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Cover illustration: Schematic representation of the HIV-1 genome. The HIV-1 genome is shown with the various structural features and genes indicated. LTR is long terminal repeat. The genes common to all retrovirus are gag, pol, env. The various auxiliary genes specific for HIV-1 are indicated. Illustration of HIV-1 genome provided by Vicente Planelles, UCLA.

Transacting Functions of Human Retroviruses

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Preface

The genome of retroviruses contains three major coding regions for virion proteins, *gag*, *pol* and *env*. *Gag* encompasses information for nonglycosylated viral proteins that form the matrix, the capsid and the nucleoprotein structures. From *pol* derive reverse transcriptase and integrase, and *env* codes for the surface glycoproteins of the virion which consist of a transmembrane and a surface domain, linked by disulfide bonds. A viral protease is derived either from the *gag* or from the *pol* coding region, depending on the virus. Simple retroviruses contain only this elementary *gag*, *pol*, and *env* coding information. Once integrated, they are able to multiply efficiently, using the cellular transcriptional and replication machineries without intervention of viral transacting factors. Most oncogenic retroviruses belong in this category.

Complex retroviruses, on the other hand, encode additional nonstructural proteins from multiply spliced messages. These proteins play important regulatory roles in the life cycle of the virus. They function as transacting factors that, in concert with cellular regulatory proteins, control viral gene expression and function and are essential components in the replication of complex retroviruses. To this category belong the lentiviruses, the spumaviruses and a group of oncogenic retroviruses that includes human T cell leukemia virus (HTLV) and bovine leukosis virus(BLV).

The additional layer of regulation found in complex retroviruses is the subject of this volume of *Current Topics in Microbiology and Immunology*. All human retroviruses isolated to date, be they lentiviruses, spumaviruses, or oncogenic retroviruses, have complex genomes. The regulatory mechanisms available to these viruses may enhance their ability to survive and persist in the host. For instance, virus is present in only a small fraction (0.1%–10%) of potential target cells in individuals infected with human immunodeficiency virus (HIV) or with HTLV. Transacting proteins such as Tat of HIV or Tax of HTLV are part of a positive feedback loop that can lead to rapidly increasing rates of viral expression and production of

progeny virus. Interruption of this feedback loop can result in an equally rapid decline of virus production. Thus, in the expression of complex retroviruses bursts of activity may be followed by periods of relative quiescence. This form of regulation may reduce immune recognition of the infected cell and still allow efficient spread of the virus.

Retroviral transacting proteins work through interaction with the cellular regulatory machinery, including various signal transduction, translocation and transcription factors. Several specific biochemical interactions between retroviral transactivating proteins and cellular factors have recently been elucidated. The Tax protein of HTLV has been shown to interact with members of the NF-κB family of transcription factors, and cellular proteins binding to the Tat protein of HIV have also been identified. Thus, the study of viral regulatory mechanisms is providing insights into control elements of the cell.

The interaction of viral factors with the cellular regulatory machinery does not merely facilitate and guide virus reproduction, it inevitably disturbs normal cellular controls. Both the Tax and the Tat proteins are known to affect the levels of cellular growth factors and of transcription factors, and these primary changes can initiate cascades of secondary effects. This interference with cellular regulation appears to play an important role in the pathogenesis of these viruses.

There are at present neither protective vaccines nor curative therapeutic agents available for human retrovirus infections. HIV infections continue to spread, and the suffering and death toll from acquired immunodeficiency syndrome (AIDS) are still on the rise worldwide. Retrovirology is responding to this challenge by concentrating great efforts on the development of a defense against HIV. The transacting proteins of complex retroviruses are attractive targets for therapeutic intervention. They are indispensable for the virus and therefore represent a point of viral vulnerability, and they appear unrelated to cellular proteins allowing, at least theoretically, for highly specific drug-virus interactions without deleterious side effects for the cell. In order to achieve this goal of shutting down a human retrovirus infection by blocking the function of an essential viral protein, a thorough understanding of that viral target will be necessary. It is hoped that this volume, in summarizing the current knowledge in this area, will contribute towards the conquest of disease.

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Regulation of Foamy Virus Gene Expression

A. Rethwilm

1 Introduction

Foamy viruses make up the third subgroup of the family of exogenous retroviruses (Teich 1984). They are far less well characterized than the onco- and lentiviruses.

The first detection of a foamy virus was reported in 1954 by Enders and Peebles in primary monkey kidney cultures. Since then, many simian foamy virus (SFV) isolates have been obtained from old- and new-world monkeys, great apes, and prosimians. Foamy viruses have also been reported in other vertebrate families such as cats, cattle, and occasionally others (for reviews see Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981; Flügel 1991; Loh 1993). The natural prevalence of foamy virus antibodies in simians, cats, and cattle is high, reaching

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values of up to 70% or more (for reviews see Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981).

In 1971 the isolation of a human foamy virus (HFV) from cells explanted from a nasopharyngeal carcinoma patient, originating from Kenya, was described (Achong et al. 1971). Although further human isolates have been reported (Young et al. 1973; Stancek et al. 1975; Cameron et al. 1978; Werner and Gelderblom 1979), the 1971 virus is the only foamy virus of human origin that is available to the scientific community. The HFV isolate and the recently sequenced foamy virus from chimpanzee (SFVcpz) are highly homologous, however, they are not identical (Herchenröder et al. 1994). Since so far nothing is known on the natural sequence variation of chimpanzee foamy viruses the HFV isolate can neither be proven nor excluded as a chimpanzee virus variant (Bieniasz et al. 1995).

There are several reports in the literature suggesting a pathogenic role for foamy viruses in human beings. The search for foamy viruses in human brain diseases was inaugurated by studies describing a fatal encephalopathy and myopathy in mice transgenic for foamy virus genes (Bothe et al. 1991; Aguzzi et al. 1992a, b; Aguzzi 1993). The diseases for which an association with foamy virus infection has been looked for include De Quervain's thyroiditis (Stancek et al. 1976), Graves' disease (Lagaye et al. 1992; Wick et al. 1992,1993), motorneuron disease (Westarp et al. 1992), multiple sclerosis (Svennigsson et al. 1992), and chronic fatigue syndrome (Landay et al. 1991; Gow et al. 1992; Flügel et al. 1992). Serological and PCR methods were applied to demonstrate the presence of foamy viruses in these disparate conditions. However, positive results were not confirmed (Debons-Guillemin et al. 1992; Neumann-Haefelin et al. 1993; Schweizer et al. 1994; H. Hahn and A.R., unpublished results).

With respect to natural HFV infections, seroepidemiological surveys have led to controversial results (Achong and Epstein 1978; Brown et al. 1978; Muller et al. 1980; Loh et al. 1980). A more recent study postulating a high seroprevalence among East Africans (Mahnke et al. 1992) has been criticized for methodical inconsistencies (Neumann-Haefelin et al. 1993) and was not confirmed by others (Schweizer et al. 1995). While natural human infections are in question, the analysis of a few cases of accidental transmission has revealed that human beings are clearly infectable by primate foamy viruses (Hooks and Gibbs 1975; Neumann-Haefelin et al. 1983,1993). Attempts to demonstrate human-to-human transmission in these rare cases have failed so far (D. Neumann-Haefelin and A.R., unpublished results). As things stand, foamy viruses remain "viruses in search of a disease" (Weiss 1988), and with respect to natural human infections, they may turn out to be "viruses in search of a host".

In their natural hosts simian foamy viruses give rise to lifelong persistence and apparently benign infections in the presence of high antibody titers (Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981; Bieniasz et al. 1995; McClure et al. 1994). In addition to saliva, milk, urine, and feces they have been isolated from almost every tissue that has been looked at, which may indicate a wide host-cell range in vivo (for reviews see Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981). It is believed that foamy viruses reside in a latent state in most organs of

the infected host (Neumann-Haefelin et al. 1993). A tissue culture system that mimics latent foamy virus infection has been established recently for SFV-3 (Schweizer et al. 1993). Expression of the provirus was found to be suppressed by methylation of the viral DNA and was overcome by the addition of demethylating agents to the culture medium and, interestingly, by transfection of an expression plasmid for the viral transcriptional transactivator (Taf, see below) into the latently infected cells (Neumann-Haefelin et al. 1993). However, a definite analysis of the nature of the viral target cells, the state of the viral DNA, and the expression levels in the infected host has unfortunately not yet been undertaken.

In tissue culture, foamy viruses replicate lytically, giving rise to multinucleated and vacuolated syncytia with a "foamy" appearance. Virus replication has been reported for a wide range of host cells derived from epithelial, fibroblastoid, and lymphoid lineages (Hooks and Gibbs 1975; Hooks and Detrick-Hooks 1981), indicating a ubiquitous presence of the virus receptor (s). Like lentiviruses, foamy viruses are able to replicate in nondividing cells of the macrophage lineage (J. Mikovits and F. Ruscetti, personal communication). During the lytic replication cycle large quantities of unintegrated linear viral DNA copies, on a per-cell basis, have been found for several virus isolates (Schweizer et al. 1989; Mergia and Luciw 1992). It is not yet clear whether these contribute to the cytopathology of the viruses. Compared with the amount of cell-bound virus, extracellular virus titers were reported to be rather low (Loh et al. 1977; Neumann-Haefelin et al. 1983). However, more recent studies suggest that this may depend on the type of host-cell line, virus isolate, and assay system used (Baunach et al. 1993; Yu and Linial 1993). All foamy viruses display a characteristic strong nuclear staining when they are reacted with the serum from an infected individual in immunofluorescence assays (Hooks and Detrick-Hooks 1981; Neumann-Haefelin et al. 1983). This nuclear fluorescence distinguishes the foamy viruses from all other retroviruses (Teich 1984) and may therefore be used as a reliable diagnostic criterion (Neumann-Haefelin et al. 1993).

The interest in retrovirology that has been raised by the AIDS pandemic since the mid 1980s has also stimulated molecular investigations on the "Cinderella group" of retroviruses. In the course of these studies a number of fascinating discoveries on foamy viruses were made and warrant being reviewed together with their more important cousins dealt with in this book.

2 General Features of Primate Foamy Virus Genomes

Foamy viruses have the longest genomes reported so far for retroviruses (Fig. 1). The genomic RNAs of HFV, SFV-1, SFV-3, and SFVcpz are 11.67, 11.52, 11.62, and 11.64 kb in length, respectively (Kupiec et al. 1991; Renne et al. 1992; O. Herchenröder et al. 1994; M. Rappold, O. Herchenröder and A.R., unpublished observation). For the related bovine foamy virus (BFV), an approximately 11-kb RNA has additionally been reported (Renshaw et al. 1991). Initially, the HFV LTR

was described to be approximately 500 bp shorter in its U3 region compared to the SFV LTRs (Rethwilm et al. 1987; Flügel et al. 1987; Maurer et al. 1988). This view was supported by the construction of HFV molecular clones with "short LTRs" that replicated in tissue culture (Rethwilm et al. 1990b; Löchelt et al. 1991). However, it was shown recently by molecular cloning of SFVcpz and studying an early passage of HFV that HFV has undergone an U3 LTR deletion upon replication in tissue culture (Herchenröder et al. 1994). The deleted sequences were PCR cloned from the early passage HFV DNA and used to construct a full-length replication-competent molecular clone for HFV (M. Rappold, O. Herchenröder and A. Rethwilm, unpublished).

Foamy viruses possess a second polypurine tract in the middle of the genome that is most likely used as a second site of intiation of plus-strand cDNA synthesis, resulting in a gapped, unintegrated DNA intermediate (Kupiec et al. 1988; Schweizer et al. 1989; Tobaly-Tapiero et al. 1991; Renshaw et al. 1991), a feature they share with lentiviruses (Harris et al. 1981; Charneau and Clavel 1991).

Like all replication-competent retroviruses, foamy viruses bear genes for the structural proteins Gag, Pol, and Env. In addition to these, two accessory open reading frames (orf) have been found in the 3' region of the genomes of SFV-1 and –3 (Mergia et al. 1991; Kupiec et al. 1991; Renne et al. 1993), while HFV harbors three additional orfs (Flügel et al. 1987,1990) (Fig. 1). A third accessory orf has also been found in SFVcpz (Herchenröder et al. 1994). However, the sequence analysis of three independent molecular clones encompassing this orf revealed an in-frame stop codon in the SFVcpz orf-3 (Herchenröder et al. 1994). The accessory orfs of HFV have been designated *bel* for their location *b*etween *e*nv and *L*TR (Flügel et al. 1987). Since the first of the accessory foamy virus orfs was identified to encode for a transactivator protein (see below), the functional name, Taf, for transactivator of foamy viruses, has been suggested by Mergia et al. (1991); I will adopt it solely for the purpose of this review.

The *pol* gene sequences have been used to incorporate the foamy viruses into the phylogenetic tree of retroviruses, clearly demonstrating that these viruses consititute a separate subgroup of retroviruses, having a reverse transcriptase (RT) which is phylogenetically more closely related to the RT of MoMuLV than to any other exogenous retrovirus (Maurer and Flügel 1988; Doolittle et al. 1990; Kupiec et al. 1991; Herchenröder et al. 1994).

Sequence comparison of the four characterized primate foamy viruses revealed the close relationship of HFV and SFVcpz sequences (Herchenröder et al. 1994). Surprisingly, the higher divergence of Env amino acid sequences which is normally found in lentiviruses does not apply to foamy virus genomes. While Pol and Env sequences are reasonably conserved between the four primate foamy viruses, the Gag and the deduced amino acid sequences of the accessory orfs, diverge highly (Flügel et al. 1987; Maurer et al. 1988; Mergia et al. 1990a; Kupiec et al. 1991; Renne et al. 1992; Herchenröder et al. 1994). Even among SFV-1 and SFV-3, Gag and 3' orf sequences show more divergence than the Env sequences. LTR nucleotide sequences show a high degree of homology of R-U5, while the U3

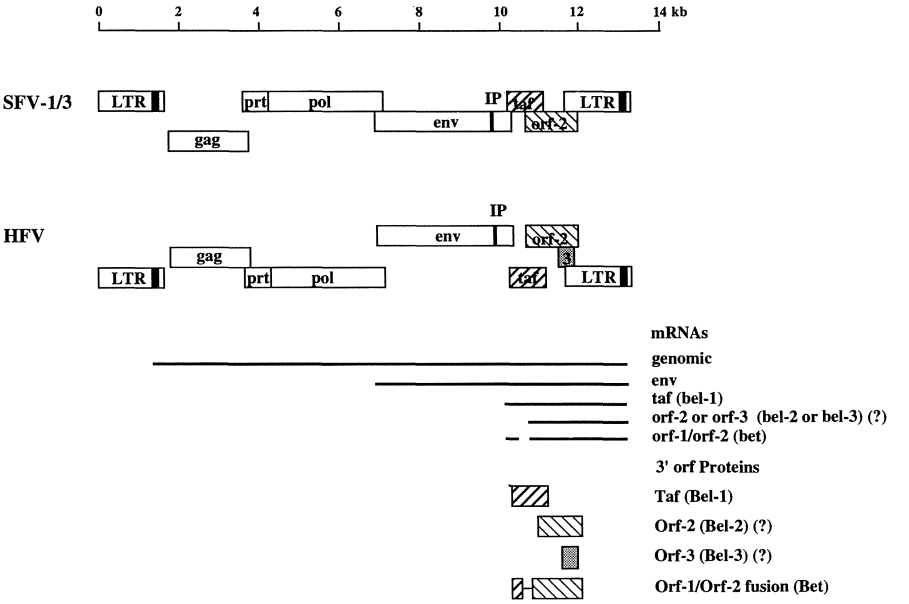

Fig. 1. Foamy virus genome organization, transcripts, and accessory proteins. A third accessory orf (*bel-3*) is present in the HFV genome, but not in SFV-1 and -3. Independent clones of SFV isolates from chimpanzee show a *bel-3* orf with an in-frame stop codon. In all sequenced foamy viruses *pol* is in the +1 frame relative to *gag*. Subgenomic transcripts starting at the LTR promoter may give rise to *env* and 3' orf proteins. In addition, spliced 3'orf transcripts may also start at an internal promoter (IP) located in the *env* gene (LÖCHELT et al. 1993; MERGIA 1994; CAMPBELL et al. 1994). For clarity, additional non-coding exons in the 3'orf transcripts (MURANYI and FLÜGEL 1991; LÖCHELT et al. 1994; HAHN et al. 1994) have been omitted from this figure. Two 3'orf transcripts and their respective proteins dominate: Taf (Bel-1) is the viral transcriptional transactivator that augments LTR and IP directed gene expression; Bet is an abundant 60-kDa cytoplasmic protein generated by an efficient splicing event after 88 (HFV) or 93 (SFV-1) codons, respectively, of the orf-1 into the orf-2. The Bet function is not known, however, it is dispensable for virus in vitro replication (BAUNACH et al. 1993; YU and LINIAL 1993; LEE et al. 1994). The HFV Bel-2 protein is expressed at very low levels if at all (BAUNACH et al. 1993). A *bel*-3 specific mRNA has yet to be identified

sequences are again highly divergent. The reason for this unusual mixture of conserved the divergent sequence stretches among foamy virus genomes is not understood.

3 Expression of Structural Virus Proteins

The HFV Gag precursor protein appears as a doublet of approximately 70/74 kD molecular weight in reducing SDS polyacrylamide gels (NETZER et al. 1990; BARTHOLOMÄ et al. 1992; AGUZZI et al. 1993). Both precursor molecules are phosphorylated (HAHN et al. 1994). HFV pr 70/74 gag was suggested to be cleaved

into a matrix protein (MA) of approximately 27 kDa and a major capsid protein (CA) of 32 kDa (Aguzzi et al. 1993). The HFV nucleocapsid (NC) protein has not been identified yet, but a 15-kD core protein with affinity for single-stranded RNA was reported for SFV-1 (Benzair et al. 1986).

Using antisera generated against recombinant HFV Gag proteins, it was shown recently that pr 70/74 synthesized in the infected cell is quantitatively translocated into the nucleus (Schliephake and Rethwilm 1994). One of three basic sequences rich in glycine and arginine (GR-box 2) in the putative NC domain, which is conserved among the foamy virus isolates sequenced to date, was found to bear a nuclear localization signal. Hence, the nuclear localization of the foamy virus Gag precursor was suggested to be responsible for the prominent nuclear fluorescence of foamy viruses with sera from infected individuals (Schliephake and Rethwilm 1994). The function of the transient nuclear pathway of foamy virus Gag proteins is not clear yet, and speculations on functional aspects may include possible roles in gene regulation, accumulation of unintegrated viral DNA, or packaging of the genomic RNA. Since all foamy viruses investigated so far show the "nuclear fluorescence" in all cell types analyzed, it is likely to assume a fundamental difference in foamy virus maturation compared to the other retroviruses.

With respect to the latter, foamy viruses are distinguished from other retroviruses by another peculiarity. HFV, as well as the SFVs, lacks cysteine-histidine boxes (CH boxes) (Maurer et al. 1988; Kupiec et al. 1991; Renne et al. 1992; Herchenröder et al. 1994), which are conserved in the NC protein of all other retroviruses and are implicated in packaging the genomic RNA and in stabilization of the RNA in the nucleoprotein complex (Linial and Miller 1990; Aronoff et al. 1993). It is reasonable to assume that the foamy virus basic GR boxes in the NC protein perform this function, which may represent an analogy to hepatitis B virus (Nassal 1992). While it is likely that the overall basic character of the putative foamy virus NC protein is a crucial factor for RNA packaging, the exact mechanism of foamy virus RNA encapsidation, in particular any specific RNA-binding properties of foamy virus NC proteins, remain to be elucidated.

The HFV *pol* gene has been described to give rise to protease (PR), RT/RNaseH, and integrase (IN) of the apparent MWs of approximately 10, 80, and 40 kDa, respectively (Netzer et al. 1993; Pahl and Flügel 1993). The RT/RNaseH of SFV-1 and the HFV integrase have been characterized to be manganese-dependent enzymes (Liu et al. 1977; Benzair et al. 1982; Pahl and Flügel 1993).

Most notably, attempts to identify a Gag-Pol precursor molecule, as present in other retroviruses, of the approximate MW of 200 kDa have failed so far for HFV, while a Pol precursor of 127 kDa was readily identified (Netzer et al. 1993; Pahl and Flügel 1993). It is not yet known whether or not the short half-life and low abundance of the putative 200-kDa intermediate molecule complicate its detection. Interestingly, the *pol* orf is in the +1 frame relative to the *gag* orf in all sequenced foamy viruses and starts with an AUG (Kupiec et al. 1991; Renne et al. 1992; Netzer et al. 1993; Herchenröder et al. 1994); this is without precedent among exogenous retroviruses (Jacks 1990). Kupiec et al. (1991) and Flügel (1993)

suggested a +1 ribosomal frame shift for foamy viruses leading to a Gag-Pol fusion protein, occurring either immediately downstream of the *gag* orf or at a purine-rich sequence upstream of the *gag* stop codon. Alternatively, one may hypothesize that foamy viruses do not synthesize a Gag-Pol precursor, but express their Pol protein similar to pararetroviruses (SCHLICHT et al. 1989; CHANG et al. 1989). In any case, the way in which the foamy virus Pol protein is generated awaits a detailed analysis which may shed new light on a fundamental step in retrovirus replication.

The use of antisera generated against recombinant Env proteins and of monoclonal antibodies has led to the identification of an intracellular gp 130 Env precursor molecule that is cleaved into a gp 70–80 surface (SU) and a gp 47 transmembrane protein (TM) (NETZER et al. 1990; AGUZZI et al. 1993; GIRON et al. 1993). An SU protein similar in size to that of HFV was previously identified for SFV-1 (BENZAIR et al. 1985). The nature of a second intracellular HFV glycoprotein of approximately 170 kDa (NETZER et al. 1990; GIRON et al. 1993) that has been reported to immunologically react with antibodies generated against the 3' accessory orfs (GIRON et al. 1993) remains to be elucidated.

Further investigations of the foamy virus Env proteins and their interaction with the ubiquitous cellular receptor(s) may finally lead to the identification of the latter, which would be of great scientific interest with respect to a future use of foamy viruses as retroviral vectors.

4 Accessory and Regulatory Proteins

4.1 Foamy Viruses Encode for a Transcriptional Transactivator (Taf) Required for Virus Replication

The complex genome structure of foamy viruses resembles that of lentiviruses and, in particular, HTLV/BLV genomes. It was therefore presumed that the regulative mechanisms directing foamy virus gene expression may be similar to those observed for the complex retroviruses (CULLEN 1991a).

Following the demonstration that LTR-directed gene expression is largely augmented in HFV-infected compared with uninfected cells (RETHWILM et al. 1990a), the Bel-1 (Taf) protein was identified as the transcriptional transactivator of HFV (RETHWILM et al. 1991; KELLER et al. 1991; VENKATESH et al. 1991). Taf_{HFV} has been reported to be a 36-kDa nuclear phosphoprotein that is required for viral replication (KELLER et al. 1991; VENKATESH et al. 1993; BAUNACH et al. 1993). As in HFV, the homologous proteins of SFV-1 and SFV-3 are encoded in the first of the accessory orfs (MERGIA et al. 1991; RENNE et al. 1993). Transactivation of the BFV LTR has also been demonstrated in BFV-infected cells (RENSHAW and CASEY 1994b). Foamy virus transactivators regulate gene expression by interacting with U3 (DNA) elements of the LTRs (RETHWILM et al. 1991; KELLER et al. 1991; VENKATESH et al. 1991; MERGIA et al. 1992; RENNE et al. 1993). Transcriptional activation of their

cognate LTRs by foamy virus Taf proteins has been described for a wide range of mammalian and even avian cells (KELLER et al. 1991; MERGIA et al. 1990b, 1991).

4.2 Accessory Proteins other than Taf

Two proteins resulting from the accessory orfs in the 3' region of the HFV genome have been readily identified by cDNA cloning or immunological methods, or by relating them to a specific function. Besides Taf (Bel-1), a second accessory protein, the Bet protein (MURANYI and FLÜGEL 1991), is generated by an efficient splicing event fusing the N-terminal 88 amino acids (aa) of orf-1 to orf-2 (Fig. 1). The approximately 60-kDa cytoplasmic Bet protein is easily identified in infected cells by immunological methods using both *bel*-1 or *bel*-2 orf-specific antisera (LÖCHELT et al. 1991; BAUNACH et al. 1993). Proteins analogous to Bet have also been identified in, or suggested for, SFV-1, SFV-3, SFVcpz and BFV (MERGIA et al. 1991; RENNE et al. 1992; RENSHAW and CASEY 1994a; MERGIA 1994; HERCHENRÖDER et al. 1994; HAHN et al. 1994).

On the basis of RT-PCR-mediated mapping of splice sites, further *bel* gene-derived proteins (Bel-2, Bel-3, Bes, Beo) have been initially postulated for HFV (MURANYI and FLÜGEL 1991); however, with regard to Bes and Beo neither the respective cDNAs nor the proteins have yet been cloned and expressed or unambiguously identified by immunological methods, respectively. The postulated Bel-2 protein would represent an N-terminal truncated Bet protein, believed to use the first AUG of the *bel*-2 orf for translational initiation, which is in a rather weak context (KOZAK 1984). A 44-kDa protein which should represent Bel-2 has been found by some investigators (LÖCHELT et al. 1991; GIRON et al. 1993) but not by others (HE et al. 1993; BAUNACH et al. 1993; YU and LINIAL 1993). While a specific mRNA for the third (*bel*-3) orf has yet to be identified, some characteristics of the putative Bel-3 protein have been published recently (WEISSENBERGER and FLÜGEL 1994). It has therefore been suggested that Bel-3 might be translated from a bicistronic virus mRNA (FLÜGEL 1993; WEISSENBERGER and FLÜGEL 1994). Since SFV-1 and -3 lack the respective reading frame and SFVcpz harbours only a mutated orf-3, the question on the expression and function of a *bel*-3 related protein remains.

Like the primate foamy viruses, BFV possesses two accessory orfs in the 3' region of the genome. Beside transcripts for the Taf and Bet homologous proteins, a variety of multispliced transcripts using additional splice donor and acceptor sites in orf-1 and orf-2 have been identified (RENSHAW and CASEY 1994a). In each case the transcripts remained in-frame and coded for a potential protein product. The existence of infectious molecular clones for BFV should enable a functional analysis of these hypothetical proteins (RENSHAW et al. 1991).

This has been done for HFV with limited success, however. Using 3' orf mutants of an infectious molecular clone (RETHWILM et al. 1990b), the necessity of the accessory genes for HFV in vitro replication has been investigated recently (BAUNACH et al. 1993; YU and LINIAL 1993; LEE et al 1994). Surprisingly, of these, only

the *taf* gene was found to be required for HFV replication in tissue culture. This finding was corroborated by another study showing that HFV mutants deficient in all 3' orfs were able to replicate in cells constitutively expressing Taf_{HFV} (Rethwilm et al. unpublished). While BAUNACH et al. (1993) reported no significant differences in the development of extracellular virus titers for the replication-competent virus mutants, the work of YU and LINIAL (1993) suggested a slight reduction in cellfree virus titers of the *bet*-mutants.

Northern blot (MERGIA et al. 1991; MURANYI and FLÜGEL 1991), Western blot (LÖCHELT et al. 1991; HE et al. 1993; BAUNACH et al. 1993), and cDNA cloning experiments (HAHN et al. 1994) suggest that the phosphorylated Bet protein is expressed at higher levels than any other virus protein, at least at some stage of viral replication. Furthermore, an immunodominant intracellular 60-kDa protein was previously shown to react with sera from foamy virus-infected primates in radio-immunoprecipitation assay (NETZER et al. 1990), and experiments from our laboratory indicate the 60-kDa protein to be Bet (HAHN et al. 1994). Interestingly, *taf*-deficient and hence replication incompetent proviruses which were derived from reverse transcribed genomic RNA from which the *bet* intron was spliced out have been identified in HFV, SFV-1, SFV-3, and SFVcpz infected cells (SAIB et al. 1993; HERCHENRÖDER et al. 1994). The biological function of these helper-virus-dependent proviruses remains to be elucidated.

At the present time, one can only speculate on a possible Bet function. In lentiviruses some proteins, in particular Nef, have only little if any consequence for viral replication in vitro (KIM et al. 1989; HAMMES et al. 1989), which contrasts to the situation in vivo. Using molecularly cloned pathogenic SIVmac, it has been shown that the *nef* gene product is a requisite for efficient virus replication and induction of disease in the host (KESTLER et al. 1991). There is some evidence for the *vpr* gene product to behave in a similar manner (LANG et al. 1993). Similar to Bet, Nef has also been shown to be highly expressed and to induce a strong humoral and cellular immune response (KIRCHHOFF et al. 1991; VENET et al. 1992; BOURGAULT et al. 1992). Like the Nef protein, Bet appears to be membrane associated (H. Hahn and A. Rethwilm, unpublished observation). However, the detailed functions of both proteins are likely to be different. Nonetheless, it is tempting to speculate on an essential role of the Bet protein for foamy virus replication in vivo. Animal experiments using the described mutant viruses (BAUNACH et al. 1993) might help to elucidate this point.

It has also been noted that there is a weak but significant amino acid homology between the deduced HFV Bel-3 and the HIV-2 Nef protein sequences (MAURER and FLÜGEL 1987). However, the apparently low level of expression, if any, of *bel*-3 and, in particular, its absence in the lower simian foamy viruses does not concur with the suggested role of Bel-3 as a foamy virus Nef equivalent. It is worthy of mention that the deduced Bel-3 protein sequence of HFV contains a characteristic motif known as the 'leucine zipper' (KOUZARIDES and ZIFF 1988). Recent experiments demonstrated that in vitro translated Bel-3 protein is able to form homodimers via this zipper motif (B. Maurer and A.R., unpublished).

5 Differential Expression of Foamy Virus Genes

5.1 No Evidence for a Post-transcriptional Regulator

The complex retroviruses essentially possess two proteins regulating gene expression at the transcriptional and the post-transcriptional level (CULLEN 1991a). While the foamy virus Taf proteins fall into the first class of regulators, attempts to identify a foamy virus Rev/Rex equivalent protein have been without success so far. Moreover, two lines of evidence indicate that foamy viruses might not possess a post-transcriptionally acting regulatory protein to express their structural proteins. First, as mentioned above, none of the known or hypothesized accessory genes except *taf* has been found to be required for HFV in vitro replication. Second, the expression of HFV structural Gag, Pol and Env proteins from heterologous promoters was found to be independent of the presence of *cis*-acting RNA sequences and *trans*-acting factors (BAUNACH et al. 1993; LEE et al. 1994; B. Cullen, B. Felber, and G. Pavlakis, personal communication).

In functional terms, the post-transcriptional regulators of complex retroviruses enable these viruses to gain some independence from the cellular machinery governing the gene expression of the 'simple' retroviruses, in particular, by allowing them to perform a switch from the expression of nonstructural to structural proteins (CULLEN 1991b). Thus, the question arises whether or not such a switch can be observed in the foamy virus system, and if so, how foamy viruses accomplish this switch.

5.2 Intragenic Start of Transcription

In a recent study, LÖCHELT et al. (1993) discovered that HFV uses a second promoter besides the 5' LTR (Fig. 1). Primer extension analysis and S1 nuclease-protection assays with RNA from HFV-infected cells revealed an internal start of transcription that is located in the *env* gene approximately 180 bp upstream of the *bel*-1 gene. The internal promoter (IP), from nucleotide position –40 to +16 relative to the cap site, shows strong homology to the LTR promoter and includes a TATA motif at position –26. Similar regions of nucleic acid homology are also present in the SFVs (KUPIEC et al. 1991; RENNE et al. 1992; HERCHENRÖDER et al. 1994), and consequently an IP has been identified in the SFV-1 genome pointing to the importance of this regulatory element for foamy virus gene regulation (MERGIA 1994; CAMPBELL et al. 1994). In transient assays using reporter gene constructs, the basal activity of the HFV and SFV-1 IPs was found to be low. Co-transfection of an HFV Taf-expressing plasmid led to an increase in promoter activity that was comparable to the transcriptional augmentation observed with the LTR promoter in the presence of Taf (LÖCHELT et al. 1993a; MERGIA 1994).

Transcription directed by the IP may give rise to the accessory proteins Taf and Bet. On this basis a bimodal mechanism of foamy virus gene expression has

been suggested, with the IP functionally replacing the requirement of the post-transcriptionally acting regulatory proteins present in the other complex retroviruses (LÖCHELT et al. 1993). According to this hypothesis, the IP would initially direct the expression of accessory genes, followed by the LTR-directed expression of structural genes. In support of this hypothesis, Löchelt et al. demonstrated the activity of the HFV IP in the early phase of viral replication (LÖCHELT et al. 1994). Furthermore, in vivo experiments on the activity of both foamy virus promoters suggest the IP to have a slightly higher basal activity compared with the LTR promoter (LÖCHELT et al. 1993b; CAMPBELL et al. 1994). On the other hand, the initial activity of the IP does not seem to be an absolute requirement for foamy virus structural gene expression since a proviral construct in which the HFV *env* gene (including the IP) was exchanged for the SIV *env* gene expresses HFV Gag protein in transient assays (A.R., unpublished). Taf (Bel-1) as well as Bet-specific transcripts have been identified to be expressed from the IP, the majority of which are spliced in the 5' untranslated region deleting a 119 nt intron (MERGIA 1994; LÖCHELT et al. 1994; HAHN et al. 1994). However, a quantita-tive discrimination between *taf* (*bel*-1) and *bet*-specific transcripts directed by the IP has not been undertaken yet. It has already been mentioned that the foamy virus Bet protein is highly expressed in infected cells and, although dispensable in tissue culture, Bet is likely to be an essential protein for in vivo replication. If this holds true, the virus might have found a way to provide sufficient Bet protein by making use of the IP. This is not without precedent among retroviruses since mouse mammary tumor virus makes use of additional promoters to direct the expression of the *sag* gene (ELLIOT et al 1988; GÜNZBURG et al. 1993).

6 Foamy Virus Gene Regulation by Taf

6.1 Characterization of Taf-Responsive Elements

Foamy Virus U3 regions lack recognition sequences for known transcriptional enhancers, except for the ubiquitous transcription factor AP-1 (Fig. 2). The HFV AP-1-binding sites, however, were found to be dispensable for transactivation (MAURER et al. 1991; LEE et al. 1993); instead, they were suggested to modulate the transcriptional activity of HFV in response to extracellular stimuli (MAURER et al. 1991). In this respect the fine structure of the BFV LTR seems to be different since a variety of binding sites for known transcription factors have been identified in the BFV U3 and R regions (RENSHAW and CASEY 1994b) (Fig. 2). It is not known yet as to what extent these sites influence the basal or inducible activity of the BFV promoter.

In order to elucidate the mechanism of foamy virus transactivation the Taf-responsive elements in the U3 regions of HFV, SFV-1, and SFV-3 have been determined (MERGIA et al. 1992; RENNE et al. 1993; LEE et al. 1993; ERLWEIN and RETHWILM 1993). This task was facilitated by analyzing LTR reporter gene constructs

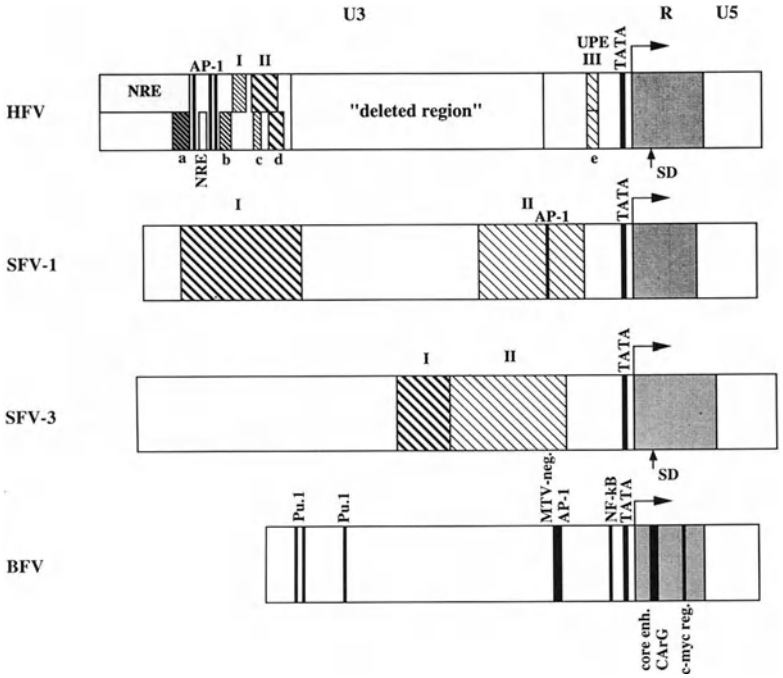

Fig. 2. Characterization of *cis*-regulatory elements in foamy virus LTRs. Whereas LTR U3 regions of the sequenced foamy viruses are highly divergent in length and nucleotide sequence, R-U5 regions show approximately 80% homology among the primate foamy viruses to which the BFV R-U5 sequences are approximately 40% homologous. R-U5 suppresses LTR directed gene expression, which has been attributed to a stable secondary structure of the R-U5 mRNA (Mergia et al. 1992). The negative role of R-U5 can only have an effect on the genomic or *gag/pol* mRNA since foamy viruses use an early splice donor (SD) at nucleotide position +51 with respect to the transcriptional start (+1). Primate foamy viruses lack recognition sequences for known transcription factors, except for the TATA box, and some viruses lack binding sites for the AP-1 factor. The latter, however, have only a minor effect on LTR-directed gene expression (Maurer et al. 1991). Crucial for the transcriptional activity of the LTR are the *taf*-responsive elements. These elements have been determined approximately in the SFV-1 and -3 LTR (Mergia et al. 1992; Renne et al. 1993). While in the SFV-1 U3 region, only element II behaves like an enhancer, both elements in the SFV-3 LTR show enhancer characteristics. Mapping of the HFV LTR *taf* response elements has led to the identification of independently acting elements (I–III by Erlwein and Rethwilm 1993; a–e by Lee et al. 1993). Element III or e, respectively, behaves like an upstream promoter element (*UPE*). In addition, different negative regulatory elements (*NRE*) have been proposed. Studies on HFV U3 regulatory elements were done with LTR constructs having a large central deletion ("deleted region") (Herchenröder et al. 1994). Regulatory elements in this region await detailed analysis. The fine structure of the BFV LTR is somewhat different from the primate viruses since it contains a variety of recognition sites for known transcription factors (for details see Renshaw and Casey 1994b). The effect of this sites on BFV promoter activity is, however, not known

for their responsiveness to the respective transactivator proteins and has been hampered mainly by the lengths of the DNA regions to be investigated.

Common to all characterized foamy viruses is the presence of *cis*-regulatory enhancer elements in their U3 regions that confer Taf responsiveness to homologous and heterologous basal promotors, irrespective of their orientation (Fig. 2). While the Taf-responsive elements in the SFV-1 and –3 U3 regions have been mapped approximately (Mergia et al. 1992; Renne et al. 1993), the respective

elements have been studied in greater detail for HFV by LEE et al. (1993) and ERLWEIN and RETHWILM (1993). As shown in Fig. 2, some differences are noted between the two studies, in particular with respect to the 5' Taf-response elements and the position of a proposed negative regulating element (NRE), which should be resolved in future studies. If we disregard these discrepancies, the overall message from both studies is consistent: multiple small *cis*-regulatory elements in the HFV U3 region confer independent and additive *taf* responsiveness on their own promoter. Whereas the upstream elements behaved like classical enhancer elements, since they act irrespective of their orientation and location relative to the promoter, the promoter-proximal element (III or e, respectively, in Fig. 2) exhibited the characteristics of an upstream promoter element (UPE) (LATCHMAN 1991), since it was found to respond only weakly to Taf when located distant from the promoter (LEE et al. 1993; ERLWEIN and RETHWILM 1993). However, both studies were done at a time when only the "short" LTR sequences of HFV were known (see above). Thus, the mapping experiments will have to be completed with the full-length LTR to elucidate additional regulatory sequences in the HFV U3 region. This may also result in the identification of the likely reason for the LTR deletion that occurred upon virus replication in tissue culture.

Interestingly, no apparent sequence homologies are noted between the *taf*-response elements and known enhancer sequences, and between the disparate response elements of the different primate foamy viruses. Consistent with this, no cross-transactivation was observed between HFV and SFV-1 (MERGIA et al. 1992; O. Erlwein and A.R., unpublished observation), while the Taf_{SFV}-1 was reported to be partially active on SFV-3 LTR-directed gene expression (RENNE et al. 1993). These findings might reflect the closer relationship of the lower simian virus *taf* proteins and LTR U3 DNA sequences compared with HFV (RENNE et al. 1992).

The situation is now even more complex, following the discovery that the IPs of HFV and SFV-1 and -3 may also be stimulated by Taf proteins (LÖCHELT et al. 1993a; MERGIA 1994; CAMPBELL et al. 1994). The Taf-responsive elements at the IPs remain to be mapped, while the core promoter sequences are the only region of nucleic acid homology between the IPs and the LTRs. Furthermore, to what extent the LTR enhancer elements influence IP-directed transcription awaits more detailed analysis (LÖCHELT et al. 1993b).

The foamy virus Taf proteins are able to weakly augment gene expression directed by the HIV/SIV LTRs (KELLER et al. 1991; VENKATESH et al. 1991; MERGIA et al. 1992). A region upstream of the NFkB enhancer-binding sites in the HIV-1 LTR was reported to be the HFV *bel*-1 target sequence (LEE et al. 1992; KELLER et al. 1992). Within this region, a nonamer motif is contained that has eight of nine nucleotides identical to an HFV LTR motif, which was therefore suggested to be an important Taf_{HFV} target (LEE et al. 1992). However, this sequence does not seem to play a major role in *taf*-mediated transactivation of the HFV LTR (LEE et al. 1993; ERLWEIN and RETHWILM 1993). Interestingly, the HIV LTR lacking functionally active NFkB sites was found to respond up to 20-fold better to Taf than the parental promoter sequence with unmutated NFkB sites (LEE et al. 1992; KELLER

et al. 1992). The NFkB complex is large when assembled at its DNA-binding site (Molitor et al. 1990). Thus, a likely explanation for this phenomenon would be that NFkB sterically hinders the action of Taf at the protein level.

6.2 Negative Effect of R-U5 on LTR-directed Gene Expression

Another interesting finding was reported for the R-U5 region, the role of which appears to be the suppression of foamy virus gene expression. In the cases of SFV-1 and –3, and in some studies on HFV, it was found that constructs harboring the R-U5 region lead to a 10- to -30-fold reduced level of reporter gene expression compared with constructs lacking R-U5 (Venkatesh et al. 1991; Mergia et al. 1992; Renne et al. 1993; Erlwein and Rethwilm 1993). On the basis of computer analysis, the inhibitory action of R-U5 has been attributed to a stable secondary structure of the nascent foamy virus mRNA (Mergia et al. 1992; Renne et al. 1993; Erlwein and Rethwilm 1993), which might execute suppressive co- or post-transcriptional effects. The introduction of R-U5 sequences derived from SFV-1 in both orientations downstream of the SV40 promoter inhibited reporter gene expression approximately tenfold (Mergia et al. 1992). When the HFV R-U5 sequences were introduced into a similar indicator gene construct, downregulation was more pronounced with plasmids harbouring R-U5 in antisense orientation (A. Rethwilm, unpublished). These results suggest that RNA secondary structure might be responsible for the observed effect. Compared with the divergent U3 sequences among foamy viruses, the highly conserved R-U5 nucleotide sequences (HFV and SFV-1 show 78% nucleotide sequence homology in R-U5 compared to 45% homology in U3) argue for a specific function of this region. However, at present it remains unclear as to what this function might be. It is noteworthy that the foamy virus 5' splice donor has been mapped to position +51 relative to the start of transcription (Muranyi and Flügel 1991; R. Renne and P. Luciw, personal communication). Hence, the putative inhibitory role of R-U5 becomes effective only for the foamy virus genomic or *gag-pol* RNA. Retroviral 5' RNA regions harbor *cis*-acting sequences, among which the packaging signals are believed to form stable secondary structures (Alford et al. 1991; Harrison and Lever 1992). It is therefore tempting to speculate on the foamy virus R-U5 regions performing a similar role, in which the nuclear localized Gag protein might also be engaged.

6.3 Dissection of the Taf Protein and Possible Mechanism of Taf Action

Complementary to the studies at the DNA level, experiments on the functional organization of the transactivator proteins have been performed which allow some more definite conclusions on the mechanism of foamy virus transactivation. Using deletion mutants and site-directed mutagenesis, as well as fusion to

the DNA-binding domain of the yeast GAl4 and the activation domain of the herpes virus VP16 protein, distinct regions of Taf_{HFV} critical for its transactivation activity have been determined by VENKATESH et al. (1993), HE et al. (1993), VENKATESH and CHINNADURAI (1993), GARRETT et al. (1993), and LEE et al. (1994). The consistent results identified the carboxyterminal approximately 60–70 aa as the activating domain of Taf_{HFV} that was found to enhance transcription when targeted to a TATA box containing minimal promoter, in addition to the LTRs of HFV and HIV via the DNA-binding domain of GAL4. This region contains a motif that is highly conserved between HFV and SFV Taf proteins (aa 273–287 in Fig. 3), which represents the minimal activation domain since it was found to augment promoter-directed gene expression as a GAL4 fusion protein (VENKATESH and CHINNADURAI 1993; GARRETT et al. 1993; LEE et al. 1994). When HFV Taf portions covering this activation motif and extending from aa 260 to 290 (GARRETT et al. 1993) or from aa 273 to 300 (G. Chinnadurai, personal communication) were targeted to a minimal promoter as GAL4 fusions, transcriptional enhancement was observed in cells of mammalian, avian, and even fungal origin, implying the conservation of the *taf* mechanism of action from lower to higher eukaryotes (GARRETT et al. 1993). The minimal activation domain does not share sequence homologies with known transcriptional activators of non-foamy virus origin and may therefore act by a novel mechanism (GARRETT et al. 1993; MERGIA et al. 1993). On the other hand, the activation domains of foamy virus Taf proteins are rich in hydrophobic amino acids (Fig. 3). It has, therefore, been suggested by LEE et al. (1994) that Taf proteins may act analogous to the acidic activators exemplified by VP16 for which hydrophobic residues within the activation domain have been identified to be critical for activation (CRESS and TRIEZENBERG 1991; REGIER et al. 1993). Interestingly, the supposed BFV Taf protein is only distantly related to the primate virus Tafs, its C-terminus is, however, rich in hydrophobic residues (Fig.3) (RENSHAW and CASEY 1994). The first experimental evidence in support of this hypothesis has recently been provided by BLAIR et al. (1994). These authors used an elegant genetic approach to demonstrate that the biological activity of Taf_{HFV} in yeast is dependent on the ADA2 transcriptional adaptor, a property Taf_{HFV} shares with the VP16 class of activators (BERGER et al. 1992). Furthermore, Taf down-mutants selected in yeast exhibited also a down phenotype in mammalian cells and frequently involved hydrophobic and/or aromatic residues of the activation domain. It therefore appears that, although lacking a 'typical' acidic activation domain, transcriptional activation by Taf_{HFV} at least in part involves a mechanism similar to the VP16-like acidic class.

Consistent with the results reported for the Taf_{HFV} protein, the dissection of the SFV-1 transactivator (Fig. 3) revealed the 68-aa C-terminal region which includes the region of homology to Taf_{HFV} representing an activation domain (MERGIA et al. 1993; GARRETT et al. 1993). This was further corroborated by functionally replacing the Taf_{HFV} C-terminal region for the respective Taf_{SFV-1} region (GARRETT et al. 1993). Mutational analysis and the use of chimeric proteins with GAL4 identified a second activation domain in the Taf protein of SFV-1, which is located in the acidic N-terminus that is also part of the Bet protein (see above,

A

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HFV     MDSYEKEESV AS----TSGI QDLQTLSELV GPENAGEGEL TIAEEPEENP   46
SFVcpz  MDSYQEEEPV AS----TSGL QDLQTLSELV GPENAGEGDL VIAEEPEENP   46
SFV-1   MASWEAQEEL RELLHHLPED DPPADLTHLL ELDEMEPKVL CGENPGDEKL   50
SFV-3   MASWEKEKEL AHL--HQPED DPLPDLSLLL DMDQFEPTEG PDSNPGAEKI   48
BFV     MAS-------------------------------------------------     3

HFV     RRPR-RYTKR EVKCVSYH-A YKEIEDKHPQ HIKLQDWIPT PEEMSKSLCK   94
SFVcpz  RRPR-RYTKR DVKCVSYH-A YKELEDKHPH HIKLQDWIPK PEEMSKSICK   94
SFV-1   KKQVIKTPPM HPSTVTWHFG YKQKEDQQDN -IKMRDWVPN PSKMSKSTCK   99
SFV-3   YLQL-QVAPG DPSEKTYKFG YEDKEAQNPD -LKMRNWVPD PEKMSKWACA   96
BVF     ---------- --------GG TPEKARVACR RVDLSSFLAQ PDDYPTAADS   35

HFV     R--LIL---- CGLYSAEKAS EILRMPFTVS WEQSDT-DPD CFIVSYTCIF  137
SFVcpz  R--LIL---- CGLYSGEKAR EILKKPFTVS WEQSET-NPD CFIVSYTCIF  137
SFV-1   R--LIL---- LGLYQACKAQ EIIKMNYDVH WEKSVV-NEQ YFEVEYNCKM  142
SFV-3   R--LIL---- CGLYNAKKAK ELLKMDYDIH WEQSKE-DSQ YFEIEYHCKM  139
BFV     KEDLILKLAC TTLFSEKHAH EIYE-NYKLH LKRDELRGGK EWVIIYSCKH   84

HFV     CDAVIHDPMP IRWDPEVGIW VKYKPLRGIV GSAVFIMHKH QRNCSLVKPS  187
SFVcpz  CDAVIHDPMP VVWDSEVEIW VKYKPLRGIV GSAVFIMEKH QKNCSLVKPS  187
SFV-1   CRTVLHEPMP IMYDPETELW VKPGRLRGPL GSAVYTLKKH YERCLLTLPS  192
SFV-3   CMTVIHEPMP VSYDKKTGLW IKMGPLRGDI GSVVHTCRRH YERCMSALPS  189
BFV     CYTVFMDNSR LTLGPS-GLF KVIRNKKGDV MLCQMLTRHL TDRC------  127

HFV     TSCSEGPKPR PRHDPVLRCD MFEKHHKPRQ KRPRR-RSID NESCASSSDT  236
SFVcpz  TSCPEGPKPR RRHDPVLRCD MFEKHHKPRP KRSRK-RSID HESCASSGDT  236
SFV-1   LK--GTRLPK RRCNPSRRYE TFREHPPTRK RRSKEGIPTD QQPSTSNGDP  240
SFV-3   SG--EPLKPR VRANPVRRYR EKSLIVADRP KRSRWGVAPR EQPNTSSGDA  237
BFV     ---------D PRTKPFQSSS SLHPNLVTEN PRGTGGGTPG QHTLGGDQDM  168

HFV     MANEPGSLCT NPLWNPGPLL SGLLEESSNL PNLEVHMSGG --PFWEEVYG  284
SFVcpz  VANESGPLCT NTFWTPGPVL QGLLGESSNL PDLEVHMSGG --PFWKEVYG  284
SFV-1   MALLSGPCGP HSIQPPGCLL QELPKPEVGS PEMAVAMSGG --PFWEEVYG  288
SFV-3   MALMPGPCGP FNMDPPGCLL ERVPGSEPGT SEMALAMSGG --PFWEQVYR  285
BFV     RVDTSGI--- ----KPLSSL CQCARDDPGR SDNPLEMAEP VQPWWTDSSL  211

HFV     DS-----ILG PP-------S GSGEHSVL                          300
SFVcpz  DS-----ILG PP-------S GSGEHSVL                          300
SFV-1   DS-----IFA TP-------L GSSEDQLLSQ FD                     308
SFV-3   DS-----ISG PP-------T GPSEN                             298
BFV     EPEITTWVLG DPDATARFWT GDDKGPQEWD FDDDLLGP               249
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B

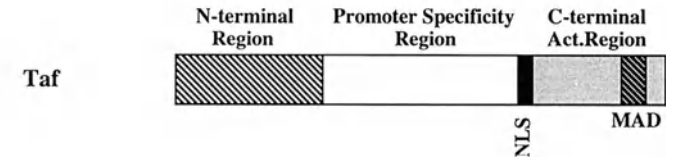

Fig. 3A,B. Amino acid sequences (**A**) and functional organization (**B**) of foamy virus Taf proteins. The acidic N-terminal domain of Taf_{SFV-1} can independently stimulate transcription when targeted to a minimal promoter as fusion protein with the GAL4 binding domain (MERGIA et al. 1993). The respective domain of HFV has only a minor effect on Taf_{HFV} stimulated gene expression (VENKATESH et al. 1993; HE et al. 1993; VENKATESH and CHINNADURAI 1993; GARRETT et al. 1993; LEE et al. 1994). The N-terminal region of both proteins is also part of the orf-1/orf-2 fusion protein (Bet). The distantly related BFV Taf protein lacks the acidic N-terminus (RENSHAW and CASEY 1994). Characteristic for foamy virus Taf proteins is the C-terminal region, which contains the minimal activation domain (*MAD*). This region is highly conserved

Sect. 4.2). This finding suggests a mechanism of action that is already known from the acidic transactivators for this part of Taf_{SFV-1} (MERGIA et al. 1993; PTASHNE 1988). The acidic N-terminal domain of Taf_{HFV}, which has minor homology with the respective domain of Taf_{SFV-1} (Fig. 3), was found to have a negligible effect on transactivation in one study (VENKATESH et al. 1993), whereas its deletion was reported to drastically reduce Taf activity in another study (HE et al. 1993). However, the fusion of this domain to GAL4 did not result in any activity of suitable reporter constructs (VENKATESH and CHINNADURAI 1993; GARRETT et al. 1993). It may therefore be that Taf_{SFV-1} has acquired, or preserved, an additional function that is not present in Taf_{HFV}. With respect to the necessity of the acidic N-terminus for Taf function, it is worthy of mention that this region is absent in the deduced protein sequence of the BFV Taf gene, which was derived from an infectious molecular clone (Fig.3) (RENSHAW et al. 1991; RENSHAW and CASEY 1994a). Therefore, the C-terminal activation domain is the discriminative feature of foamy virus transactivators.

Transcriptional activators generally comprise activation domain(s) and motifs directing the protein to the DNA (PTASHNE 1988). Given that the activation domain(s) of *taf* is located at the C-terminus, and possibly an additional one at the N-terminus in the case of SFV-1, it is conceivable to locate the 'DNA binding' and promoter specificity domain to the middle portion of the protein (Fig. 3). This view is corroborated by studies using $Taf_{HFV/VP16}$ chimeras, showing that the activation domain of VP16 has augmenting activity when directed to the DNA by a Taf protein deleted in the C-terminal activation domain (HE et al. 1993) and by the $Taf_{HFV/SFV-1}$ activation domain replacement experiments already mentioned (GARRETT et al. 1993).

Most mutations introduced into conserved regions of the middle part of HFV or SFV-1 Taf proteins, including the nuclear localization signal which marks the border between 'DNA binding' and C-terminal activation domains (Fig. 3), were deleterious for transactivator function (VENKATESH et al. 1993; HE et al. 1993; MERGIA et al. 1993; LEE et al. 1994; CHANG et al. 1995). However, attempts to demonstrate direct binding of Taf_{HFV} protein, expressed as a fusion protein in bacteria or in vitro translated in rabbit reticulocyte lysate to its cognate LTR or fragments thereof have failed so far (VENKATESH et al. 1993; ERLWEIN and RETHWILM 1993; B. Cullen, personal communication; I. Jordan and A.R. unpublished). Furthermore, when an oligonucleotide spanning the BRE II (Fig. 2) was incubated with proteins from nuclear extracts of uninfected cells, this DNA fragment was specifically retarded in electrophoretic mobility shift assays (EMSA) (I. Jordan and A.R., unpublished results), indicating that cellular proteins recognize the foamy virus enhancer elements.

Taf is phosphorylated; this is, at least in part, facilitated by a nuclear kinase

◀

among the primate foamy viruses. The C-terminal activation region is able to augment gene expression when targeted to a minimal promoter in lower and higher eukaryotes. The basic nuclear localization signal (*NLS*) precedes the C-terminal region. This motif is not present in the BFV Taf protein (RENSHAW and CASEY 1994). The middle portion of the protein is believed to direct the transactivator to its cognate LTR promoter

(Venkatesh et al. 1993). Phosphorylation may have an inhibiting or a stimulating effect on both the DNA-binding and -transactivating activity of transcription factors (for review see Hunter and Karin 1992). Current knowledge on the activity of (un)phosphorylated Taf is too limited (Venkatesh and Chinnadurai 1993) for us to argue about what effect, if any, phosphorylation might have on Taf. However, when Bel-1 protein from recombinant baculovirus (Liu et al. 1993) was used in EMSA a direct binding to DNA could not be detected (I. Jordan and A.R., unpublished). Furthermore, the 'binding domain' lacks motifs recognized for DNA-binding activity from other proteins.

It will therefore be the task of future investigations to resolve whether or not Taf binds directly to the identified enhancer elements or via intermediate cellular proteins. Taking the plethora of response elements into account, it is difficult to imagine a promiscuous Taf that interacts directly with all of these. However, it remains to be seen whether there are as yet undiscovered cellular factors which must be conserved from avian through mammalian species mediating this binding. Furthermore, these cellular factors would have to account for the promoter specificity of the different foamy virus Taf proteins. A third possibility would be that Taf has DNA-binding activity itself and makes use of various cellular proteins enhancing the binding. According to this model, cellular factors that bind to the enhancer elements would direct Taf to the LTR. Taf in turn leads to the recruitment of proteins of the basal transcriptional machinery. This hypothesis could explain the dilemma that results from the disparate enhancer elements on the one hand and the apparent specificity of foamy virus Taf proteins for their cognate LTR promoter on the other.

It is noteworthy that the mutational analysis of the middle portion of Taf_{HFV} also identified various mutants of a dominant negative (DN) phenotype (Venkatesh et al. 1993; He et al. 1993; Venkatesh and Chinnadurai 1993; Lee et al. 1994). While the nuclear localized DN mutants are likely to act via sequestration of cellular co-factors of Taf, a cytoplasmic localized DN mutant indicated that Taf can dimerize (Venkatesh et al. 1993; Venkatesh and Chinnadurai 1993). Most recently it has been demonstrated that triple multimerization domains are located at the Taf N-terminus in the region that is shared between Taf and Bet proteins (Chang et al. 1995). It is not known yet, however, whether Taf can form complexes with Bet or wether Taf multimerization is required for transactivation function.

7 Conclusions

Research on the molecular biology of foamy viruses in the past few years has lead to some interesting discoveries indicating that foamy viruses "do some things different from all other retroviruses." We are now in the situation where specific questions can be asked (and hopefully answered) addressing what these apparent differences in the replication strategy to other retroviruses mean. Beginning at the 5' end of the genome, some of these questions include: Why do foamy

viruses localize their Gag protein into the nucleus? How do they package their genome? How is the Pol protein expressed? What is the foamy virus receptor(s)? How is the internal promoter regulated? How does Taf interact with its cognate LTR and with the proteins of the transcriptional machinery? What is the function of the Bet protein? How is the Bel-3 protein expressed and what is its function? Answers to these questions will not only be beneficial, or essential, to the future use of foamy viruses as retroviral vectors, but may also lead to a better understanding of the principle molecular biology of retroviruses.

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Transactivation of Cellular Genes by Human Retroviruses

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1 Introduction

Discovery of the link between human T-cell leukemia virus type I (HTLV-I) and adult T-cell leukemia, and the emergence of the acquired immunodeficiency syndrome, in association with human immunodeficiency virus type 1 (HIV-1) has led to intense investigation of underlying mechanisms of pathogenesis and retroviral gene regulation. In contrast to avian and murine RNA viruses previously studied, the human oncogenic retroviruses HTLV-I/II, as well as the Lentivirus

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family, represented by HIV-1, HIV-2, and SIV, demonstrated genomes of increased complexity, encoding multiple mRNA species in contrast to the simpler avian/murine species. "Simple" retroviruses generally encode two mRNA species, unspliced gag/pol, and singly spliced env mRNA, while additional splicing events result in as many as eight identifiable mRNA species for HTLV-1, encoding at least two, and potentially up to five, additional proteins (Myers and Pavlakis 1991). For HIV-1, an exceptionally complex pattern of transcripts is observed, resulting in up to 20 different mRNAs and ten or more different proteins (Myers and Pavlakis 1991; Schwartz et al. 1990). Several of the proteins encoded by the novel spliced mRNAs in HTLV-I/II and HIV-1/HIV-2 act as regulators, both transcriptional and posttranscriptional, of viral gene expression. By definition, these *trans*-acting regulatory proteins interact with both *cis*-acting viral sequences, as well as with cellular regulatory machinery to alter viral gene expression. Researchers have therefore focused attention on possible cellular regulatory consequences of retroviral gene expression.

Rather than attempting an exhaustive review of the published literature on this subject, we have chosen to highlight two of the better characterized regulatory genes, for which substantial evidence for dysregulation of cellular gene expression has accumulated. The first, the Tax gene in HTLV-I/II, acts to increase viral transcription and has been implicated in T-cell transformation leading to leukemia, as well as in paraneoplastic syndromes seen in ATL such as hypercalcemia. The second, the Tat gene, fulfills an analogous functional role, albeit by an entirely different mechanism in HIV-1, and has been linked directly and indirectly to various sequelae of HIV-1 infection. The Tax and Tat proteins are considered here as two of the better studied examples of dysregulation of cellular gene expression by retroviral transacting genes. Undoubtedly, the complexity of the viral genomes and the large variety of proteins produced will yield additional examples of viral dysregulation of cellular gene expression, whether for the "purpose" of viral replication, or as an inadvertent by-product of viral gene expression.

2 Transacting Regulators and Human T-Cell Leukemia Viruses Types I and II

The human T-cell leukemia virus type I (HTLV-I) was the first human retrovirus identified in T-cell lines derived from a patient with a cutaneous T-cell lymphoma/lymphoma (Poeisz et al. 1980, 1981; Hinuma et al. 1981; Yoshida et al. 1982). Shortly thereafter, the closely related but distinct HTLV-II was identified in another T-cell line derived from a patient with a chronic lymphoproliferative disorder (Kalyanaraman et al. 1982), and an infectious HTLV-II molecular clone was isolated (Chen et al. 1983a). Both HTLV-I and -II have also been linked to chronic neurologic diseases designated as HTLV-I/II-associated myelopathy (Gessain et al. 1985; Yoshida et al. 1987; Berger et al. 1991; Hjelle et al. 1991; Rosenblatt et al. 1992).

The two viruses were the first examples of retroviruses directly linked to human malignancy, despite the numerous tumorigenic animal retroviruses identified. However, unlike previously identified animal retroviruses, neither HTLV-I/II nor the related oncogenic retrovirus bovine leukemia virus (BLV), a cause of B-cell leukemia in cattle, was found to integrate with any specificity adjacent to cellular proto-oncogenes (Seiki et al. 1984). In addition, neither HTLV-I/II nor BLV contain transduced cellular sequences similar to transduced oncogenes observed in acutely transforming retroviruses. Both HTLV-I and -II transform T-cells in vitro, yielding immortalized T-cell lines capable of sustained growth in the absence of IL-2 (Miyoshi et al. 1981; Yamamoto et al. 1982; Chen et al. 1983b; Popovic et al. 1983). In the case of HTLV-I these lines were generally of $CD4^+$ phenotype, while for HTLV-II $CD8^+$ cell lines are frequently obtained, similar to the observed phenotype of the virally induced malignancies (Rosenblatt et al. 1986, 1990; Ijichi et al. 1992). The lack of specific proviral integration or of a viral oncogene led to an intensive effort to identify alternative mechanisms for transformation. The 3' region of the HTLV-I/II genome was found to contain coding sequences for additional viral regulatory proteins (Seiki et al. 1983; Lee et al. 1984; Shimotohno et al. 1985; Sodroski et al. 1985; Slamon et al. 1984; Chen et al. 1983a, 1985; Felber et al. 1985). The first of these, Tax, is a 40-kD nuclear protein in HTLV-I and a 37-kD nuclear protein in HTLV-II (Lee et al. 1984; Slamon et al. 1985; Beimling and Moelling 1989). Tax serves as a transcriptional regulator of viral expression, increasing transcription from the viral long terminal repeats (LTR) (Cann et al. 1985; Felber et al. 1985; Sodroski et al. 1985; Shimotohno et al. 1985; Seiki et al. 1986). Mutations within Tax abrogate transcription in infectious HTLV-II clones (Chen et al. 1985).

Further investigation of the coding region of HTLV-I, HTLV-II, and BLV disclosed the presence of an additional protein(s), encoded by the same spliced mRNA as Tax, on an alternate open reading frame, designated Rex (Kiyokawa et al. 1985; Sagata et al. 1985; Shima et al. 1986; Inoue et al. 1987). Rex appears to be required for nuclear to cytoplasmic export of full length gag mRNA and partially spliced env mRNA. The function of Rex is analogous to that of the rev gene in HIV-I/II, and virion production does not proceed in the adsence of Rex (Rimsky et al. 1988). An arginine–lysine-rich motif important for nuclear localization is also required for RNA binding and Rex function (Nosaka et al. 1989). The Rex protein in HTLV-I migrates with an apparent MW of 27 kD, and a truncated protein, 21 kD in size, is encoded using an alternate methionine initiation codon. Only p27 Rex has a known function (Inoue et al. 1987). HTLV-II Rex has an apparent MW of 26 kD while a dephosphorylated form has a MW of 24 kD (Green et al. 1991). Both HTLV-I and -II Rex bind with high specificity to a cognate mRNA recognition element located within R and reiterated on the 3'LTR (Yip et al. 1991; Black et al. 1991a, b). It is not known whether Rex affects nuclear to cytoplasmic transport, assembly of the spliceosome complex, or both. HTLV-I/II Rex bind with decreased avidity to the HIV-1 Rev response element (RRE); nevertheless, Rex from either HTLV-I or -II can rescue HIV-1 Rev-deficient mutants, suggesting that Rex may also affect expression of other non-HTLV-I/II mRNAs (Rimsky et al. 1988; Yip et al. 1991).

However, to date, Rex effects on cellular mRNAs have been noted only in the case of interleukin-2 and interleukin-2-receptor α chain expression (KANAMORI et al. 1990; MCGUIRE et al. 1993) and have otherwise been poorly characterized. We will primarily review the literature regarding Tax transactivation of cellular genes and focus on two more recently identified genes found to be transactivated—PTHrP and the early response gene *egr*-1—and on their potential relation to the malignant phenotype in ATL.

3 HTLV-I/II Tax—a Promiscuous Transactivator

Because HTLV-I does not integrate with precision adjacent to cellular proto-oncogenes and has not transduced cellular proto-oncogene sequences, interest has focused on a potential role for Tax in pathogenesis. Tax effects are believed to be mediated through interactions with cellular transcription factors. These interactions can also alter expression for a variety of cellular gene promoters. Transcriptional dysregulation by Tax may be responsible for numerous phenotypic characteristics of the HTLV-I-infected T cell. Evidence is accumulating which implicates Tax in the process of T-cell transformation and in genesis of the malignant phenotype.

The mechanism by which Tax induces HTLV-I/II transcription is still poorly understood. The discovery of a transactivating protein in HTLV-I/II was unexpected. Prior to Tax such transacting genes had been demonstrated only in DNA viruses. Early on, comparative analysis of the LTRs of HTLV-I and HTLV-II indicated the presence of three imperfect 21-bp repeated elements in the U3 portion of the LTR, which subsequently proved to be enhancer elements necessary for transactivation by Tax (OHTANI et al. 1987; SHIMOTOHNO et al. 1986; KITADO et al. 1987). Mutagenesis studies indicate that Tax effects are mediated through a TGACGT motif within the 21-bp repeats. Tax does not bind directly to the 21-bp elements (FUJISAWA et al. 1991; SHIMOTOHNO et al. 1986; XU et al. 1990); rather it affects transactivation through interaction with cellular transcription factors which bind DNA (FUJISAWA et al. 1991). Recently, HTLV-I Tax has been noted to increase the in vitro DNA binding activity of multiple CRE/ATF proteins, as well as several other proteins encoding a bZIP dimerization domain (WAGNER and GREEN 1993). Tax is thought to interact with bZIP-containing transcription factors in CRE/ATF-like proteins prior to DNA binding, increasing stability of the homodimer (WAGNER and GREEN 1993). This may lead to an elevated concentration of the bZIP homodimer, which may increase DNA binding activity of bZIP-containing transcription factors. This may also be characteristic of Tax interactions with other transcription factors that may oligomerize, although so far this has been described only for CRE/ATF-like factors. The requirements for such interaction both in Tax and in bZIP-containing proteins have not been mapped, although such a mechanism allows for promiscuous effects on a variety of transcription factors with minimal bZIP domains and stabilization of both homodimer and heterodimer

formation. The recent demonstration of functional associations between $NF_\kappa B$ containing a so-called rel domain, and the leucine zipper containing C/CAAT enhancer binding protein C/EBPα, containing a bZIP domain, suggests a mechanism by which Tax binding to one family of factors (e.g., CRE/ATF) might affect interactions with other factors such as C/EBP or $NF_\kappa B$-like factors (Stein et al. 1993). Such interactions could lead to altered rates of transcription from the HTLV-I/II viral LTRs. Intuitively, investigators suspected that interactions with host transcription factors could lead to a variety of effects on susceptible cellular genes. Numerous examples of cellular genes directly or indirectly transactivated by Tax now exist. Many of these examples help to explain the unusual phenotypic characteristics of HTLV-transformed T cells. As the mechanisms of Tax and Tax interactions with the LTR are extensively reviewed elsewhere in this volume by Dr. Mitsuaki Yoshida et al., we will focus on Tax interactions with cellular genes.

3.1 Cellular Promoters Affected by Tax

In addition to transcriptional enhancement of the viral LTR, Tax affects transcriptional activity of a wide variety of cellular promoters. These include several genes intimately linked to T-cell growth, such as the IL-2 gene, the alpha chain of the IL-2 receptor (IL2Rα), and several cellular proto-oncogenes including c-fos (Depper et al. 1984; Greene1986; Fujii et al. 1988; Leung and Nabel 1988; Ballard et al. 1989; Green et al. 1989), as well as genes involved in DNA repair, such as β-polymerase (Jeang et al. 1990). Although the latter is an example of trans-repression of expression by Tax, most Tax interactions described to date result in increased expression of the affected cellular genes.

Induction of both the IL2Rα chain and the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) has been seen not only in HTLV-I/II-infected cell lines, but also in transgenic mice expressing the HTLV-I Tax gene (Green et al. 1989; Shinohara1991). Transactivation of the viral LTR proceeds through effects on factors that bind to a cAMP response element-like (CRE) sequences contained within the 21-bp repeats (Radonovich and Jeang 1989; Giam and Xu 1989; Marriott et al. 1990; Nyborg et al. 1988,1990; Beraud et al. 1991). As described above, binding of Tax to several members of the CRE/ATF family of transcription factors has been observed (M. Yoshida, personal communication). In contrast, IL2Rα expression is increased by Tax through induction of $NF_\kappa B$, a member of the rel family of proto-oncogenes (Hoyos et al. 1989; Bohnlein et al. 1988; Ballard et al. 1988). The transcriptionally active form of $NF_\kappa B$ is a 50-kD protein, synthesized from a 105-kD precursor, p105. Nuclear translocation of p50 is abetted by a second protein p65, which binds to p50, and is inhibited by a cytoplasmic inhibitor known as $I_\kappa B$. $I_\kappa B$ appears to interact with the p65 subunit of the $NF_\kappa B$ complex, and disassembly of $I_\kappa B$ from the $NF_\kappa B$ complex allows translocation of p50/p65 to the nucleus and induction via $NF_\kappa B$-binding motifs. Tax does not bind directly to $NF_\kappa B$ DNA motifs (Hoyos et al. 1989; Sun et al. 1993). Yoshida and co-workers have demonstrated direct Tax binding to the p105 $NF_\kappa B$ precursor (Hirai et al.

1992), and other $NF_{\kappa}B$-like proteins may also be bound by Tax (W. Greene, personal communication). Arima and Greene have demonstrated Tax induction of several $NF_{\kappa}B$-like DNA-binding proteins, suggesting interactions with additional members of the c-rel family in T cells (Arima et al. 1991). Although Tax binding to the p105 precursor and other members of the family has been demonstrated, the requirements for Tax binding and/or interaction with $NF_{\kappa}B$ are poorly understood.

Cellular genes induced via the $NF_{\kappa}B$ pathway include IL-2 and the IL2Rα genes, potentially leading to autocrine T-cell stimulation (Cross et al. 1987; Greene et al. 1989; Leung and Nabel1988; Bohnlein et al. 1989; Arima et al. 1991). The pronounced level of expression of high-affinity IL-2 receptors observed on HTLV-I-transformed T-cell lines and ATL (Depper et al. 1984) may be due to IL2Rα induction by Tax via $NF_{\kappa}B$. The IL-2 and IL2Rα autocrine "loop" may lead to early T-cell expansion following HTLV-I infection and has therefore been implicated in leukemogenesis (Greene 1986). However, many ATL cells express very little, if any Tax, and many HTLV-I/II-transformed cell lines do not make appreciable levels of IL-2 (Katoh et al. 1986). Constitutive expression of IL2Rα in ATL cells in the absence of co-existent Tax expression indicates that transient Tax expression may result in sustained transcriptional and phenotypic alterations through "long-term" transcriptional effects that are not understood. Thus Tax may have both proximal and secondary, "downstream" effects on cellular gene expression.

Induction of $NF_{\kappa}B$ may also account for activation of other cellular genes, such as the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNFβ, although activation of the GM-CSF promoter via both $NF_{\kappa}B$-dependent and -independent pathways has been described (Nimer et al. 1989; Nimer 1991; Greene et al. 1989; Lindholm et al. 1991; Ishibashi et al. 1991). More recently, Duyao et al. (1992) have suggested that Tax could transactivate both the human and murine c-myc promoter through $NF_{\kappa}B$ binding sites. Whether the interaction with c-myc is important in immortalization and/or proliferation of T cells has not been demonstrated. In addition to IL2Rα, Tax transactivation of the HIV-1 LTR has also been observed to be mediated through the $NF_{\kappa}B$ pathway (Leung and Nabel 1991; Bohnlein et al. 1988). Although some investigators have assigned a hypothetical role for HTLV-I/II in abetting HIV-1 replication in vivo, clinical evidence for HTLV-I as a co-factor in progression of HIV-1 infection is lacking.

The 21-bp enhancer elements of the HTLV-I/II LTRs, required for trans-activation by Tax (Rosen et al. 1985; Shimotohno et al. 1986; Ohtani et al. 1987; Fujisawa et al. 1991), can be subdivided into so-called A-, B-, and C-motifs which interact with a variety of transcription factors (Muchardt et al. 1992; Nyborg et al. 1990; Tsujimoto et al. 1991; Yoshimura et al. 1990). The core sequence TGAGC is conserved in the B-motif and serves as a binding site for members of the CREB/ATF family of transcription factors (Nyborg et al. 1988; Tsujimoto et al. 1991; Yoshimura et al. 1990). At present it is unclear which if any of the CRE-like binding proteins identified in T cells and/or HeLa extracts are actually involved in Tax transactivation. The cellular proto-oncogene c-fos also appears to be activated by Tax via the CRE/ATF pathway, and the Tax-responsive element within the c-fos

Table 1. Transcription factors linked to Tax transactivation

Factor(s)	Affected genes
$NF_{\kappa}B$, c-rel	IL-2, IL2Rα, GM-CSF?, c-myc, HIV-1
ets-1, ets-2	HTLV-1 LTR, PTHrP?
CRE/ATF	HTLV-I/II LTR, c-fos, EGR-1
AP-2	HTLV-I LTR, PTHrP
CATTA/T binding proteins	GM-CSF

promoter contains a CRE motif (Fujii et al. 1988). Therefore, Tax may affect cellular transcription through two or more distinct transcriptional pathways, $NF_{\kappa}B$ and CRE/ATF, leading to distinct transcriptional consequences. Transformation of rat fibroblasts in vitro appears to involve primarily the CRE/ATF pathway (Smith and Green 1991). Whether this is the case for T-cell transformation remains to be clarified.

The HTLV-I 21-bp enhancer elements also contain within the so-called A domain octamer sequences with strong homology to the recognition motif (CC^{C}/G^{CA}/G^{GC}) for the AP-2 transcription factor (Muchardt et al. 1992a). AP-2 reportedly can directly activate expression from the LTR, as can Tax (Muchardt et al. 1992b). However, AP-2 and Tax have demonstrated antagonistic effects on HTLV-I LTR expression when co-transfected. Tax appears to inhibit AP-2 binding to the 21-bp repeats, through a potential interaction between the amino terminus of Tax and the amino terminus of AP-2 (Muchardt et al. 1992). AP-2 has unique structural features, including carboxyterminal DNA binding and dimerization domains and a proline-rich amino terminal transactivating domain. Although the role of Tax and AP-2 in HTLV-I/II regulation remains unclear, at least one cellular promoter, that of the PTHrP gene, is potentially transactivated by Tax through an interaction with AP-2 (D. Prager, personal communication, see below). Examples of transcription factors and families of factors implicated in Tax transactivation of a variety of cellular genes are indicated in Table 1.

3.2 Transactivation of the PTHrP Gene by Tax; a Link to Hypercalcemia?

A unique example of Tax effects upon cellular genes of potential clinical importance is the effect on expression of parathyroid hormone-related protein (PTHrP). Adult T-cell leukemia (ATL) linked to human T-cell leukemia virus type I (HTLV-I) infection is associated with the development of hypercalcemia in 50–70% of patients. A leading candidate gene involved in ATL-linked hypercalcemia is PTHrP. The PTHrP gene was cloned following purification and partial sequencing of a peptide from solid tumors associated with hypercalcemia. The PTHrP gene is located on chromosome 12 and consists of eight exons and two known promoters (Suva et al. 1987). Numerous transcripts arise as a result of alternate splicing; however, no distinct pattern is evident in malignancy (Mangin et al. 1990).

Although the gene was initially isolated from tumor tissue, it is now known to be widely expressed at low levels in normal tissues. These include skin, lactating mammary tissue, gravid uterus, endocrine pancreas, central nervous system, smooth muscle, bone marrow, fibroblasts, thyroid, parathyroid, adrenal, and pituitary glands (Merendino et al. 1986; Thiede et al. 1988; Thiede and Rodan 1990; Drucker et al. 1989; Weir et al. 1990; Kermer et al. 1991; Ikeda et al. 1988). The PTHrP gene is predicted to encode novel peptides of 139–173 amino acids, with eight of the first 13 amino acids being identical to parathyroid hormone (PTH) at the amino terminus (Goltzman et al. 1989). Humoral hypercalcemia of malignancy represents the only syndrome clearly documented to date in which PTHrP enters the circulation in sufficient quantities to mediate hypercalcemia.

Patients with humoral hypercalcemia of malignancy exhibit signs of abnormal calcium metabolism: increased renal tubular resorption of calcium, elevated nephrogenous cyclic AMP, and increased bone resorption (Broadus et al. 1988). Another candidate hypercalcemic factor, TNFβ—a cytokine with bone-resorbing activity, is unlikely to contribute to the hypercalcemia, as not all ATL tumor cells produce TNFβ and levels may be normal in acute ATL (Ishibashi et al. 1991). Fresh leukemic cells from patients with ATL have been shown to produce TGFβ (Kim et al. 1990) and TGFβ induces expression of rat PTHrP in keratinocytes (Allison and Drucker 1992). Watanabe et al. (1990) have demonstrated PTHrP expression in peripheral blood mononuclear cells of patients with ATL, HAM, or HTLV-I infection by reverse PCR. In addition, pleural and ascitic fluid obtained from patients with ATL also contains detectable levels of PTHrP (Motokura et al. 1989). These findings, as well as the prominent role played by PTHrP in other forms of humoral hypercalcemia of malignancy, suggest a similar role for PTHrP in hypercalcemia associated with ATL.

To determine if the Tax gene of HTLV-I and -II might directly transactivate PTHrP gene expression, a 2.8-kb downstream PTHrP promoter fragment was subcloned upstream of a chloramphenol acetyl transferase (CAT) indicator gene. The downstream PTHrP promoter is preferentially utilized in T cells (E. Ejima, personal communication). Co-transfection of this plasmid together with either an HTLV-I or -II Tax expression vector enhanced PTHrPCAT expression up to 50-fold (Ejima et al. 1993) in T-cell lines. Transactivation of the downstream PTHrP promoter occurred despite the absence of classical $NF_{\kappa}B$ or CRE DNA-binding motifs.

A series of 5′PTHrP promoter deletion mutants were transactivated by both HTLV-I and -II Tax with the longest construct exhibiting the greatest induction by Tax proteins (Ejima et al. 1993). To map the PTHrP Tax responsive sequences, site-directed mutants were generated to specifically remove putative AP1, AP2, EGR-1, "CRE-like", or Sp1 transcription factor recognition sequences. Transient co-transfection studies with HTLV-I Tax identified a 12-bp AP2 sequence at -212/-200 with respect to the exon 2 transcription start site, which is critical in Tax transactivation of PTHrP (D. Prager, personal communication).

Ruben and Rosen (1990) have previously documented that deletion of 30 carboxy terminal amino acids of the HTLV-1 Tax protein results in loss of Tax transactivation of IL-2Rα via the $NF_{\kappa}B$ pathway. Utilizing this Tax mutant with the

IL-2Rα and HTLV-I LTR as controls, the carboxy terminal domain of the Tax protein, but not $NF_{\kappa}B$, was shown to be required for PTHrP transactivation (EJIMA et al. 1993). Therefore, Tax transactivation of PTHrP likely involves protein-protein interactions with the acidic carboxy terminal region. Although the defect in Tax Δ984 is related to failure of $NF_{\kappa}B$ induction in the case of the IL-2Rα promoter, (an)other factor(s), perhaps AP-2, appears to be involved in the case of PTHrP (EJIMA et al. 1993). Several additional AP-2 sites are scattered throughout the PTHrP promoter, suggesting that the context of AP-2 binding is equally important with respect to Tax transactivation.

Transactivation of PTHrP was cell-type specific and was observed in co-transfections of T cells but not B cells, fibroblasts, or F9 cells (EJIMA et al. 1993). In contrast, the HTLV-I LTR was transactivated by Tax in all cell types tested. Furthermore, Northern analysis indicated that only HTLV-I productively infected T cells (MT2) produce PTHrP mRNA. A B-cell line that is productively infected with HTLV-II (729 pH6Neo) (CHEN et al. 1985) and constitutively makes HTLV-II Tax still fails to express PTHrP mRNA. These data suggest that Tax expression alone is insufficient for PTHrP induction and that T-cell specific cellular factor(s) are required. The effects on PTHrP may be an example of the pleomorphic manifestations of HTLV-I infection and specifically Tax expression in T cells. It is presently unclear why hypercalcemia should be principally observed in the setting of ATL, and not in HTLV-I/II carriers or myelopathy patients. Careful correlation of PTHrP levels with disease state needs to be performed. One possible explanation may simply be cell load, as higher numbers of HTLV-I-infected cells are observed in ATL patients than in carriers. In addition, JACOBSON and co-workers (1988,1990) have observed high levels of cytotoxic T-cell (CTL) activity in myelopathy patients, with CTL directed primarily against the Tax protein. It is possible that high "Tax"-producing cells may be selected against in HAM compared with ATL, leading to lower levels of PTHrP in the circulation. Nevertheless, the high degree of Tax transactivation of the PTHrP promoter and the strong association of PTHrP with humoral hypercalcemia in other malignancies suggest a link between Tax and hypercalcemia in ATL.

3.3 EGR-1 Gene: an Early Response Gene Induced by Tax

The EGR-1 gene is a member of a family of growth-inducible "early response genes". EGR-1, 2, and -3 are zinc finger-containing transcription factors which activate gene expression through a common *cis*-element (SUKHATME et al. 1988). The products of these genes are thought to be nuclear transducers of growth signals which elicit cellular proliferation. HTLV-I or -II productively infected cell lines constitutively express EGR-1 (WRIGHT et al. 1990). Furthermore, Jurkat cells stably transfected with an HTLV-I Tax gene under control of the metallothionine promoter demonstrated induction of EGR-1 expression (FUJII et al. 1992).

It is speculated that the HTLV-I Tax protein may elicit aberrant cell growth through induction of "early response genes" as an initial step in the development

of ATL or HAM (Tendler et al. 1990). This may abolish the usual requirement for mitogenic signals to the early immediate genes. Thus, deregulated expression of EGR-1 could contribute to the uncontrolled growth and transformation occurring in HTLV-infected T cells in the early stages of T-cell activation.

Fujii recently demonstrated Tax-I and -II activation of the c-fos, EGR-1, and -2 promoters mediated through so-called CArG boxes (Fujii et al. 1991). CArG-mediated transcription occurred in the absence of mitogenic signals through interaction with the CArG binding factor $p67^{SRF}$. Both in vitro and in vivo data demonstrate that $p67^{SRF}$ is also the target molecule for Tax 1 activation of c-fos. Therefore, like E1A, Tax may act as a "bridge" for $p67^{SRF}$ by interacting with basal transcription factors. The domain of Tax-I (2-312) interacting with $p67^{SRF}$ coincides with that determining viral enhancer specificity (Fujii et al. 1992). This suggests either that Tax-I-induced immediate early genes participate in viral enhancer activation, or that $p67^{SRF}$ and other transcription factors which mediate Tax activation share similar motifs for Tax interactions.

Transient co-transfections of human EGR-1 promoter CAT deletion mutants with HTLV-I and -II Tax into human T-cell lines demonstrate transactivation of the recombinant EGR-1 promoter (Sakamoto et al. 1992). The human EGR-1 promoter contains two CREs and five SREs (serum response elements) (Sakamoto et al. 1992). Consensus oligonucleotides representative of the SRE, CRE, or EGR-1 binding sites (EBS) on the upstream EGR-1 promoter were cloned upstream of a thymidine kinase promoter-driven CAT reporter gene. Transient co-transfections together with HTLV-I or -II Tax into MLA-144 T cells showed a six- to sevenfold transactivation of the CRE construct with minimal induction obtained with the EBS or SRE elements. Similar results have been obtained using HTLV-I Tax and the murine EGR-1 promoter, where the Tax response also mapped to a CRE element (Alexandre et al. 1991). Therefore, Tax transactivation of the human EGR-1 promoter likely occurs through its CRE, but not its SRE sequences (Table 1). EGR-1 transactivation was also cell-type dependent, as observed for PTHrP.

Tax interactions with the EGR-1 promoter may be responsible for the constitutive expression of EGR-1 in HTLV-I-transformed cells. The consequences of EGR-1 overexpression are unclear. However, ectopic expression of egr-1, c-fos, c-myc, and other transcriptional regulators in HTLV-I-infected T cells may play a direct role in transformation and contribute to the transformed phenotype.

Finally, Jacobson, and others have implicated the immune response to Tax in pathogenesis of myelopathy associated with HTLV-I/HTLV-II (Jacobson et al. 1988, 1990; Koenig et al. 1993; Elovaara et al. 1993). Investigators note the unusually high frequency of cytotoxic T lymphocytes (CTL) directed against a variety of epitopes within Tax in patients with myelopathy. The frequency of Tax-specific $CD8^+$ CTL may be several hundredfold increased in patients with HAM (Jacobson et al. 1990). A variety of Tax epitopes appear to be recognized. The relationship of Tax-directed CTL to neurologic disease is unclear. Whether Tax-mediated transactivation of cellular genes plays a role in HAM is also presently unknown.

3.4 Rex Effects on Cellular Gene Expression

In contrast to Tax, little is known about potential effects of the Rex protein on cellular genes. Rex is an RNA-binding protein which influences splicing and/or transport of viral mRNAs, leading to a net export of structural gag and env mRNAs to the cytoplasm (YIP et al. 1991; BLACK et al. 1991; GRASSMAN et al. 1991). HTLV-II LTR-driven gene expression is dependent on HTLV-II Tax for transcription and on provision of Rex to facilitate RNA export from the nucleus (ROSENBLATT et al. 1988; BLACK et al. 1991).

RIMSKY and GREENE (1988) have reported that HTLV-I Rex can substitute for the *Rev* gene of HIV-1 in trans, providing the first evidence for a potential role of Rex in regulation of heterologous RNA transport/processing. Similarly, we observed that HTLV-II Rex can also substitute for HIV-1 Rev (YIP et al. 1991). McGuire and Haseltine have observed that IL-2 promoter-driven expression is synergistically enhanced by Rex (MCGUIRE et al. 1993). How this effect is mediated by Rex is unclear, although indirect effects such as activation of the CD28 T-cell activation pathway have been invoked. Rex has also been associated with enhanced stability of the IL2Rα-chain mRNA in HTLV-I-infected cells (KANAMORI et al. 1990). These studies provide preliminary evidence that Rex might also affect expression of cellular genes and may play a role in potentiating an autocrine IL-2/IL-2 receptor loop.

4 Transactivation of Cellular Genes by HIV-1

The major transcriptional activating protein of the human immunodeficiency virus is Tat. This protein is formed from a multiply spliced messenger made shortly after activation of the HIV long terminal repeat (HIV LTR). It is one of the first messenger RNAs made in HIV-infected cells. The protein can be made as a one- or two-exon product and can also be made with a fusion involving the *env* and *rev* genes, referred to as tev (ROBERT-GUROFF et al. 1990). All three Tat proteins have similar biologic activities. However, there are differences in the cellular uptake and binding, depending upon the presence of fibronectin-binding domains found in the second exon. Tat exerts its transactivation activity through both transcriptional and post-transcriptional mechanisms (SOUTHGATE and GREEN 1991; KATO et al. 1992; HELLAND et al. 1991; LASPIA et al. 1989; GREENE 1990; JAKOBOVITS et al. 1990; CULLEN 1990;MARCINIAK et al. 1990; MARCINIAK and SHARP 1991). Tat binds to a cognate recognition element found in the messenger RNA of all HIV-specific messages. This binding to the U-rich bulge in an RNA stem-loop structure referred to as TAR (transactivation response) results in the recruitment of other transcriptional components (Tat-binding proteins as well as transcription initiator complexes) and an increase in viral gene expression (NELBOCK et al. 1990; GUTEKUNST et al. 1993; KESSLER and MATHEWS 1991).

A major function of HIV Tat is to markedly increase the rate of transcription of HIV-specific messenger RNAs. The specificity of this reaction is determined by the binding of Tat protein in concert with cellular proteins to a specific RNA sequence found in the response element, referred to as the Tat response element or TAR. Thus, it is believed that Tat's principal function is to allow for specific transactivation of HIV messages in preference to other cellular messages. Nonetheless, a growing body of evidence that has accumulated over the past 2 years suggests that HIV Tat can directly or indirectly regulate the expression of a variety of cytokines, differentiation antigens, and adhesion molecules, as well as modulate the immunologic function of human macrophages and T cells. In addition, there is some evidence that HIV can transactivate heterologous promoters of several viruses which may play a role in the pathogenesis of HIV infection. However, as opposed to HTLV-I/II Tax, the mechanism by which Tat alters cellular gene expression is poorly understood. To date, only one human mRNA containing a TAR-like sequence has been identified. Nevertheless, myriad Tat effects on cellular phenotype have been observed and are summarized below.

5 Modulation of Cytokine Expression by HIV Tat

Several investigators have shown that the expression of HIV Tat in T cells (Buonaguro et al. 1992), monocytes (Seth et al. 1991), endothelial cells, AIDS Kaposi's sarcoma-derived cells (Miles et al. 1991; Buonaguro et al. 1992; Ensoli et al. 1990), or epithelial cells can alter the expression of important immunoregulatory cytokines. Using HIV Tat expression constructs and recombinant protein, it has been shown that HIV Tat can alter the ability of human primary T cells to respond to soluble antigen. Moreover, this alteration in T-cell responsiveness appears to be the result of an increase of TGFβ1 production. This has suggested an additional mechanism for immunodeficiency seen in patients with HIV (Kekow et al. 1991). Similarly, recombinant Tat protein elicited the production of factors by bone marrow-derived macrophages which inhibit the production of hematopoietic progenitor cells (Zauli et al. 1992). Using neutralizing antibodies to TGFβ1, they demonstrated that the bone marrow inhibitory properties of recombinant Tat protein were due to the elaboration of TGFβ1 from bone marrow-derived macrophage cells. Thus, in both primary human T cells and macrophages, TGFβ1 expression is increased by recombinant HIV Tat.

Disturbances in TGF-β production may have other deleterious effects. After exposure to recombinant Tat protein, monocytes have an altered ability to control vaccinia virus production. Using cultured primary human monocytes, both recombinant and synthetic HIV Tat were shown to induce TGFβ in monocytes (Seth et al. 1991). In addition, HIV Tat was able to block the normal inhibitory effects of gamma interferon. This was associated with an enhanced expression of F_c receptors and CD16 antigen, suggesting that the monocytes had differentiated in response to HIV Tat (Seth et al. 1991).

HIV Tat has been shown to be a mitogen for AIDS Kaposi's sarcoma (KS)-derived cells (Miles et al. 1991; Ensoli et al. 1990; Vogel et al. 1988). These cells are presumably of mesenchymal origin and have characteristics of both smooth muscle cells (Weich et al. 1991) and endothelial cells. Several groups have shown that either Tat expression vectors, recombinant Tat protein, or synthetic Tat peptides can induce proliferation of AIDS KS-derived cells. This increase in proliferation is associated with an increase in IL-6 production as well as IL-1, bFGF (basic fibroblast growth factor), and granulocyte-monocyte colony-stimulating factor production (Nakamura et al. 1988; Ensoli et al. 1990, 1991). Since many of these cytokines are also mitogens for these cells, it is unclear whether HIV Tat is directly mitogenic or works indirectly through the induction of one or more cytokines (Miles et al. 1990, 1991, 1992).

In Jurkat T-cell lines, high-level expression of HIV Tat down-regulates expression of interleukin-2 as well as the high-affinity interleukin-2 receptor (Purvis et al. 1992). Since interleukin-2 is critical for the proliferation of cytotoxic T cells as well as provision of T-cell "help", down-modulation of IL-2 or its receptor could have pathologic consequences. It has also been shown that the first exon of the Tat gene is responsible for the increased production of TNFβ found in T cells chronically infected with a reverse transcriptase-defective HIV-1 provirus (Buonaguro et al. 1992). Indirect evidence from this group demonstrated that HIV can directly up-regulate TNFβ production.

HIV Tat also increased TNFβ production in B-lymphoblastoid Raji cells (Pocsik et al. 1992) and decreased TNFβ receptor number fivefold without a significant change in receptor affinity. Down-regulation of TNFβ receptors was not due to occupancy of the receptor by autocrine production of TNFβ, nor was it due to a decrease in gene expression. Instead, it appeared that the receptor remained intracellular in *tat* transfected cells.

6 Tat Modulation of Cellular Adhesion Molecules, Matrix Proteins, and Surface Cytokine Receptors

As previously mentioned, HIV Tat has the capacity in some model systems to down-regulate the interleukin-2 receptor as well as the TNF receptor. Other groups have demonstrated that HIV Tat can down-regulate CD28 expression in T-cell lines (Kashiwamura et al. 1990). These effects are relatively specific to Tat, as T-cell lines expressing other HIV-regulatory proteins (Nef or Rev) did not have similar effects. The decrease in CD28 expression is associated with a decreased ability of the cells to be triggered by the co-stimulatory C28 ligands, the B7-1/B7-2 molecules. This suggests that some of the T-cell proliferative defects seen in HIV *tat* transfected T cells and the inability of primary cells to respond to soluble antigen in the presence of Tat (Kekow et al. 1991) could be related either to production of TGFβ1 or to down-modulation of CD28.

Another potential mechanism for immunologic escape and immune dysregulation by HIV Tat is the apparently decreased expression of MHC class-1 antigen in cytotoxic T cells expressing HIV Tat (Howcroft et al. 1993). This repression of MHC class-1 activity appeared to be related to the presence of two-exon Tat, as single-exon Tat was incapable of mediating this effect. Coupled with the observation that single-exon Tat altered IL-2 and IL-2 receptor expression, these data suggest that there may be specific immunologic effects related to the different Tat species detected in HIV-infected cells (Purvis et al. 1992; Howcroft et al. 1993).

6.1 Effects of Extracellular Tat

In addition to acting directly through intracellular production of Tat, a number of groups have demonstrated that soluble HIV Tat can affect the function of other cells. These can include the effects of recombinant Tat protein on T cells and T-cell proliferation, as well as on monocytes and on cells of the central nervous system (Taylor et al. 1992a,b; Chowdhury et al. 1990). The mechanism of cellular uptake of recombinant Tat has recently been delineated. Recent studies have shown that vitronectin-binding integrin, $\alpha V\beta 5$, binds the basic domain of Tat (Vogel et al. 1993). It appears that the Tat basic domain, containing the arginine-rich sequence RKKRRQRRR, is homologous to the heparin-binding domain of the $\alpha V\beta 5$ ligand, vitronectin, and can compete for the binding of vitronectin to the surface of cells. This binding is specific, as peptides containing polyarginine or polylysine do not bind to the receptor and do not compete for Tat binding (Vogel et al. 1993). A second group has mapped the cellular uptake region to Tat aa 49–57 and demonstrated a cell-surface integrin receptor which is capable of binding HIV-1 Tat (Weeks et al. 1991, 1993). Thus, it appears that Tat can act in two ways: directly, using intracellular alteration of gene expression, as well as indirectly, by exiting the cell and binding to adjacent cells through integrin receptors, where it is taken up, thereby affecting cellular function of nearby cells.

In addition to T-cell and macrophage defects, Tat may also be involved in pathogenesis of AIDS-related Kaposi's sarcoma; Tat transgenic mice developed KS-like lesions (Vogel et al. 1988). These lesions are not due to the direct production of HIV Tat within the KS- like cells, as Tat expression is restricted to perilesional cells. Furthermore, CV1 cells expressing HIV Tat have the capacity to increase the proliferation of AIDS KS-derived cells (Ensoli et al. 1990). The proliferation is associated with a modulation of multiple cytokines that are known to be involved in the proliferation of AIDS KS-derived cells (Ensoli et al. 1989, 1990, 1993; Barillari et al. 1992; Nakamura et al. 1988). Thus, the paracrine effects of extracellular Tat may be as important as direct effects on HIV-1 and cellular gene expression.

6.2 Tat Effects in the Central Nervous System

In addition to altering the expression of cellular receptors and adhesion molecules in macrophages and T cells, HIV Tat can also alter matrix protein and cellular receptors in cells of central nervous system origin. Using recombinant Tat protein, there was a significant increase in mRNA levels of both fibronectin and collagen types 1 and 3 in glial cells of human origin (TAYLOR et al. 1992a). Transient transfection assays showed that there is an increased level of transcription of both the fibronectin and the alpha 1 type-1 collagen promoters. In addition, Tat has direct cytotoxic effects on rat brain-derived cells, and the basic regions of HIV Tat proteins can act as potent neurotoxins (HAYMAN et al. 1993; PHILIPPON et al. 1992). Moreover, the toxicity of these proteins can be ameliorated by blocking nitric oxide synthetase as well as *N*-methyl-D-aspartate (NMDA) channel opening. A direct cytotoxic effect of HIV Tat on T cells has been suggested by another group as well (SABATIER et al. 1991; BENJOUAD et al. 1993). Basic peptides from region of aa 49–57 inhibited mitogen-induced T-cell proliferation. Moreover, using ^{51}Cr release and trypan blue exclusion, Tat altered cell membrane permeability and viability in a dose-dependent manner. Thus, in addition to functional defects, there may be direct cytotoxic effects in the central nervous system and on T cells.

6.3 Modulation of Cell Differentiation and Function by HIV Tat

Some of the effects on cellular adhesion molecules and functional activity of both T cells and monocytes may be attributable to the partial differentiation of these cells in the presence of HIV Tat. Production of HIV Tat in monocyte-derived cell lines (U937) results in an increased expression of several oncogenes, including C-fos and C-fms (MACE et al. 1991). Expression of these oncogenes is associated with an alteration in the differentiation of the cells, with an increased expression of CD16 antigen and Fc receptors. In AIDS-related Kaposi's sarcoma cells, HIV Tat expression is associated with an increase in smooth muscle alpha actin expression as well as morphological change with spindle cell formation (WEICH et al. 1991). In mouse monocyte cell lines, these alterations and cellular differentiation can result in a loss of tumoricidal activity, induced by gamma interferon and LPS (SETH et al. 1991).

6.4 Transactivation of Heterologous Viral Promoters by HIV Tat

The mechanism underlying most alterations in gene expression reported after exposure to recombinant HIV Tat or *tat* expression vectors is not understood. For example, in the case of increased IL-6 expression in AIDS-related Kaposi's sarcoma cells, there is no evidence that HIV Tat directly increases IL-6 expression. Studies of the IL-6 promoter in monocyte-derived cell lines have shown that

there is no direct effect of HIV Tat on the IL-6 promoter (Chen et al. 1992). Thus, it is assumed that many of the alterations in expression of cytokines, cellular adhesion molecules, cell-surface receptors, and alterations in functional activities are indirect and the result of some other activity of HIV Tat, rather than direct modulation of promoters of specific genes and gene products. However, for some viral promoters it has been possible to demonstrate direct transactivation by HIV Tat. For example, using the human papilloma virus 16 upstream regulatory region (URR) linked to a chloramphenicol acetyl transferase (CAT) expression construct, single-exon Tat significantly increased CAT activity (Vernon et al. 1993). Moreover, HIV Tat reverses E2-mediated repression. This increased URR-directed gene expression was observed after transfection with HIV Tat and following co-cultivation with HIV-1-producing cells. Similarly, Tat increased late gene expression of the human neurotrophic JC virus in glial cells (Chowdhury et al. 1993). Using deletion mutation analysis, they have identified a region upstream of the JC virus late RNA start sites which can respond to Tat in human glial cells. Using synthetic oligonucleotides and a heterologous promoter, a GA/GC region (GAGGCGGAGGC) was shown to confer Tat responsiveness in glial cells. Since this sequence is not identical to the U-rich bulge region found in the HIV TAR, this indicates Tat responsiveness in a heterologous promoter that is not due to the presence of a TAR homologous sequence.

HIV Tat also transactivates the murine CMV major late promoter, which also lacks a sequence similar to the TAR region (Kim and Risser 1993). The response region appears to be in the upstream enhancer region, as deletion of this region completely abrogated Tat responsiveness. Moreover, since Tat stimulates expression at both transcriptional and translational levels, there appears to be a complex interplay between the enhancer region and a sequence upstream from the known enhancer which negatively affects expression. HIV-1 Tat has been shown to increase prion gene expression in human astrocytes (Muller et al. 1991). Presumably, this is due to the pentanucleotide CUGGG found in the stem-loop structure of prion proteins. This sequence is similar to sequences within the HIV *cis*-acting TAR sequence found in the HIV LTR. Moreover, infection of human astrocytes with HIV-1 increased expression of DNA topoisomerase-II activity (Muller et al. 1991) as well as prion gene expression. It is possible that HIV-1 could alter prion gene expression in patients, resulting in progression of neurologic disease. Finally, investigators groups have demonstrated reciprocal interactions between HIV-1 Tat and human herpes virus type 6 (Di Luca et al. 1991). Both HIV-1 and HHV-6 can infect human CD4 cells, so it is possible that this interaction may occur directly in co-infected patients.

6.5 Pleiotropic Transactivation by HIV Tat

In virtually all of the model systems studied to date, HIV Tat has been shown to modulate one or more of a variety of genes which may be important in immunologic recognition or function. It is becoming clear that HIV Tat plays a much broader role in the pathogenesis of HIV infection than simply increasing expres-

sion of HIV-1 through binding to the *cis*-acting TAR sequence. However, many of the activities ascribed to HIV Tat could be the result of cellular differentiation or de-differentiation. Thus, for many of the alterations described to date (modulation of IL-6, TGFβ1, CD28, integrin receptors, etc.) there is little if any direct evidence that the promoter for any of these gene products is directly transactivated. This is in contrast to heterologous viral promoters; here the evidence for direct transactivation of these gene products is clear. Since alteration of cytokines, their receptors, and adhesion molecules can profoundly affect immune function, it is possible that immunologic paracrine effects of HIV Tat, as well as the direct biologic effects of HIV-1 on T-cell function and cell number, may contribute to immunopathogenesis.

7 Summary

We have focused this chapter on interactions with two of the best characterized transregulatory genes, *tax* for HTLV-I/II and Tat for HIV-1. Both genes illustrate the complex interplay between retroviral regulatory genes and cellular gene regulation. In both instances a viral gene of relatively straightforward function in the viral context appears to cause extensive dysregulation of cellular genes, either directly or as a consequence of altered cellular differentiation. Understanding this viral/cellular gene cross-talk may elucidate mechanisms leading to malignant transformation autoimmune disease and to neurologic and paraneoplastic complications such as hypercalcemia for HTLV-I/II, as well as the pathogenesis of immune dysfunction and opportunistic malignancy in HIV-I/II-infected individuals. An understanding of functional mechanisms of these transregulatory viral genes will undoubtedly afford better explanations for the myriad manifestations of retroviral infection.

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Regulation of HIV-1 Gene Expression by the Transactivator Protein Tat

R.B. GAYNOR

1 Introduction

The regulation of HIV-1 gene expression is an area of intense investigation. Like all retroviruses, HIV-1 integrates into cellular DNA, and its gene expression is dependent on host cell transcription factors and polymerases (GAYNOR 1992). Cellular transcription factors are able to interact with distinct regulatory regions in the long terminal repeat to modulate gene expression (GAYNOR 1992). In addition, viral-encoded proteins are critical to the control of HIV-1 gene expression. A number of important questions exist concerning the mechanisms which regulate HIV-1 gene expression. What cellular factors are responsible for increases in HIV-1 gene expression in response to T-lymphocyte activation? What are the

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mechanisms that repress HIV-1 gene expression in quiescent cells? How do HIV-1 transactivator proteins stimulate increases in gene expression? An understanding of these questions is critical to determining the interplay between viral and cellular factors which regulate HIV-1 gene expression.

After the discovery of HIV-1 (Barre et al. 1983; Gallo et al. 1984; Ratner et al. 1985; Sanchez et al. 1985; Wain et al. 1985), extensive mutagenesis of the virus was performed to determine the *cis*- and *trans*-acting elements which regulate viral gene expression. An 86 amino acid viral protein, Tat, was identified and found to be required for HIV-1 gene expression and for subsequent viral replication (Sodroski et al. 1985a,b; Dayton et al. 1986; Fisher et al. 1986). *tat* is unique among viral transactivators. Unlike E1A and Tax, which activate a number of viral and cellular genes, Tat activation is relatively specific for HIV-1 (Cullen 1986; Peterlin et al. 1986; Wright et al. 1986; Berkhout et al. 1989). A *cis*-acting element in the HIV-1 LTR, located downstream of the RNA initiation site, was critical for high-level gene expression. This element, which extends from +1 to +60 in the HIV-1 LTR, was designated the *trans*-acting response element, or TAR (Rosen et al. 1985). TAR forms a double-stranded RNA structure which is required for high-level gene expression in response to Tat (Okamoto and Wong 1986; Muesing et al. 1987). Since *tat* is essential for HIV-1 gene expression and replication, an understanding of its function is necessary to determine the factors that modulate the HIV-1 life cycle. Furthermore, given the specificity of Tat for activating HIV-1 gene expression, it serves as a target of potential therapeutic interventions to inhibit HIV-1 infection. This review explores the structure of the Tat protein, its role in the viral life cycle, the cellular factors which are involved in regulating *tat* function, and the mechanisms by which Tat activates HIV-1 gene expression.

2 Regulation of Gene Expression by *tat*

The mRNAs which are transcribed from HIV-1 include unspliced, singly spliced, and multiply spliced precursors (Feinberg et al. 1986; Kim et al. 1989) of 9.2 kb, 4.3 kb, and 2.0 kb, respectively. The unspliced RNA encodes the *gag* and *gag-pol* precursors, the singly spliced RNA encodes the *env* gene products, and the multiply spliced RNA encodes the accessory proteins *tat*, *rev*, and *nef*. Early after HIV-1 infection, multiply spliced RNAs that encode viral regulatory proteins are the predominant RNAs which are transcribed (Kim et al. 1989). Later, the abundance of singly spliced and unspliced RNAs that encode HIV-1 structural proteins become predominant (Kim et al.1989). Thus, the Tat protein, which is encoded by multiply spliced RNAs, is produced during the early phase of the HIV-1 life cycle.

The *tat* gene of a variety of HIV-1 strains is extremely conserved at the amino acid level (Meyers 1988). The *tat* gene is encoded by two exons (Arya et al. 1984; Sodroski et al. 1985a, b). The first exon, preceding the *env* gene, codes for 72 amino acids, while the second exon, within the *env* gene, codes for an additional 14 amino acids (Arya et al. 1984; Sodroski et al. 1985a,b). In HIV-1-infected cells,

the predominant form of Tat is 16 kDa and is comprised of 86 amino acids (ALDOVINI et al. 1986; MUESING et al. 1987). A minor form of Tat is 14 kDa, comprised of only 72 amino acids (WRIGHT et al. 1986). In addition, a third form of 28-kDa Tat is also seen in HIV-1-infected cells (BENKO et al. 1990). This protein, designated Tev, contains 72 amino acids from *tat*, 38 amino acids from *env*, and 91 amino acids from *rev* (BENKO et al. 1990). The Tev protein has functions of both the *tat* and *rev* proteins. Thus, several different forms of the Tat protein are capable of being translated from a variety of multiply spliced RNAs. It is not clear if these different forms of *tat* have different functional properties, though the transcriptional activating properties of the 72- and 86-amino acid *tat* proteins have been elucidated (SODROSKI et al. 1985a, b; CULLEN 1986).

3 Structure of the Tat Protein

Examination of the 86-amino acid sequences of Tat reveals a number of interesting structural characteristics, as shown in Fig. 1 (MEYERS 1988). The amino-terminus of Tat which extends from residues 1 to 21 contains three repeats of proline XXX proline in addition to acidic amino acids at positions 2, 5 and 9. This region is followed by a domain extending from residues 22 to 37, which contains seven cysteine residues potentially capable of binding divalent ions such as cadmium and zinc. A domain located between residues 38 and 48, with no obvious transcriptional activating domains, is critical for *tat* activation of HIV-1 gene expression. A region extending between residues 49 and 57 contains eight basic amino acids, including both lysine and arginine. The residues between 58 and 72 do not have obvious unique structural features, while the carboxyl terminus of Tat, extending from residues 73 to 86, contains the highly conserved tripeptide sequence Arg-Gly-Asp, known as an RGD sequence, which may be important in Tat binding to cell surfaces via integrin-mediated cell adhesion (BRAKE et al. 1990). Thus, the *tat* protein contains a number of unique structural motifs which likely mediate its transcriptional regulatory properties.

The purification of recombinant Tat protein using bacterial expression systems has allowed a characterization of its biochemical properties (FRANKEL et al.

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1                                   10                                  20
Met Glu Pro Val Asp Pro Asn Leu Glu Pro Trp Lys His Pro Gly Ser Gin Pro Arg Thr

21                                  30                                  40
Ala Cys Asn Asn Cys Tyr Cys Lys Lys Cys Cys Phe His Cys Tyr Ala Cys Phe Thr Arg

41                                  50                                  60
Lys Gly Leu Gly Ile Ser Tyr Gly Arg Lys Lys Arg Arg Gin Arg Arg Arg Ala Pro Gin

61                                  70                                  80
Asp Ser Gin Thr His Gin Ala Ser Leu Ser Lys Gin Pro Thr Ser Glu Ser Arg Gly Asp

81                  86
Pro Thr Gly Pro Lys Glu
```

Fig. 1. The 86 amino acid sequence of *tat*

1988; Gentz et al. 1989; Dingwall et al. 1990; Wu et al. 1991). Tat has been difficult to purify because it is avidly bound to bacterial nucleic acids. Furthermore, oxidation of the cysteine residues potentially alters the structural integrity of this protein (Frankel et al. 1988). Even with these difficulties, several methods have been used to purify recombinant *tat* protein from *Escherichia coli*; Tat has been purified from bacteria following denaturation with urea or guanidine to liberate it from nucleic acids (Frankel et al. 1988; Gentz et al. 1989; Dingwell et al. 1990). Following renaturation, Tat has been purified via either conventional column chromatography or affinity chromatography, using nickle columns which bind histidine-tagged Tat. Protease cleavage of fusions of Tat with glutathione S-transferase has been used successfully to purify native Tat (Wu et al. 1991). This protocol avoided denaturation and subsequent renaturation steps, which can decrease the activity of Tat. Other systems yielding high levels of Tat include expression in baculovirus, yeast, and vaccinia. The purification of Tat from these expression systems has many of the same difficulties as those seen with bacterial-produced Tat.

It was necessary to develop assays for determining the activity of recombinant Tat proteins. The ability of Tat to be taken up from tissue culture media and subsequently transactivate HIV-1 LTR CAT reporter constructs has been used to assay the activity of Tat (Frankel and Pabo 1988; Gentz et al. 1989). Several recent studies have demonstrated that recombinant Tat was able to stimulate gene expression using in vitro transcription assays with the HIV-1 LTR (Marciniak et al. 1990a; Marciniak and Sharp 1991). Another assay for recombinant *tat* protein was based on its ability to bind to labeled in vitro transcribed TAR RNA in gel retardation assays (Dingwall et al. 1989, 1990; Wu et al. 1991). Further studies of Tat, including NMR and crystallization, will be required to determine its molecular structure.

4 Functional Motifs of Tat

Several techniques have been used to identify which domains in Tat mediate its transactivating properties. The most commonly used method involves co-transfection into tissue culture cells of expression constructs containing wild-type or mutated *tat* genes, along with an HIV-1 LTR construct fused to either chloramphenicol acetyltransferase or luciferase reporter genes (Hauber et al. 1987; Garcia et al. 1988; Kuppuswamy et al. 1989; Ruben et al. 1989; Rice and Carlotti 1990). This co-transfection assay has also been used to determine which regulatory regions in the HIV-1 LTR are required for *tat* function. Another assay of *tat* function involved inserting mutated *tat* genes into HIV-1 proviral constructs and assaying the production of infectious virus (Sadaie et al. 1988). Finally, recombinant wild-type and mutant Tat proteins can be tested for their ability to stimulate HIV-1 LTR gene expression using in vitro transcription assays (Marciniak et al. 1990a; Marciniak and Sharp 1991).

Mutations of the amino terminus of Tat that disrupted proline residues which were part of three proline XXX proline repeats did not severely alter *tat* function (Garcia et al. 1988). However, internal deletions of four amino acids between positions 3 and 6 inhibited the function of *tat*, as did mutation of three acidic amino acids at positions 2, 5, and 9 (Kuppuswamy et al. 1989; Rappaport et al. 1989; Tiley et al. 1992). Though these latter mutations demonstrated the importance of the amino terminus, a number of other mutations in this region did not severely inhibit *tat* function. Thus the structural characteristics of the amino terminus which regulates *tat* function remain to be determined. The cysteine-rich domain is critical for *tat* function. Mutagenesis of six of seven specific cysteine residues in the cysteine-rich portion of *tat* between amino acids 22 and 37 severely decreased *tat* transactivation (Garcia et al. 1988; Sadaie et al. 1988; Kuppuswamy et al. 1989; Rice and Carlotti 1990). Changes in the remaining cysteine at amino acid 31 in this domain did not markedly alter the ability of *tat* to transactivate the HIV-1 LTR. Whether these cysteine residues function to mediate cadmium or zinc dimers of *tat* is not known (Frankel et al. 1988). Several specific amino acid substitutions in the so-called core domain of *tat*, between amino acids 38 and 48, resulted in severe defects in *tat* transactivation (Kuppuswamy et al. 1989; Rice and Carlotti 1990). For instance, substitution of alanine for phenylalanine at residue 38, or alanine for lysine at residue 41 prevented *tat* activation. The structure of this domain, critical in mediating *tat* activation, is not known.

The basic domain of *tat*, which extends between amino acids 49 and 57, is required for the nuclear and likely nucleolar localization of Tat (Hauber et al. 1987; Garcia et al. 1988; Kuppuswamy et al. 1989; Ruben et al. 1989; Ensoli et al. 1990; Siomi et al. 1990). This domain is also essential in mediating Tat binding to TAR RNA (Dingwall et al. 1989, 1990; Cordingley et al. 1990; Weeks et al. 1990; Calnan et al. 1991a,b; Weeks and Crothers 1991; Wu et al. 1991). Mutation of individual basic amino acids in this domain did not alter Tat nuclear localization or binding to TAR RNA, or the ability of *tat* to transactivate HIV-1 (Garcia et al. 1988; Ruben et al. 1989; Dingwall et al. 1989, 1990; Roy et al. 1990a; Siomi et al. 1990; Modesti et al. 1991). However, mutations which change the overall basic charge in this domain dramatically decrease the transactivating properties of Tat (Garcia et al. 1988; Ruben et al. 1989; Dingwall et al. 1989, 1990; Roy et al. 1990a; Calnan et al. 1991 a,b). The region of *tat* extending from amino acids 58 to 72 has an augmenting function on *tat* activation (Garcia et al. 1988; Kuppuswamy et al. 1989; Ruben et al. 1989). Deletion of this region resulted in two- to threefold decreases in *tat* transactivation. The second exon of Tat, which contains 14 additional amino acids, does not appear to alter the degree of *tat* activation. This region of *tat* contains an RGD sequence that may be involved in Tat binding to sites required for integrin-mediated cell adhesion. However, recombinant Tat lacking the second exon can be taken up by adsorbative endocytosis and is able to transactivate an HIV-1 LTR construct (Mann and Frankel 1991). The role of the RGD sequence in *tat* is not clear. *Tat* proteins containing either 72 or 86 amino acids did not appear to differ significantly in their ability to transactivate the HIV-1 LTR (Kuppuswamy et al. 1989). The functional domains of Tat are shown in

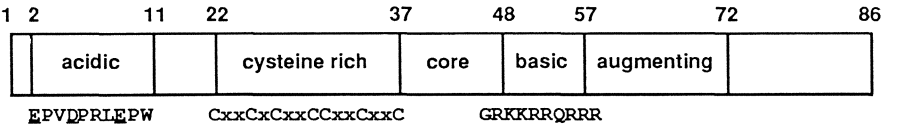

Fig. 2. Structural aspects of Tat. Functional domains in Tat including the amino terminus extending from amino acids 2 to 11, the cysteine-rich domain extending from amino acids 22 to 37, the core domain extending from amino acids 38 to 48, the basic domain extending from amino acids 49 to 57, the augmenting domain extending from amino acids 58 to 72, and the second exon of *tat* extending from amino acids 73 to 86

Fig. 2. Thus, the Tat protein contains a number of functional domains which modulate its transcriptional activating properties.

5 Role of *cis*-acting DNA Regulatory Elements in *tat* Function

Though the TAR element is critical in mediating *tat* activation, a number of *cis*-acting regulatory elements in the HIV-1 LTR are important in modulating HIV-1 gene expression in both the presence and the absence of *tat*. The cellular factors which have been demonstrated to bind different elements in the HIV-1 LTR are shown in Fig. 3 (Gaynor 1992). For simplicity, the HIV-1 LTR can be divided into three functional regions. These include the modulatory region that extends from –454 to –78, the core region that extends from –78 to the transcription initiation site, and TAR, which extends from +1 to +60 (Gaynor 1992). The modulatory region contains a number of regulatory elements that stimulate HIV-I gene expression in response to activating T-lymphocyte proliferation (Siekevitz et al. 1987; Tong et al. 1987; Crabtree 1989). The best studied of these elements is the enhancer element, containing two NF-κB motifs that bind the members of the *rel* or NF-κB family (Nabel and Baltimore 1987). Though NF-κB motifs are important in regulating HIV-1 gene expression, this element is not essential for high levels of *tat* activation, nor is it required for relatively normal viral growth properties (Leonard et al. 1989; Ross et al. 1991).

The core element in the HIV-1 LTR is critical for *tat* activation. This region contains three SP1 binding sites (Jones et al. 1986; Harrich et al. 1989; Kammine et al. 1991; Kamine and Chinnadurai 1992) and the TATA element (Garcia et al. 1989; Berkhout and Jeang 1992; Olsen and Rosen 1992; Lu et al. 1993). Mutation of individual SP1 binding sites in HIV- 1 LTR CAT constructs was fully activated by *tat*, while constructs with mutations of all three SP1 binding sites exhibited markedly reduced degrees of *tat* activation (Jones et al. 1986; Harrich et al. 1989). However, the SP1 binding sites are not essential for *tat* activation. The replacement of all three SP1 binding sites with binding sites from other upstream activator proteins was able to partially restore the level of *tat*-activation (Southgate and Green 1991; Berkhout and Jeang 1992). These studies demonstrated that the

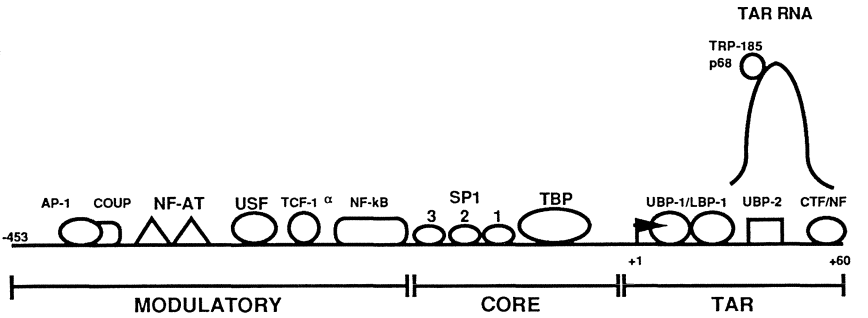

Fig. 3. Cellular factors that bind to the HIV-1 LTR. The region of the HIV-1 LTR extending from –453 to +60 is shown. The HIV-1 LTR can be divided into three functional regions designated the *modulatory*, *core*, and *TAR* elements. Cellular factors binding to the modulatory elements include AP-1, COUP, NF-AT, USF, TCF1α, and NF-κB. The core element contains three SP1-binding sites, and the TATA element binds the cellular factor TBP. The TAR DNA element binds several cellular factors including UBP-1/LBP-1, UBP-2, and CTF/NF1, while TAR RNA binds several factors including TRP-185 and p68

fold-activation by *tat* was inversely proportional to the basal level of HIV-1 gene expression (Southgate and Green 1991). Thus, the binding of strong upstream activators such as GAL4-VP16 fusions to GAL4 binding sites inserted in place of the SP1 binding sites raised the basal level of HIV-1 gene expression but lowered the fold of *tat* activation. However, SP1 binding sites were demonstrated to be the optimal upstream element for mediating high-level *tat* activation.

Several studies have demonstrated that the structure of the HIV-1 TATA element was critical for *tat* activation (Garcia et al. 1989; Berkhout and Jeang 1992; Olsen and Rosen 1992; Lu et al. 1993). Mutation of the TATA sequence or substitution with the TATA elements of SV40, the adenovirus major late promoter, or the cellular DRA promoter each dramatically decreased the level of *tat* activation (Garcia et al. 1989; Berkhout and Jeang 1992; Olsen and Rosen 1992; Lu et al. 1993). However, these changes in the TATA sequence did not affect the basal expression from the HIV-1 promoter (Garcia et al. 1989; Berkhout and Jeang 1992; Lu et al. 1993). The replacement of the HIV-1 TATA element with the terminal transferase initiator element eliminated *tat* transactivation (Olsen and Rosen 1992; Lu et al. 1993). These studies indicate that the structure of the TATA element may specify so-called nonprocessive transcription complexes that are the target for *tat* activation.

DNA regulatory elements downstream of the TATA element also influence the level of *tat* activation. These DNA elements between –38 and –16 and between –16 and +27 bind the cellular transcription factor UBP-1/LBP-1. The region between –16 and +27 is a high-affinity UBP-1/LBP-1 (Garcia et al. 1987; Jones et al. 1988; Wu et al. 1988; Kato et al. 1991) binding site, while the region between –38 and –16 is a lower affinity site for this factor. When UBP-1/LBP-1 was present in high levels in in vitro transcription assays, it repressed HIV-1 gene expression by preventing cellular factors from associating with the TATA element (Kato et al. 1991). A number of other cellular factors such as upstream regulatory factor (USF) can also bind to downstream regulatory regions that include

pyrimidine-rich initiator elements (Du et al. 1993). Though specific upstream DNA regulatory elements are important for *tat* activation, maximal levels of *tat* activation require the presence of both specific upstream regulatory elements and TAR (Garcia et al. 1987). *tat* activation likely involves interactions either directly or indirectly with cellular transcription factors that bind to both these upstream and downstream regulatory elements.

6 Heterologous Fusions to Determine *tat* Function

Transfection studies with the HIV-1 LTR did not clarify whether *tat* activation was a consequence of interactions with cellular factors that bound to HIV-1 DNA or RNA elements. In fact, it was considered possible that *tat* could act as a bridging molecule between DNA and RNA bound cellular factors. To address whether Tat functioned when bound to either DNA or RNA templates, heterologous fusions were constructed in which Tat was fused to either known DNA (Berkhout et al. 1990; Southgate and Green 1991) or RNA (Selby and Peterlin 1990; Southgate et al. 1990) binding proteins. The recognition sites for these proteins were then inserted into HIV-1 LTR reporter constructs. Transfection of Tat fusion proteins with the corresponding HIV-1 reporter constructs was then performed.

Several studies indicated that Tat could activate gene expression when bound to HIV-1 LTR DNA elements (Berkhout et al. 1990; Southgate and Green 1991). Fusions of Tat with the cellular transcription factor c-*jun* indicated that a Tat/c-*jun* fusion was capable of activating gene expression when bound to an HIV-1 template containing AP-1 binding sites (Berkhout et al. 1990). Mutation of the AP-1 binding sites eliminated activation by the Tat/c-*jun* construct. Similar studies were performed by inserting multiple GAL-4 DNA binding sites in place of the NF-κB motifs in the context of an HIV-1 LTR (Southgate and Green 1991) construct with a mutated TAR element. Transfection experiments indicated that a fusion of Tat with the DNA binding domain of the yeast transcription factor GAL4 was able to activate HIV-1 gene expression (Southgate and Green 1991). Optimal activation by *tat* required the presence of functional SP1 binding sites and a TATA box but not an intact TAR element (Southgate and Green 1991). Mutations in critical functional domains of Tat eliminated this activation. These results indicated that Tat can function when bound to HIV-1 DNA templates in the absence of a functional TAR element.

Other heterologous fusions have been constructed in which Tat was fused to known RNA binding proteins (Selby and Peterlin 1990; Southgate et al. 1990). These included fusions of Tat with either the RNA binding domain of the *rev* protein (Southgate et al. 1990) or the RNA binding domain of the prokaryotic R17 phage coat protein (Selby and Peterlin 1990). In these experiments, the native TAR element was replaced with either the *rev* response element (RRE) (Southgate et al. 1990) or the RNA binding site of the R17 phage coat protein (Selby and Peterlin 1990). Transfection of these *tat* fusions with the appropriate reporter

gene demonstrated that each Tat fusion was able to activate HIV-1 gene expression when bound to its respective downstream RNA binding site (Selby and Peterlin 1990; Southgate et al. 1990). The Tat/R17 coat protein fusion activated its reporter construct even when the basic domain of Tat was deleted (Selby and Peterlin 1990). Since the basic domain of *tat* functions in binding to TAR RNA, this latter result established that the *tat* fusion bound through the R17 coat protein binding site. However, the level of activation by the R17 coat protein fusion with Tat was only 5–10% of the level obtained by wild-type *tat* activation of an HIV-1 LTR construct containing an intact TAR element (Selby and Peterlin 1990).

These studies with heterologous Tat fusions suggested that they could activate gene expression when bound to either a DNA or an RNA binding site. Similar results have been obtained with the viral transactivator VP-16. VP-16 has also been demonstrated to activate HIV-1 gene expression as a heterologous fusion with either GAL-4 or *rev*. These VP-16 fusions will activate the gene expression of HIV-1 LTR CAT reporter constructs that contain either the DNA (Southgate and Green 1991) or RNA (Tiley et al. 1992) binding sites for the respective heterologous *tat* fusion construct. Since VP-16 has been demonstrated to bind to the general transcription factors such as the TATA-binding protein (TBP) (Stringer et al. 1990), it was suggested that both VP16 and *tat* likely targeted components of the basal transcription complex to activate gene expression (Tiley et al. 1992). It is unclear whether both *tat* and VP16 target the same cellular factors present in the transcription complex.

7 Tat Binding to TAR RNA

A number of studies have indicated that the TAR element was the major target for *tat* activation (Rosen et al. 1985; Feng and Holland 1988; Hauber and Cullen 1988; Jakobovits et al. 1988; Berkhout and Jeang 1989; Garcia et al. 1989; Selby et al. 1989; Roy et al. 1990b). Since computer modeling demonstrated that TAR RNA was capable of forming a stable RNA secondary structure (Muesing et al. 1987; Feng and Holland 1988), mutagenesis was performed to elucidate whether TAR RNA secondary structure was required for *tat* activation. A variety of mutant TAR elements were constructed in the context of HIV-1 LTR CAT constructs. Co-transfection of these constructs with the *tat* gene indicated that several structural features of TAR were critical for *tat* activation (Rosen et al. 1985; Feng and Holland 1988; Hauber and Cullen 1988; Jakobovits et al. 1988; Berkhout and Jeang 1989; Garcia et al. 1989; Selby et al. 1989; Roy et al. 1990b).

One major element required for *tat* activation was the preservation of the TAR RNA stem structure. Mutations in the lower portion of the TAR RNA that disrupted stem base pairing resulted in decreased levels of *tat* activation (Hauber and Cullen 1988; Jakobovits et al. 1988; Garcia et al. 1989; Selby et al. 1989; Roy et al. 1990b). For instance, several different mutations between +1 and +18 and +44 and +60 resulted in only two- to threefold decreases in *tat* activation.

However, mutations in the upper portion of the stem structure, between +18 and +43, that disrupted stem base pairing resulted in dramatic decreases in *tat* activation (Feng and Holland 1988; Hauber and Cullen 1988; Jakobovits et al. 1988; Garcia et al. 1989; Selby et al. 1989; Roy et al. 1990b). Compensatory mutations that restored stem base pairing in either the upper or lower portion of the TAR RNA stem were able to nearly restore the degree of *tat* activation.

The upper portion of TAR RNA between +18 and +43 contained two other important regulatory elements. One structure that was important for *tat* activation was the three-nucleotide bulge between positions +23 and +25 (Berkhout and Jeang 1989; Dingwall et al. 1990; Roy et al. 1990a,b; Calnan et al. 1991a). For instance, mutation of the uridine residue at position +23 in the bulge resulted in marked decreases in *tat* activation (Berkhout and Jeang 1989; Cordingley et al. 1990; Dingwall et al. 1990; Roy et al. 1990a,b; Calnan et al. 1991a). The six-nucleotide loop between positions +30 and +35 was also critical for *tat* activation (Feng and Holland 1988; Berkhout and Jeang 1989; Garcia et al. 1989; Roy et al. 1990a,b; Wu et al. 1991). Mutation of individual nucleotides in this region resulted in decreases in *tat* activation, while changes in multiple nucleotides in the loop region between +31 and +34 eliminated *tat* activation (Feng and Holland 1988; Berkhout and Jeang 1989; Garcia et al. 1989; Roy et al. 1990a,b; Wu et al. 1991). The positions of the TAR RNA loop and bulge regions are shown in Fig. 4. Though a number of HIV-1 LTR DNA regulatory elements have been identified that modulate the level of HIV-1 gene expression (Garcia et al. 1987; Jones et al. 1988; Wu et al. 1988; Kato et al. 1991), the above results established a critical role for TAR RNA in mediating *tat* activation.

These studies suggested that *tat* activation may be the result of its binding to TAR RNA. To determine whether Tat bound directly to TAR RNA, RNA gel retardation analysis was performed to correlate the transactivating properties of *tat* with its ability to bind to TAR RNA. In vitro transcribed and ^{32}P-labeled TAR RNA was incubated with purified Tat protein, followed by polyacrylamide gel electrophoresis and autoradiography. Gel retardation experiments demonstrated

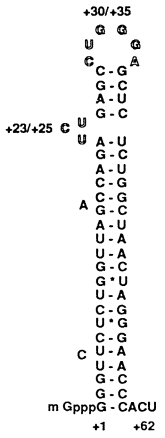

Fig. 4. The stem-loop structure of TAR RNA extending from +1 to +60. Two important regulatory elements, the 3-bp bulge between +23 and +25 and the 6-bp loop between +30 and +35, are indicated

that Tat bound specifically to TAR RNA (Dingwall et al. 1989; Cordingley et al. 1990; Dingwall et al. 1990; Weeks et al. 1990; Calnan et al. 1991a,b; Weeks and Crothers 1991; Wu et al. 1991). To determine the region of TAR RNA that bound Tat, binding to various TAR RNA mutants was performed, and the effects of various TAR RNA mutations on Tat binding were determined. This analysis indicated that deletion of the TAR RNA bulge eliminated Tat binding (Cordingley et al. 1990; Dingwall et al. 1990; Weeks et al. 1990; Calnan et al. 1991a,b; Weeks and Crothers 1991; Wu et al. 1991). In fact, mutation of the U residue at +23 in the bulge was sufficient to eliminate Tat binding (Cordingley et al. 1990; Dingwall et al. 1990; Weeks et al. 1990; Calnan et al. 1991a,b; Weeks and Crothers 1991; Wu et al. 1991). Mutation of individual groups of nucleotides in the loop had only small effects on Tat binding (Cordingley et al. 1990; Dingwall et al. 1990; Weeks et al. 1990; Calnan et al. 1991a,b; Weeks and Crothers 1991; Wu et al. 1991). Disruption of the upper portion of the TAR RNA stem structure between +18 and +43 also markedly decreased Tat binding, likely by destroying the integrity of the bulge structure. However, mutations of the lower portion of the TAR RNA stem structure did not markedly influence Tat binding. Though three portions of TAR RNA, including the upper portion of the stem, the bulge, and the loop, were all involved in influencing the degree of *tat* activation, only the preservation of the bulge structure in the context of an intact TAR RNA stem appeared critical for Tat binding.

It was also critical to determine which domains in the Tat protein were involved in its RNA binding. One domain of Tat, the basic domain, contains eight lysine and arginine residues (Cordingley et al. 1990; Dingwall et al. 1989, 1990; Weeks et al. 1990; Calnan et al. 1991a,b; Weeks and Crothers 1991). This is similar to a domain found in several prokaryotic RNA binding proteins, such as the lambda phage N protein (Lazinski et al. 1989; Roberts 1993). Mutations in the basic domain of Tat eliminated its ability to bind to TAR RNA in gel retardation assays (Dingwall et al. 1989; Cordingley et al. 1990; Dingwall et al. 1990; Weeks et al. 1990; Calnan et al. 1991a,b; Weeks and Crothers 1991; Wu et al. 1991). *Tat* proteins with mutations in other domains, including the amino terminus or the cysteine-rich region, were still able to bind efficiently to TAR RNA. To further explore the binding properties of the Tat basic domain, a variety of small peptides corresponding to the basic domain of Tat were tested in gel retardation analysis with TAR RNA (Cordingley et al. 1990; Weeks et al. 1990; Calnan et al. 1991a,b; Weeks and Crothers 1991). Peptides containing eight arginine residues, but not a similar arrangement of lysine residues, bound specifically to TAR RNA (Calnan et al. 1991b). The position of an arginine residue at amino acid 52 or 53, surrounded by three or four basic amino acids, was required for specific binding of Tat peptides to TAR RNA (Calnan et al. 1991b). The binding properties of a number of peptides corresponding to the basic domain with TAR RNA were then tested using gel retardation analysis. The ability of these Tat peptides correlated with the ability of these amino acid sequences to bind to TAR RNA when placed in the context of full-length Tat proteins to transactivate HIV-1 gene expression (Calnan et al. 1991b).

Tat peptides have also been used to more clearly define the regions of TAR RNA that were critical in Tat binding. These studies mapped the critical sequences for Tat binding to nucleotides extending from +20 to +27 in TAR RNA (Calnan et al. 1991b; Weeks and Crothers 1991). Again, the uridine residue at +23 in the bulge region was found to be essential for Tat binding (Calnan et al. 1991b; Weeks and Crothers 1991). The bulge region was thought to alter TAR RNA structure in a manner that allowed the RNA major groove to be accessible for Tat binding. Ethylation interference and molecular modeling demonstrated that the arginine at positions 52 and 53 of Tat interacted by hydrogen bonding with phosphates surrounding the adenine residue at +22 in the bulge. This model of Tat binding was referred to as "the arginine fork" (Calnan et al. 1991b; Puglisi et al. 1992). Though these studies pointed to an important interaction of Tat with TAR, the lack of stringent specificity of this interaction suggested that *tat* activation involved subsequent interactions with cellular transcription factors.

8 Cellular Factors Bind to TAR RNA

The studies reviewed in the previous section indicated that Tat binding to the TAR RNA bulge was critical in regulating HIV-1 gene expression. Though the loop sequences have been demonstrated to be important for *tat* activation, mutation of these sequences did not markedly alter Tat binding. This suggested that the loop sequences may be involved in the binding of cellular proteins. Though the major function of the stem secondary structure was likely to preserve the loop and the bulge elements, this element could also serve as the binding site for cellular factors. A number of studies have been performed to identify cellular factors that bound to the TAR RNA and may be involved in regulating HIV-1 gene expression (Gaynor et al. 1989; Marciniak et al. 1990b; Gatignol et al. 1991; Sheline et al. 1991; Wu et al. 1991).

To identify cellular factors that bound to TAR RNA, gel retardation using 32p-labeled TAR RNA was performed as previously described for Tat-binding studies. HeLa nuclear extract was fractionated using column chromatography, and these fractions were subsequently assayed by gel retardation analysis with TAR RNA. To determine the specificity of cellular protein binding to TAR RNA, competition analysis was performed with a variety of unlabeled wild-type and mutated TAR RNAs. Using these techniques, a 185-kDa protein designated TRP-185 (TAR RNA binding protein-185) or TRP-1 was identified (Sheline et al. 1991; Wu et al. 1991). TRP-185 bound with marked specificity and high affinity to the TAR RNA loop sequences (Wu et al. 1991), and this required another set of proteins known as co-factors (Sheline et al. 1991; Wu et al. 1991). The mechanism by which these co-factors modulated TRP-185 binding to TAR RNA has not been determined. TRP-185 stimulated basal expression from the HIV-1 LTR in one study and was found to be synergistic with Tat in stimulating in vitro transcription from the HIV-1 LTR in another study (Sheline et al. 1991; Wu et al. 1991). Gel retardation analysis

was performed to determine whether both Tat and TRP-185 might form a complex with TAR RNA. This analysis demonstrated that TRP-185 and Tat excluded each other's binding to TAR RNA (Sheline et al. 1991; Wu et al. 1991), suggesting that Tat and TRP-185 might regulate different steps in a transcriptional pathway required to activate HIV-1 gene expression.

Other cellular factors have also been demonstrated to bind to TAR RNA (Marciniak et al. 1990b; Gatignol et al. 1991). A factor known as p68 was found to bind to the TAR RNA loop sequences (Marciniak et al. 1990b). The role of this factor in regulating HIV-1 gene expression remains to be determined. Another cellular factor designated TRP-2 was also identified by TAR RNA gel retardation analysis. TRP-2 had a binding specificity similar to that of Tat, in that it bound to the TAR RNA bulge sequences (Sheline et al. 1991). Whether TRP-2 is a negative regulator of *tat* function remains to be determined. Several stem-binding proteins, including one designated TRBP, have been demonstrated to bind to TAR RNA (Gatignol et al. 1991). Though the expression of TRBP stimulated HIV-1 gene expression in co-transfection assays, it was not clear whether this effect was due to transcriptional or to translational effects. These results indicate that one or more cellular factors are likely to be critical in modulating both the binding and transcriptional activating properties of Tat.

9 Mechanism of *tat*-mediated Transcriptional Activation

Early studies performed by co-transfection of *tat* and HIV-1 LTR reporter constructs were consistent with a model in which *tat* activated gene expression by increasing both the level and the efficiency of HIV-1 RNA translation (Cullen 1986; Wright et al. 1986; Tong et al. 1987; Berkhout et al. 1989). The effects of *tat* on translation of HIV-1 RNA were confirmed by microinjection studies of *tat* into *Xenopus* oocytes (Braddock et al. 1989). Though *tat* may somewhat increase the translation of HIV-1 RNA (Cullen 1986; Wright et al. 1986; Braddock et al. 1989), this effect does not appear to be the primary mode of *tat* activation (Garcia et al. 1987; Berkhout et al. 1989; Mann and Frankel 1991). Rather, the primary effects of *tat* appear to be at the level of transcription initiation and elongation.

A detailed analysis was performed to determine the mechanism by which *tat* altered HIV-1 RNA levels (Kao et al. 1987; Muesing et al. 1987; Rice and Mathews 1988; Laspia et al. 1989, 1990; Ratnasabapathy et al. 1990; Feinberg et al. 1991). Different probes were used to analyze changes in the amount of RNA present at increasing distances from the HIV-1 transcription initiation site in both the presence and the absence of *tat*. In the absence of *tat*, the predominant RNAs generated from the HIV-1 promoter were short transcripts which terminated at +60 (Kao et al. 1987; Braddock et al. 1989; Laspia et al. 1989, 1990; Ratnasabapathy et al. 1990; Feinberg et al. 1991). In the presence of *tat*, the level of short transcripts generated from the HIV-1 promoter decreased, while the level of elongated transcripts was found to increase (Kao et al. 1987; Laspia et al. 1989, 1990; Ratnasabapathy et al. 1990; Feinberg et al. 1991).

Mutagenesis of TAR indicated that a region between –5 and +82 was critical for the generation of short transcripts (RATNASABAPATHY et al. 1990). In fact, these short transcripts were also generated from heterologous promoter constructs that were fused to these latter HIV-1 sequences (RATNASABAPATHY et al. 1990). This element within TAR, designated the inducer of short transcripts, was found to activate the overall transcriptional rate of promoters to which it was fused. A further characterization of this region suggested that a DNA element, between –5 and +26, which bound cellular transcription factors likely was critical for the generation of short transcripts (SHELDON et al. 1993). The fact that mutation of the loop sequences did not alter the generation of short transcripts indicated that the presence of short transcripts was not sufficient for *tat* activation (RATNASABAPATHY et al. 1990; SHELDON et al. 1993). However, it is possible that the process which generates short transcripts identifies a transcriptional pathway which is important in *tat* activation.

The role of *tat* as a potential anti-terminator of transcription has been analyzed by nuclear run-on experiments. HIV-1 LTR constructs containing the SV40 origin were transfected into COS cells in both the presence and the absence of *tat* and the HIV-1 RNAs transcribed were analyzed (KAO et al. 1987; LASPIA et al. 1989). *Tat* was found to have no effects on promoter-proximal transcription, but it markedly increased promoter distal transcription (KAO et al. 1987; LASPIA et al. 1989). These results suggested that *tat* acted as an anti-terminator to elongate transcripts that usually terminated at +60 in TAR RNA. However, attempts to identify a distinct terminator sequence in TAR were unsuccessful (KAO et al. 1987; LASPIA et al. 1989). In another series of experiments, an adenovirus vector lacking E1A but containing an HIV-1 LTR CAT construct was used to infect HeLa cells in either the presence or the absence of *tat* (LASPIA et al. 1989, 1990). Nuclear run-on experiments were performed to determine whether *tat* altered the level of RNA transcribed from the HIV-1 promoter. In the absence of *tat*, the majority of transcripts hybridized primarily to probes derived from regions located within 60 nucleotides of the HIV-1 transcription initiation site, but not to probes located several hundred nucleotides downstream (LASPIA et al. 1989). In the presence of *tat*, there was an increase in the level of HIV-1 transcripts in both the promoter proximal and downstream regions (LASPIA et al. 1989, 1990). The effects of *tat* were more pronounced on increasing the levels of long RNAs than an increasing those of short RNAs. The role of *tat* on transcriptional activation was compared with that of the viral transactivator E1A, which is also capable of activating HIV-1 gene expression. In contrast to *tat*, the primary effect of E1A was on transcriptional initiation rather than on elongation (LASPIA et al. 1990). Thus, *tat* appears to be unique among viral transactivators, in that its primary effect is on stimulating transcriptional elongation rather than transcriptional initiation.

To confirm the results found with transfection of *tat* and HIV-1 LTR CAT reporter constructs, nuclear run-on studies were also performed with HIV-1 proviral constructs (FEINBERG et al. 1991). HIV-1 proviruses which deleted the *tat* gene were constructed. A T-lymphocyte cell line containing this provirus was isolated. Addition of recombinant Tat protein to cells containing this HIV-1 proviral construct increased the RNA levels at promoter-distal but not promoter-proximal

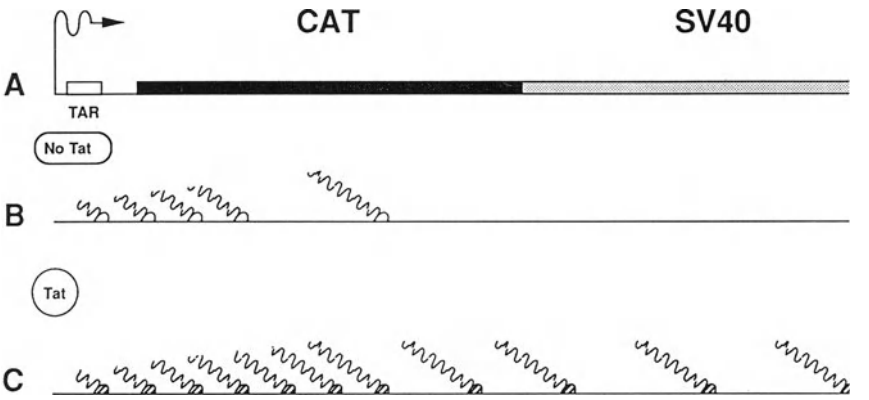

Fig. 5A–C. Tat alters transcriptional elongation. **A** The role of *tat* has been tested on reporter constructs containing HIV-1 sequences between +1 and +80 fused to the chloramphenicol acetytransferase gene and SV40 polyedenylation sequences. **B** In the absence of *tat* there is poor extension of elongating RNA polymerase molecules down the DNA template, resulting in only promoter-proximal transcripts. **C** In the presence of *tat* the number of RNA polymerase molecules near the transcription initiation site is similar to that in the absence of *tat*. However, a greater percentage of these molecules are now able to more efficiently elongate down the DNA template

sites (Feinberg et al. 1991). This result was in agreement with transient assay experiments indicating that the primary effect of Tat was to stimulate transcriptional elongation, as shown in Fig. 5. Heterologous fusions of TAR with the U2 small nuclear RNA promoter (U2 snRNA) were also constructed to investigate the role of *tat* on transcriptional elongation (Ratnasabapathy et al. 1990). The TAR element was inserted between the U2 promoter and sequences which terminate RNA polymerase II transcripts (Ratnasabapathy et al. 1990). Transfection of this construct in the absence of *tat* resulted in the generation of short transcripts, with termination occurring at the U2 terminator sequences. However, in the presence of *tat*, there was a decrease in the number of short transcripts and extension of these transcripts past the U2 terminator sequences (Ratnasabapathy et al. 1990). Though no discrete transcriptional terminators have been identified in TAR, these results suggested that *tat* either directly or indirectly modifies the activity of the transcriptional elongation complex to allow RNA polymerase II to elongate through distinct terminator sequences.

10 Role of Tat in In Vitro Transcriptional Stimulation

To determine the biochemical pathways by which Tat stimulated transcriptional elongation, it was important to develop in vitro transcription assays to test its function. Using such assays (Dignam et al. 1983), biochemical fractionation allowed the identification of the TATA-binding factor (TBP) and TATA-associated cellular factors (TAFs), in addition to the general transcription factors TFIIB, TFIIE, TFIIA, TFIIH, and TFIIF (Reinberg and Roeder 1987; Buratowski et al. 1989;

Mitchell and Tjian 1989). The development of an in vitro transcription assay that reflected Tat activation function was difficult because there was a high basal level of HIV-1 LTR gene expression using nuclear extract alone. Given this high level of transcription, it was difficult to further stimulate in vitro transcription of the HIV-1 LTR in the presence of Tat. However, it was demonstrated that preincubation of nuclear extract with the template for 30 min prior to the addition of labeled ribonucleotides to in vitro transcription assays with the HIV-1 LTR dramatically lowered basal gene expression (Marciniak et al. 1990a; Marciniak and Sharp 1991). Under these conditions, the addition of bacterial-produced Tat protein stimulated in vitro transcription from the HIV-1 LTR approximately tenfold as compared with assays that contained mutant Tat proteins (Marciniak et al. 1990a; Marciniak and Sharp 1991). Furthermore, HIV-1 LTR templates mutated in either the TAR RNA bulge or loop regions were not stimulated by the addition of recombinant Tat protein (Marciniak et al. 1990a; Marciniak and Sharp 1991). Also, mutant Tat proteins did not increase the level of HIV-1 gene expression. This stimulation by Tat involved an increase in the number of transcripts that extended greater than 500 bp from the promoter, but it did not increase the number of transcripts that extended short distances (less than 60 nucleotides) from the HIV-1 transcription initiation site (Marciniak and Sharp 1991).

Another in vitro transcription assay was also used to analyze the effects of Tat on HIV-1 LTR gene expression (Kato et al. 1992). No preincubation of nuclear extract with the HIV-1 LTR template was necessary, because sodium citrate, included in the reaction assay, decreased the high basal level of HIV-1 gene expression. When Tat was included in this in vitro transcription assay, it again increased the number of long run-off transcripts from the HIV-1 LTR promoter but not the number of promoter proximal transcripts (Kato et al. 1992). Mutations of critical domains in either Tat or the TAR RNA loop or bulge regions eliminated in vitro stimulation of HIV-1 transcription. To determine whether *tat* stimulated HIV-1 transcriptional elongation by modifying the activity of either of two previously described cellular elongation factors, TFIIS or TFIIF, these purified factors and Tat were tested (Spencer and Groudine 1990). The addition of TFIIS to in vitro transcription assays with the HIV-1 LTR stimulated the level of elongated transcripts in both the presence and the absence of Tat, probably by releasing paused RNA polymerase complexes from the HIV-1 promoter. TFIIF, which is comprised of 30- and 74-kDa subunits, has previously been demonstrated to stimulate both transcriptional initiation and elongation (Sopta el al. 1985). TFIIF increased the level of in vitro transcription from the HIV-1 LTR, but the addition of Tat did not further increase the level of elongated transcripts (Kato et al. 1992). To determine whether the effects of Tat were exerted through modulation of TFIIF activity, antisera to the 74-kDa subunit of TFIIF was added to the in vitro transcription assay. TFIIF antibody suppressed Tat activation of the HIV-1 LTR but did not alter basal gene expression (Kato et al. 1992). This suggested that Tat-mediated transcriptional activation was itself mediated either directly or indirectly by effects on TFIIF activity.

In vitro transcription assays allowed a study of the kinetics of Tat function. In the absence of Tat, only 2% of the transcription complexes that reached 82

nucleotides from the HIV-1 transcription initiation site were able to extend 1495 nucleotides downstream of the promoter (Marciniak and Sharp 1991). Transcripts that extended to +1495 proceeded through the entire plasmid template (Marciniak and Sharp 1991). However, in the presence of Tat, 20% of the transcription complexes that transcribed past +82 continued past +1495. *Tat* did not alter the level of transcripts between the transcription initiation site and +82, nor did it alter the degradation of HIV-1 RNA. The effects of the purine nucleoside analogue DRB on basal and *tat* activation were also tested (Marciniak and Sharp 1991). DRB, which had previously been demonstrated to inhibit RNA polymerase II elongation of a variety of viral and cellular promoters, was found to markedly inhibit Tat-induced effects on transcriptional elongation but not basal HIV-1 gene expression (Marciniak and Sharp 1991). These studies were consistent with a model in which basal expression from the HIV-1 promoter generated a majority of poorly processive transcription complexes, which naturally terminated at short distances downstream of the HIV-1 transcription initiation site. *Tat* probably stimulated the properties of a type of transcription complex that was capable of a greater degree of transcriptional elongation. Given our lack of knowledge concerning the cellular factors involved in transcriptional elongation, it is likely that a variety of cellular factors in addition to TFIIS and TFIIF will be identified that are important in regulating *tat* effects on transcriptional elongation.

11 Identification of Tat-associated Proteins

It is likely that Tat functions by direct interactions with specific cellular factors that mediate the assembly and activity of the transcriptional complex on the HIV-1 promoter. Thus, it will be important to identify cellular proteins that directly interact with Tat and to determine how they function to activate HIV-1 gene expression. A number of approaches have been used to identify cellular factors that modulate *tat* function. *tat* efficiently transactivates expression from the HIV-1 LTR in human but not murine cells. This result was consistent with a role for specific human cellular factors which were required for *tat* activation (Hart et al. 1989; Newstein et al. 1990; Alonso et al. 1992). In an attempt to identify these factors, mouse-human somatic cell hybrids were constructed. Mouse-human somatic cell fusions that contained human chromosome 12, but not other human chromosomes, gave high levels of HIV-1 gene expression in the presence of *tat* (Hart et al. 1989; Newstein et al. 1990; Alonso et al. 1991). This suggested that human chromosome 12 encoded a cellular factor or factors that were involved in *tat* activation. By studying a variety of HIV-1 LTR regulatory region mutants, it was demonstrated that the gene product encoded by chromosome 12 was likely to be involved in TAR element function (Alonso et al. 1992). Whether this factor or factors directly interacted with *tat* or potentially bound to TAR RNA was not determined. Somatic cell genetics may provide a method for potentially identifying cellular proteins that are involved in *tat* function.

Techniques such as far Western analysis have been used to determine whether Tat could bind directly to cellular regulatory factors. Since other viral transactivator proteins such as E1A and VP-16 have been demonstrated to directly interact with general transcription factors, like TBP (Stringer et al. 1990), it seemed likely that *tat* might also bind to cellular transcription factors. Labeled Tat protein has been used to screen lambda gt 11 cDNA expression library (Nelbock et al. 1990). A cellular factor, TBP-1 or *tat*-binding protein, was identified by this screening technique (Nelbock et al. 1990). TBP-1 was found to be a nuclear protein and its expression was highest in T lymphocytes (Nelbock et al. 1990). Though TBP-1 was initially reported to suppress *tat* activation, recent studies indicated that the full-length TBP-1 protein is an activator of HIV-1 gene expression (Nelbock et al. 1990; Ohana et al. 1993). A number of other clones have been isolated from human cDNA libraries which are very homologous to TBP-1 (Shibuya et al. 1992; Swaffield et al. 1992; Ohana et al. 1993). Studies of yeast cell-cycle growth control resulted in the isolation of a human gene *MSS1* which complemented the yeast mutant *sgv1* (Shibuya et al. 1992). The *MSS1* gene product has 42% sequence identity with TBP-1 and enhanced *tat*-mediated activation of HIV-1 in co-transfection assays (Shibuya et al. 1992). Another yeast regulatory gene, *Sug 1*, was identified and found to have sequence homology with TBP-1 (Swaffield et al. 1992). These results suggest that TBP-1 is an evolutionary conserved gene which may be involved in *tat* activation. It will be important to determine whether Tat and TBP-1 or related proteins associate in HIV-1-infected cells to determine the relevance of this interaction in regulating HIV-1 gene expression. Finally, other approaches such as the yeast two-hybrid system or the use of Tat affinity columns to chromatograph nuclear extracts will be critical in identifying cellular factors that modulate *tat* activity.

12 Role of Tat in Activating Viral and Cellular Genes

A number of studies have demonstrated that the TAR element is critical for *tat* activation. However, *tat* can also activate gene expression from the HIV-1 promoter in the presence of a mutated TAR element in both stimulated T lymphocytes and astrocytes, suggesting that it can potentially interact with upstream DNA regulatory elements to activate gene expression (Harrich et al. 1990; Bagasra et al. 1992). This was demonstrated by the fact that mutations of the NF-κB motifs in the enhancer element eliminated TAR-independent activation by *tat*. Though this TAR-independent activation was not as efficient as *tat* activation in the presence of TAR, it raised the possibility that *tat* may potentially be able to activate gene expression from viral and cellular genes lacking TAR elements.

Several studies have explored whether *tat* is capable of activating gene expression from both viral and cellular promoters. Transfection experiments demonstrate that *tat* was able to activate expression of the murine cytomegalo-

virus promoter (Kim and Risser 1993). This activation of gene expression was manifested by both increases in both steady-state RNA and protein levels of the reporter gene. The target for *tat* activation was an upstream enhancer element that contained NF-κB- and SP1-binding sites (Kim and Risser 1993). *tat* has also been demonstrated to activate gene expression from the JC virus, a human papovavirus responsible for the demyelinating central nervous system PML (Tada et al. 1990). Since this disease is more frequent in AIDS patients, the mechanism of activation of JC virus gene expression was studied. *tat* was found to stimulate JC virus gene expression in glial cells but not in cells of non-neural origin (Tada et al. 1990). The steady-state level of JC-encoded RNA increased in the presence of *tat* (Tada et al. 1990). Though the target of *tat* activation in the JC virus promoter was not determined, the JC virus promoter does not contain an element with homology to TAR. These studies suggest that *tat* can alter the expression from other viruses, probably through interaction with upstream enhancer elements.

A number of studies have also been directed at determining whether *tat* is able to activate the expression of cellular genes. HIV-1 infection increases the levels of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6) (Buonaguro et al. 1992). The role of *tat* on the expression of these cytokine genes was studied. *Tat* was demonstrated to increase the levels of TNF gene expression using transient expression assays (Buonaguro et al. 1992). *Tat* also activated the expression of the endogenous TNF gene but not the expression of the endogenous IL-1 and IL-6 genes (Buonaguro et al. 1992). In addition to directly altering T-cell and monocyte cytokine production, *tat* has been found to increase the expression of specific cellular genes in glial-derived cells (Taylor et al. 1992). Stable glial cell lines expressing *tat* were found to have marked elevations in the steady-state RNA and protein levels of fibronectin and type-I and -II collagen. Transient expression assays confirmed that *tat* was capable of activating gene expression of the fibronectin and type-I collagen promoter (Taylor et al. 1992). This brings to mind the possibility that *tat* may play a role in the CNS dysfunction seen in HIV-1 infections.

Finally, *tat* has been found to modulate the growth properties of Kaposi's sarcoma (KS) cell lines (Ensoli et al. 1990). Supernatants from either HIV-1-infected cells, *tat*-transfected COS-1 cells, or recombinant bacterial Tat were found to stimulate the growth properties of KS cells (Ensoli et al. 1990). These results would be consistent with a model in which extracellular Tat could be internalized and alter cellular growth properties by regulating the expression of specific cellular genes. Further studies to determine the viral and cellular promoter elements that mediate *tat* activation will shed light on cellular genes that are potential targets for *tat* activation.

13 Potential Therapeutic Implications of *tat* Function

Since *tat* is critical for the regulation of the HIV life cycle, inhibition of its function offers potential therapeutic possibilities in the treatment of HIV-1 infection. The use of multimerized TAR RNA decoys (Sullenger et al. 1990), the creation of transdominant Tat proteins (Pearson et al. 1990; Modesti et al. 1991), and the development of chemical inhibitors (Hsu et al. 1991) that prevent Tat binding to TAR RNA or interaction with associated cellular factors seem potentially useful. Since the presence of the TAR element is required for high-level Tat activation, attempts have been made to titrate cellular factors necessary for *tat* function and/or *tat* itself by overexpression of TAR RNA sequences. Retroviral vectors that produce multiple copies of TAR RNA have been used to infect lymphoid cell lines (Sullenger et al. 1990). Cell lines producing these so-called TAR RNA decoys were resistant to HIV-1 replication as compared with control cell lines. Cell lines that contained TAR RNAs with mutations in the loop sequence or that disrupted the stem structure were susceptible to HIV-1 replication. These results suggested that the overproduction of TAR RNA resulted in the removal of cellular factors required for HIV-1 gene expression, or which directly bound newly synthesized Tat. Such an approach could provide a means of inhibiting HIV-1 gene expression.

Studies of the HIV Rev protein have demonstrated that so-called dominant negative or transdominant Rev proteins inhibited wild-type Rev function (Malim et al. 1989). This suggested that transdominant *rev* mutants could be used to construct stable cell lines that would be resistant to HIV-1 replication. This methodology has been called "intracellular immunization" (Malim et al. 1989). CEM cells which stably expressed a *rev* transdominant mutant have been demonstrated to be resistant to HIV-1 replication (Malim et al. 1992). Transdominant *tat* proteins have also been constructed. These mutants contained deletions or substitutions in the Tat basic domain (Pearson et al. 1990; Modesti et al. 1991). Several of these mutants were capable of inhibiting wild-type *tat* activation of the HIV-1 LTR when present in equimolar concentrations with wild-type *tat*. Though the mechanism by which these mutants inhibited wild-type *tat* function is not known, interaction with a common cellular factor required for *tat* activation seems likely. The creation of lymphoid cell lines containing these transdominant *tat* mutants will be required to determine their efficacy in inhibiting HIV-1 infection. The creation of stable cell lines containing both transdominant *tat* and *rev* genes could potentially provide protection from HIV-1 replication that will exceed the protection conferred by each protein individually.

There is also the intriguing possibility that anti-viral agents can be found that inhibit *tat* function. One agent known as R05-3335, a benzodiazepine derivative, has been reported to inhibit *tat*-mediated transactivation (Hsu et al. 1991). The mechanism by which R05-3335 inhibited *tat* activation has not been elucidated. However, our knowledge of *tat* function suggests that several properties of Tat could serve as targets of this agent. These include either alteration in the cysteine

residues of Tat, inhibition of the ability of the Tat basic domain to bind to TAR RNA, prevention of Tat transport to the nucleus, or alteration of Tat interactions with cellular regulatory factors. *Tat* is potentially a good target for novel drug therapy that would inhibit HIV-1 gene expression. Since Tat is a small protein and many mutations in the *tat* gene are deleterious to its function, it is unlikely that resistance to potential *tat* inhibitors would be as frequent as with currently used reverse transcriptase inhibitors.

14 Perspective

Over the past several years, an understanding of the cellular and viral factors that regulate HIV-1 gene expression has been a major research focus of many laboratories. The Tat protein is a critical regulator of HIV-1 gene expression and it may have effects on both transcriptional initiation and elongation. The mechanisms by which Tat exerts its action remain to be determined. It will be necessary to understand the cellular factors that are responsible for regulating both the transcriptional initiation and elongation properties of RNA polymerase II to identify potential cellular targets of Tat. However, it is likely that a study of Tat function itself will provide clues about the cellular factors involved in regulating transcriptional activation.

The role of Tat on transcriptional elongation is in some aspects similar to that of prokaryotic regulatory proteins that function as anti-terminators. The N protein is a small basic RNA binding protein produced by the bacteriophage which associates with a variety of *E. coli* host factors at a discrete RNA element known as a *nut* site (Horwitz et al. 1987; Lazinski et al. 1989; Roberts 1993). The *nut* site is a 25-bp RNA hairpin structure, and thus it has similarities to TAR RNA. The complex of N and associated *E. coli* host factors associates with the RNA polymerase and facilitates its elongation through distinct downstream terminator elements. *Tat* activation differs from activation by N in that there are no distinct terminator sequences in HIV-1 (Horwitz et al. 1987; Lazinski et al. 1989; Roberts 1993).

Gene expression of a variety of higher organisms is also regulated by effects on transcriptional elongation (Spencer and Groudine 1990). Studies of the *Drosophila* heat shock promoter (hsp70) indicate that its gene expression is regulated by the stimulation of paused transcriptional complexes near the promoter (Rougvie and Lis 1988). In the absence of heat shock, RNA polymerase synthesizes a paused RNA situated near the transcription initiation site. Following heat shock, this paused RNA polymerase is released and moves down the template. A number of mammalian genes such as *c-myc* and *c-fos* are also regulated by effects on transcriptional elongation (Spencer and Groudine 1990). Thus, *tat* may function by catalyzing a normal process which is important in the transcriptional activity of a variety of higher eukaryotic organisms. A postulated mechanism of *tat* activation is shown in Fig. 6.

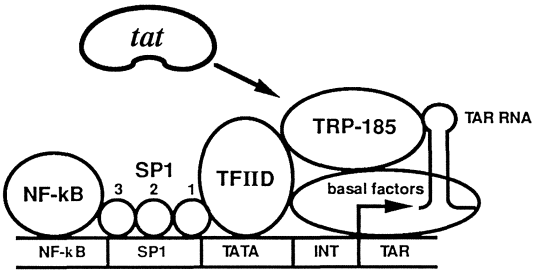

Fig. 6. Potential mechanism of *tat* activation. One model to explain *tat* activation involves the generation of a transcription complex composed of TFIID and basal transcription factors that is not able to efficiently elongate at marked distances from the HIV-1 promoter. The function of the TAR RNA-binding protein TRP-185 would be to facilitate the assembly of a transcription complex that is capable of interacting with *tat*. This complex would enhance the ability of RNA polymerase II, to elongate at prolonged distances from the HIV-1 promoter

The Tat protein function could be involved in coupling the initiation and elongation steps of transcription. It is likely that a study of Tat will increase our understanding of the host factors that assemble and regulate the transcriptional apparatus. Based on the recent identification of a number of different cellular factors that regulate transcriptional initiation and elongation, it is probable that significant progress will be made in determining the mechanisms by which Tat regulates HIV-1 gene expression. Such progress will be important for both furthering our scientific knowledge and developing novel approaches to inhibiting HIV-1 gene expression.

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HTLV-1 Oncoprotein Tax and Cellular Transcription Factors

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1 Introduction

Human T cell leukemia virus (HTLV) type 1 (POIESZ et al. 1980; YOSHIDA et al. 1982) has two regulatory genes, *tax* and *rex*, that control the viral gene expression and replication positively and negatively (SEIKI et al. 1983; INOUE et al. 1987; HIDAKA et al. 1988). In these regulations, *tax* activates transcription of the viral genome, whereas *rex* modulates the processing of the viral RNA expressing unspliced forms of the viral mRNA; therefore, both regulators are indispensable for efficient viral replication.

HTLV-1 is a causative agent of adult T cell leukemia (ATL) (UCHIYAMA et al. 1977) and a neurological disease of the spinal cord, HTLV-1-associated myelopathy or tropical spastic paraparesis (HAM/TSP) (GESSAIN et al. 1985; OSAME et al. 1986). Because of its activator function in transcription, *tax* has been proposed as a pathogenic factor for these diseases (YOSHIDA et al. 1984). Supporting this idea is the fact that *tax* stimulates expression of specific lymphokines, lymphokine receptors, and proto-oncogenes and, furthermore, immortalizes normal human T cells (GRASSMAN et al. 1989), transforms rodent

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fibroblasts (TANAKA et al. 1990), and induces mesenchymal tumors in transgenic mice (NERENBERG et al. 1987).

tax is an activator of transcriptional enhancer: a 21-bp enhancer of HTLV-1 is responsible for the activation of the viral LTR, NF-κB binding site for the IL-2 receptor α gene, and CArG box for the c-*fos* gene. However, these enhancers do not share any sequence homology; furthermore, *tax* does not bind to these enhancer DNAs. To explain these findings, several mechanisms have been proposed: (a) indirect binding of *tax* protein to enhancer DNA through interaction with DNA binding proteins, (b) activation of transcription factors by phosphorylation, (c) modulation of protein–protein interaction generating an active transcription factor, and (d) indirect activation of transcription factors by active oxygen as a second messenger. Recent studies have proposed that mechanisms a and c seem to be operating in HTLV-1-infected cells. The latest information on these two mechanisms is discussed in this chapter.

2 Activation of Enhancers by *tax*

The *tax trans*-activates transcription of the viral genome from the LTR (SODROSKI et al. 1984; FUJISAWA et al. 1985; FELBER et al. 1985) and thus is essential for viral replication (CHEN et al. 1985) responding to a transcription enhancer in the LTR, which consists of three direct repeats of a 21-bp sequence containing a cAMP-responsive element (CRE) (FUJISAWA et al. 1986; SHIMOTOHNO et al. 1986; see Fig. 1). In addition to the 21-bp enhancer, *tax* also activates the NF-κB binding site, which leads to the activation of the cellular genes for interleukin-2 (IL-2) receptor α (IL-2Rα) (INOUE et al. 1986; LEUNG and NABEL 1988; LOWENTHAL et al. 1988; MARUYAMA et al. 1987) and also activates the CArG box enhancer that activates proto-oncogenes c-*fos* and c-*egr* (FUJII et al. 1992; ALEXANDER et al. 1991; see Fig. 2). Many other genes are also known to be activated by *tax*; thus, it is possible that some other enhancers are also responsive to *tax*. In contrast, *tax trans*-suppresses the expression of DNA polymerase β (JEANG et al. 1988), which is involved in the repair synthesis of DNA, but no essential element for this suppression has been identified.

3 A General Mode of *tax* Action

Evidence for an indirect binding of *tax* to DNA has been obtained by DNA affinity precipitation (SUZUKI et al. 1993a; BERAUD et al. 1991). In DNA affinity precipitation assays, biotinylated DNA was used as a probe, and protein complexes formed on the DNA were isolated using streptavidin-conjugated beads. Using the 21-bp enhancer DNA, *tax* protein was co-precipitated with DNA in the presence of the nuclear extract of HTLV-1-infected cells, but not in the presence of the cytoplasm

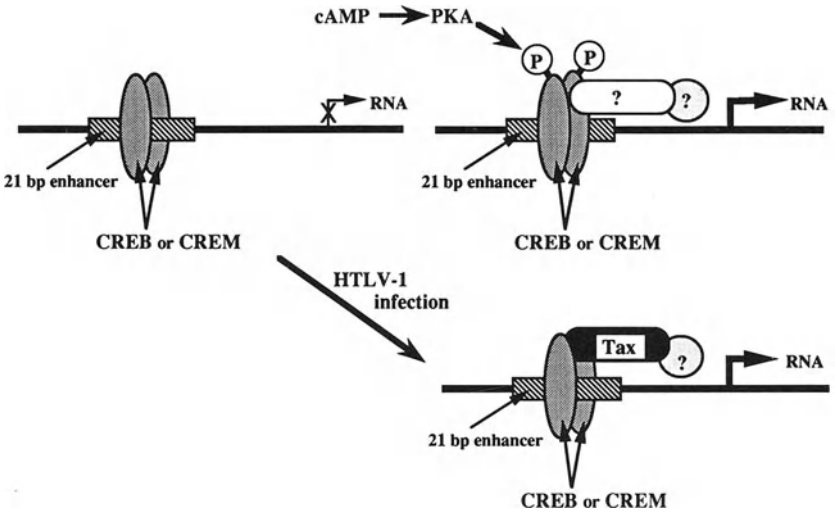

Fig. 1. *tax* binding to CREB protein and possible mechanism by which the complex *trans*-activates the transcription. *tax* binding to the CREB might bypass a cellular regulation by phosphorylation

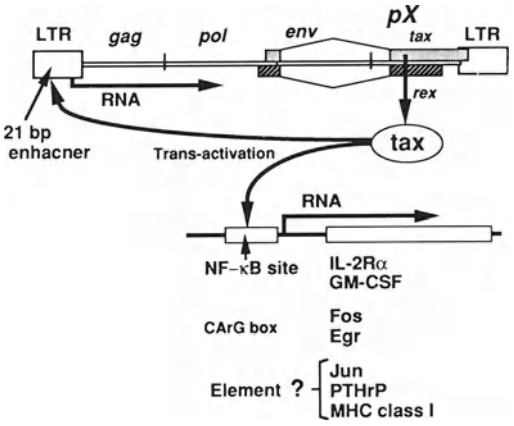

Fig. 2. *tax trans*-activation of the viral and various cellular genes. Typical enhancers for these transactivations are also indicated

(Suzuki et al. 1993a). These results indicate that *tax* can bind to DNA, and that its binding requires a nuclear factor(s). Furthermore, a mutant of the 21-bp enhancer which is inactive in activation of transcription did not bind to *tax* protein, clearly indicating specific formation of DNA-protein complexes and its biological significance in transcriptional activation.

Similarly, studies using DNA probes of the NF-κB binding sequence and of the CArG box sequence have shown that *tax* also binds to the corresponding enhancer DNAs, and its binding is dependent on the presence of the nuclear

extract of infected cells (SUZUKI et al. 1993b). Therefore, indirect binding of *tax* to enhancer DNA in the presence of nuclear factors is general on three unrelated enhancers and thus seems to be a common mechanism of *trans*-activation of transcription.

4 Specific Binding of *tax* to DNA-binding Proteins

tax protein binds to enhancer DNA in the presence of nuclear factors. Identification of this or these factors responsive to the transcriptional activation induced by *tax* would be of interest for understanding the mechanism of the *trans*-activation and also of transcriptional regulation in the target cells. Factors involved in each enhancer are different, as discussed in the following sections.

4.1 The HTLV-1 Enhancer

The 21-bp enhancer of HTLV-1 contains CRE sequences; thus, the CRE-binding proteins can bind to the 21-bp enhancer. These proteins include the CREB/ATF family, which belong to leucine-zipper proteins. Among these, *tax*-responsive element-binding proteins (TREBs) (YOSHIMURA et al. 1989), activating transcription factors (ATFs) (HAI et al. 1989), cyclic-AMP response element-binding protein (CREB) (GONZALEZ et al. 1989), cyclic-AMP response element modulator (CREM) (FOULKES et al. 1991), and the 21-bp binding proteins (HEBs) (SUZUKI et al. 1993b) have been shown to bind directly to the 21-bp enhancer.

The CREB and CREM proteins have been shown to bind to *tax* protein by gel shift assay (SUZUKI et al. 1993a; ZHAO and GIAM 1991) and also by DNA affinity precipitation assay (SUZUKI et al. 1993a; Fig. 3). Other proteins, such as TREB-5, TREB-7 (CRE-BP1), and TREB-36 (ATF-1), did not interact with *tax* indicating that the interaction was specific. The binding of *tax* to the CREB or CREM proteins can take place in the absence of the 21-bp DNA. Bacterially made proteins also interact with each other, suggesting that two proteins interact directly without any specific modification such as phosphorylation. Together, these findings indicate that *tax* binds to the CREB and/or CREM protein, and the complex then binds to the 21-bp enhancer to activate transcriptional initiation.

The *tax*-CREB complex was also detected in a nuclear extract of HTLV-1-infected cells, indicating that the complex is not an artifact formed by purified proteins at high concentration (SUZUKI et al. 1993a). Furthermore, depletion of CREB protein from a nuclear extract of HTLV-1-infected cells results in a drastic decrease in the level of *tax* binding to the 21-bp enhancer (SUZUKI et al. 1993a). Therefore, the *tax*-CREB complex is the greatest part of the *tax* that can bind to the 21-bp DNA in infected cells. Thus, this protein complex is expected to play a role in *trans*-activation of transcriptional enhancer.

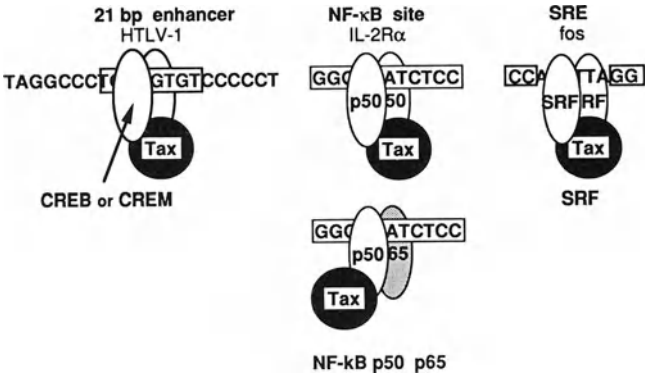

Fig. 3. Binding of *tax* to the enhancer-binding proteins CREB, CREM, NF-κB p50, and SRF. Indirect association of *tax* protein to the enhancer DNA through each enhancer-binding protein, or increase of the active NF-κB p50 through binding to the precursor seems to be the activation mechanism of *tax*

How does *tax* binding to CREB induce transcriptional activation ? The answer is not clear yet; however, it is possible to speculate: We have proposed another protein that interacts with *tax* on DNA: *gal*4-*tax* fusion protein has been found to activate the transcription by binding to the *gal*4 binding site, but it competes efficiently with the free from of *tax*, which does not affect the transcription of this reporter gene (FUJISAWA et al. 1991). This observation suggests that *tax* may interact with another molecule on the DNA for activation (Fig. 3). This factor could be another transcriptional regulator or a basic transcription factor. Alternatively, *tax* may stabilize the CREB-DNA complex as proposed by other group (ZHAO and GIAM 1991); however, stabilization of the complex does not seem sufficient to explain our competition experiment of *gal*4-*tax* fusion protein with free *tax* protein.

The *tax*-CREB complex formation may have significance in breaking the normal regulation of transcription. The CREB protein is known to mediate transcriptional activation by responding to cAMP signaling, which activates protein kinase A to phosphorylate various proteins including CREB itself (GONZALEZ et al. 1989). Phosphorylated and unphosphorylated CREB can bind equally to the CRE, but only phosphorylated CREB can activate specific transcription. On the other hand, *tax* binds to unphosphorylated CREB protein; thus, it is conceivable that *tax* binding to unphosphorylated CREB may bypass the normal regulation by phosphorylation with protein kinase A.

4.2 The NF-κB-binding Enhancer

The NF-κB regulates many genes including IL-2Rα, GM-CSF, TNFβ, and HIV LTR, and its binding sites are present in the regulatory region of these genes. *tax* activates these genes, probably through the NF-κB-binding sequence. The NF-κB protein consists of various binding proteins including NF-κB p50, p65, c-*rel* and

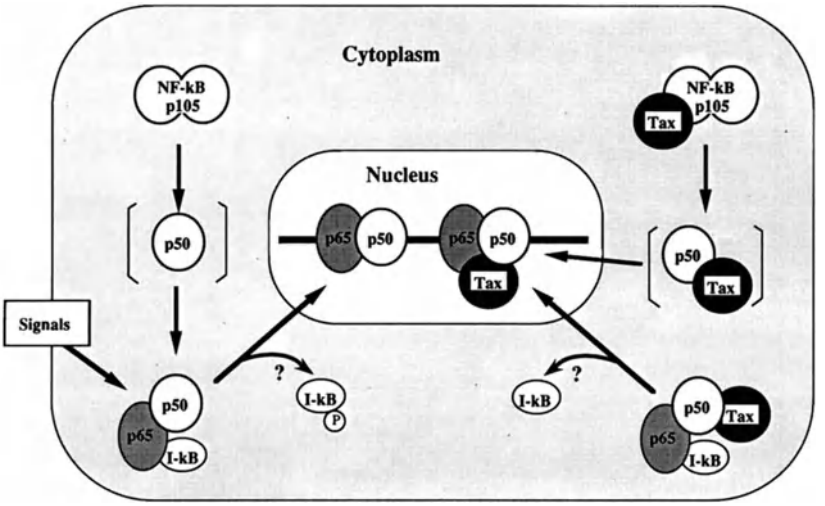

Fig. 4. Interaction of *tax* with NF-κB p105 and p50 and its effect on induction of nuclear translocation of p50 and p65. *Right*: uninfected cells; *left*: HTLV-1-infected cells

*lyt*10 (SCHMITZ et al. 1991). Data in our laboratory have revealed that NF-κB p50 can bind to *tax* and mediate the *tax* binding to DNA (SUZUKI et al. 1993b) (Fig. 4). Therefore, it is reasonable to postulate that *tax* can bind to p50–p50 homodimer and p50–p65 heterodimer and can bind to DNA. The homodimer p50–p50 is rather thought to suppress the gene expression; thus, it is possible either that *tax* binding to the homodimer switches its function into activation, or that the heterodimer p50–p65 plays a major role in *trans*-activation by *tax* protein. It would be of great interest to know whether other members of the NF-κB family are involved in *tax* interaction or not.

4.3 Serum Response Element

tax also binds to the CArG box sequence of the c-*fos* and c-*egr* genes in the presence of a nuclear factor, as discussed in the previous section. It was found that a CArG box-binding factor $p67^{SRF}$ (serum response element-binding protein) mediates *tax* binding to the specific DNA (FUJII et al. 1992; SUZUKI et al. 1993b). Biologically inactive mutants of *tax* did not bind to the $p67^{SRF}$ (SUZUKI et al. 1993b), indicating that the complex formation is important for *trans*-activation (Fig. 3).

4.4 TRE-2

tax Trans-activation requires at least two copies of the 21-bp enhancer. However, another sequence adjacent to the 21-bp sequence in the LTR was identified to enhance the *trans*-activation of one copy of the 21-bp sequence (BRADY et al.

1987). This sequence is called *tax*-responsive element 2 (TRE-2), but it alone did not respond to *tax* and was inactive as an enhancer, even in its repeated form. A protein of 36kDa which binds to TRE-2 was isolated and reported to bind to *tax* (Marriott et al. 1990). However, no further information is available about the nature of this protein or its significance in *trans*-activation. The TRE-2 sequence contains DNA motifs related to the binding sequences for the NF-κB and also for the oncogene product Ets (Bosselut et al. 1990), but our results indicated that these two elements are not required for the *trans*-activation by *tax* protein.

We have recently identified a GLI binding site in the TRE sequence and two cDNA clones that code for proteins that bind to this site: THP-1 and -2 (Tanimura et al. 1993). The THP proteins contain five zinc finger motifs and appeared to be a member of the oncogene GLI family that was amplified in glyoma cells (Kinzler et al. 1988). Preliminary data indicate that *tax* also interacts with THP proteins; therefore, it seems that *tax* binding to two unrelated proteins, CREB and THP, on two unrelated DNA sequences can activate the transcription and mimic the direct repeats of the 21-bp enhancer. The interaction of these two proteins, CREB and THP, would be of interest for understanding the *tax* activation of the LTR and the significance of repeats of the enhancers.

The interaction of *tax* with the 21-bp sequence, the NF-κB enhancer, the CArG box sequence,and TRE-2 through their respective binding proteins seems to be a general mechanism for the activation of transcription by *tax*.

5 *tax* Also Binds to Cytoplasmic Proteins

Independent of the enhancer DNA-associated complexes, we have been studying proteins that bind to *tax*. Using anti-*tax* antibodies, the NF-κB precursor p105 has been co-immunoprecipitated with *tax* (Hirai et al. 1992). The ability of *tax* to bind to the p105 correlates well with the functional activities of *tax* mutants, indicating its significance in *trans*-activation of the NF-κB enhancer. However, p105 and *tax*-p105 complex do not bind to DNA, suggesting that there is another mechanism in addition to DNA binding.

The p105 is a precursor of NF-κB p50 and is localized in the cytoplasm (Ghosh et al. 1990; Kieran et al. 1990). The p50 newly formed by proteolytic cleavage is complexed with I-κB, forming a ternary complex p50/p65/I-κB kept in the cytoplasm. Signals for cellular proliferation or differentiation phosphorylate the I-κB and dissociate the complex, allowing translocation of p50/p65 heterodimer into nucleus (Ghosh and Baltimore 1990; Baeuerle and Baltimore 1988). Recently, it has been proposed that the precursor p105 functions similarly to I-κB and complexes to the NF-κB p50/p65 in the cytoplasm (Rice et al. 1992). Therefore, it is possible that *tax* binding to p105 may dissociate the ternary complexes p105/p50/p65, releasing active p50/p65 dimer and allowing its nuclear translocation. Such *tax*-dependent nuclear translocation was in fact demonstrated by analyzing localization of p65 in the cytoplasm and nucleus. Such an effect of the *tax* binding might be also explained by the *tax* binding to p50, either newly formed or pre-

existing in the I-κB/p50/p65 complexes. If the ankyrin motif in p105 is a site for *tax* binding, *tax* may also interact with I-κB to dissociate the ternary complex. This possibility is under investigation

tax now appears to function by two different mechanisms: (a) *tax* in the nucleus binds to the NF-κB p50 and to DNA, and (b) *tax* in the cytoplasm binds to the NF-κB p50 precursor and/or to other related proteins and induces nuclear translocation of active p50/p65.

6 Functional Domains of *tax* Protein

At the present time, *tax* is known to interact with at least six proteins (CREB, CREM, NF-κB p105, NF-κB p50, SRF, and 36 KDa TRE-2-binding protein). Certainly, more target proteins will be found. No homologous sequences common to all of these proteins have been identified, thus suggesting that more than two domains of *tax* may be involved in interactions with these proteins. However, the functional domains of *tax* protein are not well defined.

Our mutant d3, which has a deletion of three amino acids at the N-terminus, does not interact with the 21-bp sequence but is almost fully active at the NF-κB site (Hirai et al. 1992), supporting the idea that two different domains of *tax* are used for activation of the 21-bp and NF-κB enhancers. A similar dissociation of the 21-bp sequence and NF-κB activation sites has been reported for two other mutants, M22 and M47 (Smith and Greene 1990). M22 has a mutation at the N-terminal region and is active at the 21-bp sequence but not at the NF-κB site. Conversely M47, which has a mutation in the middle region, is inactive at the 21-bp sequence but active at the NF-κB site.

A cluster of acidic amino acids of enhancer activators, yeast Gal4 and herpes virus VP16, has been identified as an activation domain (Ptashne 1988). Similar to these activators, the C-terminal region of *tax* contains a cluster of acidic amino acids. Deletion of the 69 amino acids that constitute this region inactivates *tax trans*-activation (Smith and Greene 1990) and supports the notion that the acidic domain activates transcription in a similar way. However, many internal deletion mutants of *tax* that retain the C-terminus are inactive even when fused to the Gal4 DNA-binding domain. Furthermore, a fusion protein of the C-terminus acidic domain with Gal4 is also inactive (M. Yoshida, unpublished data); thus, it has been strongly suggested that *tax* functions by just not providing an acidic domain, thus somehow differently from the VP16 or Gal4.

We propose here two independent mechanisms for *tax trans*-activation of transcription: *tax* binds to enhancer-binding proteins in the nucleus and to enhancer DNA, or *tax* binds to NF-κB p50 and its precursor p105 in the cytoplasm and induces nuclear translocation of active transcription factors. These properties in binding to positive regulators are in contrast to oncoproteins of DNA tumor viruses, T antigen of SV40, E6 and E7 of papillomaviruses, and E1A of adenoviruses, which are bound to tumor-suppressor proteins, Rb and p53 (Whyte et al. 1988; DeCaprio et al. 1988; Dyson et al. 1989). Therefore, it is proposed that

tax functions in a different mode, which, when elucidated, will provide new insights into the molecular mechanisms of transcriptional regulation and tumor development.

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The Human Immunodeficiency Virus Type 1 Rev Protein: A Pivotal Protein in the Viral Life Cycle

T. Hope[1] and R.J. Pomerantz[2]

1 Introduction

The human immunodeficiency virus type 1 (HIV-1) and related lentiviruses have more complex genomes than typical retroviruses (Cullen 1991). HIV-1 expresses at least nine different genes in a temporally regulated manner (Kim et al. 1989). In addition to the *gag*, *pol*, and *env* genes common to all retroviruses, HIV-1 also encodes genes for *tat*, *rev*, *nef*, *vif*, *vpu*, and *vpr* (Rosenblat et al., this volume). To encode nine different genes in a small, approximately 9-kb genome, the virus employs alternative reading frames and complex patterns of RNA splicing (Gallo et al. 1988; Schwartz et al. 1990a). The HIV-1 protein Rev (regulator of expression of the virion) plays an essential role in the temporal regulation of virus gene expression during a replication cycle (Kim et al. 1989; Pomerantz et al. 1990). The genes expressed by HIV-1 can be separated into two distinct groups based on whether their expression is Rev-dependent or not (Schwartz et al. 1990b; Hammerskjöld et al. 1989; Malim et al. 1989; Garrett et al. 1991). The Rev-independent or early genes encode Tat, Rev, and Nef. The Rev-dependent or late

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genes are important for virion production and encode the structural proteins Gag, Pol, and Env and the accessory products Vif, Vpu, and Vpr. Rev is absolutely required for HIV-1 replication. Proviruses that lack Rev function remain transcriptionally active, but fail to generate new viral particles.

2 RNA Splicing and Retroviruses

The compact genomes of retroviruses contain RNA splice sites which allow the expression of multiple gene products from the single primary retroviral transcript (Cullen 1991; Kim et al. 1989; Gallo et al. 1988; Schwartz et al. 1990a). Typically, incompletely spliced mRNA do not exit the nucleus to be subsequently translated into protein in the cytoplasm (Green 1991). However, retroviruses can clearly bypass this regulatory checkpoint. The splice sites of retroviruses are typically very inefficient substrates for splicing (Katz and Skalka 1990; Berberich and Stoltzfus 1991). This is an important characteristic, since introns are usually quickly excised from the primary transcripts of endogenous cellular genes. Inefficient splicing of retroviruses is necessary to allow the accumulation of unspliced mRNA, which is essential both as the genomic information in each viral particle as well as the message-encoding essential viral proteins. Complex retroviruses, typified by HIV-1, have multiple introns and splice sites, allowing the production of a large number of mRNA species (Gallo et al. 1988; Schwartz et al. 1990a). Alternative splicing allows the generation of at least 35 distinct mRNA species from the approximately 9-kb genome of HIV-1 (Gallo et al. 1988; Schwartz et al. 1990a). In addition to alternative patterns of splicing, complex retroviruses express a regulatory protein, commonly known as Rev, to regulate the expression of the diverse mRNA species that are generated (Kim et al. 1989; Pomerantz et al. 1990).

3 Identification of HIV-1 Rev

The HIV-1 regulatory protein Rev was first identified when it was determined that certain mutations downstream of the *tat* gene were found to abolish viral replication (Feinberg et al. 1986; Sodroski et al. 1986). These mutant viruses were further shown to be transcriptionally active, but unable to produce the viral structural proteins Gag, Pol, and Env. Upon closer inspection it was shown that the mutations specifically interrupted an open reading frame that had not previously been demonstrated to encode a protein product (Feinberg et al. 1986; Sodroski et al. 1986). This new open reading frame, encoded by two exons, partially overlaps with and shares common splice sites with the *tat* coding region (Robert-Guroff et al. 1990). The new open reading frame encoded a 116-amino

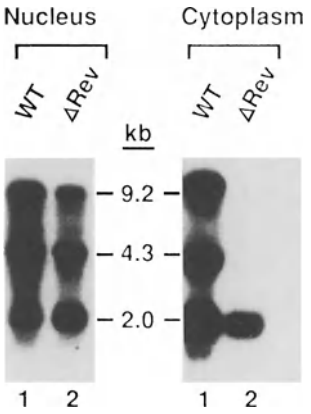

Fig. 1. Northern blotting of nuclear and cytoplasmic RNA from human immunodeficiency virus (HIV)-1-infected cells. Both wild-type virus and Rev-mutant virus were utilized in these studies. (Reprinted with permission from Trono and Baltimore 1990.)

acid protein. The unique mode of action of this viral regulatory protein was revealed when the cytoplasmic mRNA expression of Rev-defective proviruses was compared to wild-type HIV-1 (Hammarskjöld et al. 1989; Malim et al. 1989; Emerman et al. 1989; Felber et al. 1989). There are three major species of mRNA expressed by HIV-1 with lengths of approximately 9, 4, and 2 kb corresponding to unspliced, singly spliced, and multiply spliced transcripts (Fig. 1). All three mRNA species can be found in the cytoplasm of HIV-1-expressing cells, whereas only increased amounts of the 2-kb class of mRNA can be identified in the cytoplasm of cells expressing a Rev-defective provirus. This difference was shown to be Rev specific, because the normal pattern of cytoplasmic mRNA could be restored by the coexpression of Rev protein expressed from a plasmid (Knight et al. 1987). It then seems that Rev works by facilitating the cytoplasmic localization and subsequent expression of genes encoded on incompletely spliced mRNA. Rev therefore regulates the cytoplasmic localization and subsequent expression of incompletely-spliced viral RNA. The mechanism that Rev employs to facilitate the expression of incompletely spliced mRNA has yet to be fully elucidated and will be discussed later.

4 Role of Rev in the Viral Life Cycle

The expression of HIV-1 genes during viral replication can be divided into two phases, as is diagrammed in Fig. 2. Initially, the virus expresses only low levels of multiply spliced transcripts that encode the viral regulatory early gene products Tat, Rev and Nef (Gallo et al. 1988; Schwartz et al. 1990a; Robert-Guroff et al. 1990). Tat then acts to greatly increase the levels of transcription from the viral long terminal repeat (LTR), and this in turn acts to greatly stimulate the level of expression of the viral early genes (Lee et al. 1986; Rice and Mathews 1988). Another viral regulatory protein, Rev, then plays an essential role in the transition

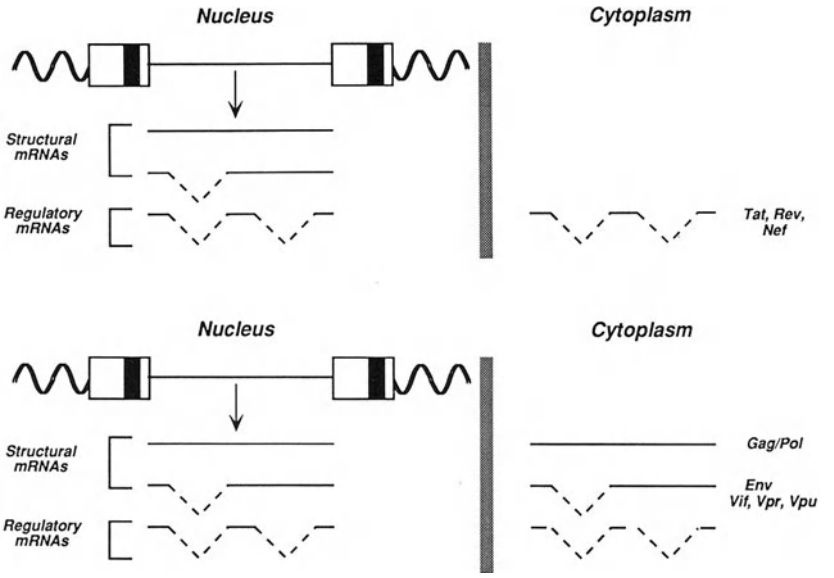

Fig. 2. Early (*top*) and late (*bottom*) phases of human immunodeficiency virus (HIV)-1 replication

from the early phase into the late phase of viral gene expression. When Rev accumulates to sufficient levels or is activated by some other mechanism, the expression of viral late genes is activated, leading to the production of viral particles (Pomerantz et al. 1990,1992). The expression of the structural proteins Gag, Pol, and Env as well as of the accessory proteins Vpu, Vpr, and Vif is dependent on the presence of a functional Rev gene product. The role of Rev in this switch from the early to the late phase of viral gene expression has led a number of groups to hypothesize that Rev plays a key role in viral latency (Pomerantz et al. 1990; Malim and Cullen 1991). Understanding the molecular basis of HIV-1 proviral latency will be critical in the effort to develop clinical therapies for patients with the acquired immune deficiency syndrome (AIDS).

5 Rev Response Element

HIV-1 Rev is a 13-kDa phosphoprotein that has a sequence-specific RNA-binding activity (Hauber et al. 1988; Cochrane et al. 1989; Fig. 3). Produced from a fully spliced message made early in the viral life cycle, Rev acts to induce the transition into the late phase of the viral life cycle. Rev accumulates within the nuclei and nucleoli of infected cells (Cochrane et al. 1990b; Perkins et al. 1989) and binds directly to the unspliced and singly spliced structural forms of HIV-1 RNA (Zapp and Green 1989; Daly et al. 1989). Binding takes place at a 240-bp region of complex RNA secondary structure, called the rev response element (RRE), that

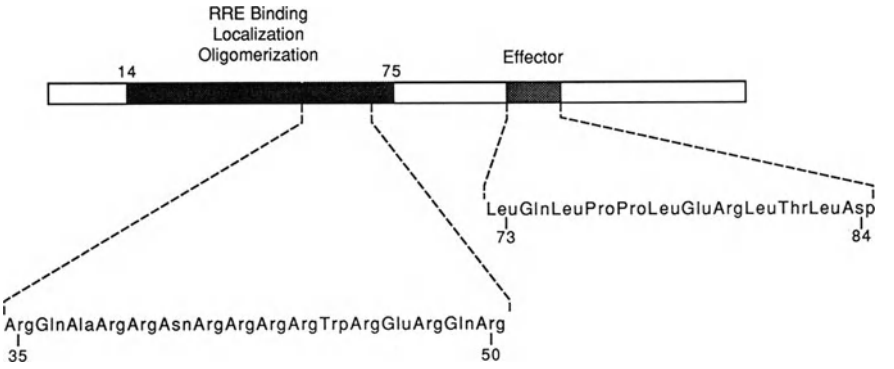

Fig. 3. Structure of Rev. *RRE*, Rev response element

lies within the envelop coding region contained in the second major intron of HIV-1 (Rosen et al. 1988; Hadzopoulou-Cladaras et al. 1989; Cochrane et al. 1990a; Huang et al. 1991). A putative structure for the RRE determined by computer folding programs and in vitro footprinting is presented in Fig. 4. Specifically, the RRE is located at the junction between the two subunits of the Env protein gp120 and gp41. This is an especially strategic location for the RRE because it is present in unspliced and singly spliced transcripts, but deleted from multiply spliced Rev-independent mRNA. A high-affinity binding site for Rev in the RRE has also been identified. This binding site consists of a sequence-specific region of nonpaired nucleotides a "bubble" located within a stretch of double-stranded RNA (Bartel et al. 1991; Heaphy et al. 1991). Studies of Rev fused to the bacteriophage RNA-binding protein MS2 indicate that Rev-specific *trans*-activation can be directed through a heterologous RNA-binding specificity (McDonald et al. 1992; Venkatesan et al. 1992). This result indicates that the RRE acts primarily as a docking site for HIV-1 Rev since it can be functionally replaced with a heterologous RNA-binding protein and RNA target. The interaction of Rev with the viral transcripts allows these structural RNA molecules to enter the cytoplasm before splicing is complete, and this in turn activates the expression of the structural proteins. At the same time, Rev tends to suppress production of fully spliced mRNA, thereby down regulating its own expression (Felber et al. 1990; Ahmad et al. 1989). Studies of HIV-1 Rev protein have indentified the regions of the protein required for its specific RNA-binding activity and other essential functions.

A RNA element that can functionally replace the Rev/RRE system of HIV-1 has recently been identified in the type D retrovirus Mason Pfizer monkey virus (MPMV) (Bray et al. 1994). This small, approximately 200-bp element, which is located adjacent to the 3'LTR of MPMV, is able to facilitate the expression of Rev-dependent genes in the absence of the RRE or Rev. It is believed that this element is probably the binding domain for a cellular analogue of HIV-1 Rev. This element represents another mechanism employed by retroviruses to bypass the complete splicing of mRNA prior to exit from the nucleus.

6 Structure/Function of Rev

Mutagenesis studies of HIV-1 Rev have revealed that Rev has at least two functional regions or domains found to be essential for function (Fig. 3) (Malim et al. 1989b; Hope et al. 1990a). First, there is a large multifunctional amino terminal region (approximately amino acids 14–56) that controls at least three important aspects of Rev function. The ability of HIV-1 Rev to properly oligomerize (Olsen et al.1990; Zapp et al. 1991), localize (Hope et al. 1990b; Venkatesh et al. 1990), and to specifically bind its RNA target (Olsen et al. 1990; Zapp et al. 1991) is encoded in the amino-terminal region. Second, a much smaller essential region known as the effector or activation domain (Malim et al. 1989c, 1991; Hope et al. 1991) is located in the C-terminal portion of HIV-1 Rev (approximately residues 70–85).

Within the amino-terminal region there is a very positively charged domain, known as the arginine-rich domain, that provides several activities required for Rev function. This cluster of positively charged amino acids is important for the proper nuclear and nucleolar localization of the HIV-1 Rev protein (Hope et al. 1990b; Venkatesh et al. 1990). The arginine-rich domain is also important for specific RNA binding and facilitates the interaction between HIV-1 Rev and the RRE. Mutations of the arginine-rich tract have been shown to disrupt the ability of HIV-1 Rev to interact with the RRE both in vivo and in vitro (Malim et al. 1989b;

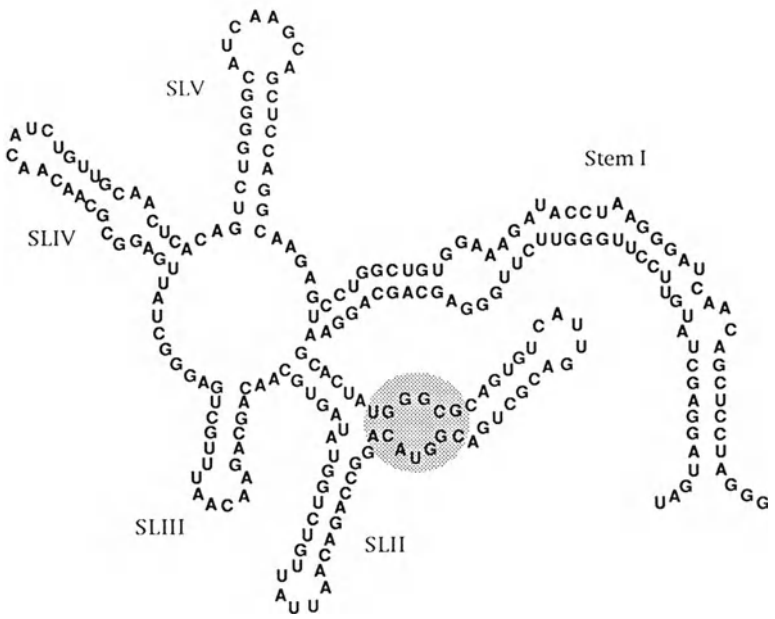

Fig. 4. Structure of the Rev response element (RRE). The *shaded area* represents the Rev-binding site. *SL*-Steve loop

Hope et al. 1990a; Olson et al. 1990; Zapp et al. 1991). The positive charges of the arginine-rich domain are believed to interact with the overall negative charge of the phosphodiester backbone of the RNA within the RRE (Lazinski et al. 1989; Calnan et al. 1991; Tao and Frankel 1993). In addition to the essential positively charged arginine residues within this domain, there are also other important residues. The tryptophan at amino acid 45 in the arginine-rich domain is also important for function. It is possible that the aromatic tryptophan may be able to intercalate into the RNA to further stabilize RNA protein interactions (Saikumar et al. 1990). Specific interactions between peptides of the arginine-rich domain and the RRE have also been demonstrated in vitro and in vivo (Kjems et al. 1992; Tan et al 1993). These results indicate that most of the specificity for the interaction between HIV-1 Rev and the RRE are encoded within the arginine-rich domain.

It has recently been demonstrated that the aminoglycoside neomycin B is able to inhibit the binding of HIV-1 Rev in vitro and inhibit viral late gene expression at millimolar concentrations in vivo (Zapp et al. 1993). These agents that can inhibit Rev function by blocking the ability of Rev to interact with RRE hold promise as the progenitors of efficient drugs that can be used in anti-HIV-1 therapies.

A second important function of the amino-terminal region is involved in the functional interactions of monomers of HIV-1 Rev. This region has been implicated in the multimerization of HIV-1 Rev, both for the generation of stable Rev tetramers as well as the higher order interaction of multiple Rev tetramers when bound to the RRE (Malim and Cullen 1991; Olsen et al. 1990; Zapp et al. 1991). There is a variety of evidence indicating that HIV-1 Rev exists as a stable oligomer in vivo. Cross-linking and gel filtration studies of recombinant, *Escherichia coli*-derived protein suggest that HIV-1 Rev exists as a stable tetramer (Olson et al. 1990; Nalin et al. 1990). Further cross-linking studies of Rev expressed in eukaryotic cells indicate that Rev is also a tetramer in vivo (Olson et al. 1990). Colocalization immunofluorescence studies provide additional support that Rev exists as an oligomer in vivo (Hope et al. 1992). Various mutations between amino acids 14 and 56 of HIV-1 Rev have been shown to specifically affect the ability of the protein to oligomerize (Malim and Cullen 1991; Olsen et al. 1990; Zapp et al. 1991; Hope et al. 1992). The region of HIV-1 Rev from amino acids 14–23 is particularly sensitive to mutations that specifically effect multimerization. Those mutants which do not oligomerize are unable to bind strongly to target RNA (Olsen et al. 1990). These observations indicate that Rev must exist as a tetramer to generate stable RNA-binding complexes. A new system to analyze Rev multimerization in vivo has recently been developed that uses a variation of the yeast two-hybrid system (Bogerd et al. 1993). However, the relevance of this system for multimerization is questionable because effector domain mutants effect multimerization in this new system while all other assays indicate that effector domain mutants of HIV-1 Rev are tetramers. It is possible, however, that the detected interactions may be mediated through the Rev cofactor or some other cellular protein. This interpretation is supported by the fact that Rev does not score positively with the two-hybrid system in yeast, indicating that this is a factor specific to higher eukaryotes (Bogerd and Greene 1993).

The effector domain is absolutely required for function and is believed to be the site of interaction between the viral Rev protein and cellular components which facilitate HIV-1 Rev function (Malim et al. 1991; Lazinski et al. 1989; Nalin et al. 1990). One possible cellular cofactor is the eukaryotic initiation factor 5A, which appears to bind to the Rev effector domain (Ruhl et al. 1993). The effector domain is composed of a leucine-rich region that can be readily indentified in several members of the family of Rev-like viral post-transcriptional transactivators (Nalin et al. 1990; Hope et al. 1992). It has been clearly demonstrated that the effector domains of this family of viral regulatory proteins can be functionally exchanged (Malim et al. 1991; Lazinski et al. 1989; Nalin et al. 1990; Hope et al. 1992). When Rev function is directed through a heterologous RNA-binding protein, the bacteriophage protein MS2, it can be demonstrated that a functional effector domain is required, while a functional amino-terminal region is not (McDonald et al. 1992). These results can be interpreted to mean that a Rev-like molecule is defined as a specific RNA-binding protein with a functional effector domain. From this prospective, Rev-like proteins are basically adapters that allow a response element containing (viral) RNA to interact with a specific cellular pathway by interactions mediated through the effector domain. Mutagenesis studies, functional characterization by complementation of effector domain mutants of HIV-1 Rev, and apparent homologies indicate that a functional effector domain is a common feature of the viral Rev-like proteins that can be found in all lentiviruses and some oncoviruses.

Mutations of the HIV-1 Rev effector domain are particularly interesting because they generate dominant negative mutants (Malim et al. 1989b; Hope et al. 1992). Nonfunctional mutants are defined as dominant negative mutants when they not only lack intrinsic activity, but can also block the activity of the wild-type protein (Herskowitz 1987). In this way dominant negative mutants of HIV-1 Rev have the ability to inhibit the function of wild-type Rev when present in excess. It was initially thought that effector domain mutants of Rev are dominant negative because they compete for binding to RRE-containing transcripts (Malim et al. 1989b). However, we have published a subsequent study that suggests that the dominant negative mutants of HIV-1 Rev work, at least in part, through the generation of nonfunctional Rev multimers (Hope et al. 1992). Functional Rev monomers can interact with mutant Rev monomers to generate nonfunctional complexes. This mechanism for inhibition of function by dominant negative Rev is further supported by the observation that secondary mutations that will effect oligomerization of a dominant negative Rev mutant destory its ability to inhibit wild-type protein function (Hope et al. 1992). As such, *trans*-dominant negative mutant proteins hold promise as "intracellular immunization" approaches to inhibit HIV-1 replication (Baltimore 1988). In addition, single-chain variable fragments (SFv) of antibodies to Rev have recently shown promise for intracellular immunization against HIV-1 infection of human cells (Duan et al. 1994).

7 Rev and Pathogenesis

Rev may also have a function in certain forms of HIV-1 proviral latency. Cell lines have been selected from the survivors of lytic HIV-1 infections that maintain HIV-1 in the restricted state and constitutively produce very low levels of the virus. These cell lines can be stimulated to increase HIV-1 expression with a variety of exogenous compounds. Most of these compounds appear to act via activation of nuclear factor-kappa B (NF-κB). Two HIV-1 latently infected cell lines have been extensively characterized, the U1 monocytic and the ACH-2 T lymphocytic lines. These cell lines have been used as model systems to explore HIV-1 postintegration latency in cell culture. In the baseline unstimulated state, these cells express mainly multiply spliced HIV-1-specific RNA, as compared to productively infected cells, in which all three HIV-1 RNA species are expressed in nearly equivalent amounts (Pomerantz et al. 1990). This RNA expression pattern undergoes a switch to mainly synthesis of unspliced viral transcripts upon stimulation of these cells. There is, as well, a concomitant upregulation of total viral RNA transcription. Time course experiments have demonstrated that after stimulation there is an initial rise of multiply spliced HIV-1 RNA species prior to the increase in unspliced viral RNA (Pomerantz et al. 1990). The unstimulated U1 and ACH-2 cells express an HIV-1-specific RNA pattern analogous to early-stage cells and appear blocked from progression to the late stage of productive infection, unless they are stimulated by activating agents. This molecular model for postintegration HIV-1 latency has recently been demonstrated in other cell types (Laughlin et al. 1993).

U1 and ACH-2 cells may be models for postintegration latency in certain cells of HIV-1-infected individuals. It is apparent that something about these cells maintains HIV-1 in the early stage of infection. Exactly what prevents progression to the late stage of infection is not clear. The relatively low levels of viral transcription in these cells will lead to a low level of Rev. We have suggested that the preponderance of multiply spliced viral RNA species in these cells may be caused by the low levels of the viral protein Rev, implying that there is a critical threshold level of Rev required for efficient Rev function. We have recently demonstrated that a functional threshold level of Rev exists in certain cells (Pomerantz et al. 1992). This Rev threshold may be secondary to the requirements for Rev multimerization to allow it to function. Thus a sub-threshold level of Rev may be the cause of the aberrant viral RNA expression pattern demonstrated in these latently infected cells, and this splicing pattern may also be detected in certain cells in the peripheral blood of HIV-1-infected individuals (Seshamma et al. 1992).In addition, recent data suggests that mutations in *rev* may be more commonly found in asymptomatic HIV-1-infected individuals than in patients with AIDS. Of note, these mutations were detected in the C-terminal effector domain of *rev* (A.K.N. Iversen and J.I. Mullins, personal communication, submitted).

8 Mechanism of Rev Function

Several studies considering the relationship between the post-transcriptional *trans*-activation by HIV-1 Rev and mRNA splicing indicate that the two pathways are, to a limited extent, interdependent. It has been shown that Rev function requires inefficient splice sites (Chang and Sharp 1989). The ability of the Rev/RRE system to function in the heterologous intron from beta globin was demonstrated to require the type of inefficient splice sites that are commonly found in retroviruses. A later study further defined this interaction with the observation that Rev activity was dependent on functional interactions between target mRNA and the small nuclear RNA U1 (Lu et al. 1990). Point mutations in the U1 binding site interfered with the ability of the studied transcript both to splice and to be *trans*-activated by HIV-1 Rev. However, the cotransfection of a U1 molecule with compensatory mutations led to the restoration of both splicing and Rev-specific transactivation. Although these results imply that HIV-1 Rev requires functional splice sites, it is difficult to interpret because it is not possible to detect the target mRNA in the absence of potential functional interactions between 5' splice sites and U1. The fact that Rev cannot facilitate the cytoplasmic localization of a RNA with a mutated 5' splice site may reflect the fact that such transcripts in a eukaryotic nucleus are targeted for degradation. HIV-1 Rev is functional in the cells of many species from humans to *Drosophila* (Ivey-Hoyle and Rosenberg 1990). The fact that the pathway(s) used by Rev are common to higher eukaryotes indicates that the cellular cofactor(s) facilitating Rev function are highly conserved and probably essential for the function of normal cells. In mice, though, the situation is more complicated. Whereas normal Rev function can be demonstrated in murine cells using Rev-based reporter systems (Malim et al. 1991), Rev function in the context of a HIV-1 provirus is defective (Trono and Baltimore 1990). It has further been shown that this defect in Rev function is not due to the inefficient function of Tat that had been previously demonstrated in mouse cells (Winslow and Trono 1993). The differences detected are probably due to the efficient splicing of HIV-1 transcripts in murine cells. This increase in splicing efficiency in mouse cells has been previously demonstrated in the case of the retrovirus Rous sarcoma virus (RSV) (Berberich et al. 1990; Quintrell et al. 1980). As described above, inefficient splice sites are required for Rev function.

The Rev protein acts by inducing the cytoplasmic accumulation and subsequent translation of incompletely spliced viral mRNA (Hammarskjöld et al. 1989; Malim et al. 1989a; Emerman et al. 1989; Felber et al. 1989). The exact mechanism of Rev action has not been determined, but the Rev-dependent appearance of incompletely spliced messages in the cytoplasm along with the Rev-dependent decrease in the expression of completely spliced transcripts presumably involves either direct inhibition of splicing or a selective induction of RNA transport (Green and Zapp 1989; Chang and Sharp 1990). There are two possible explanations for the decrease in the amount of splicing that has been observed. Rev may specifically inhibit mRNA splicing by directly interfering with the splicing machin-

ery. This specific inhibition, in turn, eventually leads to cytoplasmic localization and expression of Rev-dependent mRNA. It is also possible that Rev may indirectly decrease the amount of fully spliced transcripts, kinetically, by the depletion of substrate mRNA available for splicing through direct transport to the cytoplasm. These observations have led to the development of two primary models for Rev function, the inhibition of splicing or facilitated transport, to explain the mechanism of Rev action.

The inhibition of splicing model is based on the observed decrease in the amount of spliced viral message in the presence of HIV-1 Rev, along with the requirement for inefficient splice sites for Rev function (Katz and Skalka 1990; Berberich and Stoltzfus 1991; Chang and Sharp 1989; Lu et al. 1990). According to this model, the interaction of Rev with the RRE leads to a disruption of splicing complexes forming on the transcript, which in turn leads to the appearance of the incompletely spliced messages in the cytoplasm. It has been previously observed in yeast that certain splice site mutations cause an increase in the expression of unspliced mRNA (Legrain and Rosbash 1989). Additionally, it has been shown that a peptide of the arginine-rich domain of Rev can inhibit splicing in vitro (Kjems et al. 1991). This study has been extended to determine that the arginine-rich peptide inhibits the entry of the U4/U6-U5 small nuclear ribonucleoprotein into an assembling splicesome complex (Kjems and Sharp 1993). However, it has not been demonstrated that the observed inhibition of splicing is required for transactivation in vivo. Also, in vivo Rev function requires an intact effector domain which is not required for the observed peptide-specific inhibition described in vitro.

It is also possible that the interaction of HIV-1 Rev with the RRE directly leads to the transport of the incompletely spliced mRNA into the cytoplast, where it is subsequently translated. In this model, Rev will allow the circumventing of the constitutive nuclear check points that normally prevent incompletely processed mRNA from exiting the nucleus. Unfortunately, little is currently known about the details of the nuclear export of processed mRNA into the cytoplasm. In fact, understanding the details of HIV-1 function should reveal many details of how mRNA is exported form the nucleus. These two models are not mutually exclusive and it may be that the actual mechanism of Rev function may include elements of both models. The splicing and eventual transport of mRNA from the nucleus may represent a single pathway that is in some way modified by Rev.

It has also been observed that Rev may effect the stability and efficiency of translation of viral mRNA. A recent study of the effect of HIV-1 Rev on the stability of viral mRNA in a T cell line demonstrates that Rev has the ability to greatly increase the half-life of viral messages within the nucleus (Malim and Cullen 1993). These results indicate that in the absence of Rev, viral mRNA is targeted for degradation. Therefore, the induction of gene expression mediated by Rev is in part the result of the redirection of viral messages from degradation pathways to transport pathways that facilitate the cytoplasmic localization and subsequent translation of incompletely spliced viral mRNA encoding late genes. Several groups have identified negative *cis*-acting sequences contained within the viral genome that can be overcome by the addition of HIV-1 Rev (Cochrane et al. 1991;

Schwartz et al. 1992a,b). These inhibitory sequences may be important for the targeted degradation of nuclear viral mRNA and changes in mRNA stability observed when viral mRNA is expressed in the absence of Rev. It has also been demonstrated that mRNA redirected to the cytoplasm by Rev is polysome associated, whereas cytoplasmic RNA detected in the absence of Rev is not associated with ribosomes (Arrigo and Chen 1991; D'Agostino et al. 1992). This observation may indicate that post-transcriptional induction of gene expression by Rev extends to the level of increasing the efficiency of translation. However, these changes in mRNA stability and translation may simply be another outcome of Rev-specific *trans*-activation. For instance, mRNA that is specifically exported to the cytoplasm by HIV-1 Rev may be more efficiently translated and more stable as a result of following the appropriate pathway.

Thus, the HIV-1 regulatory protein Rev is a prime example of the complexity of replication in this unique lentivirus. Understanding Rev structure and function, in its intricacy, promises to yield critical clues in the study of HIV-1 pathogenesis and novel therapeutic modalities.

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The Human Immunodeficiency Virus Type 1 Vpu Protein: Roles in Virus Release and CD4 Downregulation

M. Abdul Jabbar

1 Introduction

Human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus that encodes a number of novel regulatory proteins (Tat, Rev, Nef, Vpr, Vif and Vpu) in addition to the canonical structural proteins (Gag, Pol, and Env) that are common to all retroviruses. HIV-1 is the causative agent of the acquired immunodeficiency syndrome (AIDS). The complexity of HIV genomes reveals the complex nature of host–virus interactions in AIDS pathogenesis (Levy 1993; Weiss 1993). Some of the HIV-1 proteins are involved in the regulation of gene expression in virus-infected cells. For example, the Tat and Rev proteins are essential for HIV replication in tissue culture (Cullen 1992). These proteins have been shown to exert their effects through specific interactions with viral RNA: *trans*-acting response element (TAR) in the case of Tat and Rev response element (RRE) in the case of Rev. The mechanism of action of these proteins will not be considered in this review, as they will be discussed elsewhere in this volume.

This chapter focuses on biosynthesis and structure–function of Vpu, as well as the role of Vpu in the HIV life cycle. Aside from the envelope gene product gp 160, Vpu is the only other HIV-encoded protein which is anchored to the membrane using a hydrophobic transmembrane sequence. However, unlike the gp120–gp41 complex which constitutes the virus envelope, Vpu is a non-structural protein which is not part of the virus particle (Strebel et al. 1989; Terwilleger et al. 1989). The Vpu protein appears to have roles in two apparently

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unrelated functions: virus release and CD4 proteolysis in the endoplasmic reticulum (ER). In this review, the results of experiments will be summarized that specifically address the role of Vpu in CD4 downregulation and virus release.

2 Synthesis and Membrane Topology of Vpu

The HIV-1 proteins are synthesized from three distinct classes of mRNA: full-length, intermediate, and small mRNA. The full mRNA produces Gag-Pol and Gag proteins, and the intermediate RNA synthesize Vpu, Env, Vif, and Vpr, whereas the multiply spliced RNA are responsible for the production of Tat, Rev, and Nef proteins (Haseltine 1991; Cullen 1992). The Vpu protein of HIV-1 is synthesized with the envelope glycoprotein precursor gp160, (Fig. 1), from the same bicistronic mRNA in infected cells (Schwartz et al. 1990). The coordinate regulation of gp160 and Vpu might have relevance in the virus life cycle, but it is not apparent from in vitro studies. However, Vpu has been shown to regulate the formation of gp160–CD4 complexes in the cell (Willey et al. 1992a).

The Vpu protein is a transmembrane phosphoprotein which is localized in the intracellular membrane compartment of HIV-infected cells. Figure 2 shows a schematic diagram of the HIV-1 Vpu protein with structural features that are critical for its biological activities in mammalian cells. Experiments with canine microsomal membranes have revealed that the 27-amino acid N-terminal region

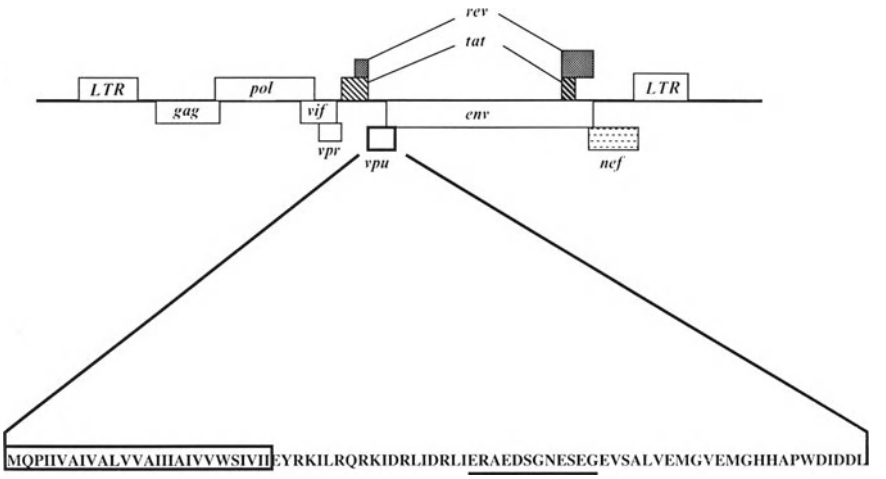

Fig. 1. Organization of the human immunodeficiency virus (HIV)-1 genome. The Vpu and gp160 (*env* gene product) proteins are coordinately synthesized from the same bicistronic messenger RNA (Schwartz et al. 1990). The amino acid sequence of the pNL43 Vpu protein is shown (Strebel et al. 1988). The Vpu protein is inserted to the membrane through the N-terminal hydrophobic region (*boxed*); (Strebel et al. 1989; Malderelli et al. 1993), and the 12-amino acid region (*underlined*) is conserved in all known HIV isolates (Chen et al. 1993). *LTR*, long terminal repeat

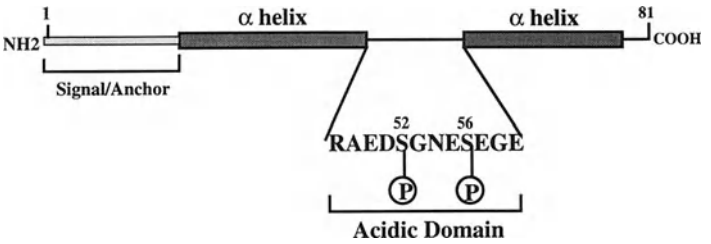

Fig. 2. Structural features of the Vpu protein. The human immunodeficiency virus (HIV)-1 Vpu protein is a transmembrane protein of 81 amino acids which is phosphorylated by casein kinase-2 at positions Ser52 and Ser56 (Schubert et al. 1994). The 12-amino acid acidic region is flanked by two amphipathic α-helical regions (amino acid positions 30–47 and 60–70). The 54-amnio acid cytoplasmic domain is preceded by the 27-amino acid hydrophobic signal/anchor region, which presumably inserts the protein to the membrane (Schubert et al. 1994)

of Vpu is responsible for membrane association (Strebel et al. 1989; Malderelli et al. 1993), suggesting that this region could function as both the ER signal sequence and the membrane anchor. Furthermore, the 54-amino acid cytoplasmic domain of Vpu is highly hydrophilic, having a number of charged amino acids (Cohen et al. 1988; Strebel et al. 1988; Schubert et al. 1994). This domain posseses a 12-amino acid region that is conserved in all known HIV isolates (Chen et al. 1993), and the two seryl residues in the conserved region serve as the phosphoacceptor sites for the ubiquitous casein kinase-2 (CK-2; Schubert et al. 1994). Furthermore, the secondary structure prediction of Vpu suggests that the two regions (amino acid positions 30–50 and 58–70) flanking the acidic domain could assume amphipathic α-helical configurations which might have roles in the assembly of Vpu in the cell. Interestingly, the Vpu protein has been shown to assemble as a homo-oligomer in the absence and presence of membranes in vitro (Malderelli et al. 1993), and it is likely that only the oligomeric form of the protein is active in mammalian cells.

3 Role of Vpu in Virus Release

Early experiments were performed to ascertain the role of Vpu in the virus life cycle, as this protein is unique to HIV-1 and is, not encoded by HIV-2 or the majority of simian immunodeficiency virus (SIV) isolates (Cohen et al. 1988; Strebel et al. 1988; Klimkait et al. 1990; Terwilleger et al. 1989). These studies have demonstrated that functional Vpu protein is necessary to release particles efficiently from the plasma membrane of infected cells, and Vpu-deficient viruses are poorly released into culture medium (Klimkait et al. 1990; Terwilleger et al. 1990, Yoo et al. 1992, 1993). The majority of mutant virus particles have been shown to bud from internal membrane compartments, mostly into exaggerated vacuoles of HIV-infected cells. These particles have the appearance of mature virions with condensed nucleoids. Furthermore, a small fraction of mutant virions

is displayed as a tether at the plasma membrane of Vpu-deficient virus-infected cells (KLIMKAIT et al. 1990; Yoo et al. 1993). Thus, the Vpu protein appears either to prevent the assembly of virus particles in inappropriate membranes (intracellular membranes) or to target the virus to a correct membrane compartment (plasma membrane).

The assembly and budding of retroviruses is a complex process involving a number of viral proteins (HUNTER 1994). For the generation of infectious particles, the envelope glycoproteins and the group-specific antigens (Gag) are targeted to the plasma membrane, where they are incorporated into virions in a highly specific fashion. Both envelope and Gag proteins reach the surface of mammalian cells by different routes. The envelope glycoproteins use the fairly well defined mammalian secretory pathway for maturation and proper delivery to the plasma membrane, whereas Gag proteins are targeted to a specific region of the plasma membrane by a poorly defined pathway in the cell (HUNTER 1994). It has been shown that myristoylation of the Gag proteins was necessary for efficient assembly and release of virus particles from HIV-infected cells (BRYANT and RATNER 1990; GOTTLINGER et al. 1989). Vpu-mediated release of virions could therefore be related to its interactions with the Gag precursor or processed Gag proteins. However, recent evidence suggests that the effect of Vpu on the release of virus particles is not specific to HIV-1. For example, viruses as diverse as visna virus, SIV, HIV-2, and Mo-MuLV are released efficiently from cells expressing functional Vpu. The Gag protein of visna virus is not myristoylated as it lacks the N-terminal glycine residue (myristoylation signal), suggesting that myristoylated Gag proteins are not required for Vpu-enhanced release of virus particles from HIV-infected cells (GOTTLINGER et al. 1993).

In addition, the C-terminal regions of the Gag precursor proteins are distinct for different retroviruses and play critical roles in virus assembly processes (HUNTER 1994). The HIV Gag precursor is proteolytically cleaved to generate the C-terminal 6-kDa protein, and mutations in the protein have generated viruses that are defective in the assembly process (GOTTLINGER et al. 1991; PAXTON et al. 1993). It is interesting to note, however, that visna virus does not possess the open reading frame (ORF) that would encode a protein analogous to the HIV p6 protein. It appears therefore that Vpu-mediated enhancement of virus particles might not be related to the presence of the *p6* gene in the HIV genome (GOTTLINGER et al. 1993). Thus, these studies have demonstrated that the Vpu protein alters a cellular pathway which is commonly used by many retroviruses for assembly and budding processes, but the mechanism of Vpu action is not clearly defined yet. GOTTLINGER et al. (1993) have further demonstrated that the virus release function of Vpu is cell type dependent, as HIV particles were efficiently released from COS-7 cells in both the absence and presence of Vpu, suggesting that a cellular factor(s) might substitute for Vpu activity responsible for virus release in certain cell types (e.g., COS-7).

Vpu-defective viruses have been shown to be highly cytopathic in tissue culture as compared to wild-type virus. This cytopathicity was due perhaps to the accumulation of viral proteins in the cell, as the ratio of intracellular to extracellular

proteins was high in cells infected with the mutant virus (KLIMKAIT et al. 1990; TERWILLEGER et al. 1990; GOTTLINGER et al. 1993). The intracellular accumulation of viral proteins could potentially be toxic to cells, thereby reducing the viability of cells infected with the mutant virus. Paradoxically, a number of studies have demonstrated an early onset of syncytia induction in Vpu-deficient virus-infected cultures as compared to those infected with wild-type virus (KLIMKAIT et al. 1990; TERWILLEGER et al. 1990). This property of Vpu-defective viruses directly correlated with the accumulation of envelope glycoproteins at the cell surface of infected cells, which might recruit uninfected cells into the giant cell complex (YAO et al. 1993). In contrast, the envelope glycoprotein was incorporated into virus particles and efficiently released into culture medium of wild-type virus-infected cells, thereby reducing the concentration of envelope glycoproteins at the cell surface (YAO et al. 1993). However, there was no difference in the rate and extent of single cell killing in wild-type and mutant-infected cultures. Recent studies of a macrophage tropic virus demonstrated the importance of functional Vpu for the establishment of productive infection in macrophages (BALLEIT et al. 1994). In this study, the authors analyzed the replication potential of a Vpu-defective virus and showed that the mutant virus was highly restricted in the macrophage, but its growth was normal in T lymphocytes (BALLEIT et al. 1994). Thus, the study of macrophage tropic viruses with defined mutations in the *vpu* gene would provide valuable information as to the role of Vpu in HIV infection of macrophages.

It has been established that the Vpu protein has two known activities in HIV-infected or -transfected cells. One of the activities is related to its function in the degradation of CD4 in the ER, and the other is to enhance the release of virus particles from infected cells. As discussed above, Vpu is a transmembrane phosphoprotein which undergoes phosphorylation in two (ser25 and ser56) of the three serine residues in its cytoplasmic domain by the ubiquitous CK-2. SCHUBERT and STREBEL (1994) have analyzed the role of phosphorylation in distinct functions of the Vpu protein. Mutations in the two phosphoacceptor sites had only moderate effects in the release of virions from the plasma membrane of infected cells. However, the unphosphorylated Vpu mutant was completely defective in inducing degradation of CD4 in the ER. It was further demonstrated that the lack of Vpu activity in the degradation process was not due to missorting of the mutant protein to a cellular compartment where it could not function, as brefeldin-A (BFA) treatment of cells expressing the Vpu mutant and CD4 did not reconstitute Vpu activity (CD4 proteolysis) in the ER environment. BFA is a fungal metabolite which blocks the transport of protein from the ER to the Golgi compartment by inducing the collapse of Golgi membranes with ER membranes and also affecting other membrane trafficking pathways in the cell (PELHAM 1991; KLAUSNER et al. 1992). The release of virus particles was defective in cells treated with BFA, suggesting that the Vpu protein exerts its effect on the assembly of viruses in a cellular compartment distinct from the ER. Since mutations in the phosphorylation sites did not appreciably reduce the release of virions from infected cells, phosphorylation per se might not regulate membrane trafficking of Vpu in the cell. However, the modification of Vpu by the cellular CK-2 is critically important in

regulating its activity in the ER compartment. Furthermore, it has been shown that phosphorylation does not confer stability to Vpu, as both unphosphorylated and wild-type Vpu proteins are equally stable in mammalian cells (Schubert and Strebel 1994). These studies have further revealed that the Vpu effect was very specific in enhancing the release of virus particles, but did not affect the release of soluble Gag proteins into the culture medium (Schubert and Strebel 1994). Thus, the HIV-1 Vpu protein appears to function in distinct membrane compartments, exhibiting two different activities (virus release and CD4 proteolysis) in mammalian cells.

4 Role of Vpu in Degradation of CD4 in the Endoplasmic Reticulum

As mentioned above, the other activity of Vpu is to induce degradation of CD4 in the ER. This was achieved in cells expressing all three proteins (gp160, CD4, and Vpu) or in conditions in which CD4 was artificially retained in the ER (Willey et al. 1992a; Vincent et al. 1993). A number of studies have shown that the envelope glycoprotein precursor gp160 binds tightly to CD4 in the ER, forming gp160–CD4 complexes (Kawamura et al. 1989; Crise et al. 1990; Jabbar and Nayak 1990; Bour et al. 1991; Willey et al. 1992b; Raja et al. 1993). One of the major consequences of the interaction between gp160 and CD4 in the intracellular compartment was the interference of normal CD4 trafficking in the cell (Kawamura et al. 1989; Crise et al. 1990; Jabbar and Nayak 1990). CD4 is a type I glycoprotein which is delivered to the cell surface via the Golgi complex undergoing endo-H-resistant oligosaccharide modifications (Crise et al. 1990; Jabbar and Nayak 1990). However, in cells expressing both gp160 and CD4, the maturation of CD4 was defective, suggesting that CD4 was not able to exit the ER. This was due to tight binding and the complex formation between CD4 and gp160 (Buonocore and Rose 1990; Crise et al. 1990; Jabbar and Nayak 1990; Raja et al. 1993). Furthermore, it has been shown that the envelope precursor, gp160, was poorly transported to the cell surface, and only a minor fraction of gp160 was endoproteolytically cleaved, generating gp120 and gp41 subunits in mammalian cells (Willey et al. 1988; Crise et al. 1990; Jabbar and Nayak 1990; Raja et al. 1993). In some experimental systems, the majority of gp160 was shown to be highly unstable and underwent degradation in lysosomes (Willey et al. 1988).

Recent experiments have elucidated the role of Vpu in the intracellular metabolism of CD4 (Willey et al. 1992a,b). It was demonstrated that the presence of Vpu in cells expressing gp160 and CD4 had resulted in the destabilization of gp160–CD4 complexes in the ER, and gp160 was able to be processed to gp120 and gp41 (Willey et al. 1992). This phenotype of Vpu expression was primarily due to selective degradation of CD4 in the gp160–CD4 complex (Willey et al. 1992b). Vpu-mediated degradation of CD4 has been measured mostly in

nonlymphoid (HeLa) cells, in which CD4 was expressed under control of either the T7 promoter or the HIV long terminal repeat (LTR; Willey et al. 1992a; Vincent et al. 1993; Lenberg and Landau 1993). The virus release assay was performed in both lymphoid and nonlymphoid cells (Klimkait et al. 1990; Terwilleger et al. 1990; Yeo et al. 1993; Geraghty and Panganiban 1993). Using proviruses that are otherwise isogenic except for the synthesis of functional Vpu, it has been shown that Vpu-mediated virus release occurred independently of both gp160 and CD4 expression (Yeo et al. 1992; Geraghty and Panganiban 1993).

As shown in Fig. 2, the Vpu protein anchors to the membrane through the N-terminal hydrophobic region, and the 54-amino acid cytoplasmic domain is projecting into the cytoplasmic face of the ER membrane. Therefore, we hypothesized that the interaction, if any, between CD4 and Vpu is presumably mediated through either the cytoplasmic or the anchor domain. Accordingly, we generated HIV envelope glycoproteins that have the extracellular domain of gp160, the cytoplasmic domains of CD4, and the gp160 anchor domain or the CD4 anchor domain (Fig. 3). These envelope glycoproteins were able to fuse HeLa $CD4^+$ cells, and the fusion assay was performed to map putative Vpu-responsive elements in the anchor and cytoplasmic domains of CD4 (Vincent et al. 1993; Raja et al. 1994). We have shown that the envelope glycoprotein E-TC38 underwent Vpu-induced degradation in the ER and hence was not available for membrane fusion events at the cell surface. Therefore, no syncytia were formed in HeLa cells expressing Vpu and E-TC38. However, a 20-amino acid deletion in the CD4 had rendered the mutant chimeric envelope glycoprotein E-TC18 resistant to Vpu-mediated degradation in the ER, and therefore was both biochemically stable and biologically active in cells expressing Vpu (Vincent et al. 1993). Thus, we mapped the putative Vpu-responsive element (*VRE*) to a six-amino acid region (LSEKKT) of the CD4 tail (Vincent et al. 1993). Others have also identified a similar or identical sequence element in the CD4 cytoplasmic domain employing mutant CD4 molecules in both in vivo and in vitro assays (Chen et al. 1993; Lenberg and Landau 1993). Thus the functional (syncytium) assay has correctly revealed the putative minimal *VRE* in the cytoplasmic domain of CD4.

Further experiments from our laboratory demonstrated that the anchor domain of CD4 played a critical role in the initial recognition event that presumably occurs in the ER membrane between Vpu and CD4. This conclusion was based on the observation that the envelope glycoprotein EA-C38 bearing the ectoanchor domains of gp160 and the full-length CD4 cytoplasmic tail was stable and functionally active in cells expressing Vpu (Raja et al. 1994). Our conclusion differs from that of recent study using CD8/CD4 chimeric proteins (Willey et al. 1994). It has been shown that the stability of CD8/cyto4 (the CD8 ectoanchor domains and the cytoplasmic domain of CD4) was comparable to that of CD8/CD4 (the extracellular domain of CD8, and the anchor-cytoplasmic domains of CD4) in the presence of Vpu (Willey et al. 1994). One interpretation of the study was that the cytoplasmic domain of CD4 alone was necessary and sufficient for conferring Vpu sensitivity. It is possible that both the CD4 and CD8 anchor domains share common structural or sequence features that are being

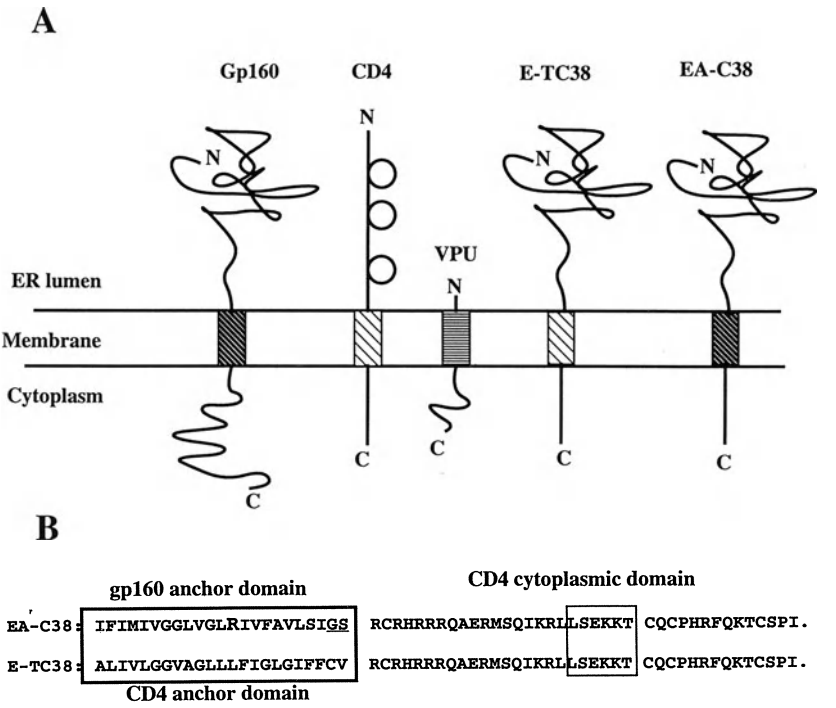

Fig. 3 A,B. Human immunodeficiency virus (HIV)-1 gp160, CD4, Vpu, and chimeric envelope glycoproteins. The chimeric envelope glycoproteins were generated by ligating DNA fragments encoding distinct domains of the proteins (Sanchez-Pascodar et al. 1985; Maddon et al. 1987; Vincent et al. 1993; Raja et al. 1993). Recombinant clones were isolated and confirmed by sequencing through the junction using dideoxy reactions. E-TC38 contains the ectodomain of gp160 and anchor-cytoplasmic domains of CD4, whereas EA-C38 contains the ecto-anchor domains of gp160 and the cytoplasmic domain of CD4. The membrane topology of Vpu is similar to that of type III transmembrane proteins (von Heijne 1988; Pinto et al. 1992). Mutational analyses have shown that the minimal Vpu-responsive element (*VRE*) maps to a six-amino acid region (*boxed*) in the CD4 cytoplasmic domain (Vincent et al. 1993; Chen et al. 1993; Lindberg and Landau 1993). *ER*, enodoplasmic reticulum

recognized by the Vpu protein with equal efficiencies in the ER membrane. It is intriguing that the two chimeric proteins CD8/cyto and CD8/CD4 underwent Vpu-dependent degradation in the ER at similar kinetics. Both proteins have conserved amino acids (e.g., the trileucine, LLL, motif) in their anchor domains, and it is likely that the trileucine motif might constitute an important Vpu recognition sequence in the ER membrane. Furthermore, we cannot rule out the possibility that other regions of the CD4 anchor domain might be involved in the initial recognition event between CD4 and Vpu. It is interesting to note that the gp160 anchor domain does not possess the trileucine motif, and envelope glycoproteins bearing this domain did not undergo degradation in cells expressing Vpu (Raja et al. 1994). Furthermore, glycosylation does not play a role in the Vpu-mediated degradation process, as both glycosylated and unglycosylated CD4 underwent proteolysis in cells expressing Vpu (Willey et al. 1992a).

Recent biochemical analyses have also revealed that the HIV-1 Vpu protein requires both the CD4 transmembrane (anchor) and cytoplasmic domains to induce the degradation of VSV G-CD4 hybrids in the ER (Buonocore et al. 1994). For example, the hybrid protein comprising the VSV G ectodomain, the anchor and cytoplasmic domains of CD4 was sensitive to the Vpu protein and underwent rapid proteolysis in the presence of Vpu. In contrast, the chimeric protein bearing the extracellular-anchor domains of VSV G and the full length CD4 tail was stable in cells expressing Vpu (Buonocore et al. 1994). Thus, this conclusion is consistent with the study of gp160/CD4 chimeric envelope proteins in the presence of Vpu (Raja et al. 1994).

The CD4 molecule plays an important role in immunoregulation of the host (Weiss and Littman 1994) and is also the major receptor for HIV (Dalgleish et al. 1984; Klatzman et al.1984; Maddon et al. 1986; McDougal et al. 1986). CD4 undergoes internalization in response to mitogenic (e.g., PMA) or antigenic stimulation (Acres et al. 1986; Shin et al. 1990). The phorbol myristate acetate (PMA)-induced internalization and subsequent degradation of CD4 in the lysosome requires a sequence element in the CD4 cytoplasmic tail (Shin et al. 1991). In an attempt to understand the role of phosphorylation in the Vpu-mediated degradation process, CD4 mutants defective in phosphorylation were tested for their stability in the presence of Vpu. Both wild-type and mutant CD4 molecules underwent degradation in cells expressing Vpu, suggesting that the phosphorylation of CD4 was not critically important for Vpu-dependent proteolysis in the ER (Lenberg and Landau 1993). These results are not surprising, as protein kinase C (PKC)-mediated phosphorylation of CD4 occurs at or very close to the plasma membrane and Vpu induces degradation of CD4 in the ER compartment. There is no evidence for the phosphorylation of CD4 trapped in the ER. CK-2 phosphorylates the two seryl residues in the cytoplasmic domain of Vpu, and this phosphorylation event is absolutely necessary to induce degradation of CD4 in the ER (Schubert and Strebel 1994). In addition, a relatively conserved region at the C terminus of the Vpu protein has been shown to be critical for Vpu-mediated proteolysis of CD4 in vitro, as deletion of the six C-terminal amino acids had completely inactivated the Vpu protein (Chen et al. 1993). It is possible that this region plays a role in oligomerization of Vpu or in other structural features important for the functional reconstitution of Vpu in vitro. It will be interesting to study the effect of this deletion on virus release and CD4 degradation functions of the Vpu protein in the mammalian cell.

We and others have demonstrated that the CD4 ectodomain did not contribute appreciably to the Vpu-dependent degradation process (Vincent et al. 1993; Lenberg and Landau 1993; Willey et al. 1994). However, the degradation kinetic rate was dependent on the nature of ectodomains to which the CD4 anchor and cytoplasmic domains were appended. Our analysis had shown that the gp160/CD4 chimeric protein E-TC38 exhibited a half-life of 60–90 min in cells expressing Vpu (Fig. 3A), whereas CD4 underwent rapid degradation (half-life, 12 min) in the presence of Vpu (Willey et al. 1992a). The CD8/CD4 chimeric proteins exhibited relatively long half-lives (4 h) in cells expressing Vpu. This was perhaps due to the

highly stable nature of CD8, which has the turnover rate of 50–60 h in the cell (Willey et al. 1994). We envisage that the syncytium inhibition assay developed in our study could be used to screen small molecules that antagonize the activity of Vpu in mammalian cells. For example, the gp160/CD4 chimeric protein E-TC38 failed to induce syncytia in the presence of Vpu, and an inhibitor or inhibitors of Vpu could potentially restore the ability of E-TC38 to form syncytia in cells expressing Vpu. Recently, Chen et al. (1993) demonstrated that Vpu induces degradation of CD4 in the presence of canine microsomal membranes, and the in vitro degradation assay will be useful to dissect cellular components that are involved in Vpu-mediated proteolysis of CD4 in the ER.

5 Perspectives

The preponderance of evidence suggests that Vpu is a bifunctional protein having a number of cellular targets in mammalian cells (Fig. 4). One of the functions of the Vpu protein is related to its activity in inducing degradation of CD4 in the cell. This activity of Vpu is sequence specific, requiring both the anchor and cytoplasmic domains of CD4, and it is also compartment specific (ER). The other activity of Vpu relates to its function in the release of virus particles from infected cells and is independent of the ER degradation reaction (gp 160 and CD4 expression independent).

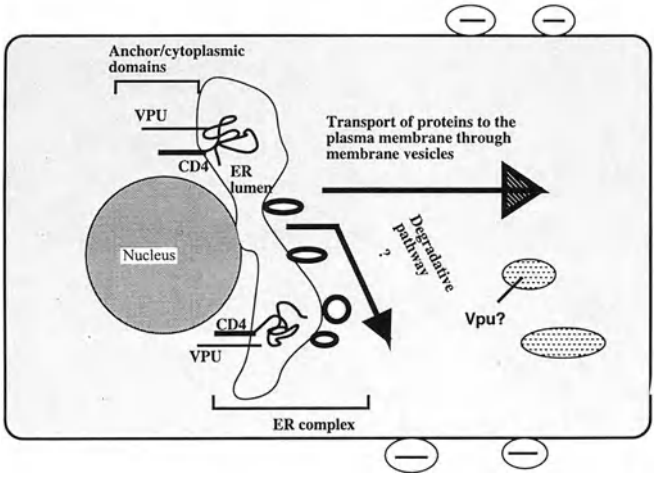

Fig. 4. A model for the role of Vpu in CD4 proteolysis and virus release. The Vpu protein induces degradation of CD4 in the endoplasmic reticulum (ER; Willey et al. 1992b). The Vpu-mediated degradation process has been shown to be sequence and compartment specific (Willey et al. 1992a; Vincent et al. 1993; Lindberg and Landau 1993). The Vpu protein also enhances the release of virus particles ⬭ from infected cells by an unknown mechanism. This activity of Vpu appears to be revealed in a distinct cellular compartment or compartments ⬭ other than the ER (Schubert and Strebel 1994)

Even though the sequence requirements for the Vpu-dependent degradation process are fairly well known, the mechanism by which the Vpu protein targets CD4 to a degradation pathway in the ER is not clearly understood at the present time. We can envisage a number of scenarios. For example, Vpu could selectively target CD4 to a microenvironment of the ER where it becomes susceptible to ER-specific proteases (selective targeting mechanism). Alternatively, the interaction between CD4 and Vpu might activate a proteolytic system which selectively degrades CD4 in the ER membrane (selective degradation mechanism). It is clear that the Vpu protein is not a protease, but it could recruit a protease or proteases to the site of Vpu–CD4 interactions at the ER membrane (protease-targeting mechanism).

The ER has long been recognized as the major folding compartment in the cell. Misfolded or unassembled proteins are prevented from reaching the cell surface by regulatory mechanisms that operate in the ER compartment (Gething and Sambrook 1992). For example, one of the major, soluble ER proteins, Ig heavy chain binding protein, (BiP) appears to have roles in the assembly of proteins in the ER lumen. Misfolded proteins are precipitated as aggregates and the majority of them have been shown to exist as BiP–protein complexes in the ER (Gething and Sambrook 1992). Thus, the ER of eukaryotes serves as a quality control check point ever vigilant in monitoring the status of environmental changes during the flux of proteins in the secretory pathway. Any abnormal folding of proteins is quickly sensed so that they could be retained for eventual degradation in the ER (Hurtley and Helenius 1987; Doms et al. 1993). Recent studies on the T cell receptor complex (TCR) subunits have revealed sequence-specific degradation of the α- or β-subunits in the ER (Bonifacino et al. 1991; Klausner and Sitia 1990; Shin et al. 1993; Young et al. 1993). Thus, the ER has also emerged as a major membrane compartment in which proteolytic systems are abundantly present. However, the proteolytic system that targets CD4 for degradation in cells expressing Vpu is presently unknown.

Furthermore, the mechanism of the Vpu effect on the release of virus particles is not clearly defined. Recent experiments have elucidated that this effect is independent of both gp 160 and CD4 expression (Yao et al. 1992; Geraghety and Panganiban 1993). It has been shown that Vpu-mediated enhancement of virus release is not specific to HIV-1, as viruses as divergent as visna virus, HIV-2, and Mo-MuLV are efficiently released from cells expressing functional Vpu (Gottliger et al. 1993). These experiments have suggested that the Vpu protein affects a common cellular pathway utilized by many retroviruses for assembly and morphogenesis. The Vpu protein is structurally related to the M2 protein of influenza viruses. M2 is an ion channel protein which appears to protect influenza virus hemagglutinin (HA) from undergoing acid-induced conformational changes in the *trans*-Golgi network of the secretory pathway (Pinto et al. 1992; Takeuchi and Lamb 1994; Holsinger et al. 1994). There is no evidence so far to suggest that the Vpu protein possesses ion channel activity. Clearly, the systematic analysis of Vpu would reveal novel modes of action of this unique protein in the HIV life cycle and CD4 downregulation.

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Viral Protein X

J.C. KAPPES

1 Introduction

Since the discovery of the human immunodeficiency viruses types 1 (HIV-1) and 2 (HIV-2) as the causative agents of acquired immunodeficiency syndrome (AIDS) (BARRE-SINOUSSI et al. 1983; CLAVEL et al. 1987; PAPOVIC et al. 1984) and the isolation of related lentiviruses from several species of Old World monkeys, much has been learned about their biology and evolution. Based on genetic analysis, five distinct phylogenetic lineages of primate lentiviruses have now been identified: HIV-1/simian immunodeficiency virus $(SIV)_{CPZ}$, HIV-2/SIV_{SM}/SIV_{MAC}, SIV_{AGM}, SIV_{MND}, and SIV_{SYK} (for review see JOHNSON et al. 1991; MYERS et al. 1992; SHARP et al. 1994). Like all retroviruses, primate lentiviruses are similar in structure and genomic organization. They contain *gag*, *pol*, and *env* genes that encode structural proteins essential for virion architecture (Gag), glycoproteins that enable the virus to recognize and infect its target (Env), and enzymatic proteins essential for processes of virus maturation, reverse transcription, and integration (Pol). While the genomic structure of all five lineages is conserved, differences exist in the composition of auxiliary genes: all five groups of viruses contain *tat*, *rev*, *nef*, and *vif*, all but SIV_{AGM} contain *vpr*, only HIV-1/SIV_{SYK} contains *vpu*, and only HIV-2/SIV_{SM}/SIV_{MAC} and SIV_{AGM} viruses contain *vpx* (CULLEN and GREEN 1990; JOHNSON et al. 1991). Primate lentiviruses have complex life cycles regulated by interactions of viral structural and accessory gene products as well as host factors (CULLEN

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1991). Viral accessory genes modulate replication, host cell tropism, chronic persistence, and pathogenicity. Defining the role of viral accessory genes is critical for understanding the natural history of infection and disease pathogenesis and thus represents a major goal in biomedical AIDS research. Moreover, knowledge of accessory protein function may facilitate the development of therapeutic and vaccine interventions. In this chapter the author reviews the present understanding of the role of Vpx in the HIV-2/SIV life cycle and the structure–function relationships that mediate incorporation of this protein into virions. Based on genetic and phylogenetic evidence indicating that in the HIV-2/SIV_{SM}/SIV_{MAC} lineage *vpx* arose from a gene duplication of *vpr* and on the proposed reclassification of SIV_{AGM} *vpx* to *vpr* (Tristem et al. 1990, 1992), for clarity this review will discuss *vpx* for only the HIV-2/SIV_{SM}/SIV_{MAC} group of viruses.

2 Association of Vpx with Virions

Viral protein X (Vpx) is a virion-associated protein of 14–16 kDa that is expressed in vitro and in vivo (Franchini et al. 1988; Henderson et al. 1988a; Kappes et al. 1988; Venet et al. 1992; Yu et al. 1988). The *vpx* coding region is located in the central region of the viral genome between *vif* and *vpr*, partially overlapping *vif* on its 5′ end (Chakrabarti et al. 1987; Franchini et al. 1987; Guyader et al. 1987). (Fig. 1). It predicts a protein of 112 amino acids and is likely encoded from a singly spliced mRNA whose expression is regulated by *rev* (Viglianti et al. 1990). Vpx was first identified when it was purified by high-performance liquid chromatography (HPLC) from sucrose-banded preparations of SIV_{MAC} (Mne strain) and characterized by N- and C-terminal amino acid sequence analysis (Henderson et al. 1988a,b). Comparison of its amino acid sequence with nucleotide sequences in the genomes of HIV-2 and SIV_{MAC} confirmed its viral origin. The *vpx* gene was also

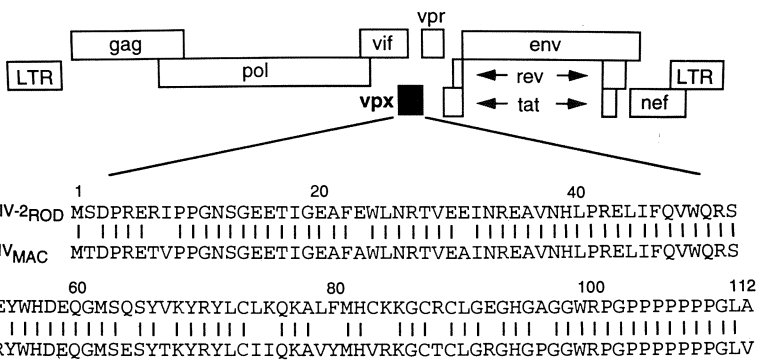

Fig. 1. Genomic organization of human immunodeficiency virus (HIV)-2/simian immunodeficiency virus $(SIV)_{MAC}$/SIV_{SM} viruses and alignment of the Vpx amino acid sequences of $HIV\text{-}2_{ROD}$ and SIV_{MAC}. *Vertical bars* indicate identity. *LTR*, long terminal repeat

shown to represent a functional gene by immunologic detection of its protein product in infected cells and purified virions (Franchini et al. 1988; Kappes et al. 1988; Yu et al. 1988). Quantitative amino acid analysis of HPLC-purified SIV_{MAC} proteins demonstrated that Vpx is present in virions in molar amounts approximately equivalent to Gag proteins (Henderson et al. 1988 b). Using semiquantitative methods, a similar relationship for virion-associated Vpx has been demonstrated for HIV-2 (Henderson et al. 1989b; Wu et al. 1994).

Although the abundance of Vpx in the virus particle could suggest that it plays an important structural role, electron microscopy studies of HIV-2-infected lymphocytes indicated there are no morphologic or ultrastructural differences between wild-type and Vpx-deficient virions (Kappes et al. 1991). Vpx mutant viruses were shown to be normal in size and exhibited mature core structures and glycoprotein spikes recognizable on their envelope surfaces. Furthermore, there were no apparent differences in budding virus structures associated with infected cells. The location of Vpx within the virus particle has been examined by analyzing the protein contents of SIV_{MAC} cores purified after destroying the envelope with detergent (Yu et al. 1993). In contrast to intact virions, substantially less core-associated Vpx was detected relative to p27 Gag. The concomitant loss of matrix (MA) protein from purified cores suggested that Vpx localizes predominately between the virus core and envelope. Incomplete loss of Vpx from purified SIV cores, and the demonstration that Vpx has single-stranded nucleic acid-binding properties in vitro (Henderson et al. 1988b), suggests that Vpx could also localize within the core in association with viral RNA. Additional studies are necessary to further define the subvirion location of Vpx. Figure 2 depicts the structure of a mature HIV-2 virion, showing the localization of its protein constituents, including that suggested for Vpx (for review see reference Gelderblom 1991).

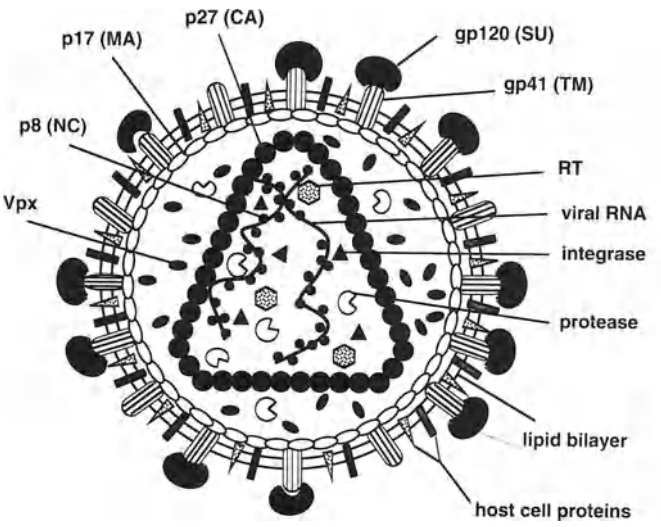

Fig. 2. The human/simian immunodeficiency (HIV/SIV) virion. The organization of the major structural proteins of a mature virion is shown. The subvirion location of Vpr and p6 Gag is presently unknown

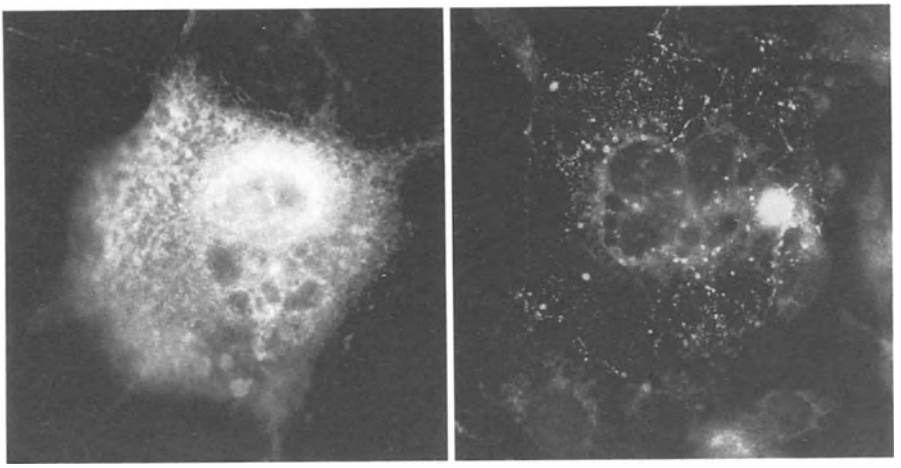

Fig. 3. Immunofluorescence microscopy of Vpx. The Vpx protein is stained within Cos-1 cells following transfection with replication-competent HIV-2 proviral DNA (*left panel*) and a eukaryotic Vpx expression plasmid (*right panel*)

3 Molecular Analysis of Vpx Incorporation into Virions

Unlike Gag, Pol, and Env proteins, Vpx is not synthesized and packaged into virions as part of a larger polyprotein precursor and therefore requires additional mechanisms to facilitate its incorporation. Early studies directed toward understanding the mechanisms involved in Vpx packaging revealed that in HIV-2-infected cells Vpx associated predominantly with the plasma membrane (Franchini et al. 1988; Kappes et al. 1993). Immunofluorescence microscopy localized Vpx on the plasma membrane in a punctate staining pattern similar to Gag (Kappes et al. 1993). In marked contrast, when Vpx was transiently expressed without other viral proteins it was not exported from cells (Horton et al. 1994) and did not localize in a punctate pattern, but rather was found to distribute broadly throughout the cell (Fig. 3) (Horton et al. 1994; Kappes et al. 1993; Wu et al. 1994).Together, these results indicate that Vpx requires the expression of other viral components for localization on the inner surface of the plasma membrane, at sites of virus assembly. Convincing evidence for this hypothesis was provided by *trans*-complementation experiments in which coexpression with HIV-2 vpx-mutant proviral DNA was shown to rescue Vpx surface targeting and colocalization with Gag (Kappes et al. 1993) (Fig. 4). Moreover, coexpression resulted in the incorporation of Vpx into progeny virions in amounts similar to that found in virus derived from productively infected cells. Identical *trans*-complementation experiments conducted with HIV-1 proviral DNA demonstrated the lack of Vpx surface localization and virion incorporation (Kappes et al. 1993). These results indicate that the incorporation of Vpx into virions is regulated by the expression of virus type-specific components.

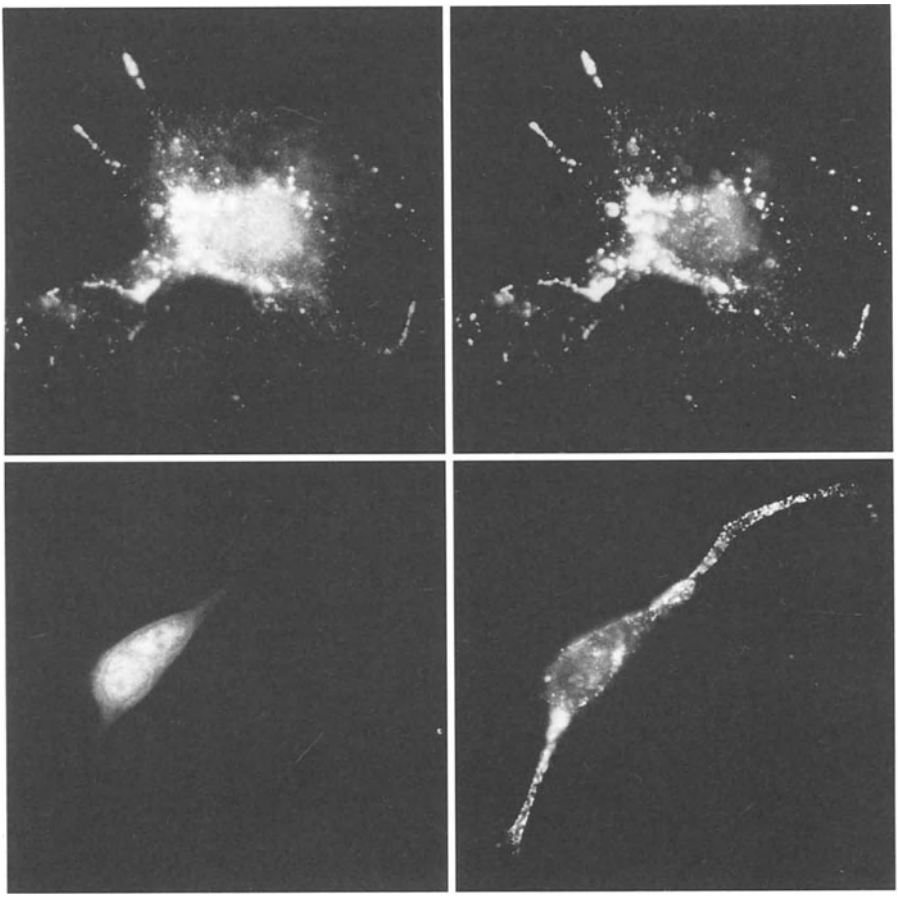

Fig. 4. Double-staining immunofluorescence microscopy of Vpx expressed in trans with HIV-2 and HIV-1. Vpx (*left panels*) and Gag (*right panels*) proteins are stained within the same cell after cotransfection with either HIV-2 vpx-mutant (*upper panels*) or HIV-1 (*lower panels*) proviral DNAs

HIV-1 does not contain a *vpx* coding region. While its genomic organization is similar to that of HIV-2, overall there is only 45%–50% identity in amino acid sequence (Ratner et al. 1985; Wain-Hobson et al. 1985), and structures responsible for the differential ability of HIV-1 and HIV-2 to efficiently package Vpx are not obvious. Similarities in Vpx and Gag surface localization, constant ratios of Vpx and p27 Gag detected on immunoblots of purified virions, regardless of whether Vpx was expressed in cis or in trans (Kappes et al. 1993), and the central role of Gag in virus assembly all suggested that the Gag precursor protein is directly involved in Vpx localization and packaging. The role of Gag in mediating incorporation of Vpx into virions has been investigated using transient T7 expression systems that enable efficient Gag expression, assembly, and extracellular release of virus-like particles (VLP) (Dong and Hunter 1993: Fuerst et al. 1986, 1987). Similar to the

findings reported using HIV-2 proviral DNA, Wu et al. demonstrated that expression of Gag alone is sufficient to induce localized cell surface expression of Vpx (Wu et al. 1994). Furthermore, association of Vpx with VLPs released into the culture medium indicated that Gag is directly responsible for incorporation of Vpx into virions (Horton et al. 1994; Wu et al. 1994).

By analyzing extracellular VLPs produced by expressing Gag deletion mutants for their capabilities to associate with Vpx, the NC region and 26 C-terminal residues of p6 were shown to be dispensable for Vpx packaging. Deletion of the entire p6 region, however, abolished the association of Vpx with VLPs, implicating a linear sequence of 59 amino acids downstream of NC (residues' 439–497) (Kumar et al. 1990) in Vpx packaging (Wu et al. 1994). The inability of Vpx to incorporate efficiently into HIV-1 (Kappes et al. 1993; Paxton et al. 1993) and VLPs produced by expressing HIV-1 Gag (Lu et al. 1993; Wu et al. 1994) provided the opportunity to investigate whether C-terminal residues contained the sole determinants necessary for interaction of Gag with Vpx. VLPs produced by expressing an HIV-2/HIV-1 gag chimera containing the coding regions for p17 and p27 of HIV-2 fused to HIV-1 p15 did not efficiently associate with Vpx. However, VLPs produced from chimeras containing p17 and p24 of HIV-1 fused to C-terminal HIV-2 Gag sequences associated with Vpx in amounts similar to HIV-2 virus (Wu et al. 1994). These results demonstrate that HIV-2 Gag mediates the incorporation of Vpx into virions and suggest the virus type-specific signal that induces packaging is located downstream of NC, within residues' 439–497 (Fig. 5).

Although these results implicate Gag for mediating incorporation of Vpx into virions, the exact mechanism by which this may occur is not clear. Direct interaction of Vpx and Gag has been suggested by experiments demonstrating anti-Vpx antibody coprecipitates p55 Gag (Horton et al. 1994). Analysis of VLPs consisting of mature Gag proteins (derived by expressing gag–pol) indicated coprecipitation of capsid (CA) with anti-Vpx antibody (Horton et al. 1994). While

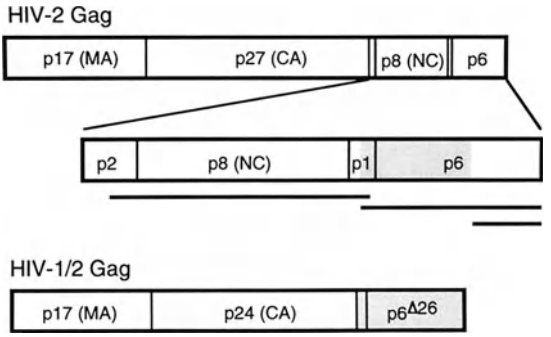

Fig. 5. The basic structure of the HIV-2 Gag polyprotein. Natural proteolytic cleavage sites and corresponding protein products of the Gag precursor are shown (Henderson et al. 1988a). C-Terminal deletion mutants are indicated by *blackened bars*. The region implicated to be important for Vpx packaging is *shaded*. The structure of the HIV-1/HIV-2 Gag capable of incorporating Vpx into virus-like particles (VLP) is also illustrated. The C-terminal HIV-2 region shown to be important for incorporation of Vpx into HIV-2 VLP (*shaded*) is shown fused to the C-terminus of p24

this result suggests binding of CA and Vpx within virions, the relevance to Vpx packaging is not clear, especially in light of data demonstrating that turncation of residues downstreams of CA abolishes incorporation of VPX into VLPs. One possible explanation for these findings could be that incorporation of Vpx into VLPs is mediated by amino acids in the C-terminus of p55 Gag and only subsequent to assembly and maturation do Vpx and CA associate. It is also possible that association with CA is mediated through interaction with other viral and cellular proteins. Experiments demonstrating Vpx does not associate with purified virus cores seems to further suggest that it does not directly bind to CA protein (Yu et al. 1993). Additional molecular genetic studies are likely to help understand the mechanisms and structural determinants that mediate Vpx Gag interaction.

The Vpx sequence has also been studied to identify structures that may be important for interaction with Gag. Computerized secondary structure analysis of HIV-2_{ST} Vpx predicted the existence of a strong amphipathic helix between residues 20 and 40 (Fig.6). This amphipathic helix is highly conserved between phylogenetically divergent HIV-2 and SIV *vpx* sequences (KAPPES et al. 1993) and by comparison with other known amphipathic helices has a relatively strong hydrophobic moment of 0.56 using the algorithm reported by JONES et al. (1992). Amphipathic helices are commonly observed structural motifs in both globular and lipid-binding proteins and are involved in protein–protein and protein–lipid interactions (SEGREST et al. 1990). The identification of a highly conserved predicted amphipathic helix in the Vpx protein raises the possibility that this region may be important for Vpx packaging. Studies mapping the requirements of Vpx for virion incorporation demonstrated that residues 20–68 are sufficient (WU et al. 1993) and support the notion that this predicted structure represents an important determinant for incorporation of Vpx into virions.

	20	40
HIV-2_{ST}	E**A**F**E**W**L**DR**T**V**E**A**I**N**R**E**A**VN**H**L
HIV-2_{ROD}	E**A**F**A**W**L**NR**T**V**E**A**I**N**R**E**A**VN**H**L
SIV_{SMPBJ}	E**A**F**D**W**L**DR**T**V**E**E**I**N**R**A**A**VN**H**L
SIV_{MAC251}	P**W**D**E**W**L**RD**M**I**E**D**I**N**Q**E**A**KM**H**F

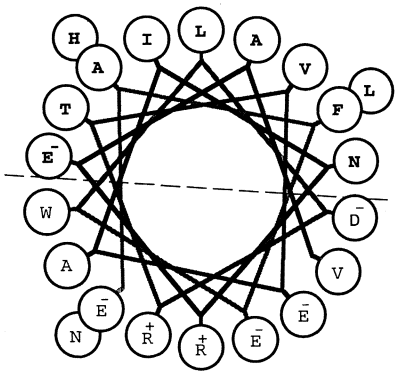

Fig 6. Computerized secondary structure analysis of the Vpx amino acid sequence. Vpx residues 20-40 are compared for human immunodeficiency virus (HIV)-2_{ST}, HIV-2_{ROD}, simian immunodeficiency virus $(SIV)_{SMPBJ}$, and SIV_{MAC251}. Conserved amino acid residues on the hydrophobic phase are in *boldface*. A helical wheel diagram of the predicted Vpx amphipathic helix of HIV-2_{ST} is presented. The wheel is a two- dimensional projection along the axis of the helix which is orientated so that the hydrophobic moment vector points to the top of the page, above the *dashed line*

4 Vpx Augments Virus Infectivity

Studies analyzing the effects of Vpx on virus replication in vitro suggest an important function in vivo. In immortalized $CD4^+$ T cell lines, vpx-mutant and wild-type viruses are equally infectious, exhibit similar replication kinetics, and produce comparable cytopathic effects. (Akari et al. 1992; Guyader et al. 1989; Hu et al. 1989; Kappes et al. 1991; Marcon et al. 1991; Shibata et al. 1990; Yu et al. 1991). In primary human peripheral blood mononuclear cells (PBMC) and lymphocytes, however, HIV-2 vpx-mutant viruses displayed a defect in replication that was most pronounced at low virus inoculums (Guyader et al. 1989; Kappes et al. 1991). To establish productive infection with Vpx-deficient HIV-2 in PBMC two to three orders of magnitude more virus was required (Kappes et al. 1991). The demonstration of reduced levels of viral DNA in PBMC newly infected (within a single round of replication) with vpx-deficient HIV-2 suggested a function for Vpx early in the virus replication cycle (Kappes et al. 1991). Normal protein composition, morphology, and ultrastructure of mutant HIV-2 virions supported this conclusion (Kappes. et al. 1991).

Although it is generally accepted that *vpx* augments virus replication in PBMC, a defect in the repication of mutant viruses is not always observed between experiments and may, in part, be influenced by the donor cells used as targets for infection (Akari et al. 1992; Hu et al. 1989; Marcon et al. 1991; Yu et al. 1991). The importance of *vpx* for virus replication in macrophages is not clear. While mutations in the *vpx* reading frame of SIV_{MAC} cause significant decreases in virus production in primary cultures of human and rhesus monkey alveolar macrophages (Gibbs et al. 1994; Yu et al. 1991); HIV-2 mutants do not appear to be impaired in replication in human macrophages or in the monocytic line U937 (Guyader et al. 1989; Hu et al. 1989; Marcon et al. 1991; Yu et al. 1991). As the complex nature by which HIV established infection in T lymphocytes and monocytes/macrophages becomes more apparent, it is possible to speculate on many distinct ways by which Vpx may facilitate virus replication. The presence of Vpx in the virion, the demonstration that it augments virus infectivity, its strong nucleophilic properties (Wu and Kappes 1993) and its association with Gag may indicate that the function of Vpx early in the virus replication cycle involves stabilization or targeting of the viral ribonucleoprotein complex.

The significance in the variation of vpx-mutant viruses to replicate in PBMC and macrophages among studies is not known. Excluding possible differences in experimental design among laboratories, the molecularly cloned virus itself is likely to influence the mutant phenotype (Sharp et al. 1994). All the biological studies on Vpx report results obtained using clones adapted to tissue culture by multiple passage and selection in immortalized T cell lines. In nearly every case, these proviruses contain mutations in other genes and/or regulatory elements (Chakrabarti et al. 1987; Guyader et al. 1987; Horton et al. 1994; Hu et al. 1989; Kappes et al. 1991). Genetic differences in molecular clones of HIV and SIV are known to affect growth characteristics, host cell tropisms, and replication properties in various macrophage/monocyte and T cell targets. Especially since

vpx serves an accessory function, it is likely to exert differing effects on the replication of viruses with distinct genetic and biological phenotypes. To more completely understand the importance of *vpx* in the virus replication cycle, studies need to be conducted using relevant viruses.

Although dispensable for replication in vitro, it is likely that *vpx* plays an important role in viral replication in vivo. Chronic viral persistence in humans and animals implicates the existence of only partially effective host immunity, but also undoubtedly reflects regulated expression of a complex lentivirus genome (Cullen 1991). The balance between viral replication and chronic persistence is orchestrated by complex interactions between viral structure and auxiliary gene products as well as host factors. In vivo, *vpx* may be important for HIV-2/SIV persistence by facilitating infection and spread during the natural course of infection, particularly during periods of low viral burden. Two biologically active SIV clones, SIV_{SMPBJ} and $SIV_{MAC-239}$, are now available and should allow for a more definitive understanding for the role of Vpx in replication and pathogenesis.

5 Genetic and Functional Similarities of Vpx and Vpr

Vpx is remarkably similar in both function and structure to Vpr. Based on sequence similarities of prototypic primate lentiviral Vpx and Vpr proteins and their adjacent position in the genomes of viruses in which both genes are found, it has been suggested that *vpx* arose by a gene duplication of *vpr* (Tristem et al. 1990, 1992). Vpr is a 15-kDa, virion-associated accessory protein (Cohen et al. 1990a,b). It is synthesized from a singly spliced mRNA late in infection (Arrigo and Chen 1991; Garette et al. 1991) and requires p6 Gag for incorporation into virions (Lavallee et al. 1994; Lu et al. 1993; Paxton et al. 1993). Reports on the effects of *vpr* on virus replication in vitro are not conclusive. There is conflicting data on its importance for virus replication in both immortalized T cell lines and macrophages (Cohen et al. 1990b; Dedera et al. 1989; Hattori et al. 1990; Ogawa et al. 1989; Westervelt et al. 1992). Computer analysis of the secondary structure of all HIV/SIV Vpr proteins also predicts a highly conserved amphipathic helix positioned near the N terminus that is highly conserved with that described above for Vpx. Despite these similarities, it is unlikely that *vpx* and *vpr* have analogous functions since both are retained in the genomes of HIV-2/SIV_{MAC}/SIV_{MN} viruses.

Elucidating *vpx* function and its role in pathogenesis may be of special importance since it likely represents a retroviral gene that has only recently evolved for adaptation of the virus to its host (Segrest et al. 1990; Tristem et al. 1992). Knowledge of the mechanisms that facilitate targeting of Vpx to the virion will contribute to our understanding of lentivirus assembly and packaging of virion-associated accessory proteins. Moreover, understanding the molecular mechanisms that mediate incorporation of Vpx and Vpr into virions may reveal novel strategies that will enable these proteins to be used (and by analogy Vpr) as

vehicles for novel capsid-targeted viral inactivation strategies (Kappes et al. 1993; Natsoulis and Boeke 1991).

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The Pathogenic Role of Human Immunodeficiency Virus Accessory Genes in Transgenic Mice

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1 Introduction

Human immunodeficiency virus (HIV) type 1, the causative agent of acquired immune deficiency syndrome (AIDS), was isolated almost a decade ago (Barré-Sinoussi et al. 1983; Popovic et al. 1984). HIV infection is mediated by the binding of the virus to the CD4 cell surface molecule and subsequent viral penetration (Dalgleish et al. 1984; Klatzmann et al. 1984; McDougal et al. 1986). Cells known to bear CD4 include the T-helper/inducer cells (Terhorst et al. 1980), immature thymocytes (Schnittman et al. 1990), Epstein-Barr virus (EBV)$^+$ B lymphoblastoid cells (Dalgleish et al. 1984; Salahuddin et al. 1987; Monroe et al. 1988), monocytes/macrophages (Wood et al. 1983; Dalgleish et al. 1984), follicular dendritic cells (Wood et al. 1983; Macatonia et al. 1990), Langerhans cells (Wood et al. 1983; Tschachler et al. 1987), astrocytes (Funke et al. 1987), neurons (Funke et al. 1987), and glomerular mesangial cells (Karlsson-Parra et al. 1989).

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A hallmark of HIV infection in humans is the functional depletion of the $CD4^+$ T-helper cells (Gottlieb et al. 1981; Rosenberg and Fauci 1991). The T-helper lymphocyte plays a critical role in controlling the immune response by communicating with other cells of this immune system via lymphokines and/or cell surface signaling. The loss of this function creates an immunodepressed state that may allow other pathogens to cause opportunistic diseases such as *Pneumocystis carinii* pneumonia (Gottlieb et al. 1981). HIV infection has also been shown to cause or to be associated with several other disorders, including Kaposi's sarcoma (KS; Mitsuyasu 1988; Boyle et al. 1993), dementia (Price et al. 1988; Merrill and Chen 1991), non-Hodgkin's lymphoma (Karp and Broder 1991; McGrath et al. 1991), and nephropathy (Cohen and Nast 1988; Bourgoignie and Pardo 1991).

HIV is a retrovirus belonging to the lentivirus (or "slow virus") genus with a clinical latency of up to several years (Lui et al. 1988; Bacchetti and Moss 1989). In addition to the structural genes *gag*, *pol*, and *env* found in all retroviruses, HIV contains at least eight other genes (Fig. 1). Of the nonstructural genes, the regulatory genes *tat* and *rev* are essential for viral replication (Sadaie et al. 1988), while the roles of *vpr*, *vpu*, *vif*, *tev/tnv*, *vpt*, and *nef* are not yet fully understood (Haseltine 1991). The complex interactions between the viral proteins and the cellular machinery may interfere with normal processes, thereby potentiating a pathogenic response. An animal model containing all, some, or even one of the viral genes that can produce clinical disease may help to determine the molecular pathways leading to disease and eventually to the design of effective antiviral therapies.

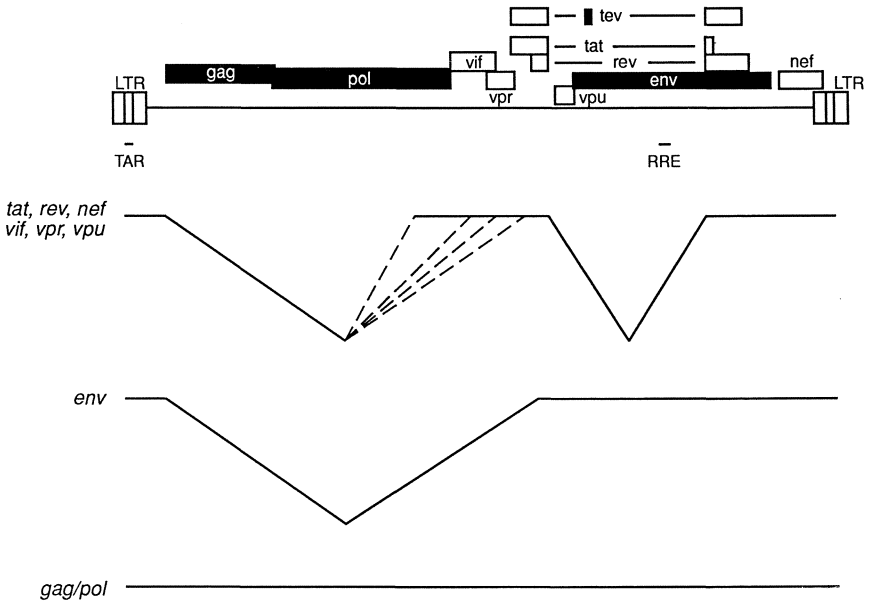

Fig. 1. Human immunodeficiency virus (HIV)-1 genome and major mRNA transcripts. *LTR*, long terminal repeat; *TAR*, *trans*-acting response element

2 Need for an Animal Model of HIV Infection and Pathogenesis

In most viral research, viruses can be propagated by infecting an appropriate cell line where certain aspects of viral reproduction can be studied. However, determining the specific immune reaction to a virus and the pathogenesis of viral-induced diseases requires the complex cellular and organismal responses of an in vivo system. Clearly, whole animals are essential experimental models for viral research.

An infectible animal, i.e., one that can support virus replication, can help determine the possible routes of viral transmission. For example, one can readily address the following critical questions: Is the virus transmitted as an air-borne pathogen, by casual contact, through an insect vector, or by exposure to bodily fluids? If transmission occurs through bodily products, is it transmissible via tissue, saliva, tears, blood, milk, or semen? Can the virus be passed vertically from mother to child? Additionally, analyses of the various tissues of an experimental animal can help determine which cell types are susceptible to infection and permissive for viral replication.

Infectible animals may also be used to develop vaccine strategies to prevent the various modes of virus uptake. Animals that are not only infectible but develop disease as a consequence of viral infection can help to define the associated disease(s) and are often invaluable for elucidating the pathogenic mechanism involved. Animals that show a specific disease phenotype may also be excellent candidates for trials of specific antiviral therapies. Many species of animals have been used successfully as models for human viral disease. What follows is a discussion of the merits and pitfalls of each of these systems with respect to HIV-1.

2.1 Great Apes

The great apes include humans, chimpanzees, gorillas, and gibbons. The closest primate to humans phylogenetically is the chimpanzee. The chimpanzee and the gibbon are endangered species and their use in medical research is limited by availability, concerns for their safety and welfare, and exorbitant costs. However, despite these potential drawbacks, both the chimpanzee and the gibbon can be infected by HIV-1 and have proven to be useful models for studying the biology of the virus.

2.1.1 Chimpanzee

The chimpanzee has been shown to be infectible by several HIV isolates (Eichberg et al. 1987; Nara et al. 1987). Infection can be achieved intravenously with cell-free viral particles, virally infected cells (cell-associated), or tissue homogenates (Fultz 1991), through application onto the vaginal mucosa but not the oral mucosa (Fultz et al. 1986a), or in utero from mother to offspring (Eichberg et al. 1988).

Additionally, casual contact of cagemates has not resulted in transmission from animal to animal (Fultz et al. 1986b). Clearly, the chimpanzee has been extremely useful in defining the various potential modes of horizontal as well as vertical transmission.

Viral particles can be transiently recovered from the plasma of infected chimpanzees for a period of up to 6 weeks and persistently by cocultivation of their peripheral blood mononuclear cells (PBMC) with a susceptible human cell line (Fultz et al. 1986a; Nara et al. 1989). Attempts to recover virus from the cerebrospinal fluid, saliva, or from monocytes/macrophage of infected chimpanzees have been unsuccessful, in contrast to human infection, in which virus is readily recovered from all three (Nara et al. 1989).

Shortly after infection, a low percentage of inoculated chimpanzees show signs of an acute response, including transient lymphadenopathy, thrombocytopenia, and hypergammaglobulinemia (Alter et al. 1984; Fultz et al. 1986b). The animals produce a strong and persistent antibody response to various HIV proteins that closely mimics the human response in degree, duration, and temporal incidence (Goudsmit et al. 1987). In addition to the humoral response, there is also a detectable cellular response; however, no appreciable change in $CD4^+$ numbers or evidence of immunosuppression in chimpanzees has been observed over a period of several years (Eichberg et al. 1987). It is speculated that the degree of immune response in the chimpanzee may be the key factor in suppressing the chronic pathological potential of HIV. To date, the chimpanzee model resembles the clinical picture of an asymptomatic human carrier, a situation which may limit its usefulness to the testing of vaccine strategies and to understanding the initial responses to viral infection.

2.1.2 Gibbon

The gibbon is the second great ape that has been shown to be infectible by an HIV isolate (Lusso et al. 1988). Its acute response, lymphadenopathy, is similar to that of the chimpanzee. Viral particles can also be recovered from gibbon PBMC for as long as 1 year after infection (Lusso et al. 1988). The immune response is both humoral and cellular, much like that of the chimpanzee. Overall, no significant difference between the chimpanzee and the gibbon with respect to the reactivity to HIV infection has been found. However, practically speaking, the gibbon is a more difficult model than the chimpanzee to study, since fewer are available for medical research and they are far more costly to acquire. Since the cost of maintaining chimpanzees and gibbons is nearly the same, the gibbon offers no distinct advantage over the chimpanzee model.

2.2 Old World Monkeys

Old World monkeys include the baboon, macaque, African green monkey, and colobus. After the great apes, the Old World monkeys are the next closest

genetic relative to man. The Old World monkeys also have an immune system that closely parallels both man and chimpanzee, which makes them a viable alternative to great apes in medical viral research.

2.2.1 Macaque

Of the Old World monkeys, only the macaque has been successfully infected with HIV-1 (Agy et al. 1992). At least five macaque species have been inoculated: rhesus, stumptail, cynomolgus, bonnet, and pigtail (Gajdusek et al. 1985; Fultz and Morrow 1987; Agy et al. 1992). Of these, only the pigtail macaque has been successfully and reproducibly infected with HIV-1 (Agy et al. 1992). Virus was recovered at various intervals up to 24 weeks from PBMC, while viral sequences were detected by polymerase chain reaction (PCR) for at least 1 year. All eight of the inoculated pigtail macaques showed signs of lymphadenopathy shortly after inoculation and a sustained antibody response. While pigtail PBMC were shown to be infected by cocultivation assays, no change in $CD4^+$ counts were observed through 80 weeks after inoculation. From these data, it appears that the pigtail macaque shows a consistent, acute clinical response but no other symptoms of HIV infection. These observations suggest that the pigtail macaque, like the chimpanzee and gibbon, may also be useful for trials of vaccine therapies but may not provide an appropriate model for HIV-associated pathogenesis.

2.3 Small Animals

Small animals are widely used in biomedical research owing to their ease of handling, relatively low costs of maintenance, and availability compared to the primates. Initial studies with small animals including rats, hamsters, guinea pigs, rabbits, musk shrews, and mice suggested that none were susceptible to HIV infection (Morrow et al. 1987). However, more recent studies showed that both rabbits and mice are susceptible to HIV infection under certain experimental conditions (Filice et al. 1988; Kulaga et al. 1989; Locardi et al. 1992).

2.3.1 Rabbit

Filice et al. (1988) demonstrated that rabbits were susceptible to HIV infection after inducing aseptic thioglycollate peritonitis and subsequently injecting infected cells intraperitoneally in the presence of interleukin-2 (IL-2). An antibody response to HIV antigens was detectable after 2 weeks and persisted for 1 year, as did circulating levels of the HIV p24 antigen. Following cocultivation of a susceptible human cell line with PBMC from infected rabbits, budding of type D viral particles, typical of HIV, was demonstrated by electron microscopy. Although viral particles were produced, the permissive cell type in the infected rabbit was not identified. Thioglycollate peritonitis predominately induces monocytes/macrophages, raising the possibility that these were the susceptible cell types.

In a second study, KULAGA et al. (1989) demonstrated that rabbits were susceptible to HIV infection when inoculated with human cells containing HIV proviruses without having to induce peritonitis or supplementing with IL-2. In this study, all of the infected rabbits seroconverted within 6 weeks. Viral particles were not demonstrated directly, but cultured peripheral blood lymphocytes contained HIV sequences as determined by PCR, showed reverse transcriptase (RT) activity, and formed syncytia after 2 weeks of growth in medium supplemented with IL-2. None of the animals developed any consistent disease other than diarrhea. It appears that the infectible cell type in the study by KULAGA et al. (1989) was the lymphocyte, whereas monocytes/macrophages were implicated previously (FILICE et al. 1988). It is obvious that further analyses regarding the permissive cell type, viral production, and any pathological effects are required to clarify the utility of the rabbit as an effective model for HIV infection.

2.3.2 Mouse

More recently, LOCARDI et al. (1992) showed mice to be infectible by intraperitoneal injections of infected human cells. The animals produced no acute response or long-term illness, but did maintain high antibody titers for 200-500 days, and 50% of the mice demonstrated p24 antigenemia. All the mice tested showed viral production from either PBMC, peritoneal cells, or splenocytes albeit, at very low levels. Furthermore, the mice contained PBMC and peritoneal cells that were positive for HIV as assayed by PCR and for RT activity. The infected cells tested negatively for human-specific sequences by PCR, suggesting that the infected cells were indeed of mouse origin.

To date, in vitro attempts to infect mouse cells have been unsuccessful (LANDAU et al. 1988). Even mouse cells transfected with the human CD4 gene were refractory to infection, suggesting that additional molecules may be needed for viral entry (MADDON et al. 1986). It may be likely that the reported HIV infection in mice was not CD4 mediated, but instead may have occurred through F_c-mediated endocytosis (TAKEDA et al. 1988; HOMSY et al. 1989). In addition, SPECTOR et al. (1990) and LUSSO et al. (1990) have shown that HIV may be pseudotyped and/or phenotypically mixed with a murine amphotropic retrovirus. In this scenario, the murine virus may have infected the inoculated human cells, phenotypically mixed with an HIV particle by providing additional surface proteins, and with its altered species tropism, the recombinant HIV virus might then have infected murine cells. As is the case for the rabbit, more information regarding the susceptibility of mice to HIV infection is required before the mouse can be considered as an appropriate model for HIV infection.

Immunodeficient mice reconstituted with human hematolymphoid tissue (SCID: hu/hematolymphoid) provide an in vivo model of the human immune system which may be used to study viral infection (MCCUNE 1991). Engrafted miniorgans of fetal liver, bone marrow, lymph nodes, or thymus produce mature human B and T cells in these mice. The human tissue can survive for several months without any incidence of graft-versus-host disease. Intranodal or intra-

thymic injection of the human tissue implants with a cloned HIV isolate in the SCID: hu/hematolymphoid mice resulted in infection and spread of the virus throughout the injected tissue, as demonstrated by in situ hybridization (Namikawa et al. 1988). Intravenous injection of a few different isolates resulted in the infection of human nodal cells, but not in the engrafted human thymus nor the murine lymph nods or spleen (Kaneshima et al. 1991). Viral RNA could be detected by in situ hybridization only in the human T cells and macrophages showing species and cell-type specificities (Kaneshima et al. 1991).

A second SCID model (hu-SCID-PBL) was developed by the intravenous or intraperitoneal injection of human peripheral blood leukocytes (Mosier 1991). Intraperitoneal injections lead to the establishment of human T cells, B cells, and macrophages in the mouse lymphoid tissue. Injection of passaged viral isolates in either cell-free or cell-associated forms resulted in infection of only human cells, as determined by in situ hybridization analysis (Mosier 1991).

Both the SCID: hu/hematolymphoid and hu-SCID-PBL chimeras showed HIV infection and suppression by 3'-azido-3'deoxythymidine (AZT), suggesting that both may be valid in vivo models for use in vaccine strategies and prophylactic antiviral therapies (McCune et al. 1990; Mosier 1991). However, neither chimera provides a permanent model for the human immune system. The hu-SCID-PBL model shows a decrease in T cell and macrophage numbers after as little as 3 months, while the SCID: hu/hematolymphoid mouse has tissue implants that eventually atrophy over a period of months, a relatively short timespan to allow for the development of disease. Additionally, both models utilize human implants which may not provide all of the cell types needed to interact to induce pathogenesis. Taken together with the fact that neither chimera produces any disease symptoms, these two models appear to have limited usefulness in the study of HIV-associated pathogenesis.

3 HIV-Transgenic Mice

The use of transgenic mice to study viral gene function and pathogenesis has been well documented (Small et al. 1986; Hinrichs et al. 1987; Green et al. 1989; Kim et al. 1991). A tremendous advantage of the transgenic approach is that the incorporation of viral sequences into every cell of the mouse bypasses the initial steps of viral infection. This effectively circumvents any species barrier that may be imposed by host-specific receptors.

Another advantage with the use of the transgenic technology is that the problems that may be associated with the route of viral administration are negated, since transgenics contain the viral sequences within every cell of the body. However, the presence of the transgene in every cell may allow expression in cell types that may not necessarily be susceptible to viral infection. This situation may result in pathologies in the transgenic mice that are not clinically

relevant to humans, since a disorder may arise in the animals as a consequence of viral gene expression in a cell type that is normally refractory to viral infection in humans. On the other hand, the transgenic mice may show pathologies that are rare in infected humans, perhaps because in humans certain cell types are difficult to infect and may only be affected in a minority of cases.

A third advantage of the transgenic mouse system is that the permanent incorporation of the transgene into the genetic material of the mouse from the beginning of development and its persistence throughout the lifetime of the animal provides a window of opportunity to monitor disease progression from the earliest stages. This is in contrast to the situation in humans, who are typically seen in a clinical setting only during the later stages of the disease. Additionally, pathological consequences of viral infection in utero or shortly after birth are not amenable to study in humans and can be difficult to reproduce. However, transgenic technology can provide fetuses harboring viral genes to model the effects of perinatal infection. Clearly, the practicality of the mouse as a model system coupled with the power of the transgenic technology provides a unique opportunity to generate the reagents necessary to dissect the complex aspects of viral gene expression in vivo.

3.1 Transgenic Approaches

Although the power of the transgenic model system is self-evident, it does have several drawbacks when applied to human viral research. Initial in vitro studies with transfected cell lines showed that mouse cells were capable of supporting HIV gene expression driven by the HIV-1 long terminal repeat (LTR; Adachi et al.1986; Levy et al. 1986). However, the transfected HIV clones produced low levels of progeny virions compared to human cells. This may be reflective of a weak or nonexistent transactivation capability of Tat on its own LTR in rodent cells (Hart et al. 1989; Khillan et al. 1988; Alonso et al. 1992). The low level of virus production could be the result of basal LTR activity in the absence of strong Tat function in rodent cells. The weaker activity of Tat may be due to a less efficient, or lack of, interaction of Tat with rodent cellular protein(s) whose counterpart(s) in human cells cooperate more effectively with Tat through the *trans*-acting response element (TAR) to increase the steady state levels of HIV mRNA.

Similar studies with HIV *rev* using various murine cells and mouse: human hybrids suggested that a human cell factor was also necessary for Rev-dependent splicing (Trono and Baltimore 1990). However, the production of mature virions hinted that the Rev protein may indeed be functional in murine cells (Adachi et al. 1986; Levy et al. 1986). As with Tat in rodent cells, the low levels of viral reproduction may be indicative of the lack of a human cellular factor or factors needed for the efficient interaction of Rev with its Rev response element (RRE).

The clear demonstration that mouse cells in vitro are capable of supporting HIV-1 gene expression set the stage for developing transgenic mouse models. A

potential concern of the transgenic mouse model is the relatively low level of expression of HIV genes seen in rodent cells in vitro compared to human cells in vitro. However, while it might be possible that the level of expression of any particular viral protein in transgenic mice may not approach the threshold level required to induce acute pathological changes, chronically or persistently low levels of expression may permit the development of phenotypes similar to those in humans but over an extended window of time. The enlarged window may provide additional opportunities for secondary effects to take place or to see disorders that appear later which may have been masked by another disease with an earlier onset.

AIDS is a syndrome resulting from viral infection, viral-related disorders, and secondary effects such as opportunistic diseases, any or all aspects of this syndrome may be present in mice harboring the HIV transgene. The use of a mouse model system may help to differentiate primary effects from secondary, characterize the multiplicity of consequences, and define related disorders. However, an early-onset disorder, not necessarily like that in humans, may mask other primary or secondary disorders or simply not allow enough time for their progression if the initial disease is life-threatening.

One approach to studying HIV in mice is to generate transgenic animals carrying the entire proviral genome. The transgene would then contain all viral transcriptional and post-transcriptional control elements such as the LTR, TAR, and RRE. In addition, the whole genomic transgene would have the potential to express all possible encoded viral proteins allowing for any interactions among the proteins, a situation that may be a necessity for the development of disease.

Studying an intact human provirus in a rodent system is not without concerns regarding safety. If indeed mice can be infected with HIV-1 (Locardi et al. 1992), then mice must be considered a potential animal reservoir for HIV. Clearly, great care must be taken in the handling of mice harboring HIV-1 and containment of these animals must be given top priority (Booth 1988). These concerns call for the containment of transgenic mice containing the HIV provirus in a level 3 (BSL3) or higher facility.

Another concern of having an intact HIV genome as a transgene is that recombination with endogenous retroviruses may occur. This may include physical recombination, such as phenotypic mixing and pseudotyping, and genetic recombination. Phenotypic mixing would occur if the envelope proteins of one virus intermingled among the envelope proteins of another virus. This would give the recombinant virus a dual tropism. Pseudotyping results when one virus provides all of the envelope proteins for a second virus. This may allow the recombinant to infect a cell type normally refractory to the core virus. However, in order to propagate the virus in the new cell type, pseudotyping must occur with every subsequent infection. Both types of physical recombinants may occur in the mouse and could be a potential health threat to humans (Lusso et al. 1990; Spector et al. 1990). A third possibility, genetic recombination between HIV and a mouse virus, may create a new hybrid virus with an unknown species specificity (Linial and Blair 1984). In all three cases, proper containment should preclude the

spread of any recombinants beyond the confines of the BSL3 facility. Of concern also is the fact that the presence of a recombinant virus, in any form, may render experimental findings uninterpretable.

Given the obvious safety concerns, one may opt to create a defective retrovirus by deleting one or more of the structural genes. In a transgenic setting, the defective retrovirus would be incapable of producing viral particles on its own. However, since most of the genome is complete, a murine retrovirus may still be capable of pseudotyping the subgenomic HIV RNA molecule. As mentioned previously, pseudotypes may allow infection of other cells but must be maintained by a subsequent pseudotyping event. In this case, the defective virus could not reproduce to yield virions, even if the pseudotyped virus infected human cells, and thus would not be a practical safety concern. Mature viral particles could, theoretically, only be produced as a result of genetic recombination. Although the subgenomic transgenic may not a priori warrant BSL4 level containment, the possibility of recombination makes additional safety measures a prudent precaution.

The subgenomic transgene may have an additional advantage over whole genomic transgenics in that it is less complex, coding for fewer viral genes. On the other hand, fewer genes may eliminate a potential phenotype that may be seen in whole genomic transgenic. This may be beneficial if other, perhaps late-onset phenotypes were unmasked by elimination of a potential phenotype that is earlier or more severe.

While whole and subgenomic HIV transgenic mice may be informative, a less complex approach is to determine the pathological consequences that result from the expression of a particular viral gene by creating single-gene transgenic mice. A disadvantage of this approach may be the fact that several viral proteins may be required to interact with each other to induce pathological changes. Additionally, as a gene is removed from its genomic context, *cis*-acting control elements may be altered or omitted, a situation that could potentially affect gene expression. However, it is possible that a single viral gene may cause a distinct phenotype and would provide a valuable experimental model to further define the etiology of one or more of the multitude of pathologies linked to HIV-1 infection.

All three approaches, whole genomic, subgenomic, and single-gene transgenics, have been successfully employed in transgenic mice. Though each approach has its own merits and pitfalls, all have yielded considerable insights. The phenotypes observed in HIV-transgenic mice, for example, renal disease and KS, often closely resemble the clinical histopathological conditions of some HIV-infected patients. In addition to providing important animal models of HIV-induced disease, these observations further substantiate the suggestion that HIV is causally linked to these disorders.

3.1.1 Whole Genomic Transgenics

Leonard et al. (1988) created transgenic mice harboring an intact HIV provirus. Seven founder-generation mice capable of germ line transmission of the trans-

gene were generated. None of these founder animals developed any sign of disease. Curiously, however, the F1 transgenic progeny of one founder consistently developed a phenotype. Of the 84 F1 animals that were screened, only 17 were identified as transgenic, suggesting that the founder may have been a germ line mosaic (Abramczuk et al. 1992). All of the transgenic progeny showed a failure to thrive, developed skin lesions and lymphadenopathy, and died before reaching sexual maturity. Viral particles were recovered from skin, spleen, and lymph nodes from all of the transgenic progeny tested but not from the founder animal. The lack of detectable levels of virions in the founder may have been due to subthreshold levels of expression as a result of the apparent genetic mosaicism. Additionally, only the founder developed an antibody response, which may have prevented viral production by antibody-dependent cellular cytotoxicity (ADCC), complement-mediated lysis, or by facilitating phagocytosis of viral particles. While viral particles could be detected in spleen, nodes, and skin of the F1 progeny, only affected skin had detectable amounts of viral RNA in the epidermis as determined by in situ hybridization analysis.

Necropsy of the F1-generation transgenic animals revealed grossly enlarged lymph nodes, splenomegaly, and an atrophic thymus. Cell counts and fluorescence-activated cell sorter (FACS) analyses of the lymph node cells revealed an increase in cell number, resulting primarily from an increase in B cells and $CD8^+$ T cells. The number of $CD4^+$ T-helper cells was normal, suggesting that the increase in cell number may have been indicative of a nonspecific reactive change. The spleens of the transgenic animals were consistently two to three times larger than control animals, reflecting a total increase in cell numbers; however, no significant change in the relative proportions of B and T cells was observed. Interestingly, splenic T cells showed a modest reduction in the proliferative response to concanavalin A (Con A), suggesting the possibility of a slight decrease in immune responsiveness. Thymuses of transgenic animals were reduced in size and cortical involution was evident, perhaps in response to stress. The general failure to thrive and ensuing stress placed on these animals could potentially have lead to the observed lymphoid changes. Unfortunately, the inability of the F1 mice to breed in conjunction with a laboratory accident (Ezzell 1988) precluded further analyses of these mice.

The most striking phenotype observed in these HIV-1 transgenic mice was a skin disease. The skin lesions were characterized by dry, thickened, scaling areas involving the tail, ears, and paws. Histological analysis revealed marked epidermal hyperplasia with numerous incidences of dyskaryotic cells, mitotic figures, hyperkeratosis, and parakeratosis, These skin lesions resemble psoriasis, which is also seen among those individuals infected with HIV.

Many distinct types of skin lesions occur among HIV-infected individuals. Some of these may be caused by opportunistic infections of viral, bacterial, or fungal agents. Examples of such infections may result in herpes simplex, herpes zoster, impetigo, or oral candidiasis (Matis et al. 1987). Other disorders with an unclear etiology which may be more prevalent, severe, or chronic in HIV-infected patients include seborrheic dermatitis (Mathes and Douglass 1985), psoriasis

(Duvic et al. 1987), Reiter's syndrome (Duvic et al. 1987; Winchester et al. 1987), and Kaposi's sarcoma (Mitsuyasu 1988; Boyle et al. 1993).

Psoriatic lesions are usually erythematous, sharply demarcated papules or plaques with an underlying chronic inflammatory response. The epidermis is hyperproliferative with incomplete maturation of keratinocytes resulting in parakeratosis. An increased risk of psoriasis may occur among certain HLA haplotypes (Duvic and Goldsmith 1983), during inflammation of the skin or in patients with an underlying T cell immune dysfunction (Krueger 1981). Although the overall incidence of psoriasis among AIDS patients may not be elevated with respect to the general population, it is clear that this disease is clinically far more severe (Duvic et al. 1987). The genomic HIV transgenic mice developed psoriatic lesions similar to those observed in AIDS patients. The distribution of affected areas in mice, principally the hairless regions which include the ear, tail, and paws, were not consistent with the areas typically affected in the human patients, although one may not expect a complete overlap given the evolutionary distance between man and mouse. Nonetheless, the transgenic mouse model of Leonard et al. (1988) has provided some insights into the relation of HIV to the induction of skin disease.

3.1.2 Subgenomic Transgenics

Iwakura et al. (1992) developed transgenic mice harboring a defective provirus with a deletion within the reverse transcriptase gene (*pol*). Four transgenic mouse strains that expressed the transgene were established. RNase protection assays demonstrated the highest level of transgene expression in eye, slightly less in spleen, and barely detectable levels in the brain and thymus. No signal was detected in the heart, kidney, liver, or bone marrow.

Consistent with the high level of expression in the eye, all of the mice in one strain developed bilateral cataracts. This observation of cataracts was confirmed in two other independent transgenic lines, albeit with a lower penetrance. In the strain with 100% penetrance, bilateral cataracts developed between 3 and 6 months of age. There was variation in the time of onset and severity of disease between animals and even between the eyes of a single animal. Histological analysis before disease onset did not reveal any abnormalities of the eye. Examination after the onset showed severe degeneration of the secondary lens fiber cells which were swollen and vacuolated. No inflammation was detected and there were no abnormalities in the cornea, iris, or retina. Immunohistochemical staining using serum from an AIDS patient revealed a positive signal within the cytoplasm of the lens fiber cells. Extraction of proteins from the eye revealed accumulation of the p24 Gag protein by western blotting. Similarly, the p24 Gag protein could be detected in the skin, brain, and eye of transgenic animals prior to disease onset. Therefore, Iwakura et al. (1992) were able to demonstrate that the presence of HIV gene expression not only coincided with the lens fiber degeneration, but preceded it. In contrast, HIV protein accumulation appeared only to affect the eye, as no effect was seen in the skin and brain where p24 was also detected.

Ocular complications increase with frequency as an HIV-infected individual becomes immunosuppressed. Commonly, ARC/AIDS patients develop retinitis due to opportunistic infections, especially cytomegalovirus (Newman et al. 1983; Pertel et al. 1992). More than half develop cotton-wool spots as a result of focal retinal ischemia caused by the occlusion of retinal arterioles and capillaries by immune complex deposition (Holland et al. 1983; Pepose et al. 1985; Jabs et al. 1989). The recovery of virus from retinal tissue and the localization of p24 and gp120 antigens to retinal vascular endothelial cells implicate HIV infection as the direct cause in the development of the cotton-wool spots (Pomerantz et al. 1987). HIV-1 has also been isolated from tears (Fujikawa et al. 1986; Ablashi et al. 1987), conjunctival tissue (Fujikawa et al. 1985; Ablashi et al. 1987), cornea (Salahuddin et al. 1986; Ablashi et al. 1987), and aqueous humor (Pepose et al. 1987). Nonetheless, HIV infection has not been shown in lens fiber cells, nor has it been associated with cataract formation. The fiber cells of the adult lens are terminally differentiated and may be refractory to HIV infection. However, it is possible that during earlier stages of lens fiber differentiation, the cells of those earlier developmental stages may be susceptible to infection. Other pathogens such as cytomegalovirus, herpesvirus, and varicella zoster virus during in utero infection may also cause neonatal cataracts (Strömland et al. 1991). While there is as yet no known clinical case of HIV-induced cataracts, the transgenic mouse model may suggest that there is a potential to cause cataracts which may be of concern particularly to the newborns of HIV-infected mothers. It is also possible that the inappropriate expression of the transgene may interfere with the lens fiber cell development. Several transgenic strains using various genes with expression in the lens fiber cells during development have also resulted in neonatal cataract formation (Khillan et al. 1987; Capetanaki et al. 1989; Griep et al. 1989; Monteiro et al. 1990).

A second series of experiments using a different HIV transgene was reported by Dickie et al. (1991). The construct used in these experiments had an internal deletion which eliminated much of the *gag/pol* coding sequence. None of the founder animals carrying this transgene developed disease during their lifespan. However, three of the founders (Tg22, Tg25, and Tg26) produced progeny that developed renal disease, and one (Tg26) developed skin lesions (Kopp et al. 1993) as well as myopathy/myositis (Adler et al. 1992).

The tissue distribution and level of expression of the viral transgene varied between the three lines. In each case, three distinct mRNA species could be detected corresponding to full-length, singly spliced, and doubly spliced messages. Northern analysis revealed highest expression in skin and muscle. Expression could also be detected in thymus (five lines), gastrointestinal tract (four lines), kidney (three lines), eye (two lines), brain (two lines), and spleen (two lines).

Interestingly, the pattern of protein expression detected by immunoblotting differed in different tissues. Analysis of protein extracts from skin demonstrated specific staining of HIV proteins, including the gp41, gp120, and gp160 env proteins, while kidney extracts were shown only to contain gp41. In contrast, skeletal muscle only showed the presence of the Nef protein. The disparate patterns of protein expression could be explained by differences in Rev function

between tissues. It is possible that some tissue, for example, skin, contains essential Rev cofactors while others do not.

All of the animals of the Tg22c, Tg25, and Tg26 lines developed proteinuria within the first month. Mice of the Tg22c and Tg25 lines died before reaching sexual maturity. These mice exhibited severe subcutaneous edema and ascites and moderate to severe edema in the mesentery, omentum, and pancreas. Both had thymuses which were reduced in size or atrophic and lymph nodes that were either normal or slightly atrophic. The kidneys were slightly enlarged, pale, and pitted. Nearly half of the hemizygous animals of the third line (Tg26) developed a nephrotic syndrome characterized by severe edema, hypoalbuminemia, and hypercholesterolemia. Almost all of the homozygous mice of the Tg26 line were runted and died within the first 30 days (Kopp et al. 1992).

Histopathological examination of the kidneys from the Tg22c and Tg25 lines revealed diffuse global glomerulosclerosis, microcystic tubular dilatation, and sparse monocytic infiltrates within the interstitum. Tg26 animals showed a spectrum of pathological changes including segmental and global glomerulosclerosis, tubular dilatation, atrophy of the tubular epithelium, proteinaceous casts, and a monocytic interstitial infiltrate (Kopp et al. 1992). Immunohistochemical staining of kidney sections were positive using patient sera. Over half of the glomeruli stained while tubular epithelium did not. Use of antibodies specific to Rev, Tat, Nef, gp41, or gp120 demonstrated the accumulation of Rev protein only within sclerotic glomeruli (Kopp et al. 1992) in contrast to the immunoblotting results, which demonstrated only the presence of gp41 in kidney lysates (Dickie et al. 1991). This apparent contradiction may be explained if the Rev protein were found in the circulation and deposited in the sclerotic glomeruli. Nevertheless, expression of HIV mRNA was documented in only the three independent lines that developed a characteristic renal nephropathology.

Following HIV infection, up to 40% of patients may develop renal complications (Pardo et al. 1984; Rao et al. 1987). Renal disease may be due to nephrotoxic drugs used in treatments, heroin use, or multiple opportunistic viral infections (Burgoignie 1990). As many as 10%-25% of HIV-infected individuals may develop renal disease with a characteristic histopathology known as HIV-associated nephropathy (HIVAN; Pardo et al. 1987).

Patients developing HIVAN have heavy proteinuria and enlarged, often edematous kidneys. Pathological changes include focal and segmental glomerulosclerosis, microcystic tubular dilatation with proteinaceous casts, and interstitial inflammation (Cohen and Nast 1988). Infection of tubular epithelial and glomerular cells have been demonstrated in some HIVAN patients (Cohen et al. 1989; Karlsson-Parra et al. 1989). The transgenic mice described by Kopp et al. (1992) not only showed the histopathological features consistent with HIVAN, but clearly implicated HIV as the causal agent.

Additionally, in the transgenic line Tg26, Kopp et al. (1993) noted skin lesions at as early as at 5 days of age. The lesions first appeared as reddish macules which, over a period of time, became nodular with marked scaling. The incidence also increased with age from 18% of the mice less than 50 days of age to 59% of mice over 300 days. The lesions often developed into papillomas with a marked

epidermal hyperplasia, hyperkeratosis, and parakeratosis with increased frequency of mitoses. The underlying dermis had no apparent lesions except a sparse mononuclear infiltrate in homozygous animals.

Northern analysis detected both the singly and doubly spliced messages in skin with a several-fold higher level in the papillomas. In situ hybridization analysis detected an increase in viral RNA expression in the epidermal cells within the papillomas. Immunohistochemical analysis of gp120 also showed increased specific staining in the epidermis of the papilloma as compared to unaffected skin from heterozygous animals. Given the increase of HIV expression within the papillomas, it is not clear whether expression leads to the formation of a papilloma or, conversely, that the changes within the papilloma induce HIV gene expression.

To study the role that HIV may play in the papilloma formation, trauma was induced by various methods in the skin of the transgenic animals. Several studies have shown that HIV-1 gene expression can be induced or enhanced in vivo using ultraviolet (UV) radiation (Cavard et al. 1990; Frucht et al. 1991; Morrey et al. 1991, 1992; Vogel et al. 1992). Kopp et al. (1993) used UV-B irradiation to enhance HIV expression in their transgenic mice. Within 2–3 weeks of exposure, 90% of transgenic mice developed papillomas. After a period of 3–4 weeks, the papillomas involuted, suggesting a sustained level of expression may be necessary. Additionally, skin trauma induced by phenol or liquid nitrogen also led to the development of papillomas. It appears that many forms of trauma may induce HIV gene expression and subsequent pathology. Perhaps physical trauma as a result of fighting or grooming between animals may have contributed to the development of some of the skin lesions. Since a sustained level of expression was needed in the UV-induced papillomas, it is probable that the induction of HIV gene expression is required to develop this skin lesion.

3.1.3 Single-Gene Transgenics

Vogel et al. (1988) used the *tat* gene, driven by its own promoter (HIV-1 LTR), to derive transgenic mice. The *tat* gene encodes an essential *trans*-regulatory protein that functions possibly through binding to the TAR region of the viral mRNA (Dingwall et al. 1989). The TAR region of the mRNA forms a stable stem-loop structure located within the first 59 nucleotides of all HIV-1 primary transcripts (Muesing et al. 1987). Tat has been shown to function by increasing the steady state levels of mRNA (Cullen 1986; Wright et al. 1986), most likely by either the stabilization of initiation complexes (Hauber et al. 1987; Jeang et al. 1988; Laspia et al. 1989) or by releasing a block to polymerase processivity, thereby allowing elongation (Laspia et al. 1989; Feinberg et al. 1991), or by both mechanisms (Cullen 1986; Wright et al. 1986).

Vogel et al. (1988) established three transgenic lines capable of expressing the LTR-*tat* construct in the tails of the animals as assayed by northern hybridization analysis. To delineate the tissues involved in expressing the transgene, additional northern analysis was performed, revealing detectable expression consistently in the skin. Histological analysis of back skin from male mice as early

as 4 months of age revealed a thickened, hypercellular epidermis and hyperkeratosis. The underlying dermis showed increased cellularity along the dermal–epidermal junction. With age, the lesions became progressively more advanced. The dermis became markedly hypercellular with spindle-shaped cells and contained scattered abnormal vascular structures. After 12 months of age, approximately 15% of the male transgenic animals developed erythematous lesions. Some animals developed multifocal tumors without any evidence of metastasis. Histological analysis of these tumors revealed a more aggressive proliferation of the spindle-shaped cells within the dermis. Irregular, disorganized vascular structures were also present, resulting in the prominent appearance of extravasated red blood cells (RBC). The lesions observed in the *tat* transgenic mice bear a striking resemblance to KS, a skin disease that debilitates a significant portion of HIV-infected individuals.

KS, a rare tumor found among men of Mediterranean descent (classical form) or in equatorial Africa (endemic), is also found in epidemic proportions among HIV-1-infected individuals (Safai et al. 1985). Indeed, KS is virtually a pathognomonic diagnosis of AIDS, it may be found in as many as 25% of AIDS patients (Curran 1983; Matis et al. 1987) and 95% among autopsy studies (Moskowitz et al. 1985). The likelihood of having KS among AIDS patients is nearly 20 000-fold higher than in the general population and 300-fold higher than other immunosuppressed groups (Beral et al. 1990). The increase specifically among AIDS patients strongly implicates HIV-1 as the causative agent. The observation of KS-like lesions in the *tat* transgenic mice further strengthens this suggestion.

The three major forms of KS share certain characteristic histological features: male preponderance and frequent multifocal involvements (Ziegler et al. 1984). The cutaneous lesions are restricted predominantly to the hands and soles of the feet in the classical and endemic forms, but are more widely dispersed in AIDS-KS, commonly afflicting the oral and anal mucosa and the lymph nodes. AIDS-KS is also more aggressive and responds poorly to treatment. The KS skin lesions may initially appear as small and diffuse. Histologically, this patch morphology is a diffuse lesion of spindle cells. The plaque form is the progression of the spindle cells to aggregate. The nodular form is a lesion of well-defined, solid nodules consisting of spindle cells with or without slit-like vascular spaces (Francis et al. 1986).

Only male LTR-*tat* transgenic mice of 3-4 months of age showed nests of spindle cells underlying the epidermis, much like that observed in the patch lesion in AIDS-KS. Older male animals developed a more extensive involvement of spindle cells between the collagen bundles of the dermis and bizarre-shaped vessels. These lesions were similar to the plaque from seen in AIDS-KS. Some of the male mice over 1 year of age developed distinct, erythematous nodules. Histological analysis demonstrated interwoven bands of spindle cells forming a well-defined nodule and extravasated RBC, as seen in the nodular form of KS.

In the LTR-*tat* transgenic mice, the hyperplastic epidermis appeared earlier than the dermal lesions and was more uniform morphologically. Expression of the *tat* gene was localized exclusively to the epidermis, but not to the dermis or tumor

cells (Vogel et al. 1992). The presence of the Tat protein in the epidermis apparently leads to proliferation which may resemble keratosis or psoriasis. The hyperplastic epidermal cells may release specific cytokines which induce a local subset of cells in the dermis to proliferate, leading to the patch-like morphology, but may progress to the plaque or nodular forms. The sustained expression of Tat may increase the chance of acquiring a genetic alteration which may give rise to a tumor. Such a mechanism may explain some of the features of the epidemic form of KS. First, the expression of HIV genes within the lesions is not required and is indeed not present within AIDS-KS lesions, although this point remains somewhat controversial (Mahoney et al. 1991; Bovi et al. 1986). Second, a cytokine induction pathway may act simultaneously to induce multiple lesions, leading to the polyclonal nature of KS. Third, the abrupt cessation of Tat expression may result in lesions that spontaneously regress, a phenomenon that is frequently observed in AIDS-KS. Fourth, the paracrine effect of the epidermal factors may affect multiple cell types within the dermis, which may be reflective of the mixed cellularity of KS lesions. Finally, the factors may act in an endocrine fashion affecting distant tissues and organs.

In keeping with a possible endocrine effect, Vogel et al. (1991) found that males of all three LTR-*tat* transgenic lines developed liver lesions, even though only one line showed detectable expression by a more sensitive assay, RT-PCR. The transgenic mice developed liver lesions including liver cell dysplasia (5%), hepatic adenoma (10%), and hepatocellular carcinoma (27%), compared to control males, which developed liver lesions with an incidence of approximately 8%. Much like the skin lesions, the liver lesions progressed through stages from dysplasia to carcinoma and may be found in multiple stages and sites within the liver of a single mouse.

Epidemiological surveys among single young men in San Francisco (Biggar et al. 1987) and New York (Biggar et al. 1989) show a slight, but not statistically significant increase in hepatoma. People at high risk for HIV infection are also at high risk for hepatitis B, and the underlying immunosuppression in AIDS may accelerate hepatitis B pathology. In addition, many of the young men in New York were immigrants of oriental and hispanic derivation to which hepatitis is endemic (Biggar 1990). However, it is possible that *tat* plays a role in the etiology of liver tumors that may be seen in more AIDS patients as the mean survival time lengthens.

Recently, another HIV single gene transgenic was created utilizing the *nef* gene. Although *nef* is not an essential gene for HIV replication in vitro (Terwilliger et al. 1986; Luciw et al. 1987; Kestler et al. 1991), it has been suggested to play a negative regulatory role in HIV gene expression (Luciw et al. 1987; Ahmad and Venkatesan 1988; Cheng-Mayer et al. 1989; Guy et al. 1990; Maitra et al. 1991); however, this may depend upon the cell type used and still remains controversial (Hammes et al. 1989; Kim et al. 1989; de Ronde et al. 1992; Zazopoulus and Haseltine 1993). Additionally, Nef has been implicated in the down-regulation of the CD4 surface antigen in established T cell lines (Guy et al. 1987; Garcia and Miller 1991; Mariani and Skowronski 1993).

Skowronski et al. (1993) created transgenic mice using each of two independent *nef* gene isolates under the control of a T cell-specific promoter. Using the CD3 promoter driving the expression of *nef* from the NL43 isolate, a decrease in the cell surface expression of both CD4 and CD8 was seen in the thymus. These transgenic mice also showed a decrease in the number of $CD4^+$ T cells in lymph nodes and in the circulation within the first 2 months. However, the number of $CD4^+$ T cells in nodes approached normal values in 8-to 12-week-old animals, demonstrating that the decrease in $CD4^+$ cells was transient. A second transgenic construct contained the *nef* gene from the HxB3 isolate under the control of CD2. In these mice, alterations in the surface phenotype or numbers of T cells were not seen, even though the protein expression level was only slightly lower than the transgenic mice harboring the *nef* gene from the NL43 isolate. This is in agreement with the in vitro data, which also demonstrated that the NL43 isolate but not the HxB3 isolate was capable of affecting CD4 surface expression (Skowronski et al. 1993).

The downmodulation of CD4 in vivo was only a transient response and was specific to the NL43 isolate. In addition, both CD4 and CD8 surface expression was downmodulated, suggesting that the effect was perhaps nonspecific. It is plausible that the CD3-*nef* construct merely delays development of the maturing T cells, which would explain the decrease in both $CD4^+$ and $CD8^+$ mature cells in the thymus and the subsequent phenotypic resolution with age.

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The Human Immunodeficiency Virus Type 1 *vif* Gene: The Road from an Accessory to an Essential Role in Human Immunodeficiency Virus Type 1 Replication

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1 Introduction

The *vif* (virion infectivity factor) gene is one of nine genes known to be expressed by human immunodeficiency virus (HIV)-1 (Wong-Staal and Gallo 1985; Haseltine 1988); *vif* and four other genes encode protein which are not structural components of virions but regulate viral replication to varying degrees (Haseltine 1988; Levy 1993). The two best-known viral regulatory genes, *tat* and *rev*, are essential for HIV-1 replication (Rosen et al. 1986; Sodroski et al. 1986b); in contrast, *vif* has been defined as an accessory gene because in initial studies its function was found to be dispensable for virus infection of transformed T cell lines (Sodroski et al. 1986a; Fisher et al. 1987; Strebel et al. 1987). After the initial description of *vif* and the effects of its ablation on virus replication (Sodroski et al. 1986a; Fisher et al. 1987; Strebel et al. 1987), very little research was done to further the understanding of the function of this viral gene product. Recently, however, a number of laboratories made the unexpected observation that *vif* is required for HIV-1 infection in its primary target cells, CD4-bearing T lymphocytes (Akari et al. 1992; Fan and Peden 1992; Gabuzda et al. 1992; Michaels et al. 1993; von Schwedler et al. 1993). This all-or-none dependence on the expression of *vif* for HIV-1 infection of peripheral blood lymphocytes (PBL) is illustrated in Fig. 1.

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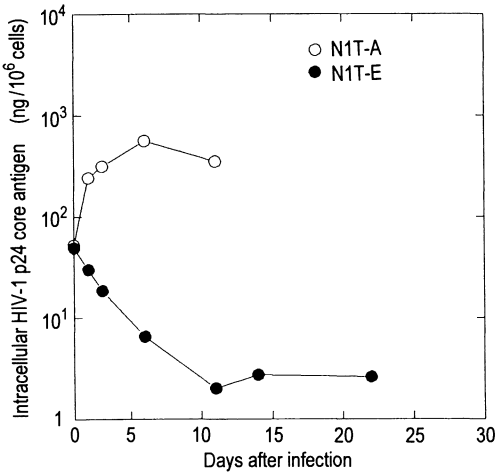

Fig. 1. Requirement for *vif* in human immunodeficiency virus (*HIV*)-1 infection of peripheral blood lymphocytes (PBL). Mitogen-stimulated PBL were infected with one of the closely related molecular coisolates *N1T-A* or *N1T-E*. The slow, non-cytopathic phenotype displayed by N1T-E during infection of transformed T cells is the result of a 35-bp deletion in *vif* which terminates translation of the protein prematurely (SAKAI et al. 1991). N1T-E did not produce detectable p24 in PBL, while N1T-A was highly productive

These findings mandate the redefinition of *vif* as an essential gene for HIV-1 replication. We shall review the studies on the role of *vif* in HIV-1 infection and shall attempt to incorporate the results from different systems in the construction of a testable model for the mechanism of action of Vif. Because the cell-type dependence of Vif activity has become apparent only recently, early studies must be interpreted with these new variables in mind.

2 Discovery of *vif* and Early Studies on Its Structure and Function and Its Role in HIV-1 Replication

The HIV-1 genome was fully sequenced well before functions were asssigned to its gene products (MUESING et al. 1985; RATNER et al. 1985; SANCHEZ-PESCADOR et al. 1985; WAIN-HOBSON et al. 1985). An open reading frame, at that time variously designated A, P, Q, ORF-1, or *sor*, was found to overlap the 3′ end of *pol* (MUESING et al. 1985; RABSON and MARTIN 1985; RATNER et al. 1985; SANCHEZ-PESCADOR et al. 1985; WAIN-HOBSON et al. 1985) and to encode a mRNA that is singly spliced (GARRETT et al. 1991; SCHWARTZ et al. 1991) and gives rise to a 23-kDa protein (KAN et al. 1986; LEE et al. 1986; SODROSKI et al. 1986a). The designation *vif* was adopted for this open reading frame to reflect the function of the gene product in modulation of virion infectivity (GALLO et al. 1988). *vif* mRNA is synthesized late in the virus life cycle, at the time of synthesis of messages encoding the viral

structural genes (Garrett et al. 1991; Schwartz et al. 1991), after the synthesis of the first messages which encode the regulatory genes *tat*, *rev*, and *nef* (reviewed in Cullen and Green 1989; Haseltine 1988), and it requires *rev* activity for translation (Garret et al. 1991; Schwartz et al. 1991). The 23-kDa *vif* protein is found in the cytoplasm, both associated with cellular membranes and as a free protein (Michaels et al. 1993; Fig. 2), but is absent from virions, at least as determined by immunoblot analysis of sedimented virions using anti-Vif antiserum (Sakai et al. 1991; Table 1). HIV-1-infected subjects have been shown to carry antibodies which recognize the bacterially derived product of *vif* (Kan et al. 1986; Wieland et al. 1991; Schwander et al. 1992), indicating both that the protein is expressed and is immunogenic during natural human infections. A *vif* homologue was found in all other lentiviruses examined, except for equine infectious anemia virus (Oberste and Gonda 1992; Tomonaga et al. 1992). The conservation of *vif* over phylogeny and during HIV-1 infection in human populations indicates the importance of *vif* over during virus replication in vivo.

To assign function to the *vif* gene, differences between the HIV-1 processes and products during infection by *vif*-expressing and *vif*-negative viruses were catalogued. Originally, three distinct phenotypes were described for three sets of *vif*-negative viruses, two of which derived from HXB-2 (Sodroski et al. 1986a; Fisher et al. 1987) and the third from NL4-3 (Strebel et al. 1987). In the study by

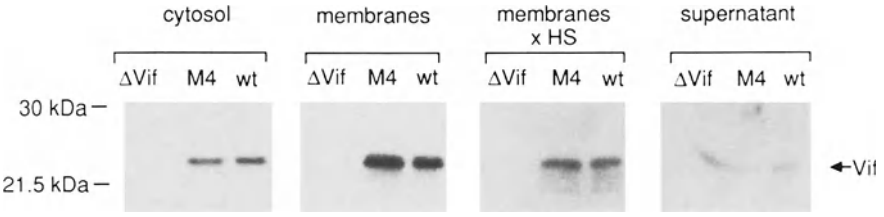

Fig. 2. Subcellular localization of Vif. CEM cells infected with wild-type human immunodeficiency-virus (HIV)-1 (*wt*), a Vif cysteine mutant (*M4*; Ma et al. 1994), or a *vif* deletion mutant (*Δvif*; Sakai et al. 1991) were fractionated and subjected to western blot analysis using rabbit anti-*vif* antiserum (Sakai et al. 1991). The membrane fraction was treated with 1 *M* NaCl buffer to elute loosely associated proteins and the remaining membrane fraction (membranes × HS) and eluted material (supernatant) were analyzed

Table 1. Properties of *vif*

Property	Reference
Coding region	
nt 5040–5618 in HXB-2	Myers et al. 1989
Size	
192 amino acids	Myers et al. 1993
23 kDa	Sodroski et al. 1986b
Localization	
Cytoplasm, membranes	Michaels et al. 1993 (see Fig. 2, this chapter)

Sodroski et al., C8166 T lymphoid cells were infected by cocultivation with a B lymphoma cell line transfected with various mutant virus constructs. *vif*-Negative viruses were found to replicate and kill C8166 cells, although at a slower rate than wild-type virus. No pronounced differences in intracellular viral proteins were observed. The conclusion of this study was that *vif* is dispensable for HIV-1 infection (Sodroski et al. 1986a).

In the report by Strebel et al., *vif*-negative virus was constructed on an NL4-3 background, was produced by transfection into colon carcinoma cells, and was tested for transmission to transformed T cells by cocultivation and through cell-free virus. In this case, cell-free virus was poorly transmitted to a subclone of CEM cells, although it could be transmitted to these cells after some lag by cocultivation with transfectants. It was shown that *vif* defects could be complemented by cotransfection of a vector encoding *vif*, indicating that *vif* operates through a diffusible product. The authors concluded that the gene product of *vif* enhanced virion infectivity by as much as 1000-fold, as measured by the levels of reverse transcriptase in infected cell supernatants, but that it was not required for cell-to-cell transmission of virus (Strebel et al. 1987).

The study by Fisher et al. (1987) was by far the most comprehensive of the original series. It employed *vif*-negative derivatives of HXB-2 and was the first attempt to determine directly the effects of mutations in *vif* on virus production by different HIV-1 target cells, including peripheral blood lymphocytes (PBL). No stable *vif*-negative virus production arising from viral DNA transfection was observed in H9, Molt-4, or PBL. Cos-1 monkey kidney cells produced *vif*-negative virus upon transfection and were used as donors in tests of virus transmissibility by cocultivation with human T cell lines. Molt-4 cells could be productively infected with *vif*-negative virus, albeit with a short lag compared to wild-type virus; however, *vif*-negative virus was very poorly transmitted to H9 cells by cocultivation. Viral RNA production was not detectably impaired in *vif*-negative virus-infected Molt-4 cells. The authors concluded that *vif* acts at a late stage of HIV-1 replication and is differentially required for virus transmission by cell-free virus or cocultivation as a function of target cell type (Fisher et al. 1987).

In detailed analyses of the life cycle of N1T-E (Sakai et al. 1988a,b; Ma et al. 1990), which subsequently was shown to be a natural *vif*-deletion mutant (Sakai et al. 1991), we studied HIV-1 infection in cells in which a *vif* deficiency has significant impact on virus replication. We found that *vif*-negative N1T-E produced in CEM cells carries viral RNA and efficiently binds to and fuses with target cells, as determined by the fluorometric assay for membrane mixing (Ma et al. 1990), implicating events following virus fusion and entry into cells as the sites affected by deficiencies in *vif*. This was further supported by an experiment in which N1T-E-infected CEM cells were cocultivated with uninfected T lymphoid HUT 102 Cells (Fig. 3). Efficient cell-to-cell fusion (giant cell formation) was observed within 24 h in cocultures of both *vif*-mutant and wild-type virus-infected cells, indicating that transmission of viral genetic information to uninfected cells occurred in both systems. However, only the coculture with wild-type HIV-1, but not with *vif*-negative virus, showed the evidence of rapid spread of viral infection (Fig. 3; Ma

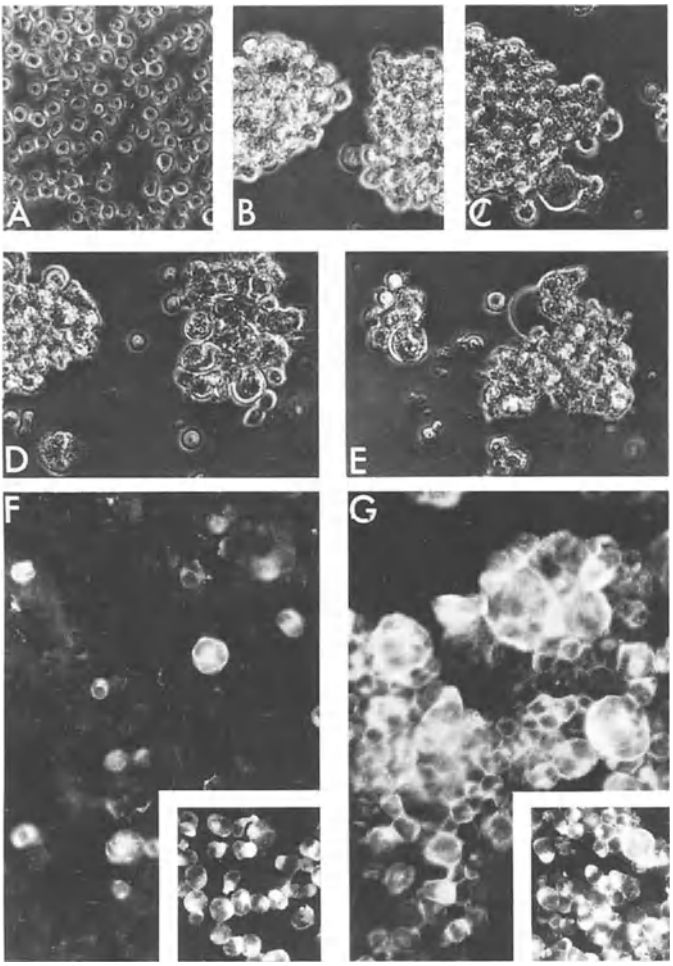

Fig. 3A–G. Syncytium formation by, and human immunodeficiency virus (HIV)-1 antigen expression of, *vif*-negative HIV-1-producing cells. **A–D** CEM cells chronically infected with *vif*-negative N1T-E (**A**) were mixed with uninfected HUT-102 cells (**B**) and cultured for 24 h (**C**) or 48 h (**D**). **E** A similar experiment was performed with CR10 cells chronically infected with wild-type N1T-A shown 24 h after cocultivation with Hut-102. **F** HIV-1 antigen expression by immunofluorescent staining of the coculture of CEM/N1T-E and Hut-102 shown in **D**; the *inset* shows the staining of CEM/NIT-E. **G** The same analysis of CR10/N1T-A and Hut-102 from **E**; the *inset* shows CR10/N1T-A. Reprinted with permission from MA et al. (1990). Copyright (1990) by Academic Press, Inc.

et al. 1990). These results indicated that virus fusion and entry are not rate-limiting steps in *vif*-negative HIV-1 infection. A recent study using *vif*-negative HIV-1 pseudotyped with murine leukemia virus arrived at the same conclusion (VON SCHWEDLER et al. 1993). Whether initiated by cell-to-cell contact as shown in Fig. 3 or by cell-free virus, infection of CEM cells, with *vif*-mutant N1T-E virus was invariably delayed, and it was characterized by prolonged hiatus in viral RNA and protein synthesis relative to wild-type virus infection. The synthesis of the viral

regulatory proteins Tat and Rev was also delayed, indicating that the apparent attenuation of N1T-E was not due to a defective regulation of viral expression (Ma et al. 1990). Indeed, other studies have demonstrated that N1T-E has intact long terminal repeat (LTR) and Tat-mediated transactivation functions (Golub et al. 1990).

In contrast to the apparent delay of N1T-E expression immediately after virus entry into CEM cells, once viral infection was established the cells were able to maintain a high-level chronic production of N1T-E (Ma et al. 1990). This indicates that *vif* is dispensable for efficient expression of viral function from proviral DNA. This notion is further supported by experiments which showed that, in transient expression assays, *vif*-negative and *vif*-positive viral DNA produce equivalent amounts of progency virus (Ma et al. 1990, 1994). Taken together, these results indicate that one major consequence of *vif* defect is a deficiency in a step of the *vif*-negative virus infection that occurs after virus entry but prior to the establishment of integrated proviral state. Another major consequence is the generally low level of cytopathicity associated with *vif*-negative virus infection. We initially described this phenomenon as a characteristic feature of N1T-E viral infection in T lymphoid and monocytoid cells (Sakai et al. 1988a,b, 1989; Ma et al. 1990) and later mapped it to N1T-E *vif* by recombinational analysis (Sakai et al. 1991). More recently, this observation has been extended to a variety of *vif* mutants from different sources (Adachi et al. 1991; Nishino et al. 1991; Kishi et al. 1992). Thus, the slow, noncytopathic phenotype is associated in a cell-dependent manner with *vif*-negative HIV-1, regardless of the viral strain of origin.

Some consensus concerning the replication of *vif*-negative HIV-1 can be drawn based upon the studies on *vif* cited above. The effects of *vif* mutations on HIV-1 replication are dependent upon the cell type infected. The most extreme phenotype results in the absence of virus spread, indicated through lack of production of viral protein or absence of stable viral DNA integration in some transformed T cell lines and in PBL. No consistent pattern of defects specifically in other viral products were observed when other products were detected. When specific phases of the life cycle were evaluated, some early stages of primary infection (binding, virus–cell fusion, entry) appeared intact in *vif*-negative virus, whereas others, including reverse transcription, RNA synthesis, and protein synthesis, occurred with delay. These findings suggest that multiple cycles of replication of moderately impaired *vif*-negative virus, each cycle being somewhat less efficient than wild-type virus, ultimately yield the delay in virus production seen, for example, in CEM cells. When delayed production of *vif*-negative virus is observed, it correlates with attenuated cytopathicity and permits the establishment of chronic productive infection in cells (such as CEM or HUT102) which would otherwise be killed by a *vif*-expressing virus. However *vif*-negative virus can spread cytopathic infection throughout cultures of certain transformed target T cells (such as C8166), with little delay apparent. The inconsistency of results of the efficiency of cell-to-cell transmission of *vif*-negative virus from different laboratories may reflect the cell donor of the virus.

Taken together, these studies led to the characterization of *vif* as an accessory gene that is produced and acts late in the virus life cycle and that increases the infectiousness of progeny virus through an unknown mechanism. Unlike the canonical retroviral genes *gag*, *pol*, and *env* or the regulatory genes first found in human lymphotropic viruses, *tat* and *rev* (WONG-STAAL and GALLO 1985; LEVY 1993), *vif* was not considered to be essential for HIV-1 replication (SODROSKI et al. 1986a), despite its conservation among lentiviruses (OBERSTE and GONDA 1992). The early evidence showing that *vif*-negative HIV-1 is infectious strictly as a function of the cell types studied (FISHER et al. 1987) was not reevaluated until 1992.

3 Redefinition of the Role of *vif* in HIV-1 Replication: Advances in *vif* Research in 1992–1994

The more recent generation of studies on the activity of Vif further explored the contribution of producer and target cell lines to the mutant phenotype. Laboratories generally selected for study transformed T cells which revealed moderately severe effects of *vif* mutations, but in which virus infection could be established. The role of *vif* in the infection of PBL was also reexamined.

The effects of both the producer cell type and the target cell type were evaluated during single-cycle infection (GABUZDA et al. 1992). There, *vif*-negative virus produced in CEM was slightly defective, while that produced in SupT1 was not, regardless of the target cell type (GABUZDA et al. 1992). In similar systems in which virus spread was possible, it was notable that the relative requirement for *vif* was also very different in different target cells (GABUZDA et al. 1992; FAN and PEDEN 1992; VON SCHWEDLER et al. 1993; SAKAI et al. 1993; SOVA and VOLSKY 1993; MICHAELS et al. 1993). HIV-1 infection of macrophages appears to require *vif* (VON SCHWEDLER et al. 1993), although the requirement is not absolute when considering HIV-2 (MICHAELS et al. 1993). A spreading infection initiated from a low input dose of cell-free virus in PBL was completely dependent on *vif*, regardless of the origin of the virus stock (GABUZDA et al. 1992; FAN and PEDEN 1992). Cell-to-cell transmission of *vif*-negative virus to PBL has been reported; however, the system used does not allow complete distinction of virus produced by the donor cell versus that transmitted to the target PBL (FAN and PEDEN 1992). When a high input dose of *vif*-negative virus was used to infect PBL, we still found an absolute block to the spread of infection (Fig. 1). These findings emphasize the distinctions between systems in which virus spread is evaluated and single-cycle systems. In the latter, the influence of the origin of the virus stock predominates. In the former, the target and host cells are effectively merged in the requirement for multiple cycles of replication. However, these systems are limited by their inability to determine the efficiency of specific phases of the virus life cycle.

An alternative method examine early events in virus replication is polymerase chain reaction (PCR) amplification of viral DNA, which is synthesized by reverse transcriptase following virion internalization in cells. It is important to note that

PCR signals reflect the efficiency of reverse transcription, but also reflect the efficiency of all the events of virus replication prior to reverse transcription, many of which are poorly resolved. We found that *vif*-negative virus derived from CEM cells was five- to seven fold less efficient in early DNA synthesis in MT-2 cells than wild-type virus, but that subsequent DNA synthesis was even less efficient, implicating multiple rounds of inefficient expression to generate the overall phenotype of nonproductive infection (Sova and Volsky 1993). Viewed with our previous finding of unimpaired virus–cell fusion using *vif*-negative HIV-1 derived from CEM cells (Ma et al. 1990), it appears that some event following virus internalization and prior to or including reverse transcription is defective. A similar study showed little difference during early DNA synthesis in CEM cells of *vif*-negative or wild-type virus and attributed defects in virus replication to other, unidentified events (Blanc et al. 1993). A third study also showed reduced early DNA synthesis by *vif*-negative virus produced by CEM cells and likewise excluded virus entry as the sole or most important event compromised by *vif* defects by showing that viral DNA synthesis was impaired when the *vif*-negative genome was introduced into cells using envelope glycoproteins of another retrovirus (von Schwedler et al. 1993).

Two recent studies addressed the question of *vif* conservation *in vivo*, rather that in virus isolates (Wieland et al. 1994; Sova et al. 1995). The consensus proviral *vif* DNA sequences obtained by PCR amplification and direct DNA sequencing from uncultured blood samples of HIV-1 positive patients contained both conserved and variable regions; however in both studies, about 90% of all the *vif*-region DNA sequences tested had intact *vif* open reaching frames, suggesting the presence of functional *vif* genes (Wieland et al. 1994; Sova et al. 1995). The two cysteines in Vif were conserved in all sequences analyzed (Wieland et al. 1994; Sova et al 1995). There was no clear correlation between the presence of *vif* open reading frames and disease status of the patients tested (Sova et al.1995). These results further support the notion that *vif* plays a fundamental role in HIV-1 replication *in vivo*.

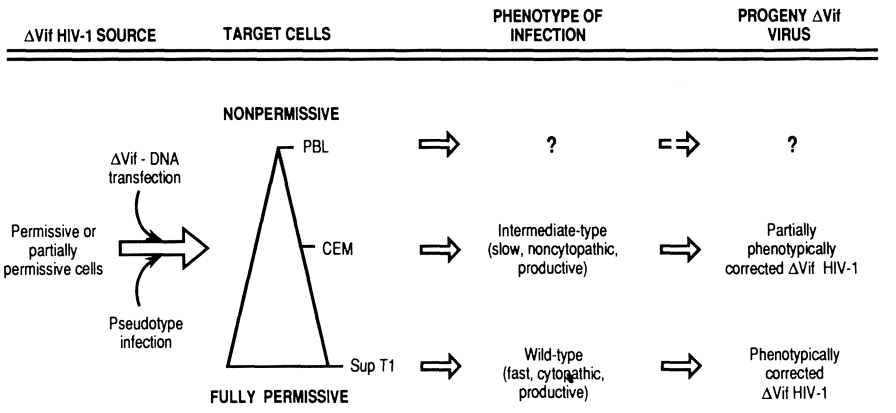

Fig. 4. Different requirement for *vif* during human immunodeficiency virus (*HIV*)-1 infection of various host cells. *PBL*, peripheral blood lymphocytes

A conclusion supported by all the recent studies of *vif* activity is that the host cells infected influence the outcome of *vif*-negative HIV-1 infection, prompting the categorization of cells as "permissive" or "nonpermissive" (Fig. 4). SupT1, C8166, Cos-1, and Molt 4, among others, are permissive; CEM, H9, and, importantly, PBL are nonpermissive to varying degrees. Depending upon the experimental system employed, either the target cell or the producer cell can determine the outcome of infection by *vif*-negative HIV-1. *vif*-Negative virus introduced into permissive cells replicates; its progeny is infectious and can spread virus throughout the culture, because the cell carries factors which compensate for the absence of *vif*. *vif*-Negative virus produced in permissive cells is competent for at least one round of infection in any target cell (Gabuzda et al. 1992; von Schwedler et al. 1993). *vif*-Negative virus derived from nonpermissive cells is minimally infectious to noninfectious in a single round of infection (Gabuzda et al. 1992).

4 Site and Proposed Mechanism of Action of Vif

Some attempts have been made to identify the site of action of Vif. Because Vif appears to influence the infectivity of progeny virus, it has been considered to act late in the virus life cycle. Examination of subcellular localization of the *vif* protein revealed that it is present mostly in the cytoplasmic fraction, with some of the protein tightly bound to cellular membranes (Fig. 2; Michaels et al. 1993; Goncalves et al.1994); Vif is not present in virions (Sakai et al. 1991), at least at levels comparable to the structural proteins or Vpr (Cohen et al. 1990; Yuan et al. 1990). The membrane-associated form of the Vif protein has been found to localize predominantly to the cytoplasmic side of cellular membranes; this association as well as the function require an intact C terminus of Vif (Goncalves et al. 1994). Assuming, therefore, that Vif may alter the processing or localization of virion components, the synthesis of viral structural proteins in the presence and absence of *vif* was examined. It has been difficult to detect any differences in intracellular viral protein profiles between wild-type and *vif*-negative HIV-1 tested in a variety of animal cells including Jurkat, SW480, MT-4, CEM, HeLa, Cos-1, and C8166 cells (Sodroski et al. 1986a; Fisher et al. 1987; Adachi et al. 1991; Nishino et al. 1991; Sakai et al. 1991; Kishi et al. 1992; Ma et al. 1994). Wild-type and *vif*-negative virions produced in CEM cells were found to show differences in envelope glycoproteins (Sakai et al. 1993), although another report disputed these findings (von Schwedler et al. 1993). It has been proposed that Vif acts as a cysteine protease to cleave the carboxyl terminus of gp41 (Guy et al. 1991), but this proposal was challenged by the finding that Vif is functional even in the absence of this region gp41 (Gabuzda et al. 1992). The gp41 present in wild-type virions can be detected by immunoblotting using antibodies directed to the C terminus of gp41 (von Schwedler et al. 1993; Ma et al. 1994), further indicating that the C terminus of gp41 is not the target for Vif activity.

A recent electron microscopy and goniometric analysis revealed that *vif*-

negative viral particles produced in CEM and Jurkat cells may be structurally defective as indicated by the non-homogeneous packing of viral cores in *vif*-negative virions harvested late after virus maturation (Hoglund et al. 1994). The *vif*-negative virions produced in SupT1 cells, a permissive cell line (Fig. 4), had a wild-type virion morphology. Although not conclusive, this study associates the reduced infectivity of *vif*-negative virus with detectable structural abnormality in extracellular virions. The altered morphology of *vif*-negative virus cores (Hoglund et al. 1994) is consistent with functional defects related to the core utilization in infection, such as the observed inefficient viral DNA synthesis (Sova et al 1993; von Schwedler et al. 1993).

Noting the potential significance of the two cysteines in Vif which are conserved in all HIV isolates reported (Myers et al. 1993) and *in vivo* (Wieland et al. 1994; Sova et al. 1995), we investigated their role in Vif function during HIV-1 replication. Using site-directed mutagenesis we replaced each cysteine by leucine in infectious provirus and found that either one or both cysteines are required for the *vif*-positive phenotype. Mutation of either cysteine resulted in an infection phenotype indistinguishable from that of the *vif* deletion mutant (Ma et al. 1994). This is the first example of a substitution point mutation ablating Vif function and indicates a region of the protein critical for its activity, which is distinct from the C terminus region required for Vif for binding to cellular membrane (Goncalves et al. 1994).

5 Conclusions and Future Prospects

As was first observed in 1987, *vif* influences the infectivity of progeny virions (Fisher et al. 1987; Strebel et al. 1987). In certain cell lines, infectious virus can be produced in the absence of *vif*. However, *vif* is essential for the production of infectious HIV-1 by PBL, the predominant target for HIV-1 infection in vivo. Unlike other observations made about the role of *vif* in HIV-1 infection, the requirement for *vif* during infection of PBL has been confirmed in every report on the matter. The ambiguity concerning the role of *vif* during HIV-1 infection in T cell lines has been superseded by its uncontested and essential activity in HIV-1-infected PBL. Thus *vif* constitutes a novel target for intervention during natural HIV-1 infection in humans.

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Nef

L. Ratner and T.M.J. Niederman

1 Introduction

The *nef* gene is present in all human and simian immunodeficiency retroviruses, suggesting an important function during natural infection (Fig. 1). A homologous gene, *bel 3*, is also present in human spumaretroviruses (Maurer and Flugel 1987). The importance of nef sequence conservation has been confirmed by studies with SIV_{mac} in rhesus macaques in which an intact *nef* gene was found to be important for continuous virus replication in vivo and for the development of AIDS. However, considerable heterogeneity is found in the *nef* gene, with levels of sequence variation comparable to that of the gp120 surface envelope protein.

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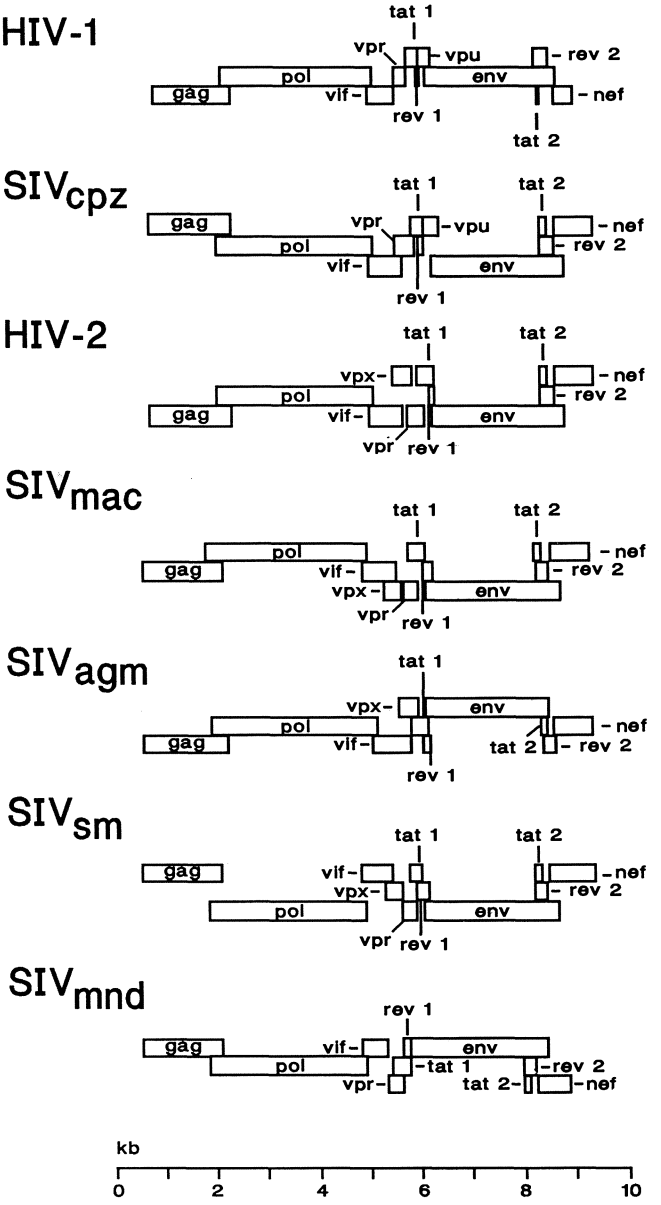

Fig. 1. Conservation of Nef. The genomic structures of HIV and SIV lentivirus genomes are shown. SIV strains were isolated from chimpanzees (*cpz*), rhesus macaques (*mac*), African green monkeys (*agm*), sootey mangabeys (*sm*), and mandrills (*mnd*). Genes encoding structural (*gag, env*) or enzymatic proteins (*pol*) proteins of the virus particles are indicated. In addition, the figure shows regulatory genes, *tat* and *rev*, and accessory genes, *vif, vpr, vpx, vpu*, and *nef*. (Modified with permission from BRYANT and RATNER 1992)

The *nef* gene is among the earliest transcribed open reading frames during HIV-1 replication in tissue culture. It encodes a 25- to 27-kDa myristylated protein with homology to GTP-binding (G) proteins. Nef is found to be at least partially anchored to cellular membranes and is not present in the virus particle.

The function of the expressed Nef protein is controversial. Considerable evidence has been presented to suggest that Nef down-regulates virus replication, though this is not agreed by all investigators. In contrast, several recent studies point to a positive effect of Nef on virus replication, particularly in quiescent cells. Several studies suggest that Nef is capable of inhibiting transcription from either the HIV-1 or the IL2 promoters, but this point is also controversial. Other data suggest a role for Nef in down-regulating the level of cell-suface CD4 expression. The experimental data to support or refute these findings will be reviewed, and potential unifying explanations will be proposed. Furthermore, the relevance (or lack thereof) of tissue culture studies of Nef will be discussed in light of the exciting data on the pathogenic role of Nef in vivo.

Nef is an immunogenic protein in HIV-1-infected individuals. Although several investigators have suggested that anti-Nef antibody reponses are diminished with HIV-1 disease progression, additional data are required. Furthermore, several investigators suggest that anti-Nef antibody responses may precede full HIV-1 seroconversion in HIV-1-infected individuals, but more recent data are in conflict with these findings. There are also considerable data to suggest that Nef is capable of eliciting T-cell proliferative and cytotoxic responses.

The *nef* gene has carried many different names, including *3' open reading frame (3' orf), open reading frame E' (orf E'), open reading frame F (orf F), and open reading frame B (orf B).* Based on functional studies in tissue culture, the gene was designated *negative factor (nef).* Certainly, when more complete information is obtained about the function of *nef*, a new name will be assigned. For this review, the *nef* gene will be indicated in italics, whereas the Nef protein will be capitalized.

2 Immune Responses to Nef

2.1 Do Antibody Responses to Nef Provide Prognostic Information?

The Nef protein is immunogenic during HIV-1 infection of humans. Sabatier and colleagues (1989) detected anti-Nef antibodies in 70% of 300 HIV-positive sera. Allan and colleagues (1985) demonstrated anti-Nef antibodies in 43% of subjects with AIDS-related complex and in 29% of subjects with AIDS. Kienzle and colleagues (1991) reported anti-Nef antibody responses in 17 of 57 (30%) HIV-1 seropositive sera. Bahraoui and colleagues (1990a) found that 16 of 32 (50%) HIV-seropositive men and five of six seropositive women had anti-Nef antibodies

detectable by a radioimmunoassay. In contrast, none of 29 seronegative women had such antibodies. When this assay was used to analyze an additional 550 sera of asymptomatic seronegative individuals, none were found to have anti-Nef reactivity (GLUCKMAN et al. 1989). In a study by CHEINGSONG-POPOV and colleagues (1990), anti-Nef antibodies were detected in 72 of 92 (78%) HIV-seropositive hemophiliacs. In an examination of the isotype restriction of antibodies to HIV-1, KHALIFE and colleagues (1988) found that, whereas the response to *gag* products was polyisotypic, including IgM, IgG1, IgG3, and IgA responses, the response to *nef* products was more restricted, to IgM, IgG1, and IgA responses.

Antibody responses to Nef may occur as a result of lysis of Nef-expressing HIV-1-infected cells or a specific export of Nef protein from infected cells. The failure to detect Nef in all HIV-1-infected individuals may be due to insensitivity of the ELISA assays for Nef, heterogeneity in the *nef* gene among different HIV-1 isolates (see below), or absence of Nef expression from some HIV-1 isolates. Alternatively, limited Nef-specific antibody responses may occur in some infected individuals.

Several investigators have also examined whether there is a correlation between the presence of anti-Nef antibodies and the stage of HIV-1 infection. REISS and colleagues (1989) reported that HIV-1-seropositive individuals with absent, disappearing, or intermittent presence of anti-Nef antibody responses were more likely to have markers of advanced HIV-1 disease than individuals with persistently positive anti-Nef responses. The disease markers which correlated inversely with anti-Nef antibody detection included detectable p24 antigenemia (44% versus 20%), absolute number of CD4 lymphocytes less than 500/mm^3 (47% vs 27%), and absent anti-p24 antibody responses (71% versus 45%). In addition, there was a trend, though it was not statistically significant for individuals with negative or transient anti-Nef antibodies, to have more frequent clinical signs of AIDS than individuals with persistent anti-Nef antibodies. RANKI and colleagues (1987) also noted declining titers of anti-Nef antibodies with HIV-1 disease progression, though insufficient numbers of patients were analyzed to identify statistically significant differences in anti-Nef titers. Similar findings were also reported by MATSUURA and colleagues (1991). In contrast, WIELAND and colleagues (1990) noted no correlation between the presence of anti-Nef antibodies and HIV-1 clinical disease stage. In this study, 86% of HIV-1-positive sera manifested reactivity with the recombinant Nef protein by immunoblot analysis. The correlation with disease stage of anti-Nef antibodies in the latter study may have been obscured by the high sensitivity of the immunoblot antibody assay used by these investigators.

Several authors have suggested that anti-Nef responses may precede full seroconversion, i.e., antibody responses to envelope and gag products. RANKI and colleagues (1987) reported antibody responses to both Nef and Vif prior to full seroconversion. However, some of these individuals were found to have p24 antigenemia, anergy to tuberculin purified protein derivative, or sporadic antibodies to HIV-1 structural proteins. These findings suggested that methods for detection of anti-envelope and anti-gag antibodies may have been insensitive.

Ameisen and colleagues (1989a,b) also reported finding anti-Nef antibodies in seven of 12 subjects prior to full seroconversion. These investigators demonstrated the specificity of their assay for anti-Nef antibodies. Blocking studies carried out with recombinant Nef proteins or synthetic Nef peptides efficiently removed the anti-Nef antibodies. Several of these individuals were also found to possess HIV-1 DNA sequences by PCR. These individuals remained negative in circulating p24 antigen, HIV-1 ELISA, and Western blot studies for the subsequent 10 months. These authors suggested that these results may be a consequence of a defective HIV-1 virus, a latent HIV-1 virus, or a different but related retrovirus.

However, these two studies were contested by de Ronde and colleagues (1988), who found only two of 72 individuals to have anti-Nef antibodies prior to full seroconversion. Furthermore, these antibodies were detectable for only a short period prior to full seroconversion. Reiss and colleagues (1989) also found anti-Nef antibodies very rarely in seronegative exposed homosexual partners of subjects with AIDS. Moreover, Bahraoui and colleagues (1990a) found no anti-Nef antibodies in 26 seronegative women who were sexual partners of seropositive hemophiliacs, even though 11 of 26 individuals were found to be HIV DNA PCR positive. However, in this study, anti-Nef antibodies were detected at the time of seroconversion in all of 12 individuals examined.

Similarly, Cheingsong-Popov and colleagues (1990) detected anti-Nef antibodies in none of 98 HIV-1-exposed seronegative individuals. These included both sexual partners of HIV index cases and hemophiliacs who received contaminated factor VIII preparations but remained seronegative. Furthermore, Cheingsong-Popov and colleagues noted that false-positive anti-Nef responses could be effectively blocked by preincubation with crude extracts of *Escherichia coli.* This suggested that there is a potential anti-Nef cross-reactive protein in *Escherichia coli,* which was used for preparation of antigens in most serological assays for Nef. This finding may account for some of the discrepancies with regard to the significance of anti-Nef antibody detection prior to full seroconversion.

Finally, Ranki and colleagues (1990) confirmed the lack of specificity of their anti-Nef detection methods by demonstrating similar serological reactions in four of 93 dermatologic patients not at risk for HIV infection.

Several studies have mapped B-cell epitopes in Nef by a variety of techniques. Bahraoui and colleagues (1990b) and Estaquier and colleagues (1992) used various peptides to identify sequences in Nef reacting with antibodies of chimpanzees inoculated with soluble recombinant vaccinia virus-expressed p27 Nef protein. Reactive sequences included amino acids 17–35, 52–66, 65–146, and 185–205, with the C-terminal peptide eliciting the highest response. Though amino acids 17–35 and 52–65 represent peaks in the hydrophilicity profiles of Nef, the C-terminal portion of the protein is not expected to be hydrophilic. The presence of a dominant B-cell epitope at the C-terminus of Nef was confirmed by Arya (1987). Bahraoui and colleagues (1990b) have also identified a B-cell epitope for Nef between amino acid residues 32 and 64 in studies with synthetic peptides used to block anti-Nef serological responses of HIV-1-infected subjects.

Gombert and colleagues (1990) used the epitope analysis method of Geysen and colleagues to analyze a set of more than 200 nonapeptides based on Nef sequences of three HIV-1 isolates: BRU, MAL, and SF2. These peptides were used to detect serological reactivities in HIV-1-infected subjects. This led to the identification of nine different homologous epitopes spread throughout the Nef sequence, including some of the residues previously identified by Bahraoui and colleagues (1990b). Similar results were reported by Schneider and colleagues (1991). In this study, amino acids 8–16 were also found to function as a B-cell epitope, a finding confirmed by Kienzle and colleagues (1991).

de Santis and colleagues (1991) demonstrated that residues 83–88 also elicit Nef-specific antibody responses by raising a murine monoclonal antibody to this sequence. This sequence was chosen in light of its homology to the N-terminus of thymosin alpha 1. Though this sequence is well conserved among North American HIV-1 isolates, less conservation is seen with African HIV-1 isolates. The resulting monoclonal antibody was capable of detection of this Nef peptide, as well as the Nef protein expressed in peripheral blood mononuclear cells (PBMCs).

In summary, these findings demonstrate anti-Nef antibody responses in 30–80% of HIV-1-infected individuals, with somewhat more frequent detection early during HIV-1 infection than during late stages of infection. However, anti-Nef antibodies are rarely found prior to full seroconversion characterized by anti-Env and anti-Gag antibodies. Several different Nef domains are immunogenic after animal inoculation or natural infection.

2.2 Is Nef a Target for Cytotoxic T-Cell Responses?

Since HIV-1 is an intracellular pathogen, it is likely that cell-mediated immune reponses are an important component of the immune defense against this pathogen. There is considerable evidence that proliferative and cytotoxic T-lymphocyte (CTL) responses are directed against HIV-1 structural proteins, including Gag, Pol, and Env (Wahren et al. 1987; Torseth et al. 1988; Ahearne et al. 1988; Schrier et al. 1989; Plata et al. 1987; Walker et al. 1987, 1988; Sethi et al. 1988; Nixon et al. 1988; Koenig et al. 1988; Shepp et al. 1988; Hoffenbach et al. 1989; Riviere et al. 1989; Robertson et al. 1993). Several investigators have also demonstrated cytotoxic T-cell responses to Nef, which are present in about 60% of HIV-1-infected individuals (Culmann et al. 1989, 1991). In both reports, the investigators demonstrated that these responses are mediated by $CD8^+$ HLA-restricted CTLs. Amino acid residues 113–147 were identified as the major T-cell epitope in the study by Culmann and colleagues (1989). The identification of this T-cell epitope was confirmed by Bahraoui and colleagues (1990b), as was the identification of the C-terminus of Nef as an additional T-cell epitope. Koenig and colleagues (1990) also identified amino acid residues 73–97 as a major target for CTLs. Identification of CTL epitopes in the central and carboxyl-terminal

regions of Nef has also been confirmed by HADIDA and colleagues (1992) using lymphocytes from lymph nodes and spleens of HIV-1-infected patients.

Three epitopes in the central portion of the SIV_{mac} Nef protein have also been identified as T-cell epitopes (BOURGAULT et al. 1992). These regions are homologous to the epitopes previously identified by KOENIG et al. (1990), CÜLMANN et al. (1989), and BAHRAOUI et al. (1990b). It is unclear, however, whether anti-Nef CTLs are protective against SIV_{mac} infection in vivo. This is particularly important to determine, if one is to consider the use of *nef*-deleted SIV_{mac} strains as attenuated vaccines (DESROSIERS and HUNTER 1991).

T-cell proliferative responses to Nef have also been induced. WINTER and colleagues (1991) expressed the HIV-1 Nef protein in *Mycobacterium bovis* and induced a T-cell proliferative response in inoculated mice.

In summary, cell-mediated immune responses to Nef are found in the majority of HIV-1-infected individuals and are directed against several different T-cell epitopes in the protein. CTL responses to an early-expressed gene product, like Nef, may be important in immune clearance of virus-infected cells prior to virus particle release.

3 Nef Expression

The complex pattern of expression of HIV-1 transcripts has been analyzed using specific RNA-PCR methods (SCHWARTZ et al. 1990). The *nef* transcript results from two or more splicing events, with uniform utilization of the splice donors at nucleotides 287 and 5625 and splice acceptors at nucleotides 5625 and 7956. Nef mRNAs represent 90% of the HIV-1-specific RNAs expressed within the first 6 hrs of infection, and *nef* mRNAs are detected as early as 1 h after infection (GUATELLI et al. 1990; KLOTMAN et al. 1992).

Tat mRNAs are also expressed early after infection, leading to a dramatic up-regulation of HIV-1 promoter expression by 6 h after infection. This results in an up-regulation of the early-expressed *rev* transcript, which is critical for the transition from the early to the late stage of gene expression (Fig. 2). Rev not only results in the increased expression of *gag-pol* and *env* transcripts, but also leads to down-regulation of the level of *nef* transcripts (AHMAD et al. 1989). Tat, Rev, and Nef represent a coordinated regulatory network involved in the conversion of a low-level persistent infection phenotype to a productive lytic infection.

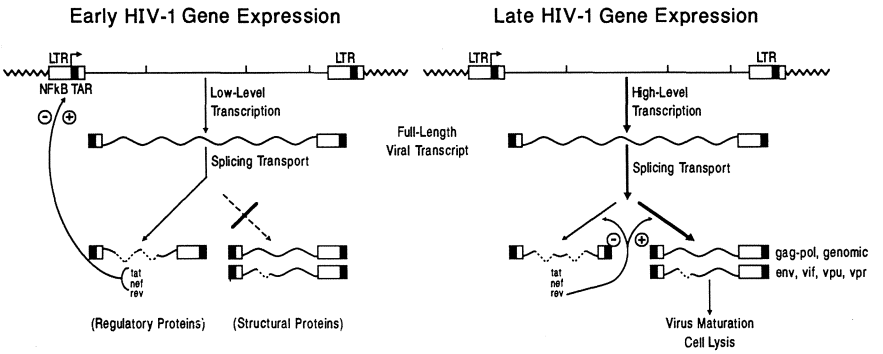

Fig. 2. Early to late switch of HIV-1 transcription. A low level of proviral transcription early in infection results in the expression of HIV-1 products Tat, Nef, and Rev. Tat and Nef have opposing effects on viral transcription. Once a threshold level of Rev expression is achieved, a switch from early to late HIV-1 transcription occurs, allowing the expression of Gag, Pol, Env, Vif, and Vpr, and Vpu products. This results in virus maturation and cell lysis. (Modified with permission from CULLEN 1991)

4 Nef Protein Structure

4.1 Why Is Nef Heterogeneous in Size and Electrophoretic Mobility?

KAMINCHIK and colleagues (1991) used the *nef* genes derived from HIV-1 isolates BH10 and LAV for in vitro translation with reticulocyte lysates and for expression with an SV40 promoter in COS-1 cells. In both cases, two protein products were identified with apparent molecular weights of 25 and 27 kDa. Pulse-chase analysis demonstrated that neither protein was a precursor to the other. However, the 27-kDa protein was found to be considerably more stable than the 25-kDa product. The 25-kDa product was demonstrated to be derived from translational initiation at the second methionine codon (codon position 20) since it was not labeled by ^{3}H-myristic acid, which is incorporated into the 27-kDa product attached to the N-terminal glycine residue after cleavage of the first methionine residue (ALLAN et al. 1985). Furthermore, mutation of the first methionine codon resulted in production of only the 25-kDa product. Deletion of the 5' 15 codons of *nef* also resulted in the synthesis of only the 25-kDa product. Mutation of the myristylation acceptor glycine residue did not alter the electrophoretic mobility of Nef, arguing against an effect of the myristic acid moiety on the apparent molecular weight of Nef.

ZWEIG and colleagues (1990) also noted two Nef Products with apparent molecular mass of 25 and 27 kDa produced in H9 lymphoid cells infected with the HIV-1 isolate HTLV-IIIB. Both of these isoforms of Nef were labeled with ^{3}H-myristic acid (BRYANT et al. 1991; RATNER et al. 1991b). Different virus subpopulations derived from the mixed HTLV-IIIB virus stock produced Nef proteins with apparent molecular mass of either 25 or 27 kDa, but not both proteins. The p25

protein was found to be more basic than the p27 protein, based on isoelectric focusing analysis (Zweig et al. 1990). These data suggested that two forms of Nef are present with different electrophoretic mobilities. The electrophoretic mobility differences may represent differences in protein folding or post-translational modification.

Obaru and colleagues (1992) provided a likely explanation for the findings reported by Zweig and colleagues (1990). They found that the presence of an aspartic acid or an alanine at residue 54 accounted for the difference in electrophoretic mobility of Nef proteins derived from HTLV-IIIB molecular clones, when expressed in reticulocyte lysates. This is likely due to a conformational change in Nef rather than to a post-translational modification. Secondary structure predictions suggest that this region of Nef is an alpha helix when residue 54 is an aspartic acid and a beta turn when alanine is present. The affinity of a monoclonal antibody is also affected by the residue at this position.

There is considerable heterogeneity in the amino acid sequence of Nef, with variation in up to 17% of amino acid residues (Ratner et al. 1985). In addition, several HIV-1 strains have deletions or insertions in the predicted Nef product (Myers et al. 1990). Prematurely truncated *nef* open reading frames have also been noticed. The HXB2 strain of HIV-1 encodes only the N-terminal 124 amino acids of Nef (Ratner et al. 1985, 1987), whereas the MN strain of HIV-1 encodes a 185 amino acid form, in contrast to the 206 amino acid form of Nef encoded by most other HIV-1 strains (Myers et al. 1990).

Delassus and colleagues (1991) examined *nef* sequence heterogeneity over the course of 4 years in HIV-1 strains infecting a single individual. Of 120 Nef protein sequences analyzed, only five were obviously defective, based on the presence of termination codons or large deletions. No particular Nef amino acid sequences predominated at a given stage of disease. However, variation was greatest in the amino- and carboxy-terminal regions of the protein product.

Shugars and colleagues (1993) examined 54 Nef sequences from 12 individuals with absolute CD4 lymphocytes counts of 10–190/mm^3. They noted that intraindividual nucleotide sequence variation ranged from 0.6 to 11.2%, whereas interindividual variation was 0.3–13.2%. Furthermore, they recognized several relatively conserved sequences, including a nearly invariant myristylation signal, a region with an acidic charge at residues 62–65, a $(Pxx)_4$ repeat sequence at residues 69–80 (where P is proline and x is any amino acid), and a potential protein kinase C phosphorylation site at residue 80. Variable duplications of residues 23–29 were found in 28 of the 54 Nef protein sequences. Premature termination codons in place of tryptophan residue 124 were noted in six of 54 sequences derived from four of the individuals, the same position at which termination codons in Nef were previously reported from other isolates (Ratner et al. 1985).

McNearney and colleagues (1995) examined 80 Nef sequences from sequential isolates over the course of disease in four individuals. Of particular interest were the high frequencies of deletions, frameshifts, and termination codons in sequences obtained at late, but not early stages of disease. Though increasing heterogeneity was noted among V3 envelope sequences obtained from these

individuals with disease progression, Nef sequences appeared to evolve independently of envelope sequences, and increasing Nef sequence heterogeneity was found in only two of three individuals examined. The functional significance of Nef sequence heterogeneity and disruption remains to be determined.

4.2 Where Is Nef Localized in the Infected Cell?

Using indirect immunofluorescence with an anti-N terminal Nef antibody and COS cells transfected with a Nef expression vector, HAMMES and colleagues (1989) found punctate cytoplasmic staining suggestive of localization at distinct intracellular sites. Using transfected astrocytoma cells, BACHELERIE and colleagues (1990) found predominant cytoplasmic localization of Nef, with perinuclear enhancement of staining. In contrast, FRANCHINI and colleagues (1986) found homogeneous staining in the cytoplasm of HIV-1-infected lymphoid cells.

With HIV-1-infected MT4 cells, OVOD and colleagues (1992) noted polar localization of Nef in the cytoplasm, suggestive of a Golgi localization. However, no other localization studies were employed to confirm this suggestion.

KAMINCHICK and colleagues (1991) examined the subcellular distribution of both the 27- and 25-kDa forms of Nef, the latter form resulting from initiation at the second methionine codon. Though the 27-kDa form was found in both the cytosolic and membrane fractions isolated by ultracentrifugation, the 25-kDa form was present only in the cytosolic fraction. This suggests that the N-terminal portion of Nef is required for membrane association, or for proper folding of Nef to allow membrane association.

In contrast, KROHN and colleagues (1991) reported transient nuclear localization of Nef in acutely infected T cells at an early stage of virus replication. In addition, these investigators found nuclear staining in chronically infected TH4-7-5 T-lymphoid cells using a monoclonal antibody directed against amino acid residues 168–175. Antibodies recognizing other regions of Nef localized the protein predominantly in the cytoplam. Stable expression of Nef in B lymphocytes also resulted in forms detectable in both the nucleus and cytoplam (KIENZLE et al. 1992). These investigators suggest that a distinct isoform of Nef is localized in the nucleus. Support for these findings comes from immunofluorescence and immunoelectron microscopy studies of MURTI and colleagues (1993), who demonstrated linear tracts of Nef protein through the nuclei of a human $CD4^+$ cell line. Although only 5–10% of cells demonstrated this staining pattern, the remainder demonstrating cytoplasmic fluorescence, the authors suggest that the low level of detection may simply represent technical considerations, in that only certain sections will be parallel to the path of the nuclear tracts. Alternatively, they raise the possibility that nuclear localization of Nef may be regulated by other parameters, e.g., the cell cycle. They also identified a potential bipartite nuclear localization signal at residues 8–15. However, functional analysis of this sequence remains to be performed, and the significance of nuclear localization of Nef remains unclear.

4.3 What Is the Role of Nef Myristylation?

ALLAN and colleagues (1985) first noted that the *nef* gene product was myristylated (Fig. 3). YU and FELSTED (1992) examined the effect of myristylation on the subcellular localization of Nef using an SV40 expression system. Both subcellular fractionation and immunohistochemical microscopy were used in this study. They used the Nef produced by the HXB3 strain of HIV-1, as well as a mutant in which the codon 2 glycine was mutated to an alanine. Upon transfection of COS cells, the myristylated form was found in the cytoplasmic membrane fraction. In contrast, most of the nonmyristylated form of Nef was found in the nucleus. These findings were surprising, since there is no identifiable nuclear localization signal in Nef analogous to those present in SV40 large T antigen, polyoma large T antigen, or adenovirus E1a proteins. Nevertheless, these findings are possibly consistent with the intriguing findings of KROHN and colleagues (1991), suggesting that one isoform of Nef may be nuclear associated. These investigators also demonstrated that nonmyristylated Nef is inactive, at least with regard to its ability to inhibit HIV-1-specific transcription.

HARRIS and COATES (1993) identified several cellular membrane proteins that bound to myristylated but not to nonmyristylated Nef. Similarly, NIEDERMAN and colleagues (1993b) noted that Nef association with the cytoskeleton was myristylation dependent (see Sect. 4.2). For these studies they utilized biochemical fractionation methods to examine the cellular localization of Nef in Jurkat and HPBALL human T-lymphoid cells. In addition, they utilized a cell-free reconstitution assay that demonstrated a requirement for Nef myristylation for maximal cytoskeleton binding activity. These findings are reminescent of studies

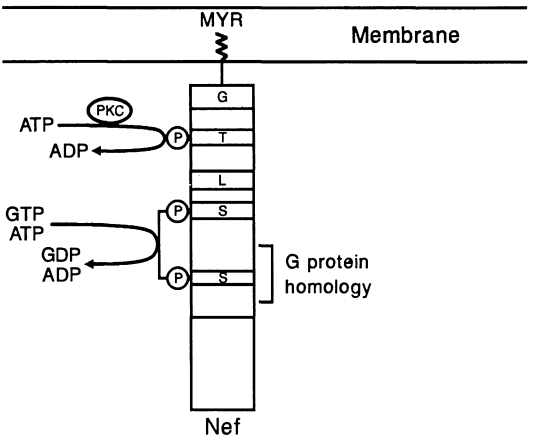

Fig. 3. Nef protein structure. The Nef protein has a myristyl anchor (*MYR*) attached to the N-terminal glycine residue (*G*), thought to be critical for cell localization near the plasma membrane. The threonine residue at amino acid position 15 (*T*) is a potential phosphorylation (*P*) acceptor for protein kinase C. Serine residues (*S*) may also be phosphorylation sites through autophosphorylation or other kinases utilizing either *GTP* or *ATP*. Leucine heptad repeat sequences (*L*) are shown with similarity to those present in several cellular transcriptional factors. The region of *G protein homology* is also indicated

performed with the myristoylated alanine-rich C kinase substrate (MARCKS) which associated with membrances in a myristylation-dependent manner (Li and Aderem 1992). In addition, $p60^{src}$ has been shown to associate with a 32-kDa plasma membrane protein in a myristylation-dependent manner (Resh and Ling 1990).

4.4 Is Nef a G Protein?

Samuel and colleagues (1987) reported sequence similarity between residues 93–112 of Nef (Fig. 3) and the nucleotide binding region of protein kinases, including cAMP- and cGMP-dependent protein kinases, calcium-dependent phosphorylase b kinase, $p60^{src}$, $p120^{gag\text{-}abl}$, $p37^{mos}$, CDC28, epidermal growth factor receptor, and insulin receptor.

Guy and colleagues (1987) also noted sequence similarity of residues 94–123 of HIV-1 Nef and residues 126–155 of HIV-2 Nef and the GTP-binding domain of ras proteins, G_s alpha, and G_t2 proteins. These investigators demonstrated GTP binding by a filter retention assay of γ ^{35}S-thioGTP and bacterially expressed Nef. They found as much as 2.5 pmol GTP bound in a reaction with 20 pmol of Nef protein. GTP binding was dependent upon magnesium and occurred under conditions in which phosphorylation of Nef also occurred. The autophosphorylation site under these conditions was thought to be a serine residue because of its lability in alkali (Fig. 3). Guy and colleagues (1987) also demonstrated GTPase activity of the bacterially expressed Nef protein. The rate of cleavage was found to be comparable to that of purified v-Ha-Ras protein.

In contrast, Kaminchik and colleagues (1990) demonstrated that neither BH10 nor LAV Nef proteins expressed in *E. coli* bound GTP or had significant GTPase activity. The discrepancy with the work of Guy and colleagues (1987) may be due to bacterial GTP-binding protein impurities in the partially purified recombinant Nef preparation used by Guy and colleagues (1987). Other methods of expression in *E. coli* resulting in soluble monomeric Nef expressed by Kaminchik and colleagues (1990) compared with the insoluble protein in the case of Guy and colleagues (1987), different N-terminal sequences in the recombinant proteins, or different internal sequences resulting from use of various cloned *nef* genes in the different studies may account for these discrepancies. Improper folding of the purified proteins obtained by Kaminchik and colleagues could also explain their negative results. EDTA present in the purification buffers may denature and inactivate Nef, as has been reported for Ras proteins (Backer et al. 1991). However, Backer and colleagues (1991) purified Nef from a bacterial expression system under conditions previously shown to allow purification of active ras proteins. Nef purified in this manner lacked GTP-binding, GTPase, or autophosphorylation activities. Similar findings to those of Backer and colleagues (1991) were reported with a baculovirus-expressed Nef protein derived from the NL4-3 strain of HIV-1 (Matsuura et al. 1991). In addition, Dubois and colleagues (1993) found no GTP-binding activity of bacterially expressed HIV-2 Nef.

Nebreda and colleagues (1991) also compared bacterially expressed Nef with bacterially expressed Ras. In this study Nef proteins from three different HIV-1 isolates (BRU, BH10, and NL432) were utilized, and all had similar activities. Though no GTP-binding activity was found with the purified Nef proteins, weak autophosphorylation activity was found with either ATP or GTP. Furthermore, Nef was incapable of inducing meiotic maturation of *Xenopus* oocytes, an activity previously reported for Ras proteins and dependent on their ability to bind and hydrolyze guanine nucleotides.

Although Nef may lack significant GTP-binding and GTPase activity, it may still represent an "ancient G protein" having evolved from this class of proteins. It may therefore function as a promiscuous competitive inhibitor, interfering with both positive and negative regulatory effects of G proteins.

4.5 Is Nef a Phosphoprotein?

Guy and colleagues (1987) noted a potential protein kinase C (PKC) phosphorylation site at threonine 15 of HIV-1 Nef and serine 10 of HIV-2 Nef proteins, homologous to those present at protein kinase C phosphorylation sites of $pp60^{src}$ and the epidermal growth factor receptor. Upon expression of HIV-1 Nef from a recombinant vaccinia virus, these investigators demonstrated incorporation of ^{32}P-orthophosphate into Nef and enhanced phosyphorylation upon treatment with a protein kinase C activator, 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Phosphate labeling was eliminated by mutation of threonine residue 15 to alanine. Furthermore, purified, bacterially expressed Nef was phosphorylated in vitro by PKC in a calcium- and phospholipid-dependent reaction.

These findings were reaffirmed by the work of Bandres and colleagues (1994), who also showed that threonine 15 was the predominant site of Nef phosphorylation in whole cells. Moreover, they demonstrated that threonine to alanine mutation at residue 15 had no effect on the ability of Nef to alter CD4 expression (Bandres et al. 1995), but that this alteration was required for the transcriptional effects of Nef (Bandres et al. 1994).

Poulin and Levy (1992) provided evidence that Nef is associated with the phosphorylation of a cellular protein. Translation of the Nef protein of HIV-1 strain SF2 in rabbit reticulocytes resulted in a major product of 27 kDa, and two minor products of 25 and 29 kDa. Using Nef immune complexes for in vitro kinase assays, they detected ATP-dependent phosphorylation of a 46-kDa protein. In these studies, no Nef autophosphorylation was seen. However, it is possible that the antibody masked a potential autophosphorylation site.

Sawai and colleagues (1994) also identified the association with a chimeric CD8-Nef fusion protein of cellular serine kinases of 62 kDa and 72 kDa. These proteins did not associated with CD8, but did associate with Nef products of HIV-1 strains SF2 and SF33. The cellular proteins coimmunoprecipitated with Nef and were phosphorylated on serine residues in in vitro kinase assays. No effect on phosphorylation was seen with inhibitors of protein kinases A or C. Furthermore,

no reaction was seen with antibodies that recognize other serine kinases including Raf, S6 kinase, GTP-associated protein (GAP), and MAP kinase. The kinases were unable to associate with forms of Nef truncated at amino acids 94 or 99.

The identity of the 46-kDa, 62-kDa, and -72 kDa proteins, their relationship to one another, the species of Nef responsible for the activity, the mechanism whereby Nef promotes phosphorylation of the serine kinases, and the significance of these findings with respect to virus replication and T-cell activation events occurring in whole cells expressing Nef with or without other HIV-1 products remain to be investigated.

4.6 Other Nef Sequence Homologies

SAMUEL and colleagues (1991) have demonstrated amino acid sequence similarity between residues 68–100 of HIV-1 Nef and residues 100–129 of HIV-2 Nef and leucine zipper motifs present in transcription factors or potential transcription factors, including L-myc, c-myc, c-fos, FRA-1, GCN4, and mXBP (Fig. 3). In addition, they noted an acidic helix-turn-helix sequence in HIV-1, HIV-2, and SIV_{mac} Nef proteins carboxyl terminal to the leucine repeat sequences, similar to those present in GCN4, VP16, CPC1, hepatitis B virus X protein, cross-pathway control gene product of *Neurospora crassa*, and heat shock transcription factor of yeast. These investigators suggested that the leucine repeat domain may be a dimerization domain. The presence of a leucine repeat domain and an acidic domain resembling the activation domain of transcriptional factors suggests that Nef may resemble such transcription factors. However, the lack of a basic DNA interaction domain and the localization of Nef in the cytoplasm would argue against a positive transcriptional activation effect. Nevertheless, these findings suggest that Nef may be capable of interacting with transcriptional factors and inhibiting their activities.

Homology of Nef to HLA class-II histocompatibility antigens has also been noted. This explains the finding that several monoclonal antibodies to Nef are also capable of reacting with H9 cells expressing HLA class-I and -II antigens (VEGA et al. 1990; GELDERBLOM et al. 1987).

Amino acid residues 91–116 of Nef share structural homology with a highly charged region within the intracytoplasmic phosphorylation domain of the human interleukin-2 receptor alpha, the ATP-binding site of the catalytic subunit of cAMP-dependent protein kinase, and other members of the protein kinase family (SAMUEL et al. 1987). Sequence homology and antigenic cross-reactivity were also noted between residues 38–118 of Nef and residues 339–414 of the human thyrotropin receptor (BURCH et al. 1991). Sequence similarity of Nef amino acid residues 91–161 with scorpion toxins were also reported (WERNER et al. 1991). Moreover, peptides based on the Nef sequence and the scorpion toxin sequence had similar electrophysiological effects on cultured cells.

5 Effects of Nef on Virus Replication

5.1 What Is the Effect of Nef on HIV-1 Replication?

Nef was shown to be dispensable for HIV-1 replication in lymphoid cell lines in studies by FISHER and colleagues (1986). This was examined in greater depth by TERWILLIGER and colleagues (1986), who came to the same conclusions. However, their data also demonstrate somewhat earlier expression of viral proteins by the *nef* mutant viruses than the HIV-1 strain with an intact *nef* gene, as determined by immunofluorescence, reverse transcriptase, and syncytia assays in both Jurkat tat III and C8166 cells. Similar effects were noted by LUCIW and colleagues (1987), who reported five-fold higher levels of replication in HUT 78 lymphoid cells of the HIV-1 strain SF2 with a frameshift mutation in *nef* compared with the parental virus with an intact *nef* gene.

NIEDERMAN and colleagues (1989) also noted that an HIV-1 strain with a deletion in *nef* replicated to higher levels in T-lymphoid cell lines than the parental clone with an intact *nef* gene. The effect of the *nef* deletion was complemented in *trans* with a *nef* expression plasmid, demonstrating that the effect of mutation of the *nef* gene of HIV-1 was due to altered expression of the Nef protein. However, no differences were noted in the infectivity of the virus particles produced from cells infected with HIV-1 strains with or without *nef*. These investigators demonstrated suppressive effects of Nef on HIV-1 gene expression (Fig. 4). Similar results were reported by AHMAD and VENKATESAN (1988).

In contrast, KIM and colleagues (1989) noted no effects of nef mutation on HIV-1 replication in H9, CEM-SS, U-937, and THP-1 cell lines and primary lymphocytes. However, their work was compromised by the use of HIV-1 strains with or without a *nef* mutation that were not isogenic. Morever, these investigators reported findings only from experiments in which HIV-1 was used at relatively high multiplicities of infection (MOI), approximately 1 tissue culture 50% infectious dose ($TCID_{50}$) per cell. Multiplicity of infection is an important parameter in assessing the effects of *nef* (our unpublished observations). At a high MOI, there is very little difference in the kinetics of replication in MOLT 3 lymphoid cells of otherwise isogenic HIV-1 strains with or without *nef*. However, with progressively lower MOIs, the magnitude of the difference in replication kinetics is magnified. Similar effects were noted by MAITRA and colleagues (1991) and TSUNETSUGU-YOKOTA and colleagues (1992). The MOI dependence of Nef activity may be due to saturation at high MOI of a cellular component required for Nef activity. Alternatively, high-level expression of another viral component may block Nef activity.

CHENG-MAYER and colleagues (1989) suggested that differential effects of Nef may be due to variation in the Nef-responsive elements of different HIV-1 strains. Alternatively, TERWILLIGER and colleagues (1991) noted differences in Nef activity dependent upon the *nef* allele utilized. Whereas the *nef* gene obtained from the HIV-1 strain BH8 virus, derived from a North American AIDS patient, was capable of suppressing virus replication in Jurkat cells, the *nef* gene obtained from African HIV-1 strain ELI accelerated virus replication in primary lymphocytes. The

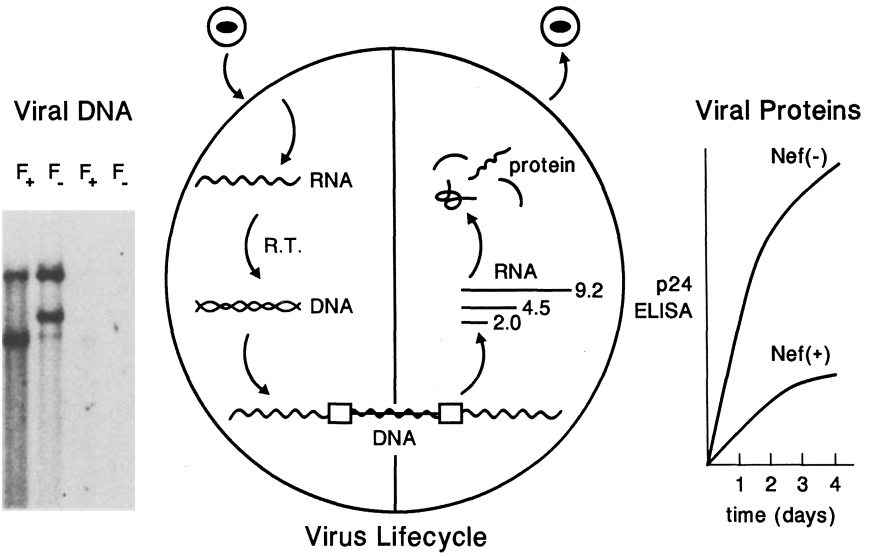

Fig. 4. Effects of Nef on virus replication. The figure summarizes the virus life cycle, divided into early and late steps. Early steps include virus entry, reverse transcription (*RT*), and integration. Late steps include transcription, translation, protein processing, and virus particle assembly. Transcription results in three classes of viral transcripts of *9.2*, *4.5* and *2.0* kb. *Left*: an assay of the early steps of virus replication, showing the results of viral DNA synthesis resulting from infection of lymphoid cells with equivalent numbers of virus particles derived from otherwise isogenic Nef+ (*F+*) and Nef– (*F–*) viruses. Viral DNA was detected after *Xho*I and *Sac*I digestion by Southern blot hybridization after infection and RNase digestion (*left two lanes*) or DNase I digestion (*right two lanes*). The difference in restriction enzyme digestion patterns results from the mutation in *nef*. The similarity in band intensities indicates that there is no significant difference in the infectivity of the virus particles. *Right*: an assay of the late steps of virus replication, showing the level of virus particles, measured by the p24 antigen ELISA, expressed from COS cells transfected with the same Nef+ and Nef– proviruses as a function of time after transfection. Two- to fivefold higher levels of virus expression were noted in cells transfected with Nef– compared with Nef+ proviruses. Similar results were obtained in assays of viral RNA levels by Nothern blot, and viral RNA synthesis by run-off assays (Niederman et al. 1989)

predicted protein products of BH8 and ELI *nef* genes differed in 25% of their amino acids. The effect of the ELI *nef* gene was identified in Jurkat cells only if the 3'portion of *env* was also derived from the HIV-1 ELI strain. Replication of virus with the BH8 *nef* gene was not analyzed in primary lymphocytes in this study. The significance of a possible interaction between the *nef* product and the *env* product remains unclear.

Zazopoulos and Haseltine (1993) extended these observations to *nef* alleles from several other HIV-1 isolates, including MAL, YU-10 and YU-21, and five primary HIV-1 sequences. In each case, the *nef* alleles were tested with HXB2 sequences in the 5'portion of the genome, ELI sequences encoding the C-terminus of envelope, and the remainder of the sequences derived from the isolates described above, with or without a frameshift mutation in *nef*. Three of these *nef* alleles produced unstable proteins that had minimal effects on virus replication, whereas the others accelerated virus replication in primary lymphocytes and Jurkat cells.

Findings similar to those of TERWILLIGER and colleagues (1991) and ZAZOPOULOS and HASELTINE (1993) were reported by DE RONDE and colleagues (1992). They noted that replacing the *nef* gene of HXB2 with that derived from PCR amplification of primary lymphocyte DNA of two HIV-1-infected individuals resulted in accelerated virus replication in primary lymphocytes. However, this work was compromised by the fact that the entire 3'LTR of HIV-1 strain HXB2 was also replaced. Therefore, it is unclear whether this result is due to a difference in nef or in LTR function.

CHOWERS and colleagues (1994) also reported that *nef* accelerated the replication of HIV-1 strain NL-4-3 100- to 10 000-fold in CEM cells. Similar results were obtained with a deletion mutation in *nef*, as with site-directed mutants, which introduce premature stop codons or alter the myristylation acceptor site. Single-cycle infection and transfection experiments, performed with the addition of neutralizing antibody, suggested that *nef* affected an early step in the virus life cycle, prior to viral integration. These findings appear to be in conflict with previous investigations, including that of AHMAD and VENKETESAN (1988), who examined *nef* activity with the same HIV-1 strain.

Significant positive effects of *nef* were reported with infection of quiescent lymphocytes and macrophages (MILLER et al. 1994; SPINA et al. 1994), but MILLER and colleagues found little or no effect of Nef in immortalized T-lymphoid cell lines. Similarly, AIKEN and colleagues (1994) reported no significant effects of Nef on HIV-1 replication in CEM cells. Using a high titer of virus (250 ng/ml), 1.0 to 3.5-fold effects were reported by MILLER and colleagues (1994), but with lower titers of virus (10 ng/ml), 8.1- to 28.0-fold effects were seen. Similar effects were reported during single-cell infection assays in HeLa-CD4-beta galactosidase cells. The latter studies suggest an effect of Nef on virus entry, or early post-entry virus replication events. SPINA and colleagues (1994) found no evidence for an effect of Nef on T-cell activation from studies of DNA synthesis, cell-cycle analysis, and expression of membrane markers for IL-2 receptor, transferrin receptor, MHC class II, and CD45 RO.

JAMIESON and colleagues (1994) analyzed the effects of *nef* on HIV-1 replication in human fetal thymus and liver implants in severe combined immunodeficient (SCID-hu) mice. Nef– and *nef*+ isogenic strains of HIV-1 JR-CSF and HIV-1 NL4-3 were utilized. Though the *nef* mutants of both strains showed similar kinetics of replication in vitro in mitogen-stimulated peripheral blood mononuclear cells, both *nef* mutants demonstrated attenuated growth properties in this in vivo model system. Furthermore, both *nef* mutants were also incapable of inducing depletion of both mature and immature thymocytes in this animal model, a property of both *nef*+ parental viruses. Since these chimeric animals have no functional immune responses towards HIV-1, the effect of *nef* on virus replication described here was most likely a direct consequence of the virological properties of the infection, rather than an immunological response to the virus. The divergence between in vitro and in vivo findings reported in this study may reflect differences in the targets of infection, i.e., mature lymphocytes versus thymocytes. Alternatively, the activation state of the cell may be a parameter critical to explaining the divergent findings.

Based on the previous findings with the ELI *nef* gene, Zazopoulos and Haseltine (1992) analyzed the effects of Nef mutations on its positive regulatory effects in Jurkat cells and primary lymphocytes. Mutation of the myristylation accepter glycine to alanine abrogated the biological activity of Nef. Mutation of alanine at position 15 to threonine, a potential protein kinase C phosphorylation site, had no effect. Mutation of cysteine residues in Nef resulted in an unstable protein (Cys-143) or in a stable protein with decreased biological activity in one case (Cys-170) and no change in activity in another case (Cys-55). Mutation of amino acid residue 54 from Asp to Ala, a change which altered the electrophoretic mobility of Nef, had no effect on biological function. Mutations in the G protein homology domain had no effect in one case (Gly-97) and resulted in an unstable protein in another case (Gly-100). Mutation of the potential N-glycosylation site (Asn-127) had no effect on Nef stability or function. These findings suggest that myristylation is critical for this biological activity of Nef. However, the other findings described above must be explored in greater depth before any conclusions can be made.

Recently, Baur and colleagues (1994) provided a possible explanation for at least some of the divergent observations of Nef activity. Their studies utilized Jurkat cells, in which they expressed a chimeric CD8-Nef protein. They identified two opposite signaling phenotypes, dependent on the intracellular localization of Nef. Expression of CD8-Nef at an intracellular location resulted in depressed T-cell activation responses. In contrast, expression of the CD8-Nef molecule at the cell surface resulted in enhanced T-cell activation markers, tyrosine phosphorylation, and nuclear translocation of NF-kB. Furthermore, the activated Jurkat cells underwent apoptosis. The investigators suggested that regulation of Nef cellular localization may result in differing effects of Nef, and that differential myristylation could account for these findings. However, nonmyristylated Nef protein has been shown to be defective in all previously characterized activities of Nef (Yu and and Felsted 1992; Aiken et al. 1994; Bandres et al. 1995). An additional limitation of this system is that comparisons of cells with Nef expressed at the cell surface that are undergoing apoptosis with viable cells not expressing Nef may not be appropriate. In addition, one may question the relevance of observations with the CD8-Nef chimeric protein to those with full length Nef. While the chimeric molecule is a homodimer, it is unclear whether Nef itself dimerizes.

In summary, the effect of Nef on HIV-1 replication remains unclear. Studies performed in vitro and in vivo with quiescent cells point to a positive effect on replication, but the mechanism for this activity remains to be defined. In cell lines, Nef has been reported to have negative or positive effects on HIV-1 replication, which are likely to reflect dual activities of the protein rather than experimental artifacts. Though recent data suggesting that site of expression of the cell may define the predominant effect of Nef, this remains to be clarified. Furthermore, it is unclear whether any of the in vitro experimental findings accounts for the pathologic effects of Nef described with SIV (see Sect. 5.2).

5.2 Effects of Nef on HIV-2 and SIV In Vitro and In Vivo

Two studies have examined the role of *nef* in HIV-2 (ZAGURY et al. 1990). In contrast to the predicted product of the HIV-1 *nef* gene, which is 206 amino acids in length, the predicted product of the HIV-2 *nef* gene is 256 amino acids in length (MYERS et al. 1990). ZAGURY and colleagues (1990) noted that HIV-2 strain NIH-Z had a deletion in the portion of the *nef* gene overlapping with the U3 region of the 3'LTR. They noted that this virus was capable of replication in human and macaque peripheral blood lymphocytes and in three different species of monkeys. Furthermore, the virus was cytopathic for primary lymphocytes. This suggested that *nef* was dispensable for HIV-2 replication and cytopathic effects in vitro. However, no comparisons were made in this study to an otherwise isogenic virus with an intact *nef* gene.

The *nef* gene is present in all SIV subtypes examined thus far (Fig. 1) and is more similar in structure to that of HIV-2 than to the *nef* gene of HIV-1. Although HIV-1 and SIV_{mac} predicted Nef protein products exhibit only 38% amino acid identity, the myristylation acceptor site is conserved, as well as amino acids found in the central, G protein-homology domain (Fig. 5) (RATNER et al. 1991a).

SHIBATA and colleagues (1990) found no effect of mutation of *nef* in SIV_{agm} with respect to virus replication. In contrast, BINNINGER and colleagues examined three different mutations in SIV_{mac} strain 251 which truncate the predicted protein product to 5, 15, or 144 amino acids. In each case, the SIV_{mac} *nef* mutant viruses

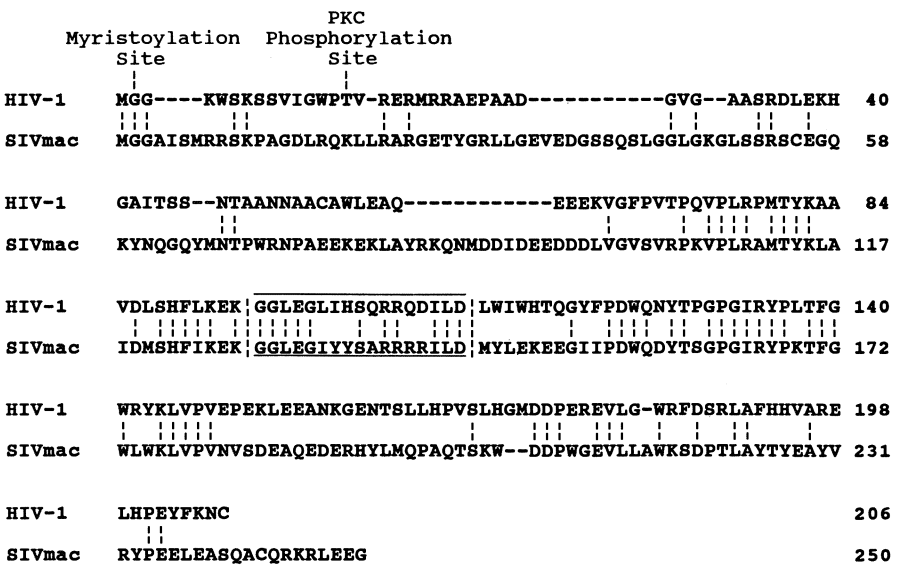

Fig. 5. Sequence comparison between HIV-1 and SIV_{mac} Nef proteins. Amino acid alignment of the Nef proteins derived from HIV-1 strain HXB2/3 and SIV_{mac} strain 102. There is a 38% sequence identity between the two Nef proteins. Both forms of Nef may be myristylated and share sequence similarity to the nucleotide-binding domain of G proteins (*boxed sequences*). (Reprinted with permission from NIEDERMAN et al. 1991)

exhibited faster replication kinetics than did the wild-type strain of virus. Similar results were obtained with HUT 78 and MT4 cells. However, they noted that the negative effects of *nef* were seen only at a very low MOI (see Sect. 5.1). Although the mutant viruses exhibited a more rapid replication rate and spread through the culture, the wild-type virus eventually exhibited a similar capacity for virus production. NIEDERMAN and colleagues (1991) also examined SIV_{mac} strain 251 and found that *nef* suppressed virus expression.

UNGER and colleagues (1992) found no effect of a 70-bp *nef* deletion on the replication properties of SIV_{mac} strain 1A11 in U937, GCT, THP-1, and HL-60 cell lines and rhesus macaque peripheral blood mononuclear cells and alveolar macrophages. In addition, no alteration of viral cytopathicity was noted.

KESTLER and colleagues (1991) focused their studies on a pathogenic molecular clone of SIV_{mac} strain 239. The parental strain was noted to have a termination codon in place of the 93rd codon of *nef* (Nef-Stop). They constructed a Nef+ strain by replacing the termination codon with a GAA codon encoding glutamic acid. In addition, they constructed a deletion mutation of *nef* (Nef-del) that did not affect sequences overlapping *nef*, including the *env* gene, the polypurine tract, or the 3'LTR. In tissue-culture studies performed with each virus strain in CEMx174 cells, primary rhesus macaque lymphocytes, and primary rhesus macaque macrophages, no significant differences in replication kinetics of each SIV virus were seen. Inoculation of rhesus macaques with each of the three SIV strains resulted in infection, as determined by anti-SIV antibody responses and recovery of virus.

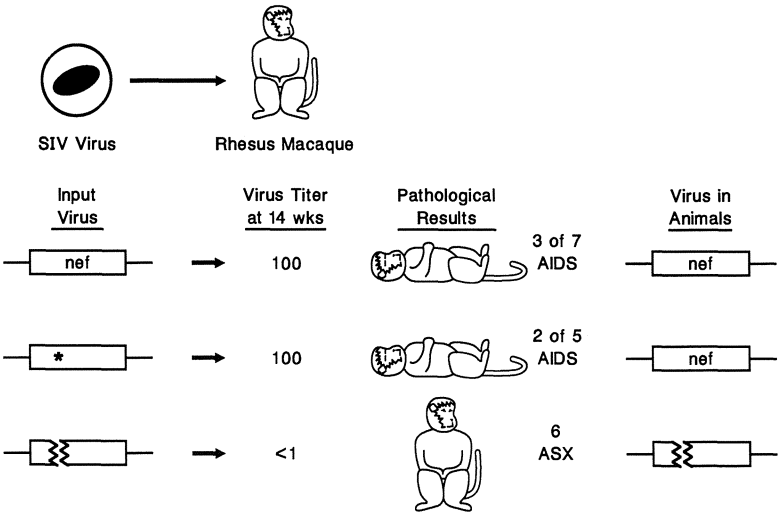

Fig. 6. Nef is a pathogenic factor in SIV_{mac}-infected monkeys. Three different otherwise isogenic strains of SIV_{mac} were used which had an intact *nef* gene, a *nef* gene with a termination codon (*), or a *nef* gene with a deletion (*broken lines*). Relative virus titers 14 weeks after infection are shown, and mortalities of infected monkeys. Polymerase chain amplification and sequence analysis of SIV DNA present in infected rhesus macaque lymphocytes revealed an intact *nef* gene for all animals infected with SIV_{mac} with an intact *nef* gene and all animals infected with SIV_{mac} with *nef* genes with a termination codon. In contrast, SIV_{mac} strains with a deletion in *nef* maintained the deletion. (Adapted from the data of KESTLER et al. 1991)

However, significant differences were identified in the number of primary lymphocytes required for virus recovery, with 100-fold more lymphocytes required to rescue Nef-del virus than either Nef+ or Nef-stop virus (Fig. 6). These findings suggest that there were significant differences in the number of infected lymphocytes and in the virus load. However, confirmatory studies are required using other virus load measures, e.g., plasma virus culture, DNA or RNA PCR studies, or serum p27 antigen levels. Differences were also noted in the absolute number of CD4+ lymphocytes in the infected animals, with lower CD4+ lymphocyte numbers in the Nef+ virus-infected animals compared with the Nef-del virus-infected animals, though it is unclear whether these differences are statistically significant.

Perhaps the most notable feature of the study by KESTLER and colleagues (1991) was the reversion of the *nef* termination codon by 2 weeks post inoculation in all Nef-stop virus-infected animals (Fig. 6). A variety of codons were found in different clones in place of the termination codon, including those encoding glutamic acid, lysine, tryosine, or glutamine. Of additional interest was the finding that AIDS-related mortality was seen only in animals inoculated with the Nef-stop virus (two of five animals died) and Nef+ virus (three of seven animals died), whereas no deaths were noted in any of the six animals inoculated with the Nef-del virus (Fig. 6).

These findings are exciting and are highly suggestive of a pathogenic role for *nef* in vivo. However, it should be noted that these studies were performed with a single strain of SIV_{mac}, and confirmatory studies should be attempted. Moreover, the mechanism of action of *nef* in vivo was not addressed in these studies. However, the findings suggest that virus load measured 14 weeks after infection

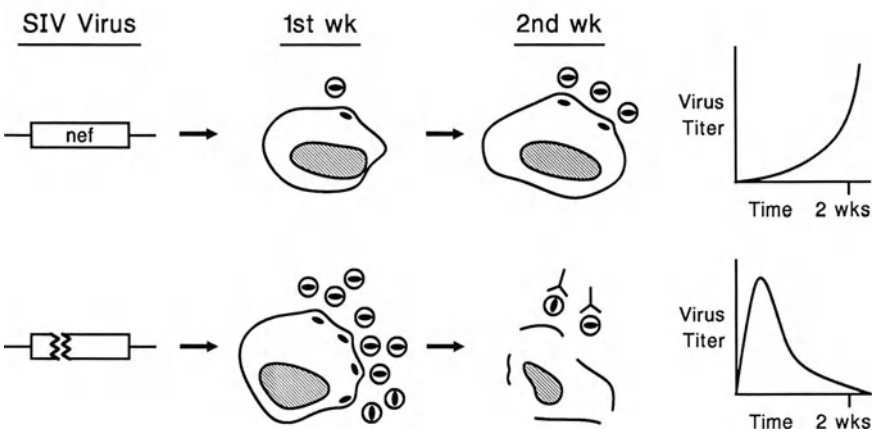

Fig. 7. Hypothetical role of Nef in SIV_{mac} pathogenicity. Nef repression of SIV infection may be required for establishment of persistent infection. Infection with a virus with an intact *nef* gene may result in repressed virus replication, and only low levels of virus expression during the initial phase of infection in vivo. This may allow establishment of a persistent infection and ultimately high titers of virus expression. In contrast, infection with a virus with a deleted *nef* gene may result in abundant virus expression early after infection. This may result in virus-induced cytolysis and a vigorous immune system capable of clearing most infected cells

correlated with the presence of an intact *nef* gene. It is possible that *nef* has a positive effect on virus replication in vivo, perhaps analogous to positive effects of *nef* noted in some tissue-culture experiments with HIV-1 (Terwilliger et al. 1991; de Ronde et al. 1992; Miller et al. 1994; Spina et al. 1994). Such a positive effect on virus replication may occur in a cell type that was not examined in vitro, or occurred under conditions that were not utilized in the tissue-culture experiments of Kestler and colleagues (1991). Conditions not examined in tissue culture might include infection at low MOI, or replication in the presence of complement or SIV-specific antibodies.

An alternative explanation is that the immune response to Nef-del virus differed from that to the Nef+ virus, although no significant differences in anti-SIV Gag and Env antibody responses were detected. Nevertheless, anti-Nef antibody responses were not examined. However, findings of Kirchoff and colleagues (1991) demonstrated no correlation between the presence of anti-Nef antibodies and the development of viral latency or disease progression or virema. Kestler and colleagues (1991) also did not examine other potentially relevant immune responses, such as cytotoxic T-cell responses or antibody-dependent, complement-mediated cytotoxicity.

Although a superficial viewpoint would suggest that the "positive" effects of Nef in vivo are in apparent contradiction to the "negative" effects of Nef seen in some tissue-culture experiments, a nonmutally exclusive relationship is still possible. As suggested in Fig. 7, high initial levels of replication of a virus defective in Nef may result in rapid clearance due to lysis of virus infected cells, or due to a rapid and effective immune response. It is possible that virus attenuated by Nef may be harbored in cells, and such cells may be inefficiently cleared by the humoral and cellular arms of the immune system. There are many example of such apparently paradoxical relationships with other virus systems.

Further analysis of the earliest effect of *nef* in this animal model and attempts to reproduce the effects in vitro are critical to careful deciphering of the mechanism of action of *nef*.

5.3 Transcriptional Effects of Nef

Structural similarities of Nef to transcription factors (see Sect. 4.6, Samuel et al. 1991) may account for the effects of Nef on virus gene expression. Niederman and colleagues (1989) used Northern blot and nuclear run-off studies to identify RNA transcription as the critical event in virus expression that is regulated by Nef. Confirmatory studies were carried out with SIV_{mac}, also demonstrating that SIV-specific transcription was down-modulated by Nef (Niederman et al. 1991). No effect on the site of RNA transcription initiation was identified, though the frequency of transcriptional initiation was suppressed by Nef. No effects were noted on other aspects of RNA processing, such as RNA turnover.

Similar findings were reported by Yu and Felsted (1992), Ahmad and Venkatesan (1988), and Mori and colleagues (1990). However, Hammes and colleagues (1989) were unable to reproduce the effects of Nef on transcription in

COS cells, nor were BACHELERIE and colleagues (1990) able to reproduce these effects in glial cells. HAMMES and colleagues (1989) suggested that the apparent down-modulation effect was an artifact due to the presence in the *nef* expression clone of downstream LTR nucleotide sequences capable of binding NF-kB and SP1. They suggested that these DNA sequences compete for binding of these positive factors with the indicator (CAT) plasmid. However, AHMAD and VENKETESAN (1988) controlled for this variable by using anti-sense *nef* vectors with the same nucleotide sequences as the sense *nef* vectors. In addition, YU and FELSTED (1991) used vectors lacking the downstream competing sequences and also demonstrated the transcriptional inhibitory effects of Nef. Similar results were reported by TSUNETSUGU-YOKOTA and colleagues (1992) in studies of Nef in U937 cells.

LURIA and colleagues (1991) noted effects of Nef on IL2 transcription. In stably transfected Jurkat cells they found markedly diminished activation of IL2 transcription in cells expressing Nef derived from HIV-1 strain NL4-3. However, when the N-terminal portion of this Nef derivative was substituted with that derived from HIV-1 strain HXB2, no effects on IL2 activation were noted. The active and inactive forms of Nef differed at positions 15 (alanine for threonine in the active form), 29 (glycine for arginine in the active form), and 33 (valine for alanine in the active form). Work by BANDRES and colleagues (1994) showed that Ala-15 was critical for this activity, but at least one of the other two residues had a contribution.

NIEDERMAN and colleagues (1992) further analyzed the effects of Nef on T-cell activation, since T-cell activation is critical for HIV-1 gene expression. For this purpose, nuclear extracts were utilized from lymphoid cells constitutively expressing *nef*, or from control cells not expressing *nef* or expessing an antisense form of *nef*. Gel-shift assays were performed with nuclear extracts from Jurkat and HPB-ALL T-lymphoid cell lines activated with phorbol 12-myristate, 13-acetate, phytohemagglutin, and/or ionomycin, or with extracts from untreated cells. Using labeled DNA probes from different portions of the HIV-1 LTR (Fig. 8), significant differences were found with only two sequences, those representing

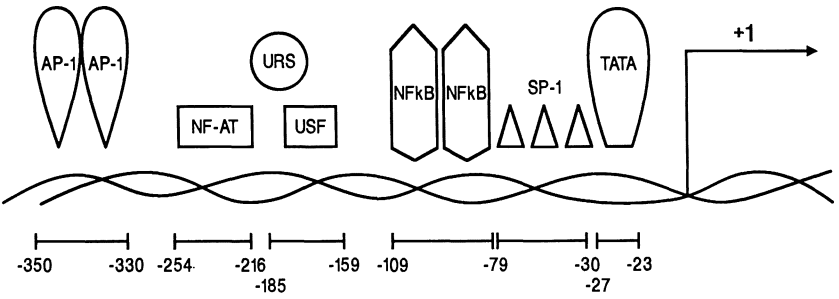

Fig. 8. *cis*-Acting elements in the HIV-1 long terminal repeat sequence. Several regions of the HIV-1 LTR are shown which are capable of binding transcriptional factor. These include binding sites for *NF-kB* and *AP-1*, as well as *SP-1*, *TATA*, *URS*, *USF*, and *NF-AT*. The positions of binding of these factors relative to the RNA initiation site are indicated

binding sites for NF-kB and AP-1 (Niederman et al. 1992, 1993a). Whereas Nef inhibited NF-kB and AP1 DNA-binding activity, it did not significantly affect the binding of SP-1, USF, URS, or NFAT to their respective DNA targets.

The NF-kB proteins whose binding activity was affected by Nef included both p50 homodimers and p50–p65 heterodimers, based on antibody inhibition and supershift assays. Functional assays were also performed with the HIV-1 LTR fused to an indicator gene, chloramphenicol acetyl transferase (CAT). In cells not expressing Nef, transcriptional activity from the HIV-1 LTR was enhanced 20-fold following T-cell activation, but only fourfold in cells expressing Nef. A deletion of the negative regulatory element, localized between nucleotides –161 and –454 relative to the RNA initiation site, did not alter the effects of Nef in down-regulation of LTR activity. In contrast, site-directed mutations in both NF-kB binding sites markedly attenuated LTR activity in the presence or absence of T-cell stimulation and independent of Nef activity. Similar findings were obtained using an IL2 promoter linked to CAT; T-cell activation of the promoter was again markedly inhibited by Nef expression.

Although Nef inhibited the recruitment of AP-1 DNA-binding activity in activated T cells, the effect of this inhibition on HIV-1 LTR-directed transcription were not significant (Niederman et al. 1993a). This is because the AP-1 binding sites within the HIV-1 LTR do not affect virus expression (Zeicher et al. 1991). However, Van-Lint and colleagues (1991) have shown that there are AP-1 binding sites located within the *pol* gene, and these sites were capable of mediating up-regulation of an indicator gene when transfected cells were stimulated with phorbol myristate acetate. The activity of this intragenic enhancer element was attenuated by Nef expression (Niederman et al. 1993a).

Guy and colleagues (1990a) also used gel retardation assays, as well as methylation interference assays, to identify DNA-binding proteins that were affected by Nef expression. In thier studies, primary lymphocytes were utilized rather than transformed T-cell lines. One factor, designated factor A_1 was found to be present in nuclear extracts of activated T lymphocytes but not quiescent T lymphocytes. It was found to bind to the negative regulatory element of the HIV-1 LTR, between nucleotides –315 and –240 upstream of the RNA initiation site. Thus, based on the site of binding, this factor is distinct from both AP-1 and NF-kB, which are also induced by T-cell activation. Moreover, the level of A_1 binding activity was suppressed by Nef. Further structural and functional characterization of A_1 remains to be performed.

In contrast to the effects described above, Murphy and colleagues (1993) reported that transfection of a *nef* expression plasmid into murine or human macrophage cell lines activated HIV-1 LTR activity but had no effect on a variety of other promoters, and no effect in COS cells. The effects of Nef were additive with those of Tat and lipopolysaccharide activation. In addition, these investigators reported a similar activity with a frameshifted mutant of Nef retaining only the N-terminal 35 amino acids. It remains unclear whether this effect is due to the activity of Nef protein or *nef* mRNA. Neither the NFkB binding sites nor the TAR region was required for Nef responses. Although similar effects were seen with

two different reporter genes, no RNA studies were presented to demonstrate that the effects occurred at a transcriptional level. Confirmation is required for these findings in primary macrophages, as well as definition of the mechanism underlying these observations.

The effects of Nef on the binding and functional activity of at least two distinct transcription factors, NF-kB and AP-1, are intriguing. It is possible that Nef directly inhibits both NF-kB and AP-1 binding to their respective DNA sites. However, there is no evidence to support this hypothesis. An alternative explanation is based on the fact that NF-kB, like AP-1, is an early response effector of T-cell activation. Thus, a common pathway involved in the activation of both transcription factors may be affected by Nef (Fig. 9). Inhibition of both factors may intensify the negative effects of Nef on HIV-1 replication in T cells. Furthermore, by inhibiting both virus replication directly and T-cell activation indirectly, through its suppressive effects on IL2 induction, Nef may provide a reservoir of persistently infected cells which may ultimately contribute to HIV-1 latency, HIV-1-mediated CD4 T-cell depletion, and AIDS.

To further investigate the mechanism of the effects of Nef on transcription, BANDRES and RATNER (1994) examined different T-cell stimuli. They found that Nef

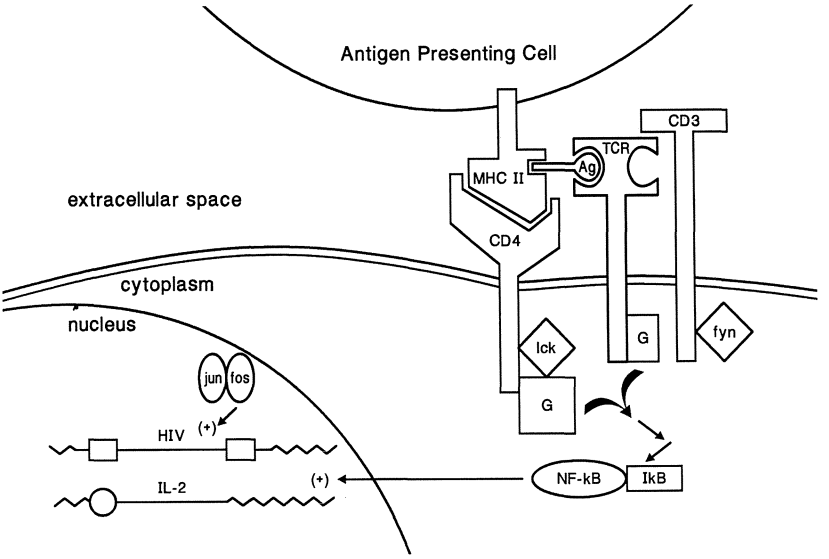

Fig. 9. Model of Nef action in suppression of HIV-1 and IL2 transcription in T lymphocytes. The Nef protein is anchored to the plasma membrane by its myristic acid modification. Nef has been shown to depress cell-surface levels of CD4 and to abrogate T-cell activation of NF-kB and AP-1 (*jun-fos*). T-cell signaling events are shown in which antigen is presented with major histocompatibility type-II proteins to the CD4–T-cell receptor–CD3 complex. Signaling through this complex is mediated by associated tyrosine kinase proteins, *fyn* and *lck*, and associated G proteins. This results in the release of NF-kB from its inhibitor I-kB and its translocation into the nucleus. T-cell activation also results in activation of AP-1 by transcriptional and post-translational mechanisms. Nef is proposed to suppress the activation of HIV-1 and IL-2 transcription via this pathway

had no effect on stimulation of Jurkat cells through interleukin-1, tumor necrosis factor, or lipopolysaccharide receptors. In contrast, stimulation of Jurkat cells by cross-linked antibodies to T-cell receptor (TCR) or CD3 was inhibited by Nef. These results are most consistent with an effect of Nef on signaling through the TCR-CD3-CD4 complex. Although Nef down-regulates CD4 (see sect. 5.4), it has no effect on the level of expression of TCR or CD3. This would suggest that the primary effect of Nef is on a molecule associated with the TCR-CD3-CD4 complex, e.g., lck, or on mediators of the signaling pathway.

5.4 Does Nef Regulate Cell-surface CD4 Expression?

GUY and colleagues (1987) first reported that vaccinia virus-expressed Nef mediated down-regulation of CD4 expression, as measured in a fluorescent flow cytometry experiment. No effect on CD4 expression was seen with a control vaccinia virus lacking *nef* sequences. Based on this effect, these investigators suggested that Nef inhibits HIV-1 replication by down-modulation of the receptor for the virus. Mutation of Nef threonine residue 15, a protein kinase C phosphorylation site (see sect. 4.5), to alanine did not alter its ability to down-regulate CD4. However, this same group of investigators noted in a separate study that phosphorylation of Nef was critical for its stability and down-regulation of cell-surface CD4 (LAURENT et al. 1990). These investigators noted that myristylation was also important for CD4 cell-surface down-regulation (GUY et al. 1990b).

However, GAMA SOSA and colleagues (1991) cautioned that CD4 down-regulation may not be causally linked to *nef* expression. In Jurkat cells transduced with vectors that do or do not express Nef, down-regulation of CD4 was comparable.

GARCIA and MILLER (1991) and GARCIA et al. (1993) reaffirmed the findings of GUY and colleagues (1987). They expressed the *nef* gene of HIV-1 strain SF2 in the correct or incorrect orientation using an amphotropic murine retrovirus vector. Expression of the sense but not the antisense *nef* expression vector in HPBALL T-lymphoid cells, AA2 B-lymphoid cells, on U937 monocytoid cells resulted in down-regulation of cell-surface CD4 expression, as measured by flouresence-activated cell sorting. No effects of Nef were seen on CD4 RNA levels or on intracellular CD4 expression examined by immunofluorescence microscopy with fixed cells. Mutation of all three serine residues in the cytoplasmic tail of CD4 that are targets for phorbol ester-induced phosphorylation had no effect on the ability of Nef to down-regulate cell-surface CD4 expression. Furthermore, these investigators demonstrated similar effects with Nef from primary HIV-1 isolates (ANDERSON et al. 1993). TSUNETSUGU-YOKOTA and colleagues (1992) also found Nef-mediated down-regulation of cell-surface CD4 expression on U937 cells that was not a result of selection of variant cell lines. Finally, BENSON and colleagues (1993) demonstrated that SIV_{mac} 239 Nef is also capable of CD4 down-regulation.

GREENWAY and colleagues (1994) reported down-regulation of surface CD4 expression with the 27-kDa form of Nef, initiated from the first ATG codon, but

not with the 25-kDa form of Nef, initiated from the second ATG codon. Similar findings have been reported by other investigators (AIKEN et al. 1994; BANDRES et al. 1995). The study by GREENWAY and colleagues (1994) utilized highly purified recombinant proteins that were electroporated across cell membranes. Furthermore, these investigators demonstrated that the 25-kDa form of Nef was capable of blocking the activity of the 27-kDa form of Nef. The significance and mechanism of this effect remain to be elucidated. GREENWAY and colleagues (1994) also noted down-regulation of interleukin-2 receptor by the 27-kDa form of Nef, but no effect on transferrin receptor, CD2, or CD7 cell-surface expression. These findings are in agreement with those of BANDRES and colleagues (1995), who demonstrated that neither the 25-kDa form of Nef nor the 27-kDa form of Nef lacking the myristylation acceptor residue were capable of CD4 down-regulation.

The effects of Nef on CD4 expression are not restricted to human cells, but were also seen in murine T-cell lymphoma lines (GARCIA et al. 1993; ANDERSON et al. 1993), as well as in T cells from Nef-expressing transgenic mice (SKOWRONSKI et al. 1993). Interestingly, GARCIA and colleagues (1993) reported Nef down-regulation of both CD4 and CD8 in murine cells, but the significance of CD8 depression in this case was unclear. Nevertheless, CD8 depression was also noted in a Nef transgenic model (see sect. 6, BRADY et al. 1993). No effects of Nef were seen on levels of murine CD3 or murine leukemia virus (MuLV) gp70 envelope expression.

RHEE and MARSH (1994) examined the effects of Nef expression in an antigen-specific murine T-cell hybridoma. They found that CD4 down-modulation did not affect CD4 synthesis or protein transport to the Golgi, the latter measured by the rate of accumulation of endoglycosidase H-resistant forms of CD4. However, the CD4 life span was decreased from 24 to 6 h. The effects of Nef on CD4 stability were blocked by ammonium chloride treatment. The effects were specific for CD4, since there was no alteration in the expression of TCR, Thy antigen, or the activation marker CD45. Similar effects of Nef on CD4 synthesis, processing, and degradation were reported by AIKEN and colleagues (1994). In human CEM lymphoid cells, CD4 half-life was decreased from 8 to 4 h by Nef expression. This was accounted for by an increase in CD4 internalization from 15–20% to 35–45% per hour. BANDRES and colleagues (1995) reaffirmed that Nef accelerated CD4 internalization. Furthermore, SANFRIDSON and colleagues (1994) demonstrated a similar effect of SIV_{mac} Nef on CD4 synthesis, processing, and degradation.

RHEE and MARSH (1994) also noted a positive effect of Nef on TCR responses to stimuli. The effect on TCR-mediated responses also occurred in a CD4- cell line, suggesting that this activity of Nef is not mediated by CD4 down-regulation. These investigators conjectured that expression of Nef in developing thymocytes may result in inappropriate signaling and induction of apoptosis.

SCHWARTZ and colleagues (1992) noted no effect of Nef on T-cell proliferative responses and lymphokine release in response to IL-2, alloantigen, phorbol ester, or antibodies directed against CD2, CD3, CD4, or CD28. However, no effects on CD4 expression were noted in this study, but when the same investigators (SCHWARTZ et al. 1993) utilized a retroviral vector achieving five-to ten fold higher levels of Nef expression, cell-surface CD4 depression was noted. Thus, the

discrepancy in effects on T-cell activation between the study by SCHWARTZ and colleagues (1992) and those of previous investigators (DE and MARSH 1994; NIEDERMAN et al. 1993a; BAUR et al. 1994) may have been due to lower levels of Nef expression.

Several studies have examined the CD4 sequences required for Nef down-regulation and the mechanism of this interaction. ANDERSON and colleagues (1994) found that the cytoplasmic tail of CD4 is necessary and sufficient for its down-regulation from the cell surface by Nef. In studies performed in murine L cells, they found that the cysteines in CD4, responsible for interaction with lck, were not required for CD4 down-regulation by Nef. In studies performed in human T cells, they found no effect of Nef on lck expression. Thus, a lower proportion of lck is associated with CD4 in Nef-expressing cells than in control cells. These investigators conjecture that the "liberated" lck may therefore associate with other signaling receptors, such as the IL-2 receptor, and thus maintain normal or enhanced responsiveness to anti-TCR stimulation. The results of these investigators were in agreement with those of RHEE and MARSH (1994) in demonstrating an accelerated turnover of CD4. However, no evidence was found in this study for a physical interaction between Nef and CD4.

Analogous approaches have been pursued by AIKEN and colleagues (1994); like ANDERSON and colleagues (1994), they utilized a chimeric molecule with CD8 surface and membrane-spanning domains and the CD4 cytoplasmic tail. They demonstrated that Nef resulted in down-regulation of surface expression of this chimeric molecule, similar to its effect on CD4. These findings suggested that the cytoplasmic tail of CD4 is *sufficient* for Nef effects. Removal of the CD4 cytoplasmic tail from either the chimeric CD8-CD4 molecule or CD4 itself, or substitution of the CD4 cytoplasmic tail with that derived from the low-density lipoprotein receptor eliminated Nef effects on cell-surface expression. These findings demonstrated that the cytoplasmic tail of CD4 is *necessary* for Nef effects.

A comprehensive panel of C-terminal deletion mutants was also analyzed by AIKEN and colleagues (1994) to more carefully define those sequences which are necessary *and* sufficient for Nef-mediated effects. In studies performed in 293 fibroblasts, it was found that deletion of 18 or fewer amino acids from the cytoplasmic tail did not alter the ability of Nef to down-regulate CD4, whereas deletion of 20 or more amino acids abrogated this effect. Thus, the two cysteines in CD4 critical for lck interactions were found to be dispensable for the effect of Nef on cell-surface expression of CD4, whereas the dileucine residues 413 and 414 of CD4 were suggested to be the critical residues mediating this effect. Confirmation for the latter findings was obtained by subsitution of the dileucine sequence with a dialanine sequence, resulting in a mutant CD4 molecule not affected by Nef. CD4 endocytosis induced by phorbol esters also requires leucines 413 and 414. However, in that case a requirement is also found for methionine residue 407, serine residue 408, and serine residue 415, whereas mutation at each of these positions had no effect on Nef-mediated depression of CD4. A dileucine sequence in the cytoplasmic tails of CD3-gamma and delta

chains has also been reported to function as an endocytosis and lysosomal targeting signal. However, Nef has no effect on CD3 expression (Bandres and Ratner 1994).

The role of the lck-binding domain of CD4 in Nef effects was re-examined by Bandres and colleagues (1995). Unlike most of the studies of previous investigators (Aiken et al. 1994; Anderson et al. 1994), Bandres and colleagues (1995) studied Nef effects on CD4 cell-surface expression using human T cells. These investigators reaffirmed the critical role of the cytoplasmic CD4 sequences in Nef effects, in this case using a chimeric protein of vesicular stomatitis virus glycoprotein G extracellular sequences with the membrane-spanning and cytoplasmic sequences of CD4. In these studies, deletion or mutation of the cysteine residues that are critical for lck binding resulted in attenuation of the effects of Nef on CD4 expression. To more carefully examine the contribution of lck binding to this effect, they examined the ability of Nef to down-regulate CD4 on U937 monocytoid cells either in the absence or in the presence of exogenous lck expression. While Nef was capable of down-regulating CD4 expression in the absence of lck, CD4 cell-surface expression was further depressed tenfold in the presence of lck and Nef.

How does Nef mediate CD4 down-regulation through endocytosis? Is there a direct interaction between Nef and CD4 that repositions the CD4 cytoplasmic tail, allowing its interaction with the cytoskeleton? Though CD4 and Nef have been shown to interact in a baculovirus expression system (M. Harris, personal communication), the failure to demonstrate such a physical interaction under more physiological conditions would point away from such a mechanism. An alternative possiblity is that Nef has indirect effects on CD4 through an interaction with a component of the endocytotic apparatus. However, the lack of effect of Nef on other cell-surface molecules would argue against this possibility.

Why does HIV-1 have multiple mechanisms for down-regulation of CD4? It is now clear that the gp160 envelope protein can trap CD4 in the endoplasmic reticulum. In addition, the Vpu protein accelerates the degradation of CD4 in the endoplasmic reticulum (Willey et al. 1992). The dileucine motif in CD4, shown to be important for the effect of Nef, is also important for Vpu sensitivity (Lenburg and Landau 1993; Vincent et al.1993). Since Nef is expressed earlier during virus replication than envelope or Vpu products, it is expected to have a more rapid effect on CD4.

Schwartz and colleagues (1993) noted that Nef down-regulation of CD4 resulted in down-regulation of surface expression of the HIV-1 envelope protein. Sequestration of gp120 by intracellular CD4 binding was found to occur in a late Golgi compartment. This resulted in a reduction of the fusion-mediated cytopathic effects of HIV-1.

Could the effects of Nef on cell-surface CD4 expression be related to the transcriptional effects of Nef? It is possible that Nef represses the transcription of a cellular factor required for stable cell-surface expression of CD4. Alternatively, Nef-induced down-regulation of CD4 may cause or result from the disruption of a signaling complex required for efficient HIV-1 and IL-2 expression (Fig. 9). It is

interesting that TREMBLAY and colleagues (1994) noted that full-length but not truncated CD4 transmits a signal that negatively regulates HIV-1 DNA expression, and lck is required for this signal. It has been shown previously that the CD4-lck-complex is required to mediate the induction of IL-2 expression in activated T cells (GIAICHENHOUS et al. 1991). AIKEN and colleagues (1994) demonstrated that Nef down-regulation of CD4 resulted in depressed levels of CD4-associated Nef.

Thus, the effects of Nef may be due to alteration in the structure and/or activity of the CD4-lck-G protein complex or the associated T-cell receptor (TCR)-*fyn*-G protein complex. This may be due to physical association of Nef with one or more of these components. Such a model is appealing, since Nef resembles both *fyn* and lck, in that they are all myristylated proteins anchored to cellular membranes. Moreover, Nef has homology to G proteins, though it lacks GTP-binding activity (see Sect. 4.4).

What is the role of CD4 down-regulation in the effects of Nef on HIV-1 replication in vitro and in vivo? It has been suggested this effect of Nef would prevent superinfection and limit the cytopathic potential of HIV-1. Although such an effect was not apparent in many studies of HIV-1 replication in T-lymphoid cell lines, the high level of CD4 expression in cell lines in vitro may not accurately recapitulate the levels of CD4 in vivo. Alternatively, CD4 depression may result in the redistribution of lck in the T cell, and thus may alter its responsiveness to various stimuli. Consistent with this possibility is the finding of BANDRES and colleagues (1995) that Nef expression resulted in decreased association of lck with CD4, but in increased association of lck with Thy-1. This possibility is also consistent with the finding of SKOWRONSKI and colleagues (1993) that Nef expression in transgenic mice resulted in elevated TCR signaling.

6 Is Nef a Superantigen?

There are several similarities between Nef and the mouse mammary tumor virus (MMTV) 3'orf protein. Both proteins are encoded by genes that overlap the 3'LTR. The MMTV 3'orf protein, like Nef, has weak transcriptional inhibitory properties. Thus, the recent exciting findings demonstrating that the MMTV 3'orf serves as a superantigen (ACHA-ORBEA et al. 1991, CHOI et al. 1991, WINSLOW et al. 1992) raise questions as to whether a similar function might be ascribed to Nef. The relevance of this question is supported by findings suggesting that HIV-1 infection in human beings results in selective deletion of specific T-cell receptor Vbeta sequences (IMBERTI et al. 1991).

A superantigen is an endogenous or exogenous antigen that is presented to the T-cell receptor by the MHC class-II complex in a manner different from most antigens. Whereas most antigens bind to both alpha and beta chains of the T-cell receptor, superantigens bind only to the Vbeta chain. The result of superantigen expression is CD4 lymphocyte proliferation and subsequent depletion of the

specific Vbeta T-cell receptor-expressing class of T lymphocytes. In the case of MMTV, it has been suggested that superantigen stimulation of lymphocytes provides an expanded cell target for virus infection. Clonal deletion of specific Vbeta T lymphocytes by an endogenous strain of MMTV makes the animal resistant to infection by the homologous MMTV strain but has no effect on infection by a heterologous strain of MMTV expressing a distinct 3'orf sequence that interacts with a different Vbeta T-cell class.

Skowronski and colleagues (1993) have noted that a high level of *nef* expression using the CD3 promoter in transgenic mice resulted in depletion of CD4+T cells from the peripheral blood. Furthermore, a depressed level of expression of both CD4 and CD8 on $CD4^+$ $CD8^+$ cells in the thymus was noted. Surprisingly, an enhanced level of proliferation of thymocytes was noted from high-level *nef*-expressing transgenic mice compared with non-transgenic mice. However, low levels of *nef* expression, or *nef* expression from other promoters, did not have the same effect. It is not clear whether high-level *nef* expression represents supra-physiological levels. Furthermore, the mechanism of *nef* effects in this model is unclear. This could be due to a superantigen effect of Nef. Alternatively, *nef* may alter the threshold for T-cell activation.

Brady and colleagues (1993) also expressed Nef in transgenic mice, but in this case, using the CD2 promoter. Similar to the report of Skowronski and colleagues (1993), Brady and colleagues (1993) found depressed CD4 expression on the surface of double-positive thymocytes, as well as a decrease in the number of single $CD4^+$ thymocytes. This was probably not due to a cytopathic effect of Nef, since Nef was also expressed in single $CD4^+$ thymocytes and in splenocytes. It is more likely that the effects of Nef in this model system were due to effects on the T-cell differentiation pathway. Since CD4 interaction with MHC class II is critical for expansion of single $CD4^+$ thymocytes, the depression of CD4 by Nef is likely to be related to the defect in these mice. In contrast to the previous studies of Skowronski and colleagues (1993), Brady and colleagues (1993) found a decrease in the activation of transgenic thymocytes by anti-CD3 epsilon antibody. These effects may be a reflection of the quantitative effects of Nef on single $CD4^+$ thymocytes. One may question whether the predominant effects of Nef on double-positive thymocytes is a particular property of the Nef gene product, or is also related to the use of the CD2 promoter. Nevertheless, the similarity of effects in these transgenic mice to those described in HIV-1-infected SCID mice (Jamieson et al. 1994) provides support for these findings. It has been suggested that dysfunction of thymopoiesis may be a pathogenic mechanism for HIV-1 (Aldrovandi et al. 1993; Bonyhadi et al. 1993).

Lindenmann and colleagues (1994) constructed transgenic mice with *nef* under the control of the murine TCR beta chain enhancer. The effects on double and single $CD4^+$ thymocytes and $CD4^+$ lymphocytes in the periphery in this animal model were quite similar to those in the previous transgenic models (Brady et al. 1993; Skowronski et al. 1993). Furthermore, defective TCR signaling was found in these mice, similar to that described by Brady and colleagues (1993). This resulted in an immunodeficiency syndrome and susceptibility to

infection by both vesicular stomatitis virus and lymphocyticchorio-meningitis virus. These effects were similar to those previously observed in retrovirus-induced immunodeficiency resulting from the murine AIDS strain of MuLV (Morse et al. 1992).

Several other disparate functions of Nef have been described. Dickie and colleagues (1990) claimed that expression of *nef* utilizing the HIV-1 LTR resulted in a high incidence of fetal abortion and a persistent form of dermatosis, marked by alopecia, papulosquamous lesions, and acanthosis.

7 Conclusions

Though the structure and function of Nef have been investigated intensively, a great deal of apparently conflicting data have resulted with respect to certain characteristics of this HIV/SIV gene product. It is generally agreed that Nef is immunogenic during natural HIV and SIV infections, and it is capable of inducting both B- and T-lymphocyte responses. Although several studies suggested that anti-Nef antibodies may precede full seroconversion to HIV-1, this is likely a result of the use of insensitive serological assays for envelope and Gag proteins and the use of nonspecific assays for Nef antibodies. The most convincing and comprehensive studies demonstrate seroconversion to Nef simultaneous with seroconversion to other HIV-1 protein products.

Nef is myristylated, and it is likely that this fatty acid modification is important for localization of Nef in the infected cell and for its biological activity. It is also clear that Nef may serve as a substrate for protein kinase C phosphorylation. Phosphorylation appears to regulate transcriptional effects of Nef, but not its effect on cell-surface CD4 expression. There is only preliminary, and thus far not convincing, evidence to suggest that Nef is a protein kinase or is capable of autophosphorylation at significant levels. However, several interesting findings suggest that Nef may associate with cellular kinases. It remains to be seen whether such associated cellular proteins mediate any of the known activities of Nef.

Although Nef has homology to G proteins, its ability to bind GTP and cleave GTP is weak or nonexistent. Nevertheless, Nef may represent an "ancient G protein", capable of disrupting the activity of stimulatory or inhibitory G proteins.

SIV and HIV Nef proteins have only 38% homology, the greatest sequence similarity being in the region of G-protein homology. Despite the limited sequence identity, SIV and HIV Nef proteins have similar activities in repressing HIV-1 replication. The inhibitory effect appears to be mediated by transcriptional down-modulation of both HIV-1 gene expression and repression of early events in T-cell activation. Recent data demonstrate specific effects of Nef on events critical for T-cell activation by NF-kB and AP-1 transcription factors. These effects may be linked to the ability of Nef to down-regulate cell-surface CD4 expression, in this case by a post-translational effect on CD4. Thus, Nef-mediated inhibition of expression or function of the CD4-TCR-tyrosine kinase-G protein complex on T

lymphocytes may be critical in providing a reservoir of persistently infected cells with repressed virus expression. It is possible that this may be essential for the establishment of infection in vivo and for the ultimate development of AIDS.

The crucial importance of Nef in the establishment of a productive cytopathic infection and the development of AIDS in a rhesus macaque model system highlights the central role of this protein in lentivirus pathogenesis. Further analysis of the early steps in infection and correlation with in vitro activities of Nef are likely to further elucidate the critical features of Nef required for its pathogenic effects. Effects of Nef on virus replication and host immune responses are likely to be important in disease progression and the development of AIDS.

Note Added in Proof

Several additional manuscripts have been published examining B cell (Catozzo et al. AIDS Res Hum Retrovir 10: 1011, 1994; Chirmule et al., AIDS 8: 733, 1994) and T cell epitopes of Nef (Couillin et al., J Exp Med 180: 1129, 1994; Culmann-Penicolelli et al., J Virol 68: 7336, 1994), Nef heterogeneity (Huang et al., J Virol 69: 93, 1995), Nef cellular distribution (Kaminchik et al., AIDS Res Hum Retrovir 10: 1003, 1994), cellular proteins associated with Nef (Harris and Neil, J Mol Biol 241: 136, 1994), positive regulatory effects of Nef (Miller and Feniberg, Trends Microbiol 2: 294, 1994; Miller et al., J Virol 69: 579, 1995), and CD4 down-regulation by Nef (Foster et al., Virol 201: 373, 1994; Poulin et al., J Gen Virol 75: 2977, 1994; Schwartz et al., J Virol 69: 528, 1995).

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The *vpr* Regulatory Gene of HIV

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1 Introduction

Virus replication is the result of a series of two-way communications between the viral parasite and its host. Since viruses are essentially inert objects in the absence of a host cell, all aspects of their natural history can be defined in terms of these interactions. For example, whether or not a cell is permissive to infection and subsequent virus replication, or whether non-productive infection results, is determined by the expression of specific cellular factors which are necessary for each stage in the viral life cycle. In turn, the virus may modulate or enhance the

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expression of these factors to suit its particular needs. Viral tropism is then similarly determined through these interactions, since the presence of the viral receptor molecule on a cell is only the first requisite for productive infection. The pathogenicity of a virus is also clearly the result of these interactions.

The complex retroviruses [all lentiviruses including human immunodeficiency virus (HIV)-1 and HIV-2 Simian immunodeficiency virus (SIV) and Visna virus, plus the non-lentiviruses human T-lymphocyte virus (HTLV)-1 and HTLV-II and the human spumaretrovirus] divide gene expression temporally into immediate-early, early, and late phases (Cullen 1991; Pavlakis et al. 1992). In the HIV immediate-early phase, viral transcription proceeds at a low rate through a dependence solely on cellular transcription factors. The early phase sees a dramatic increase in transcription as a result of accumulation of the primary transactive protein Tat, and during this phase fully spliced mRNA are exported from the cell nucleus and translated into the Tat, Rev and Nef regulatory proteins. The late phase is also Tat-dependent and as a result of accumulation of a critical threshold level of Rev protein, the singly spliced and unspliced mRNA species are exported and translated into structural proteins, as well as the Vif, Vpu and Vpr regulatory proteins.

This high level of sophistication in genetic regulation allows for a complex interplay between the virus, via its regulatory proteins, and host systems. Since HIV is directly or indirectly responsible for a myriad of disorders in vivo, understanding these interrelationships will be vital to developing means to control HIV replication and pathogenesis.

2 Review of *vpr*

The original observation that the R open reading frame (orf) of HIV-1 actually encodes a protein was made in 1987. Wong-Staal et al. (1987) translated the *vpr* orf in bacteria and found that about 40% of HIV infected individuals had antibodies which reacted with it, indicating that a Vpr polypeptide is made in vivo. Two subsequent studies which examined the reactivity of HIV patients with synthetic or bacterially made Vpr have found similar reactivity rates, i.e., about 25%–49% of HIV-infected individuals reacted with them, irrespective of disease stage or prognosis (Gras-Masse et al. 1990; Reiss et al. 1990).

The *vpr* gene of HIV-1 is a single orf located between and slightly overlapping the *vif* orf at its 5' end and the *tat* at its 3' end (Myers et al. 1991). The *vpr* gene encodes a 96 amino acid protein in most HIV-1 strains. The *vpr* orf in the viral genome is translated from a singly spliced message and its translation is dependent on Rev expression (Arrigo and Chen 1991; Garrett et al. 1991); thus Vpr is made as a late protein, along with the HIV structural proteins and Vif and Vpu.

HIV-2 and SIV also contain *vpr* genes (Fukasawa et al. 1988; Shibata et al. 1990). The Vpr protein of these viruses is generally a little larger than HIV-1 Vpr, containing between 102 and 106 amino acids. Most HIV-2 and SIV strains also

contain *vpx*, whose orf abuts *vpr* and is 5′ to it (Francini et al. 1988; Kappes et al. 1988; Yu et al. 1988). Based on sequence similarity between the two genes, Tristem et al. (1990,1992) have proposed that *vpr* and *vpx* are evolutionarily related, having probably arisen through gene duplication.

Relatively little has been reported on the function of Vpr. Six published studies examining the replication of *vpr* mutants have yielded varying results with different viruses and different cells, although the general conclusion has been that *vpr* is a positive factor for HIV replication (Balliet et al. 1994; Cohen et al. 1990a; Dedera et al. 1989; Hattori et al. 1990; Ogawa et al. 1989; Shibata et al. 1990). In 1989, Dedera et al. reported that *vpr* mutants of HIV-1 and HIV-2 replicated as well as wild-type virus in transformed T cell and monocytic lines, and in peripheral blood leukocytes (PBL). It has subsequently been demonstrated that the 79 amino acid Vpr protein of the HIV-1 pX clone (HXB2) used in this study was not functional (Cohen et al. 1990b), indicating that the carboxyl terminus is important for Vpr function. Ogawa et al. (1989) observed slower replication kinetics and a lower maximum titer of HIV-1 *vpr* mutant virus in four transformed T cell lines, but only when low doses of virus were used to infect. This was the first indication that Vpr may be a positive factor in HIV replication.

However, Shibata et al. (1990) reported no difference in the replication of *vpr* mutant HIV-2 in the transformed T lymphocytic line Molt-4. Cohen et al. (1990b) observed very similar differences between wild-type and *vpr* mutant HIV-1 as Ogawa et al. had. They also reported that transfection of *vpr* into cells containing CAT reporter constructs linked to the HIV long terminal repeats (LTR) and other promoters acted to increase transcription three- to tenfold. No further reports concerning the effects of Vpr on transcription or translation have appeared since.

Westervelt et al. (1992) reported that a *vpr* mutant monotrophic HIV-1 virus replicated only slightly less well than wild-type in primary monocytes. In striking contrast to these results, Hattori et al. (1990) found that a *vpr* mutant HIV-2 replicated very poorly in primary adherent cells, although these cells were likely to be more monocyte-like, since they were not treated with agents such as granulocyte/macrophage-colony stimulating factor (GM-CSF), which increase HIV replication through cell differentiation. Most recently, Balliet et al. (1994) found that introduction of a *vpr* mutation into a monotropic molecular clone of HIV-1 severely compromised its replication in primary differentiated macrophages, with some variation from donor to donor, but did not perturb its replication in primary PBL. Balotta et al. (1993) have reported that antisense phosphorothioate oligodeoxynucleotides against the *vpr* gene of the monotrophic HIV-1 strain Ba-L could inhibit its replication in primary macrophages. Taken together, Vpr from both HIV-1 and HIV-2 appears to not be necessary for HIV replication in T cells in vitro, but it can increase HIV replication in most cell types in vitro, particularly when using low-input virus. On the other hand, Vpr is clearly required for efficient replication in primary monocyte/macrophages in vitro.

The first evidence for the importance of Vpr in vivo came from Lang et al. (1993) who infected rhesus monkeys with *vpr-nef* mutants of a highly pathogenic SIV strain ($SIV_{MAC}239$). Previously Kestler et al. (1991) had shown that *nef* point

mutants of $SIV_{MAC}239$ would revert in vivo and that reversion to wild type was necessary for high viral loads and for the development of disease. When a point mutation in the *vpr* gene was added, nef reversion was observed in five out of five animals; however, *vpr* reversion was found in only three. Of the three animals who reverted both *nef* and *vpr*, two developed disease, but one did not. Neither of the animals in which *vpr* failed to revert developed high virus loads or disease. Thus, at least in SIV infection of monkeys, Vpr may be important for efficient replication in vivo and also for the development of disease. Since $SIV_{MAC}239$ contains both *vpr* and *vpx* genes, which may have similar functions and which may either overlap in function, assist each other, or antagonize the other, inferences from this system regarding the role of HIV-1 Vpr in vivo must be tentative.

One of the most interesting observations concerning Vpr has been that Vpr protein is incorporated into viral particles in HIV-1, HIV-2, and SIV (Cohen et al. 1990a; Hattori et al. 1990; Yuan et al. 1990). Based on immunoprecipitation experiments, the amount of Vpr in the virions appeared to be stoichiometrically similar to the quantity of gag proteins incorporated, which for p24 gag is about 1200 molecules per virion. Cohen et al. (1990a) and Yuan et al. (1990) proposed that virion-associated Vpr might assist early events in HIV replication following virus fusion with the target cell. Because they had detected a transactive potential for *vpr*, albeit a weak one, Cohen et al. made in interesting speculation that Vpr might function to transactivate the HIV LTR in the early tat-independent phase of HIV transcription. This remains to be tested, but the nuclear localization of Vpr is consistent with a transactive function (Heinzinger et al. 1994; Lu et al. 1993; Sato et al. 1990). Vpx has also been found in association with viral particles of HIV-2 and SIV; it is the only other primate immunodeficiency virus (PIV) regulatory protein consistently found in the virion (Yu et al. 1988). Vpx has been reported to assist HIV-2 replication at the early stage of infection (Yu et al. 1991), so if Vpr is similar in function to Vpx, which has yet to be proved, by analogy this would support their model.

It has recently been shown by several groups that Gag proteins are necessary for incorporation of Vpr into nascent viral particles (Lavallee et al. 1994; Lu et al. 1993; Paxton et al. 1993), and that mutation in the p6 coding region of the Gag polypeptide precursor eliminates vpr incorporation (Paxton et al. 1993). This has led to the proposition that vpr primarily assists gag functions such as virus assembly. A direct association between Vpr and a mature Gag protein has not been demonstrated to date.

We have reported several properties of Vpr, described below, which indicate that Vpr exerts a positive influence on HIV-1 expression via an interaction with the cellular targets of infection, assisting replication in resting primary cells (Levy et al. 1994, 1995a). Vpr controls cellular proliferation and can affect cell differentiation (Levy et al. 1993), through Vpr protein can enhance HIV-1 replication without affecting cell proliferation. These properties of the Vpr protein require specific interactions with cellular factors involved in regulation of fundamental cellular pathways. Consistent with this, Vpr has recently been reported to bind a cellular protein. This interaction required Vpr amino acids 60–81, which contain a leucine/

isoleucine rich region (ZHAO et al. 1994). We have found a specific interaction between Vpr and a 41-kDa cellular protein (REFAELI et al. 1995). Recently it has been reported that Vpr can complement the nucleophilic function of the martix protein of HIV-1 (HEINZINGER et al. 1994), contributing to the ability to replicate in nonproliferating cells. Vpr has not been shown to contain a nuclear localization signal, however, so the mechanism by which vpr assists migration of the preintegration complex is not clear and could be indirect via interaction with host factors.

3 Regulation of Cell Proliferation and Differentiation

3.1 HIV-1-Induced Cell Growth Arrest and Differentiation

Our studies of Vpr began with a serendipitous observation. Following transfection of a drug-selectable HIV-1 genomic construct (pNLpuro) into TE671 embryonal rhabdomyosarcoma cells, a cell line we routinely use for expression of exogenous proteins, surprisingly nearly complete cell growth arrest was observed within 3 days (LEVY et al. 1993; LEVY 1994). After about 6 days, the drug-selected transfectants had increased in size by up to 100 fold, did not replicate, but were fully viable. In fact, these cells remained very active morphologically, extending then retracting long processes continuously (LEVY and WEINER 1993). The cells remained viable in tissue culture for at least 3 weeks, after which time the culture would be overgrown with the small minority (< 1%) of the cells which had not stopped proliferating.

TE671 and its parent line RD have been used extensively as models of muscle development (AGUANNO et al. 1990; HITI et al. 1989; Siegel and LUKAS 1988; STRATTON et al. 1989). These cell lines are committed to the muscle lineage, resemble presumptive myoblasts (muscle satellite cells), and express low levels of contractile proteins such as fast-twitch myosin. Growth inhibition and differentiation can be induced using low serum medium, phorbol ester, or retinoic acid, with a resulting expression of increased levels of contractile apparatus proteins. In addition, several groups have shown that TE671 and RD are infectible at very low efficiency with HIV-1 and HIV-2 through a non-CD4 mechanism (CLAPHAM 1991; CLAPHAM et al. 1989,1991; HAGGERTY et al. 1991; SRINIVASAN et al. 1988; TATENO et al. 1989; WEISS et al. 1988; WERNER et al. 1990).

At the time of this observation there were no reports that HIV could induce such profound cell cycle arrest without cell death, and no reports of cellular differentiation by HIV. In these lines, cell differentiation can result from agents which arrest cell replication, so the differentiation effect may be a secondary phenomenon to the inhibition of proliferation. PMA and serum starvation-induced differentiation in RD and TE671 is reversed when normal culture medium is applied. In addition, complete cell growth arrest is usually not obtained with these agents. HIV expression on the other hand induced complete growth arrest that was not reversed when the culture medium was replaced daily. HIV transfected

TE671 cells expressed high levels of fast-twitch myosin, indicating muscle-lineage differentiation and not differentiation along a novel pathway.

3.2 *vpr* as a Cytostatic Gene

We reasoned that a regulatory gene of HIV was likely responsible for the observed effects since these genes operate as an interface between HIV elements and the cellular machinery for viral replication (reviewed in TERWILLIGER 1992). Accordingly, we cloned each regulatory gene into expression vectors, then transfected them individually into TE671. The *vpr* gene induced cell growth arrest and morphological differentiation, while the other regulatory genes, *vif, vpu, tat, rev,* and *nef*, failed to do so. *vpr* did not have a toxic effect on these cells, as the cells remained fully viable for many days, and transfection efficiency with the *vpr* vector was equal to the transfection efficiency obtained with each of the other vectors. In addition, *vpr* transfectants expressed increased myosin, indicating resumption of the committed muscle differentiation program. Thus *vpr* reverted the transformed phenotype in these cells in a manner reminiscent of tumor suppressor genes such as p53 or RB. Mutation of the p53 gene is a characteristic of rhabdomyosarcoma transformation (FELIX et al. 1992), as are mutation in a putative tumor suppressor gene on chromosome 11, expression of an activated *ras* oncogene and expression of autocrine transforming growth factor β (TGF-β).

One additional interesting observation was that when *vpr* was deleted from the HIV-1 genome, TE671 cells failed to stop proliferating or to differentiate to any appreciable degree. In addition, HIV production from these cells continued at high levels; thus the cells were relatively unaffected by HIV expression in the absence of *vpr* (Fig. 1) (LEVY et al. 1993). On the other hand, when TE671 cells were transfected with the HIV vector containing *vpr*, only the large differentiated cells released viral antigens. When the differentiated cells were removed by subcul-

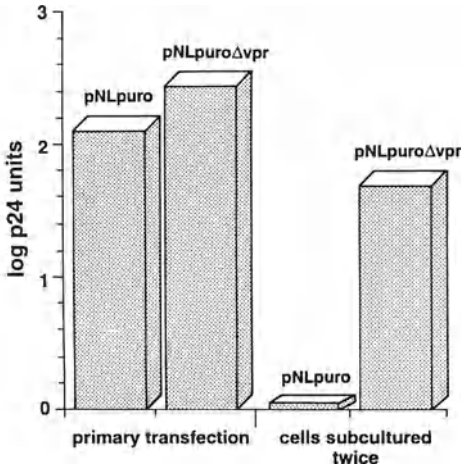


Fig. 1. HIV expression in the absence of *vpr* is compatible with TE671 replication. *Gray bars* show p24 production from TE671 cells transfected with either pNLpuro or pNLpuroΔvpr 3 days after transfection. *Black bars* show p24 production following two subcultures of the puromycin resistent cells over a 2-week period

ture and the few cells (<1%) that continued to proliferate were allowed to grow out, these proliferating cells expressed no virus. Therefore, in the context of the virus, *vpr* prevented maintenance of the cell population, but when *vpr* was deleted the virus was relatively innocuous, and virus expression could continue in the presence of cell replication. We predict that vpr expression prevents maintenance of infected cell populations in vivo and that this contributes to T cell depletion as well as loss of other types such as Langerhans/dendritic cells. In addition, these findings support the notion that Vpr mutants, combined with other genetic alterations, might be useful as live attenuated vaccines, such as proposed and tested by Desrosiers and colleagues (Daniel and Desrosiers 1989; Kestler et al. 1991; Lang et al. 1993) Alternatively, *vpr* deletion may be useful in HIV-based vectors for gene transduction, although without *vpr* intact, HIV expression may be diminished in some tissues or become repressed (see below).

To investigate the generality of the Vpr regulation of cells, *vpr* was transfected into other transformed lines. Human and canine osteosarcomas were growth arrested and underwent great enlargement, but failed to express increased levels of alkaline phosphatase, which is a marker for bone maturation (Levy et al. 1993). An interesting feature was that the perinuclear regions of many of the *vpr*-transfected cells rotate in a cyclone-like fashion with a period of about 2 h. These cells remained fully viable in cell culture for at least 2 weeks.

Lentiviral infection is commonly associated with encephalopathies and various other neurological disorders through undefined direct or indirect viral cytopathic mechanisms (reviewed in Levy 1993). Infection of microglia and of peripheral macrophages which can cross the blood–brain barrier probably contributes to the dissemination of HIV to the central nervous system (CNS). Three glioblastoma/astrocytoma cell lines (U87MG, U373MG, U138MG) were transfected with the *vpr* vector with the result that each cell line was profoundly inhibited in replication and underwent gross morphological changes (Fig. 2) (Levy 1994). The cells increased in size by 10-to-20-fold and extended pseudopods with neurite-like morphology. It is tempting to speculate that Vpr might dysregulate astrocyte function following infection in vivo and thus contribute to neuropathology.

These results (Levy 1994; Levy et al. 1993; Levy and Weiner 1993) demonstrated for the first time that Vpr regulates basic cellular processes, and in fact that *vpr* has properties reminiscent of a tumor suppressor gene. What they did not do was establish a link between the regulation of cell events by Vpr and the replication of HIV. However, circumstantial evidence supports the proposition that Vpr regulation of cellular events may influence cellular permissiveness to HIV replication. The strongest circumstantial argument is the following: *vpr* can promote cell differentiation, and cell differentiation is linked to increased permissiveness of monocyte/macrophages to HIV replication (Kitano et al. 1990; Meylan et al. 1993; Perno et al. 1989; Rich et al. 1992; Schuitemaker et al. 1992; Turpin et al. 1992; Valentin et al. 1991). HIV infection of monocytic lines can potentiate cell differentiation (Gazzolo and Macé 1990; Pautrat et al. 1990; Roulston et al. 1992), and *vpr* is required for efficient replication in macrophages. Also, since HIV

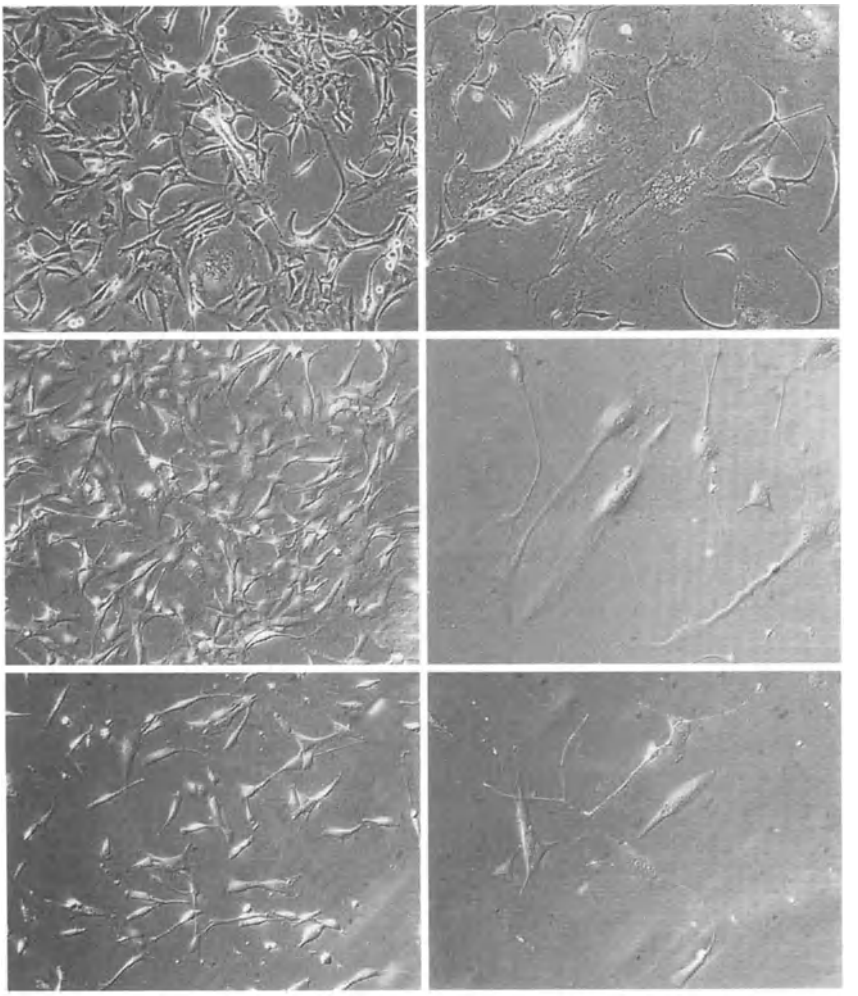


Fig. 2. Effect of *vpr* on glioblastoma cell lines (*top four panels*, U87MG; *bottom* panels, U138MG; *left panels*, cells transfected with vector alone; *right panels*, cells transfected with *vpr*). *Top two panels* were photographed with phase-contrast optics, the *bottom four panels* were photographed using Hoffman Optics (Modulation Contrast). Magnification in all panels × 250

replicates efficiently in nonproliferating differentiated macrophages, Vpr may contribute to the ability of HIV to replicate in nonproliferating cells. Together with the observation of COHEN et al. (1990b) that the *vpr* gene weakly and promiscuously increased transcription, the simplest explanation for *vpr* activity is that Vpr activates cellular genetic programs linked to cell growth, cell differentiation, and virus replication. Since Vpr protein is part of the viral particle, delivery of Vpr into cells during infection may assist early events in replication, such as reverse transcription and integration, or early LTR transcription.

4 Expression of Biologically Active Recombinant Vpr

4.1 Effect of Extracellular Vpr on Cell Proliferation

In order to test some of the hypotheses resulting from the previous study, several unsuccessful attempts were made to establish stable *vpr* transfectants of monocytic and T lymphocytic cell lines. The most likely explanation for the failure to establish stable cell lines is that Vpr may inhibit proliferation of these cells, preventing selection and maintenance of transfectants. Therefore an alternative strategy was employed, namely, recombinant protein expression, in order to further investigate Vpr function.

Native full length HIV-1 Vpr was produced in the baculovirus system (Refaeli et al. 1995; Levy et al.1995a). Interestingly, the vast majority of Vpr protein was found to be released from the producer insect cells into the culture medium. Secretion of novel proteins is not unusual in this system; however, it raised the possibility that Vpr may be active as an extracellular molecule. As assessed by capture enzyme-linked immunosorbent assay (ELISA), Vpr protein was exported in low amounts from several transformed cell lines representing T lymphocytes (H9, SupT-1), monocytes (HL60, THP-1), muscle cells (TE671, RD, A204, A673), bone (D17), astrocytoma/glioblastoma cell lines (U-373MG, U-138MG, U-87MG), and a neuroblastoma line (SK-N-MC) following transfection with the *vpr* gene (Levy 1994).

When applied to rhabdomyosarcoma cells, dialyzed Vpr-containing baculovirus supernatants induced growth inhibition and cellular differentiation identical to that observed following transection with the *vpr* gene, including high myosin expression, while control insect cell supernatants failed to do so (Levy 1994). Vpr-containing supernatants dramatically inhibited the proliferation of peripheral blood mononuclear cells (PBMC) in response to various cell stimulators including phytohemagglutinin, concanavalin A, and staphylococcal enterotoxin B. The

Table 1. Extracellular Vpr-induced cell growth inhibition[a]

Period of cell culture (days)	Growth Inhibition/Activation[b]		
	SupT-1	THP-1	HL60
1	81	82	332
2	68	60	189
3	24	26	169
4	12	1	134

[a] Cells were in culture in 5% Vpr supernatant or 5% control supernatant. Proliferation was assessed by incorporating ^{3}H-thymidine. Typical control counts per minute (CPM) were $5x10^4$–10^5 for 50×10^4 cells. Control supernatant yielded CPM within 10% of cells cultured in normal growth medium

[b] Values represent means of triplicate wells and are given as percentages of control CPM. Similar growth inhibition was obtained for H9, U937, and KG-1 as for THP-1 and SupT-1

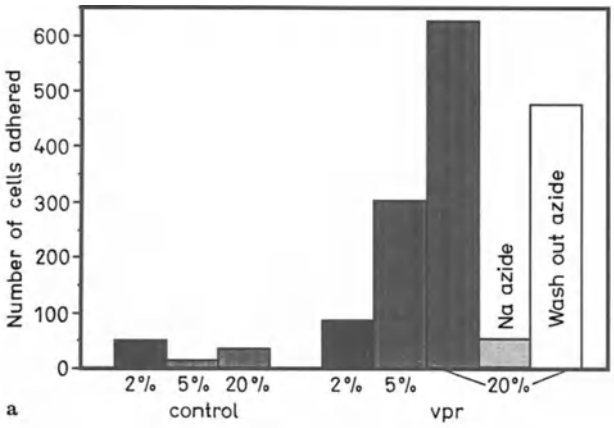
contribution of cellular toxicity of this treatment remains to be resolved. This supports the possibility that Vpr may contribute to immune depletion in vivo. Vpr-containing supernatants also profoundly inhibited the proliferation of several transformed hematopoietic cell lines, but these cells remained fully viable (Table 1).

The immature myeloid/erythroid cell line HL60, on the other hand, was enhanced for replication by Vpr supernatants, but only when cultured in suboptimal conditions such as in depleted medium or at high cell density ($>1 \times 10^6$ cells/ml). In this respect, Vpr displays activity similar to the cytokine interleukin (IL)-1, which has been reported to overcome the proliferative inhibition of HL60 in serum-depleted medium (Silvennolnen and Hure 1990). This effect of vpr on HL60 cells is cell density-dependent and dose-dependent, and was removed by specific depletion of Vpr from the supernatant. In contrast, in protein kinase C activating phorbol ester PMA induced growth inhibition in all Six cell lines tested, including HL60.

Vpr supernatants also dramatically activated the adherence to plastic tissue culture flasks of the monocytic cell lines which were growth inhibited (Fig. 3). This effect was inhibited by sodium azide, indicating an energy-dependent mechanism of adherence rather than an effect on cell membrane charge induced by Vpr. This result was consistent with the hypothesis that Vpr, through an effect on cell differentiation, may regulate cellular permissiveness to HIV replication. On the other hand, expression of differentiation-linked surface antigen expression (CD11, CD14, FcRI, FcRII, Class I, Class II, CD4, Mo1, Mac-1) produced no consistent results indicating macrophage differentiation. Expression of transferrin receptor, which is an activation antigen in these cells, increased in HL60 and KG-1 following Vpr exposure.

This straightforward picture for Vpr activity was complicated by the failure of anti-Vpr antibodies to inhibit Vpr-dependent cell growth inhibition or adherence. It may be that a second factor is present in the Vpr supernatants which depends on Vpr for activity, but is not inhibited by anti-Vpr antibodies. Vpr might modify or otherwise interact with this putative factor prior to the addition of anti-Vpr antibodies. If this factor were physically associated with Vpr, then removal of Vpr by immunoaffinity chromatography may also remove this factor. This hypothesis remains to be directly addressed. Consistent with this notion is the failure of purified Vpr to inhibit cellular proliferation or induce adherence. This observation is important for, as described below, purified Vpr is highly active in assisting HIV

Fig. 3. **a** Titration of Vpr for monocyte adherence and inhibition with azide. THP-1 cells were cultured in the presence of Vpr in Costar cell culture flasks overnight and the number of cells adhered in representative fields were counted. In the indicated cultures Na azide at 50 m*M* was added simultaneously with Vpr. In the "wash out azide" well, Vpr and azide were washed from the cells 4 h after addition and then the cells were incubated overnight. **b** Vpr-induced adherence of THP-1, U937 and KG-1. Vpr supernatants (*right panels*) or control supernatants (*left panels*) were added to cells in six well plates overnight. Nonadherent cells were removed for photography (*top*, U937; middle, THP-1; *bottom*, KG-1)


Number of cells adhered
600
500
400
300
200
100
0
2% 5% 20%
control
2% 5%
20%
vpr
Na azide
Wash out azide
a

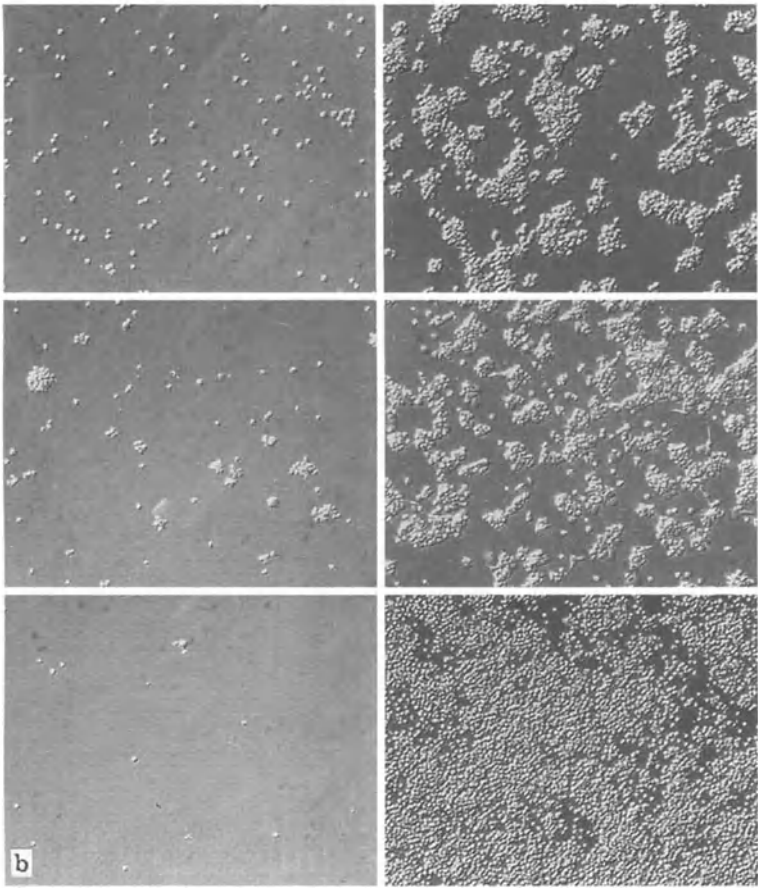

b

Table 2. Cells which are inhibited or enhanced in proliferation by *vpr* or Vpr supernatants[a]

Primary lymphoid	Monocytoid	T lymphoid	Muscle	Brain	Bone
PBMC[bc]	U937[b] THP-1[b] KG-1[b] HL60[b]	H9[b] SupT-1[b]	TE671[bd] RD[b]	U138 MG[d] U373 MG[d] U87 MG[d]	D-17[d] DAN[d] HOS[d]

[a] All cells were growth inhibited except HL60
[b] Treated with baculovirus supernatant
[c] Significant cytotoxicity
[d] Transfected with *vpr* vector

replication, indicating a dissociation between vpr effects on cell growth and HIV replication. Nevertheless, Vpr is clearly a regulator of cell proliferation, and it inhibits proliferation in the majority of cell types (Table 2). The ability of Vpr to enhance the proliferation of the HL60 cell line is very interesting since it demonstrates that Vpr operates through a mechanism linked to cell growth, but not just growth arrest (Levy et al. 1995b).

4.2 Increase in HIV Replication via Extracellular Vpr

4.2.1 Extracellular Vpr in New Infection

Very small quantities of Vpr, when applied at the time of infection, induced a dramatic increase in HIV replication (Levy et al. 1995a) (Fig. 4). Measurable levels of virus were produced in the Vpr-treated cultures 1 or 2 days earlier than in untreated or control cultures, and the levels of virus produced were at least 10 times greater by a given day and were often 100-fold. In this respect Vpr was seen to increase productive versus nonproductive infection. The response to Vpr was dose-dependent, and the promonocytic lines were sensitive to lower amounts of Vpr (100–200 pg/ml) than were the T cell lines (200–300 pg/ml). This result is consistent with the previous studies examining *vpr* mutant HIV, which found that *vpr* increased HIV replication kinetics and was most active in monocytes. However, we observed an increase in the replication of wild-type viruses which contained intact *vpr* genes; therefore, extracellular Vpr added its effects to endogenously made Vpr. Although a *vpr* mutant HIV-1 replicated equally to wild-type virus in T lymphoid cells, each virus was boosted equally by extracellular Vpr; thus extracellular Vpr functioned more powerfully than endogenously synthesized Vpr. Extracellular Vpr also restored monocyte replication competence to the *vpr* mutant NL43Δvpr (Levy et al. 1995a), demonstrating that recombinant Vpr displayed true Vpr activity.

One prediction resulting from the work with the rhabdomyosarcomas and osteosarcomas was that Vpr is a regulator of cellular permissiveness to HIV

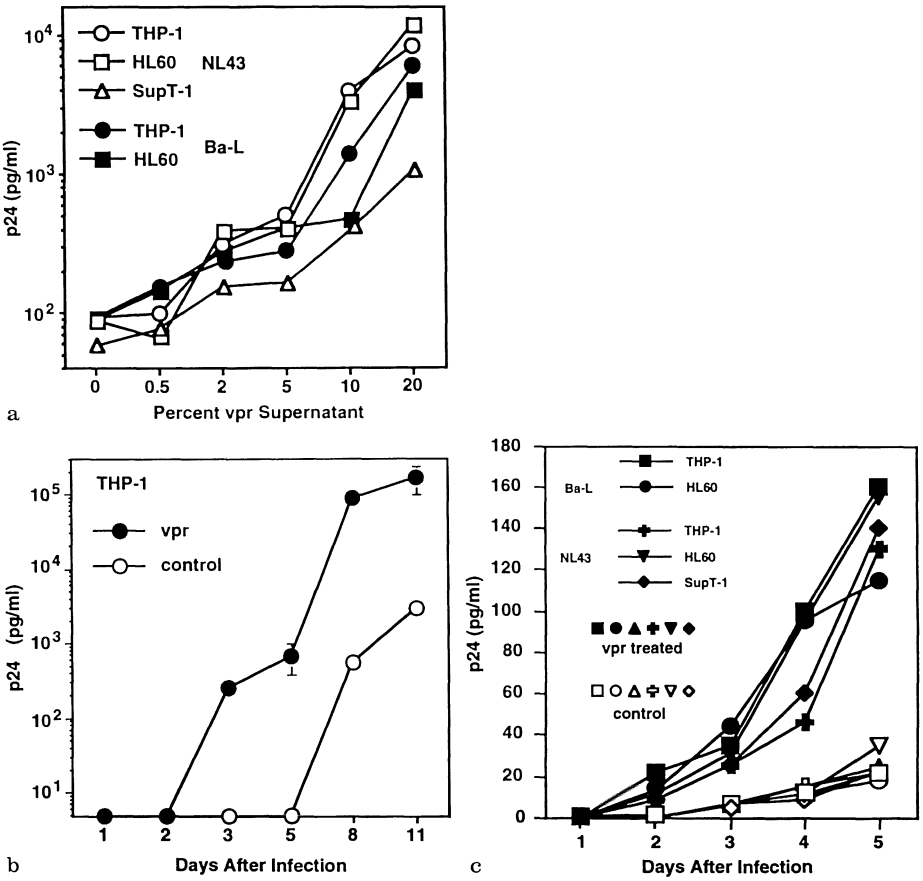


Fig. 4a-c. Extracellular Vpr increases HIV replication. **a** Titration of Vpr. Indicated cell lines were infected with HIV-1 NL43 of Ba-L in the presence of Vpr or control supernatants; 12 h after infection and addition of agents, the cells were washed to remove residual virus and Vpr, then cultured under standard conditions. p24 Gag was measured on day 16 following infection. Control supernatants had no effect on infection versus untreated cells in any of more than 25 independent experiments. **b,c** Time course infection in presence of Vpr. **b** THP-1 cells were infected as in **a**. **c** Vpr increases productive infection of several cell lines by monotropic and lymphotropic isolates. Each cell line was infected with HIV-1 Ba-L or HIV-1 NL43 as in **a**

replication. That is, that Vpr exerts its biochemical effects through interactions when the cellular target of infection, rather than through its interactions with other HIV components. Cells which had been transiently exposed to Vpr several days prior to infection displayed the same increase in virus production as was observed when Vpr was added at the time of infection, confirming the notion that Vpr regulates cellular permissiveness to productive infection (Levy et al. 1995a).

This finding suggested that Vpr in bodily fluids might generate an in vivo pool of susceptible cells. In this way Vpr could enhance the spread of HIV infection. Another potential route for Vpr activity is through virion-borne protein. The

number of virions infecting a cell could determine the initial dose of Vpr delivered to the cell, and in this way regulate whether infection is productive or nonproductive. This model would fit the observation that high and low input doses of HIV can result in productive or abortive infection, respectively, in THP-1 cells (Mikovits et al. 1990). We have not confirmed this model, however, we do find that soluble extracellular Vpr can determine that infection will be productive rather than nonproductive (see above and below), and this establishes a novel and direct mechanism for determining the outcome of infection.

4.2.2 Increase in HIV Protein Production

Vpr could increase the efficiency of infection, allowing more cells to be infected earlier. On the other hand, Vpr could increase virus replication within infected cells. If the first mechanism is true, then staining of newly infected cells for virus would show a greater proportion of positive cells. If Vpr is activating virus expression, then most or all infected cells should show an increase in staining, while the proportion of positive cells remains the same. Shortly after infection with HIV-1, cells were fixed then stained for membrane associated and intracellular p24 antigen. When analyzed by flow cytometry, the fluorescence profiles showed a shift in the virus expression in virtually the total cell population rather than an increase in the percentage of infected cells; thus Vpr increased HIV protein expression on a cell-by-cell basis. Vpr may increases translation of HIV mRNA, or it may increase HIV transcription. For the reasons stated above, we favor the notion that Vpr activates HIV transcription.

4.2.3 Activity of Extracellular Vpr in Primary Hematopoietic Cells

PBMC infection was investigated, and fractionation of the PBMC population was performed in order to examine Vpr activity on PBL and macrophages (Levy 1994; Levy et al. 1995a). We found that vpr increased HIV replication in primary PBMC, GM-CSF-generated macrophages as well as resting or phytohemagglutinin (PHA)-stimulated non-adherent PBL. HIV replication in GM-CSF stimulated macrophages by monotropic (Ba-L) and lymphotropic (NL43) was assisted by Vpr, but the monotropic strain replicated to higher titer following Vpr stimulation than did the lymphotropic strain.

An important observation was that PBL, when treated with Vpr, did not require mitogen stimulation to produce significant quantities of virus. Purified Vpr did not induce proliferation of these resting PBL (nor did it inhibit mitogen-induced proliferation). Thus Vpr allowed resting PBL (primarily T lymphocytes) as well as macrophages to replicate HIV efficiently in the relative absence of cell proliferation. Additional studies using more highly purified populations of cells will further address these issues; nevertheless, consistent with other reports (Heinzinger et al. 1994), these results support the notion that Vpr contributes to the ability of HIV to replicate in nonproliferating cells. It also indicates that extracellular Vpr can allow replication in nonproliferating cells, including PBL, where virion-borne or endogenously synthesized Vpr will not.

Following an initial decline in virus production in the macrophages, a second dose of Vpr could induce a second round of viral replication. The decline in HIV production seen after day 5 in Fig. 5 is a frequent occurrence in HIV infection. The lag in response of 2 days is an interesting feature of Vpr activity and may reflect a necessity for the accumulation of some factors in the cells (Tat, Rev, cellular transcription factors) following Vpr stimulation.

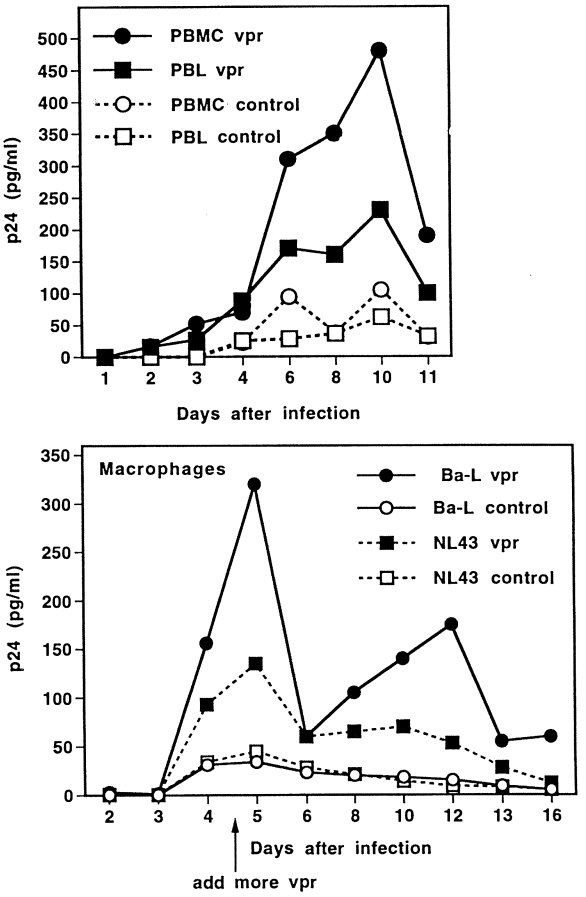


Fig. 5. Extracellular Vpr assists HIV replication in primary hematopoietic cells. *Top* infection of primary peripheral blood mononuclear cells (*PBMC*) and peripheral blood leukocytes (*PBL*). PBMC from a HIV-negative donor were prepared over Ficoll-Hypaque and PBL were isolated by two rounds of depletion of adherent cells in plastic tissue culture flasks. Cells were plated with phytohemagglutinin (PHA) (5 μg/ml) for 48 h then infected with HIV-1 NL43 in the presence of Vpr or control supernatants. *Bottom* Infection of monocyte-derived macrophages. Adherent PBMC were cultured with gronulocyte/macrophage colony-stimulating factor (GM-CSF) for 8 days prior to infection. Cells were >95% macrophages by morphology. Vpr and control supernatants were applied at time of infection

5 HIV Cellular Latency vs. Productive Infection

Cellular latency provides a means for many viruses to evade immune elimination (Oldstone 1991). Thus latency can contribute to viral persistence in vivo. Following initial infection, the host immune response is responsible for the clearance of virus from the periphery and entry into the asymptomatic stage of infection (for a review of the natural history of HIV infection in vivo see Levy 1993; McCune 1991; Schüpbach 1989). The mean length of the asymptomatic or latent phase is approximately 9 years in HIV-1 infection, and until recently, this period was thought to be a time of clinical latency. Recent evidence suggests, however, that virus replication persists in the lymphoid tissues throughout the course of infection (Embretson et al. 1993a,b; Pantaleo et al. 1993); thus true clinical latency probably does not exist in HIV infection.

Paradoxically, HIV infection in vivo has been reported to be predominantly latent at the cellular level (Embretson et al. 1993a,b). In this respect HIV infection resembles the natural history of the other lentiviruses. Peripheral blood cells isolated from HIV-positive individuals typically do not express significant levels of virus, although viral DNA can be detected in up to 1% of peripheral $CD4^+$ Tcells. Viral RNA can be undetectable in primary monocytes from HIV-positive individuals, even though DNA is present (Mikovits et al. 1992) Based on polymerase chain reaction (PCR) analysis, Embretson et al. (1993a,b) calculated that 99%–99.75% of cells which are infected in the lymph nodes in patients with acquired immunodeficiency syndrome (AIDS) are not expressing viral RNA. In the peripheral blood and in asymptomatic individuals, the proportion of non-productively infected cells may be even higher (Embretson et al. 1993b; Pantaleo et al 1991,1993).

Activation of T cells and macrophages in vitro with mitogens or with other cell activators such as phorbol esters will induce high levels of virus expression in vitro (Barre-Sinoussi et al. 1984; Bukrinsky et al. 1991; Gallo et al. 1984; Levy et al. 1984; Schnittman et al. 1989). Infection with various pathogens can lead to reactivation of HIV expression and may be an important mechanism in the progression to AIDS (Gendelman et al. 1986; Mosca et al. 1987). Following infection of cells in vitro, virus expression often declines to very low levels spontaneously over several days to weeks, but virus expression can be reactivated by coculturing with uninfected cells which provide new targets of infection. Clonal cell lines have been developed for study as in vitro models of non-productively infected T cells, B cells, and macrophages (reviewed in Bednarik and Folks 1992; Levy 1993). Each harbors integrated provirus, but expresses low levels of viral RNA and protein. Export of mature virions is very low or undetectable in these lines but virus expression can be reactivated by various cytokines (e.g., IL-1, IL-6. GM-CSF, or TNF-α) or by phorbol ester.

5.1 Reactivation of HIV Expression from Non-productively Infected Cell Lines

Based on our findings as outlined above, it seemed possible that Vpr would also increase HIV replication in infected cells which were not expressing virus. This hypothesis was tested. Five latently infected cell lines (U1 (Folks et al. 1987), OM.10.1 (Butera et al. 1991), J1.1 (Perez et al. 1991), ACH-2 (Folks et al. 1989), and LL58 (Dahl et al. 1990) were stimulated with Vpr. Virus protein production resumed or was increased in all five lines with the result that large amounts of viral antigens were exported into the culture medium (Levy et al. 1995a). Reactivation of virus replication by the phorbol ester PMA was within threefold of the induction by Vpr, either higher or lower depending on the cell line. Anti-vpr antibodies completely inhibited Vpr activity. LL58 has previously been shown by electron microscopy to express intracytoplasmic viral particles (Dahl et al. 1990), and clusters of HIV Gag antigen were revealed by immunofluorescence. Following induction with Vpr, viral antigen was detectable at a very high level, especially at the cell membrane (Fig. 6). It would be interesting to analyze the Vpr genes in these cell lines, for it might be found that Vpr mutations within the proviruses contribute to the observed latency and cell survival.

The establishment and regulation of latency in these lines is not well understood. Depending on the cell line examined and the experimental approach taken, latency has been reported to be controlled at several levels; these include failure to complete reverse transcription in primary cells (Zack et al. 1990,1992). In transformed cell lines developed as models of HIV latency, several possible mechanisms have been reported including the site of proviral integration (Winslow et al. 1993), methylation of the proviral DNA (Bednarik et al. 1987), the regulation of HIV messenger RNA splicing (Pomerantz et al. 1990), the relative levels of viral regulatory proteins (Winslow et al. 1993), and the expression of cellular factors which transactivate the HIV promoter/enhancer, particularly the transcription factor NF-κB (Felber and Pavlakis 1993). When examined, gene expression from latent provirus has been characterized by the expression of low levels of multiply spliced transcripts and very low levels of Tat and Rev proteins (Pomerantz et al. 1990). Vpr reactivation is the first demonstration of an HIV element directly regulating viral reactivation and provides a unifying mechanism for the control productive versus non-productive infection.

The relevance of these lines to in vivo latency is controversial for several reasons. First, each is derived from tumor cell lines which may or may not recapitulate intracellular signaling and other events present in untransformed cells. Second, these lines were derived from the cells which survived presumably cytopathic infection in vitro. The clone which survived may have done so as a result of some defect(s) in the cell or in the virus which has remained undetected, perhaps Vpr mutation. Third, tissue culture conditions are inherently not perfect reproductions of in vivo conditions with respect to the cytokines present and also the other cell types which provide various stimuli in vivo. Fourth, in vivo cellular latency may or may not be the result of silencing proviral transcription, but may

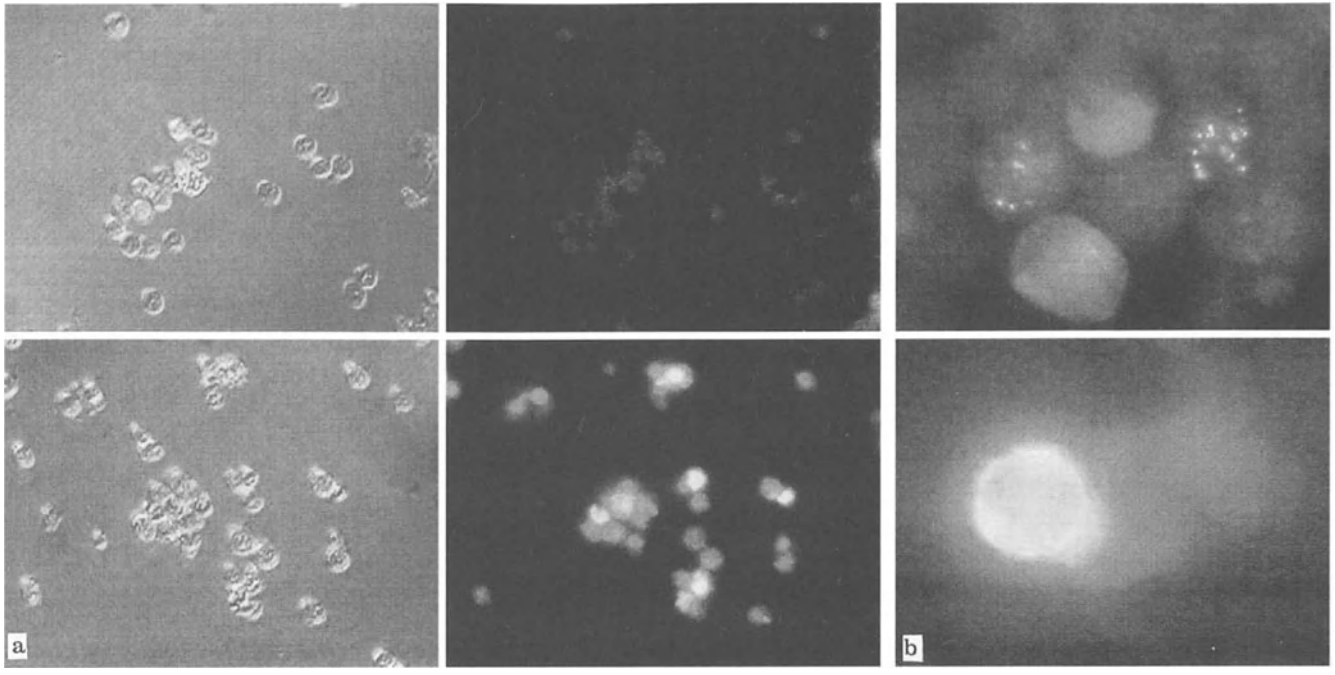


Fig. 6a,b. Vpr induction of HIV expression in LL58 chronically infected B lymphoblastoid cell line. LL58 cells were exposed to extracellular Vpr, fixed then stained for membrane and intracellular p24 Gag. **a** *TOP*, control-treated cells photographed under white light (*left*) and for p24 fluorescence (*right*); *bottom*, Vpr-treated cells. Magnification × 100 **b** Close up of individual cells. Note the point-like, compartmentalized antigen in the control cells (*top panel*) compared with the global and high cell membrane expression of viral antigen in the Vpr-treated cells (*bottom panel*). Low depth of field makes cells which are out of the plane of focus appear negative for fluorescence

reflect infection of resting cells which do not support reverse transcription and integration (ZACK et al. 1990,1992). Our next series of studies was undertaken in an attempt to extend the relevance of extracellular Vpr activity to the in vivo situation.

6 Vpr In Vivo

If Vpr is operating in vivo, it should be present in bodily fluids, and it should be possible to isolate the protein and examine its function. Therefore, the presence of Vpr in the serum and the cerebrospinal fluid (CSF) of HIV-infected individuals was measured, Vpr was purified from serum, and its ability to influence HIV replication in vitro was assessed on transformed cell lines and primary cells.

6.1 Identification of Vpr in Body Fluids

Using a capture ELISA, Vpr protein was found in the serum and also in the CSF of HIV-infected individuals in amounts that directly correlated with the level of p24 Gag present (LEVY et al. 1994). Since Vpr is a constituent of the viral particle, this was an expected finding. When high levels of virus replication produce high viremia, then Vpr levels in serum or CSF rise concomitantly. What this study did not do was to measure the amount of protein that is free and available for biological activity. Vpr may be bound up in the virus or it may be released free into the serum or CSF as a result of virus disintegration. Vpr ELISA of blood collected 3-4 h prior to use and performed without detergents yielded results identical to frozen samples or to assays done using virus-disrupting detergents such as Triton X-100. In addition, antibodies against various regions of the Vpr or against the whole molecule were used in all possible combinations to detect Vpr in ELISA to examine if epitopes of Vpr might be blocked by association with other proteins such as Gag or serum antibodies. The experiment yielded estimations of Vpr that varied by no more that 2.5 fold from each other, supporting the notion than significant quantities of Vpr may be available for activity in vivo. We estimate that total Vpr levels in serum of AIDS patients is similar to levels p24 Gag (10–1000 pg/ml, depending on the individual), which is within the range of Vpr activity observed in vitro (100 pg/ml lower limit). The identification of Vpr in the CSF of AIDS patients with neurological disease opens the possibility that this protein may contribute to the replication of HIV in the CNS and that Vpr also might contribute directly to neural pathology through effects on cell regulation.

6.2 Biological Activity of Purified Serum Vpr

Using the methods developed for recombinant Vpr purification, Vpr was purified from the serum of AIDS patients (Fig. 7). When added to the latently infected lines (at concentrations equal to or below those found in the serum), virus was reactivated in each case in a manner identical to that observed for recombinant Vpr protein (Levy et al. 1994). A further experiment was performed in which all agents, virus and cells were of primary human origin, in fact used ex vivo from HIV-positive patients. PBMC from three infected individuals were placed in tissue culture with serum Vpr, recombinant, Vpr, PHA, or PMA. Virus expression in the absence of stimulation was negligible, as typically observed. Each of the stimulating agents induced HIV replication in the PBMC of all patients with similar kinetics, although Vpr reactivation was usually two to five times greater in terms of the p24 levels achieved overall than it was on PMA or PHA stimulation. On two of three patients' PBMC, serum Vpr and recombinant Vpr displayed identical induction, but in a third patient recombinant Vpr was inferior to serum Vpr and only induced virus levels on the same order as PMA and PHA. This difference between serum-derived and recombinant Vpr opens the possibility that Vpr molecules from various strains of HIV may possess qualitatively different activities.

The notion of different activities for different Vpr molecules was further supported by the observation that serum Vpr could potentiate replication of a monocyte trophic strain of HIV (Ba-L) on monocytic cell lines (U937 and THP-1), but did not increase HIV replication of Ba-L on T cell lines (H9, SupT-1) nor did it increase the baseline replication of a T trophic strain (NL43) on either the monocytic lines or the T cell lines (Levy 1994; Levy et al. 1995b). This observation indicates that Vpr may also regulate virus tropism in vivo through differential activities. Vpr from the serum of late stage patients is likely to represent strains of

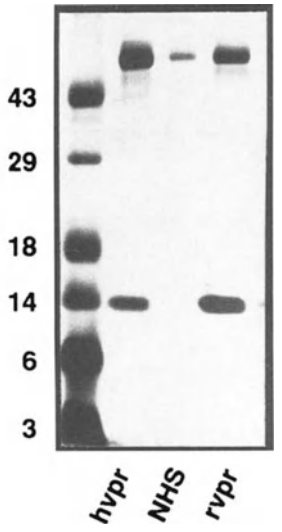


Fig. 7. Comparison of serum Vpr and recombinant Vpr. Partially purified human serum Vpr (*hvpr*), eluate from the serum of an uninfected donor (*NHS*) and partially purified recombinant Vpr (*rvpr*) were analyzed by silver staining of a 15% SDS-PAGE gel. Protein standards are shown in *left lane*

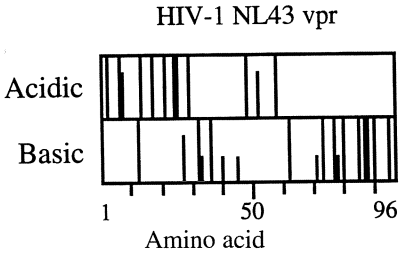


Fig. 8. Acid-basic map of HIV-1 NL43 Vpr protein. Amino acid sequence of Vpr was analyzed using the program DNA Strider 1.2 (MARCK 1988) and modified for publication. Verticle lines designate charged amino acid residues

HIV with ability to replicate in both T cells and monocytes in vivo; understanding this phenomenon as it relates to in vivo infection will thus require further correlation of patient status, primary virus isolates, and purification of primary cell types and serum Vpr from a characterized panel of patients.

6.3 Detection of Anti-Vpr Antibodies in HIV-Positive Individuals

Since anti-Vpr antibodies inhibit Vpr potentiation of HIV replication, it was interesting to examine the presence of anti-Vpr antibodies in HIV-infected individuals, as humoral immunity might regulate Vpr activity in vivo. Previous reports have demonstrated anti-Vpr reactivity in a subset of HIV-positive individuals (GRAS-MASSE et al. 1990; REISS et al. 1990; WONG-STAAL et al. 1987). These studies reported between 25% and 49% of HIV-positive individuals reacted with chemically synthesized, bacterially produced Vpr, and Vpr peptides. Baculovirus, unlike the systems used in the above reports, is a eukaryotic system for protein production, and therefore is more likely to produce proteins which are processed and folded similarly to their native counterparts than are systems based on prokaryotic expression or chemical synthesis. Using partially purified recombinant Vpr as a target in ELISA, the presence of anti-Vpr antibodies in the serum of HIV-positive individuals was investigated (LEVY 1994). Interestingly, most anti-p24 antibody-positive individuals examined displayed anti-recombinant Vpr reactivity, while 0 out of 11 uninfected individuals reacted (Fig. 8). Previous studies have not found a correlation between anti-Vpr reactivity and disease state; however, use of baculovirus-produced Vpr, native viral Vpr or patient-derived Vpr would allow further examination of this issue.

7 Discussion

Vpr is clearly a positive regulator of HIV expression, and is active in a cell-free form. Others have previously reported that *vpr* mutants replicate less well in T cell lines and very poorly in primary macrophages (HATTORI et al. 1990; BALLIET et al. 1994). Extracellular Vpr increases the synthesis of HIV proteins. We favor the idea that it acts through transcriptional activation, although translation and post-

translation events may also be influenced by Vpr, and Vpr can assist early preintegration events (Heinzinger et al. 1994). Extracellular Vpr increases HIV replication in newly infected cells, and it activates virus expression from chronically infected cells which otherwise express low levels of HIV RNA and proteins. Vpr from the serum of AIDS patients activates HIV expression from the resting PBMC of HIV-positive individuals in vitro. Anti-Vpr antibodies neutralize Vpr activation of HIV expression in both new infection and in latently infected cells. Vpr increases the ability of the cells to support HIV replication, and Vpr enhancement of HIV replication is not dependent on other HIV elements. Vpr-exposed cells are more or less permanently rendered more permissive to productive infection and high levels of HIV production. Vpr controls cell proliferation, but enhancement of HIV replication does not depend on cell cycle arrest. Vpr can cause complete cell growth arrest and gross morphological changes in cells, and/or cell differentiation in cells such as muscle and bone tumors, astrocytomas, T lymphocytic, and promonocyte lines.

The dissociation of cell growth inhibition/enhancement and virus enhancement may be important for understanding the biochemical nature of Vpr activity and for developing agents to modulate Vpr activity in vivo. Ideally such agents would inhibit both aspects of Vpr action, for cell growth arrest/enhancement may contribute to pathogenesis and virus activation may contribute to viral load and the spread of HIV. The cellular pathway for Vpr activity is not clear from these essentially phenomenological studies. Binding of Vpr to target cell membranes has not been observed by us, but others have found nuclear localization of Vpr in infected cells (Heinzinger et al. 1994; Lu et al. 1993; Sato et al. 1990). This is consistent with a transcriptional activity. The dissociation of cell growth arrest and virus replication suggests dual activities. Vpr is a small protein: HIV-1 vpr contains 96 amino acids, but it has some interesting features. The protein is highly alpha-helical in predicted structure, and it is highly charged. Using a computer analysis of amino acid composition (Marck 1988), the amino end is seen to be quite basic while the carboxyl end is highly acidic (Fig. 8). This suggests that Vpr may interact strongly with other proteins in cells, consistent with reports of such interactions (Zhao et al. 1994, Refaeli et al. 1995). The functions of Vpr described by our studies require interaction Vpr with basic cellular pathways governing cell proliferation and differentiation. Examination of these potential interactions is a subject of ongoing research in this laboratory.

7.1 A Model for Vpr Activity In Vivo

Following virus fusion with a target cell, Vpr from the viral particle is delivered into the cytoplasm along with other viral constituents such as RNA and polymerase enzymes. Through so far undefined pathways, Vpr increases the activity or expression of cellular factors which assist virus expression and/or it decreases the activity or expression of factors which otherwise would inhibit HIV replication. Perhaps through these interactions, or in addition to them, Vpr assists migration

of the preintegration complex to the cell nucleus (HEINZINGER et al. 1994). Vpr tends to direct the outcome of infection towards productive infection as opposed to nonproductive or latent infection. As a consequence of Vpr action in these cells, cell proliferation and differentiation could be affected, perhaps to the detriment of the cell's growth or effector functions. In the case of abortive infection of cells that do not permit HIV replication, Vpr protein delivered into these cells might nevertheless disregulate them, contributing to tissue-specific disease. Superinfection of latently infected cells by viruses containing Vpr may activate expression of the initial virus, contributing to virus load and the possibility of phenotypic pseudotyping and genetic recombination between viruses.

In the late phase of viral gene expression, Vpr protein is made (ARRIGO and CHEN 1991; GARRETT et al. 1991), and may contribute to the cellular dysregulation described above. Vpr is also packaged into the nascent viral particles. At the same time, some Vpr leaves the infected cell by virtue of an inherent ability to be exported, and would be immediately present in a potentially active form in the extracellular environment. Vpr present in viral particles may be eventually released by virus disintegration and by immune lysis of viral particles.

In the initial stage of virus infection of an individual, prior to the development of an effective antibody response, extracellular Vpr protein would be free to exert its effects on targets of HIV infection and on other tissues which are not infected. Extracellular Vpr also could generate a positive feedback loop in which it increases infection and replication; thus it would increase its own expression, thereby contributing to the fast increase and high titer in virus load at this stage. Vpr can create a reservoir of cells that are highly permissive for HIV infection and replication. Vpr increases HIV expression from monocytes and macrophages and, via these cells, HIV is disseminated throughout the body, including through the blood–brain barrier to the CNS.

Macrophage and glial production of Vpr in the CNS may contribute to neurological disorders and dementia. Vpr dysregulation of muscle cells, particularly muscle satellite cells that replenish mature populations, may contribute to wasting frequently observed in HIV-positive individuals. With respect to HIV replication, extracellular Vpr may be particularly effective in centers of virus replication such as the lymphoid tissues where high concentrations of HIV antigens are found. Local effects of Vpr could contribute to the generation of "hotspots" of HIV replication seen in these areas, which would in turn contribute to T cell depletion and perhaps depletion of Langerhans/dendritic cells which occurs early in HIV infection.

Following clearance of extracellular virus by the immune system, Vpr activity would be rendered minimal. Viral load and concomitant Vpr levels remain low throughout the asymptomatic period except in areas where high levels of viral antigens persist such as in the lymphoid tissues. In these areas the balance between Vpr levels and neutralization of Vpr by antibodies might favor Vpr activity, contributing to the continued HIV replication observed there.

Among many other factors, we predict that Vpr contributes to the destruction of immune systems. Failure to replenish mature effector populations, owing to

defective proliferation and differentiation of stem cells, is one way in which Vpr might aid destruction of these tissues. It will be informative to further examine the inhibition of T cell replication and function as well as macrophage function and differentiation in response to endogenously expressed and extracellular Vpr because the potential of this regulatory protein to unify some concepts of HIV pathogenesis is significant.

When anti-Vpr and anti-HIV immune responses are reduced enough, HIV and consequently Vpr levels rise. Increased Vpr levels may increase viral replication. Thus extracellular Vpr might provide a positive feedback mechanism that facilitates the high replication, increased virus loads, and progressive morbidity observed in the later stages of AIDS.

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