives chronic inflammation. Thus, by extension, antiviral therapy may be effective for treating IPF in humans (Mora et al., 2007). IFNγR–/– mice infected with M1-deficient MHV68 fail to expand Vβ4 + CD8 + T cells and are protected from fibrotic disease, indicating an immunopathological role for the Vβ4 + CD8 + T cells (Clambey et al., 2000; Evans et al., 2007).

Vasculitis. Infection of IFNγR–/– mice results in a failure to control persistent virus replication, and is associated with the development of severe inflammation of the large elastic arteries. These lesions are strikingly similar to lesions in several forms of human vasculitis – most notably, Takayasu's arteritis, the nongranulomatous variant of temporal arteritis, and Kawasaki's disease (Weck et al., 1997). Increasing the dose of MHV68 in wild-type mice resulted in arteritis that persisted up to 10 weeks, but 50-fold less virus led to chronic arteritis in IFNγR–/– mice (Dal Canto et al., 2000). IFNγ had antiviral activity in smooth muscle cells and other primary cells of the aorta in tissue culture (Dal

Canto et al., 2000). Notably, arteritic lesions were substantially resolved upon clearance of viral antigen from the media of the large elastic vessels upon antiviral treatment, demonstrating a role for persistent virus replication in the maintenance of vasculitis (Dal Canto et al., 2000). Importantly, the loss of fibrosis, but not arteritis, in mice infected with M1-deficient MHV68 unlinks the immune pathology of these virus-driven disorders (Clambey et al., 2000). In related studies, it has been shown that MHV68 infection of the aorta in apolipoprotein E-deficient mice accelerated atherosclerosis and, consistent with the observations of Dal Canto et al. (2000), disease was reduced upon antiviral therapy (Alber et al., 2000).

Arthritis. In a mouse model of transient arthritis described by Yarilin et al. (2004), MHV68 infection increased the severity and duration of disease in immune competent and RAG1–/– mice. Viral DNA, antigens and viral particles were detected in the inflamed joints. MHV68 also induced a second wave of arthritis that was suggested to represent the import and reactivation of infected cells into the synovium. The enhanced arthritis involved ongoing lytic replication because the antiviral drug cidofovir blocked the second peak of arthritis, but the immunosuppressive drug Cytoxan increased joint swelling (Yarilin et al., 2004). The possible reactivation of MHV68 in mice undergoing immunosuppressive therapy in this arthritis model is interesting in light of the occurrence of EBV-related lymphomas in rheumatoid arthritis patients treated with methotrexate (Kamel, 1997; Mariette et al., 2002).

Neurologic disease. Intracerebral inoculation of mice with MHV68 is fatal and leads to virus spread and mononuclear inflammatory infiltrates within the CNS, with a more rapid onset of clinical symptoms and death evident in younger mice (Gangappa et al., 2002a; Terry et al., 2000). M3-deficient MHV68 is 100-fold less virulent upon intracerebral infection, loss of this chemokine-binding protein prevented a neutrophilic meningeal inflammatory infiltrate (van Berkel et al., 2002). In a more relevant biologic setting, MHV68 is detected in multiple tissues at late times after intranasal infection of adult mice, most frequently in lymphoid organs and rarely in non-lymphoid tissues such as the brain (Flano et al., 2003). However, the infection of IFN-I knock-out mice results in rapid spread to the CNS (Terry et al., 2000). The intranasal infection of newborn mice with MHV68 leads to cerebral infection with severe inflammation in 50% of animals; viral antigen was detected in the brain tissues of symptomatic mice (Hausler et al., 2005). In contrast, although MHV68 exacerbated experimental autoimmune encephalomyelitis in adult rodents, virus was not detected in the CNS of adult mice (Peacock et al., 2003).

28.8 Vaccination and Immunotherapy

Latency prevention by vaccination is the ultimate challenge for gammaherpesviruses since, at least in the case of EBV infection, the serious diseases associated with gammaherpesvirus infection are lymphomas and other tumors that largely

involve latent infection. As such, drugs or vaccines that target virus replication may have little impact – unless that can successfully generate sterilizing immunity. In the case of MHV68 infection, multiple vaccination strategies have been utilized to prime the immune system to prevent lytic replication and/or latency establishment upon challenge with wild-type virus.

Subunit vaccination. Vaccinating mice with lytic cycle proteins (Stewart et al., 1999) or known MHC class I- or II-restricted lytic protein epitopes reduce lung titers and peak splenic latency upon wild-type challenge (Liu et al., 1999; Stevenson et al., 1999b). Vaccination with the latency-associated M2 antigen had no affect on acute lung infection and transiently reduced splenic latency (Usherwood et al., 2001). This transient reduction in splenic latency was also evident upon the adoptive transfer of an M2-specific T-cell line (Usherwood et al., 2000). M3 DNA vaccination resulted in a T-cell-dependent reduction in lung titers (Obar et al., 2004). Antibody responses were increased upon wild-type virus challenge of mice that had been administered heat-inactivated virus subdermally, but afforded only a mild decrease in acute replication (Arico et al., 2002). Infection of type I IFN receptor knock-out mice resulted in greater than 60% mortality within 3 weeks of infection, but subdermal injections of heat-inactivated virus completely protected these mice from death upon wild-type challenge in a 3-week period of examination (Arico et al., 2004).

Attenuated virus vaccination. Vaccination strategies with live attenuated viruses that undergo lytic replication have afforded mice greater protection against wild-type virus challenge than administration of viral proteins and known T-cell epitopes. Upon intraperitoneal infection, a replication-defective virus lacking the single-stranded DNA-binding protein established latency in peritoneal cells and prevented splenic latency upon wild-type challenge (Tibbetts et al., 2006). However, a replication-defective virus lacking the lytic transactivator RTA was detected in the lungs late after intranasal infection, but did not spread to the spleen nor did it afford any protection against wild-type virus challenge (as assessed by acute virus replication and establishment of latency) (Moser et al., 2006). The latter result suggests that in the absence of at least early viral antigen expression, there is little activation of the host immune system. In contrast, recombinant viruses with deregulated expression of ORF50/RTA are quite effective at protecting against wild-type challenge. Intranasal infection with these ORF50-overexpressing viruses nearly ablated acute replication of challenge virus in the lungs and dramatically reduced multiple indicators of splenic latency including splenomegaly and splenic reactivation; viral DNA was nearly undetectable late after infection (Boname et al., 2004a; Rickabaugh et al., 2004). Successful protection was also afforded by vaccination with a latency-deficient recombinant virus that lacks ORF73, the KSHV LANA homolog (Fowler and Efstathiou, 2004).

Long-term latency by wild-type virus is also severely impaired upon vaccination with a reactivation-defective virus (Tibbetts et al., 2003b). Intraperitoneal inoculation with a v-cyclin null MHV68 (vcyclin.LacZ) prevented lytic replication and drastically reduced latency in the peritoneum and spleen to below the

limit of detection. Tibbetts et al. (2003a) utilized knock-out and transgenic mice to further characterize the immune components of vaccination. CD8+ T-cell knock-out mice were still protected by vaccination and passive transfer of immune serum from vaccinated mice was protective against splenic latency (Tibbetts et al., 2003b). The absolute requirement for antibodies in vaccination was further investigated in mice that contain normal B-cell numbers, but lack antigen-specific antibody responses (McClellan et al., 2004). Vaccination of these mice (HELMET) still afforded protection, indicating that virus-specific antibodies are dispensable for immune control. Interestingly, the depletion of CD4+ T cells or CD8+ T cells in HELMET mice demonstrated that CD4+ T cells are required for blocking latency in the peritoneum, but either CD4+ T cells or CD8+ T cells were sufficient to protect against splenic latency (McClellan et al., 2004).

Immunotherapy. EBV and KSHV are associated with the development of lymphomas and neoplasms in immunocompromised patients. The restoration of immune control upon HAART therapy in HIV+ patients has drastically reduced the incidence of KS disease. Adoptive immunotherapy with allogeneic EBV-specific CD8+ T cells is clinically effective for reducing the incidence of EBV-associated lymphoproliferative disease in transplant recipients (Davis and Moss, 2004). As the incidence of lymphomagenesis is low when inbred mice are infected with MHV68, investigating the role of vaccination and immunotherapy in the prevention or resolution of disease in immunodeficient mice may give insight into the mechanisms of effective immune control. CD4-deficient mice (I-Ab–/–) have low levels of persistent replication in the lung and exhibit chronic wasting syndrome and succumb 3–4 months after infection (Cardin et al., 1996). Belz et al. (2000) demonstrated that postexposure vaccination with vaccinia virus expressing MHV68 lytic cycle epitopes increased virus-specific CD8+ T cells and extended the lifespan of the infected mice, but ultimately did not reduce mortality in MHV68-infected CD4-deficient mice. Notably, these virus-specific CD8+ T cells have functional defects – lower levels of CD44, decreased CTL activity and diminished IFNγ and TNF-α production (Liu et al., 2002).

Vaccine studies to date emphasize that an optimal vaccine against MHV68 infection needs to generate both a protective cellular and a humoral-adaptive immune response. Given the ability of MHV68 to utilize multiple cell reservoirs and seemingly recover over time from gross deficits in latent viral load early after infection, sensitive detection assays at late timepoints in multiple latency reservoirs for both viral DNA and reactivation are warranted to better evaluate protection afforded by the next generation of vaccines. The mechanisms by which B and T cells mediate protection remain largely unknown, but are experimentally approachable in the MHV68 system. Thus, vaccination efforts for MHV68 have generated protection against acute replication and alleviated disease associated with low-level persistent replication. Most significantly, vaccination strategies for MHV68 have dramatically reduced early and late

forms of splenic latency. As such, preventing latency and the lymphoproliferative component associated with latency establishment seems possible.

28.9 Concluding Comments

Virus-specific genes. It is certainly notable that a number of gammaherpesvirus genes associated with chronic infection do not appear to be conserved among distantly related family members. However, it has repeatedly been shown that these pathogens share common strategies for manipulating (1) key lymphocyte-signaling pathways involved in proliferation and differentiation and (2) the host immune response. The membrane-associated proteins of EBV, HVS, and KSHV that are implicated in latency and chronic infection are not well conserved, yet (1) HVS STP, KSHV K15 and EBV LMP1 signal through TRAF family members to activate NF-κB; (2) KSHV K1 and RRV R1, which share only limited sequence homology, both contain an ITAM motif and common signaling functions; (3) RRV R1 can replace the transforming function of the unrelated STP in HVS immortalization of primary T cells; and (4) HVS Tip, EBV LMP2A and KSHV K15 all contain SH2-binding and/or SH3-binding motifs capable of interacting with either the Src or Syk family of kinases and appear to be involved in either downregulating and/or mimicking aspects of antigen receptor signaling [for review see (Damania, 2004; Damania et al., 2000)]. Thus, many of these latency-associated viral factors appear to share common functions during infection despite a lack of obvious sequence conservation.

With respect to MHV68, while the M genes are unique to this rodent pathogen, we already have some insight into strategies encoded by these unique genes that recapitulate functions found in the human gammaherpesviruses. For example, as discussed above, the recent characterization of the MHV68 M2 gene function has demonstrated that expression of M2 alone in primary murine B cells leads to (1) proliferation and B cell differentiation; (2) greatly increased IL-10 and IL-6 expression; and (3) a demonstration that IL-10 is required for the observed M2-driven B-cell proliferation. In addition, these studies have been extended to demonstrate that mice infected with an M2-null MHV68 have near background levels of IL10 in their serum, while the levels of IL-10 in serum are very elevated in mice infected with wt virus. Notably, EBV encodes an IL-10 homolog and also upregulates cellular IL-10 expression during infection, while KSHV encodes an IL-6 homolog. Although there has been significant interest and speculation about the role of the cytokine homologs in EBV and KSHV pathogenesis, their importance remains unclear. The analysis of the MHV68 M2 gene product points toward a critical role for modulating these cytokines in the establishment of latency, and perhaps during virus reactivation. Similarly, the identification of a superantigen encoded by MHV68 (M1 gene product) may provide new insights into the role during EBV pathogenesis of the induction by LMP2A of the endogenous retrovirus HERV-K18 superantigen in human B

cells (Sutkowski et al., 2001; Sutkowski et al., 1996). Finally, modulation of chemokine function by the MHV68 M3 antigen has clear parallels in EBV and KSHV where both viruses encode chemokine and/or chemokine receptor homologs which very likely serve to manipulate and/or interfere with host chemokine function. Overall, these findings underscore the importance of focusing on the entire pathogen of interest, and not just "conserved" genes – since the latter may lead to missing key observations that in a broader context contribute to our understanding of the pathogenesis by this family of viruses.

What needs to be done. So what are the important issues that need to be addressed in the MHV68 model system? A primary concern is that we still have only a rudimentary understanding of the progression of MHV68 latency – particularly in different subsets of B cells. If there are close parallels to EBV infection, we would anticipate that there are distinct programs of MHV68 latency-associated gene expression in naïve, germinal center and memory B cells (Thorley-Lawson, 2001). There is already a suggestion of this based on the analysis of the M2 gene, where expression appears to be limited to a subset of latently infected B cells in vivo (Allen et al., 2006; Marques et al., 2003; Simas et al., 2004). In addition, to date a careful genome-wide search for latency-associated genes has not been completed. For example, there is one report indicating the presence of a spliced transcript arising from the terminal repeats of MHV genome (Husain et al., 1999) – which may encode a functional/positional homolog of the EBV LMP2a and/or KSHV K15 gene products. However, there has been no further analysis/characterization of this transcript reported. It is also notable that the region of the MHV68 genome encoding M1–M4 genes is relatively open with respect to potential coding capacity, compared to the rest of the viral genome. This region does also encode several tRNA-like genes and a number of candidate miRNAs. The function(s) of the tRNA-like and miRNAs encoded in this region are unknown. Ultimately, identifying viral genes expressed during latency in different cell populations, as well as the characterization of individual gene functions, will lead to a better understanding of how MHV68 may modulate host function and/or differentiation. In addition, defining when and where specific latency-associated gene products are expressed may shed light on the host immune response to virus infection. As such, this system offers great promise for defining critical cellular pathways and host responses that are targeted by this family of viruses to establish and maintain chronic infection.

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