Advances in VIRUS RESEARCH

VOLUME 54

ACADEMIC PRESS

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VOLUME 54

ACADEMIC PRESS
San Diego New York Boston
London Sydney Tokyo Toronto

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Academic Press
a division of Harcourt Brace & Company
525 B Street, Suite 1900, San Diego, California 92101-4495
http://www.apnet.com

Academic Press Limited
24-28 Oval Road, London NW1 7DX, UK
http://www.hbuk.co.uk/ap/

International Standard Serial Number: 0065-3527

International Standard Book Number: 0-12-039854-0

PRINTED IN THE UNITED STATES OF AMERICA
99 00 01 02 03 04 SB 9 8 7 6 5 4 3 2 1

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THE LIFE AND TIMES OF ADENOVIRUSES

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The development of a productive and enjoyable career rests in large part on being in the right place at the right time. I had the extremely good fortune to meet this criterion. On being released from the Army after World War II, I began a postdoctoral fellowship at the Rockefeller Institute (now Rockefeller University) under the guidance of a superb mentor, Dr. Frank Horsfall. In addition, I had the very good fortune to be constantly exposed to many outstanding scientists at the Institute at that time, such as Dr. Thomas Rivers, René Dubos, Wallace Goebel, P. K. Olitsky, Jordi Casals, and many others (fellows, members, and others would mix freely at the tables in the Institute's lunchroom). I also had the good fortune to be constantly associated with a brilliant group of other postdoctoral fellows in the laboratory, such as Igor Tamm, Peter Ahrens, George Cotzias, and too many others to list. In Dr. Horsfall's laboratory a virus had been discovered to be latent in mice; it was termed "pneumonia virus of mice" (PVM). It is now known to to be a mouse respiratory syncytial virus, and it directed my interest to the investigation of viral latency.

After my training at the Rockefeller Institute, I assumed my initial independent position at the Western Reserve School of Medicine (now Case Western Reserve), and my research focused on trying to determine the mechanism by which PVM remained latent. Then began more of my good fortune. Dr. Robert Huebner of the National Institutes of Health visited Western Reserve to present a seminar. Afterward he came to my laboratory to hear about my research, but before I could begin, he told the following exciting news: Dr. Wallace Rowe, a postdoctoral fellow in his laboratory, had made a thrilling discovery. In an attempt to isolate "the virus of the common cold," he had used an explant of an adenoid in cell culture, into which he inoculated secretions from a patient with a cold. Dr. Rowe being a very smart young scientist also used control uninoculated cultures. After only a few days he noted that the cells in the control uninoculated cultures, as well as the cells in those inoculated with patient secretions, became rounded and formed grape-like clusters, but the cells did not lyse. If he disrupted the cells,

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he could obtain and pass on the cytopathic agent to other cells. Hence, the adenoids were obviously infected with a latent virus. This seemed to me to be the ideal virus to use in investigating the mechanism of viral latency, also termed "persistence." I asked Dr. Huebner if he would send me the virus after publication of this exciting discovery. He immediately responded that prior publication was not necessary. He then called Dr. Rowe and told him to send me samples from his adenoid cultures. (Can you imagine that occurring today?). On receipt of the sample, I demonstrated that it could replicate and be passaged in HeLa cells.

Shortly, thereafter (1954), Dr. Maurice Hilleman, who was then in the U.S. Army, came to Western Reserve to see Dr. John Dingle, who was Chairman of the Department of Preventive Medicine but who during World War II had been Director of the Commission of Acute Respiratory Diseases at Fort Bragg, North Carolina. I had been sent to Fort Bragg after I returned from England, where I had been stationed in the Seventh General Hospital. At Fort Bragg I was assigned to the infectious diseases service of the Medical Service at the General Hospital. This assignment gave me many opportunities to take care of patients with a wide variety of infectious diseases, including human volunteers that the Commission on Acute Respiratory Diseases was using to investigate "acute respiratory diseases of recruits" (ARD). Fortunately, the laboratories where the Commission was located and the General Hospital were adjoining structures. Therefore, it was convenient for me to have have constant contact with Dr. Dingle and his Commission colleagues while taking care of their volunteer patients. I knew Dr. Dingle from my days as an intern and resident on the Harvard Medical Service at Boston City Hospital, where he was a professor.

Dr. Hilleman and Dr. Werner, at Fort Leonard Wood, had isolated a virus from Army recruits who were thought to have influenza. They could not isolate an influenza virus, however, and Dr. Hilleman, who was then a medical officer in the Army, was certain that he knew how to do this. Therefore, he came to Cleveland to ask Dr. Dingle to determine whether it was the ARD virus, which, as noted above, Dr. Dingle and his colleagues had investigated extensively in human volunteers at Fort Bragg; and all of the samples from these studies were stored at Western Reserve. As the basic virologist at Western Reserve, I was asked to determine whether the virus that Drs. Hilleman and Werner had isolated was related to the etiologic agent of ARD studied at Fort Bragg, as well as from the human volunteers who had received extracts from patients and in turn had contracted the disease. I soon showed

that the Hilleman-Werner viral isolate, according to neutralization assays, was identical to the virus isolated at Fort Bragg (1), and this was confirmed relative to a number of viral properties investigated in cell cultures (2). I also showed that immunologically it was distinctly different from the virus that Rowe and Huebner (3) had isolated according to neutralization assays, but that the two viruses were related according to the complement fixation assay (An aside: there are presently 49 viruses in the adenovirus family; they are all related by complement fixation assays but are distinctly different according to the results of neutralization assay.)

Dr. Dingle and his colleagues, including a statistician, Dr. George F. Badger, and an excellent group of physicians and epidemiologists including William S. Jordan, Eli Gold, and Samuel Katz in the Department of Preventive Medicine, were doing an extensive study of illnesses in families. They found that acute respiratory diseases were the predominant illnesses found in children. I was asked to determine whether either or both of the viruses that Rowe *et al.* (3) and Hilleman and Werner (4) had isolated was the etiologic agent of any of the acute respiratory diseases in the children. I readily showed that the virus that Rowe *et al.* had isolated was indeed the etiologic agent of many of these acute respiratory diseases. The virus was particularly the cause of nasopharyngitis, as well as pharnygoconjunctivitis (5,6). We subsequently determined that it was a type 5 adenovirus that was the etiologic agent of so many respiratory infections in children. The Hilleman-Werner virus, the etiologic agent of ARD, was also later shown to be a type 4 adenovirus.

The life of adenoviruses began to expand as we and a few others initiated investigations of the structure of the virion and the mechanism of its replication. After purification of the virus and electron microscopic examination, Horne *et al.* (7) revealed the virion to be a highly structured icosahedron with a fiber protein at each apex (Fig. 1). It was then found that adenoviruses contain DNA. It was readily demonstrated that the virion attached to susceptible cells via the fibers. It is important to note, however, that the hexon, the major capsid protein, not the fiber attachment protein, contains the antigen that stimulates development of virus-specific, neutralizing antibodies (8). This is essential to state because many virologists apparently believe that neutralizing antibodies merely inhibit virion attachment to susceptible cells. It has also been shown that the influenza virus neutralizing antibody-stimulating antigen does not prevent attachment to susceptible cells, although this critical antigen is on the stem of the hemagglutinin structure.

Investigation of the replication of adenoviruses quickly followed the above experiments. Indeed, the field rapidly began to expand as more

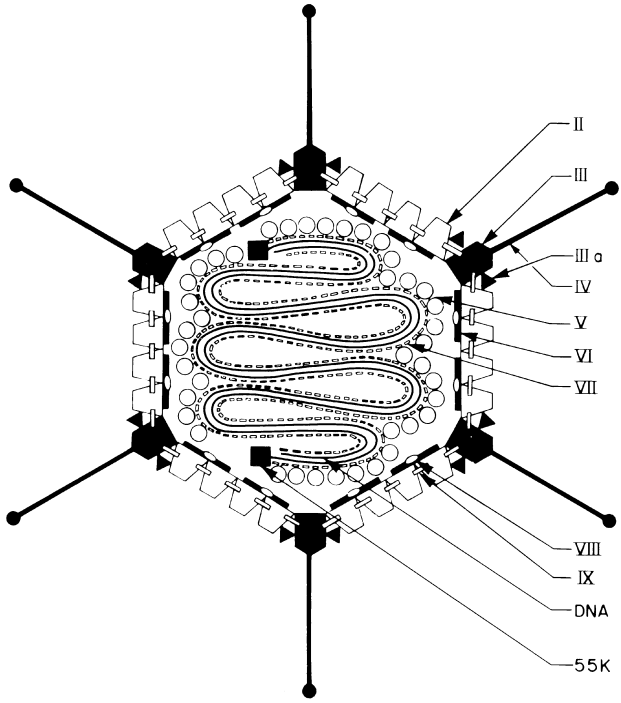

FIG 1. Model of an adenovirus particle showing the apparent architectural interrelationships of the structural proteins (roman numerals) and the nucleoprotein core in the virion. The hexon (II), penton base (III), and fiber (IV) and the hexon-associated proteins (IIIa < VI, VIII, and IX) make up the capsid. Proteins V and VII are core proteins associated with the viral DNA: the 55K is covalently linked to the 5′ end of the DNA (From B. D. Davis, R. Dulbecco, H. N. Eisen, and H. S. Ginsberg, *Microbiology,* 4th ed., Lippincott, Philadelphia, 1990, with permission.)

virologists became interested in adenoviruses. Our research led to the determination of the steps in viral replication (9). We showed (Fig. 2) that the uncoated viral DNA traveled into the nucleus, where early mRNAs were made. The mRNAs were transported into the cytoplasm to synthesize their encoded early proteins, which then traveled back into the nucleus to induce synthesis of viral DNA, from which the late mRNAs were transcribed. The late mRNAs then entered the cytoplasm for synthesis of the virion structural proteins. These proteins were transferred back into the nucleus, where they formed almost the complete viral capsid, allowing space only for the viral DNA to enter before the capsid structure was completed (10). The localization of the steps in viral replication; the effects of this replication on the host cells,

which resulted in the turning off of their DNA and protein synthesis; and the effect of these events in causing cell death and cytopathic changes were revealed. It must be emphasized, however, that although these cellular cytopathic events and cell death occurred, the infected cells *were not lysed.* Hence, it is incorrect to call adenovirus infection a lytic infection. This research was pursued with an outstanding group of graduate students and postdoctoral fellows too numerous to list (many of their names, however, do appear in the references).

In addition to ourselves, a number of other investigators studied the structure of the virion, the genes in the genome, the genome's sequence, its replication, and even the mechanism(s) by which the viral infection damaged the cell in culture. Strikingly, however, none of us investigated the actual mechanism by which adenoviruses produce disease *in vivo.* I had been trying to find an approach to this problem for some time, but there was no animal model available. From out of the blue, however, there appeared a note from Dr. Wallace Clyde of the University of North Carolina (11), whose main interest was in the respiratory syncytial virus. He had been employing cotton rats received from Dr. Robert Chanock of the National Institute of Allergy and Infectious Diseases (NIH) for this research. Dr. Clyde, however, had some extra animals, and because he had previously been interested in adenoviruses, he inoculated cotton rats intranasally with type 5 adenovirus (Ad5) and found that the lungs developed a pneumonia similar to that in humans. This clearly answered my needs. I immediately telephoned Dr. Chanock and asked if I could come down on a sabbatical to study this exciting problem. The NIH gave its permission, and in 1984 I obtained a sabbatical leave from Columbia University's College of Physicians and Surgeons, where I was Chairman of the Department of Microbiology, and began the research to determine the mechanism by which Ad5 produced pneumonia. To me and my colleagues, the results of this research endeavor proved to be very exciting.

At that time, Dr. Gregory Prince was working as a pathologist with Dr. Chanock, in whose laboratory cotton rats were being bred. Dr. Prince was accustomed to working with cotton rats (Fig. 3) and therefore was assigned to work with me. This excellent collaboration has continued up to the present. (Dr. Prince is presently Vice-President of Virion Systems, Inc., which breeds cotton rats and pursues research activities.) At that time, it was known that cotton rats were highly susceptible to many viruses, such as poliomyelitis virus, influenza virus, and smallpox virus.

The pneumonia that Ad5 induced in cotton rat lungs was very similar to that produced in humans. The virus replicated primarily in the

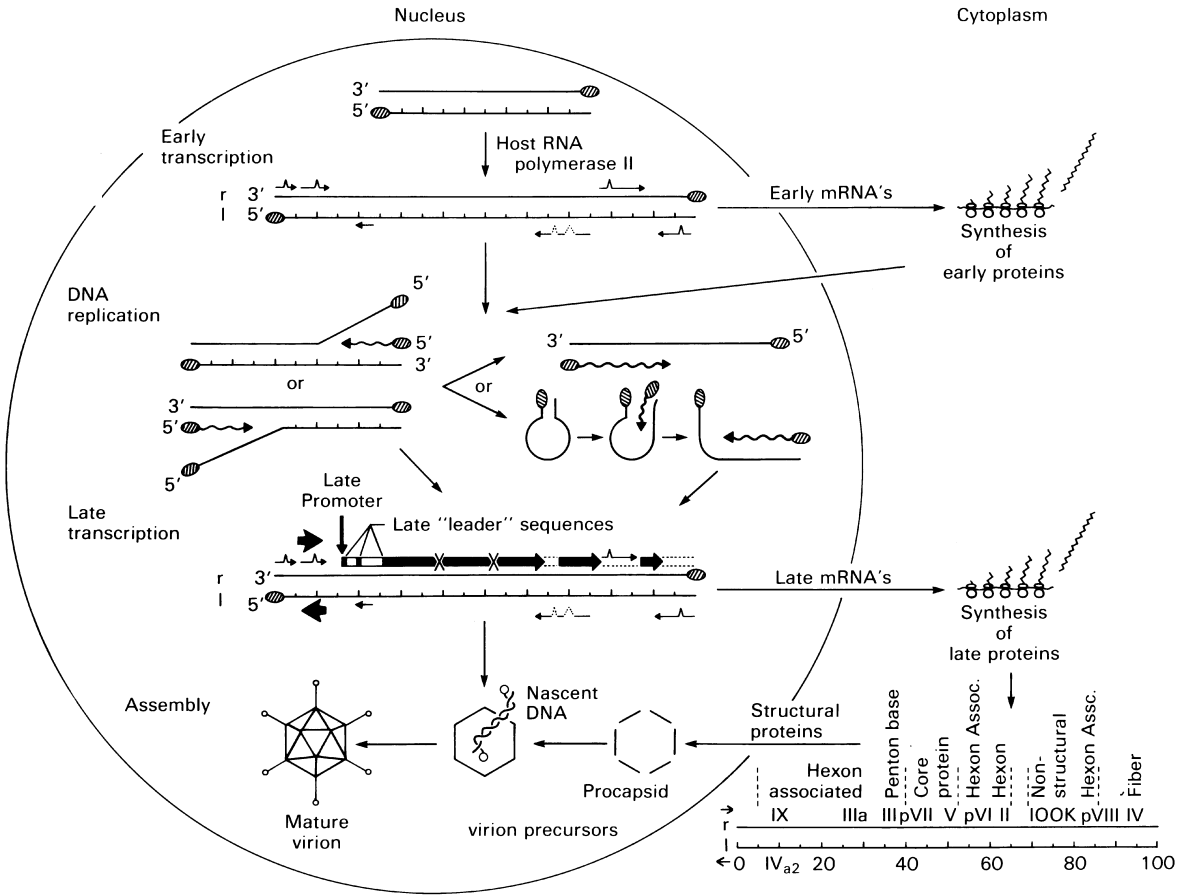
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FIG 3. (A) The cotton rat as seen in an Audobon Society painting. (B) The real cotton rat employed for experiments described in text. (For color reproduction, see color section.)

FIG 2. Diagram of the biosynthetic events in the multiplication of type 2 adenovirus (used as a model since its transcription has been studied in greatest detail). Early mRNAs are transcribed from five separated regions of the genome. Like late messages, they are processed from larger transcripts, and they have leader sequences transcribed from noncontiguous regions [the intervening absent sequences are indicated by a connecting caret (^)]. The semiconservative asymmetric DNA replication is shown. A mechanism for replication of the displaced single strands, with the use of the inverted terminal repetition to form a circle-like intermediate, is also suggested. The function of the 5′ terminal 55K protein in DNA replication is unknown; the model presented, however, shows that it could serve as a primer for DNA replication. Late mRNAs, except that for protein IVa_2, have a single promoter at 16 map units on the r (rightward) strand. Note that each late "megatranscript" contains only one set of leader sequences, and therefore only a single mRNA can be derived from each transcript. Also indicated are the regions of the genome in which the late viral proteins are encoded and their known functions. (From B. D. Davis, R. Dulbecco, H. N. Eisen, and H. S. Ginsberg, *Microbiology,* 4th ed., Lippincott, Philadelphia, 1990, with permission.)

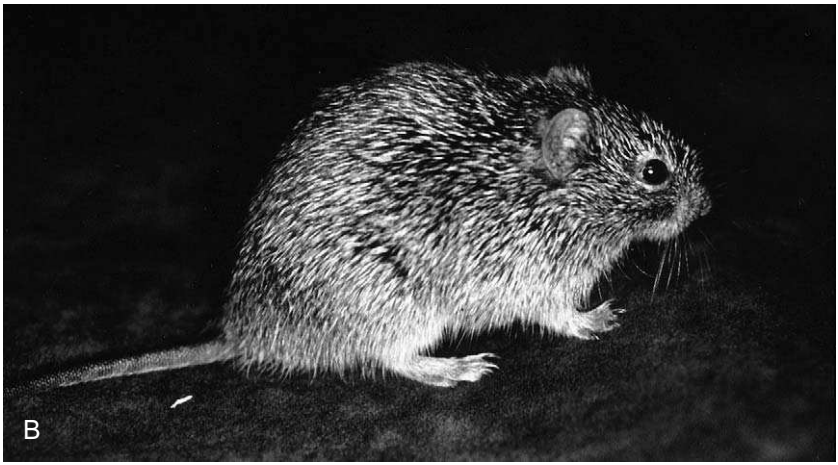

FIG 3 *Continued*

bronchiolar epithelial cells, and it is important to note again that the infected cells died but were not lysed (12). The next step was to determine whether the production of pathogenesis required early or late genes or both. *In vitro* cell culture experiments had indicated that the fiber protein alone could markedly damage cells (13). Dr. Arnold Levine, now President of Rockefeller University, carried out this research when he was a graduate student in my laboratory at the University of Pennsylvania School of Medicine, where I was Chairman of the Department of Microbiology. To determine whether early or late genes were required to induce the inflammatory response, we infected cotton rats with an Ad5 temperature-sensitive mutant, *H5ts125,* which cannot replicate at 39.2°C, the temperature of the cotton rat, and extensive pneumonia was produced. These experiments clearly demonstrated that only the early Ad5 genes are required to produce the full pneumonia (12). The use of H5ts149, a temperature-sensitive mutant in the viral DNA polymerase gene, yielded the same results. These data are another clear demonstration of the differences that may be found between *in vitro* and *in vivo* research findings.

Next, we examined the early region 3 (E3), which comprises about 10% of the genome but has been termed "nonessential" because it is not required for viral replication. It seemed most unlikely that in a genome of limited size, 10% of its DNA would have no function. We therefore infected cotton rats intranasally with a mutant from which the entire E3 region had been deleted. The mutant replicated the same

as wild-type Ad5, but the pneumonia produced was markedly increased (14). When single mutants were used in which individual E3 genes were deleted, it was found that with the mutant in which the 19-kDa gene alone had been deleted near the 5′ end of the E3 region, the virus produced a pneumonia that was as extensive as that produced by the mutant in which the entire E3 region had been deleted. Deletion of none of the four other E3 genes yielded a mutant that increased the extent of the inflammatory response. When the 14.7-kDa gene at the 3′ end of the E3 region was deleted, however, increased numbers of polymorphonuclear leukocytes appeared in the inflammation, as though this had resulted in an elaboration of the cytokine tumor necrosis factor alpha (TNF-α). (Dr. William Wold from the Institute of Virology at St. Louis University kindly supplied the E3 region mutants that he had constructed.) To test this hypothesis, however, it was necessary to obtain a new susceptible host in which cytokines could be assayed.

The mouse was the natural host selected because we had previously shown that although the virus could not replicate in mouse cells, early viral genes were expressed (15). As described above, only early genes are required to produce maximum pneumonia (12). A number of mouse strains were tested, and it was found that the C57B1/6N mice were the most susceptible to Ad5 pulmonary infection, producing a pneumonia similar to that produced in cotton rats. As predicted, the virus did not replicate in the mouse lungs, but early genes were expressed. Moreover, the anticipated cytokine TNF-α was elaborated in the Ad5-infected lungs, but interleukin-1 (IL-1) and IL-6 were also produced. It is also noteworthy that interferon was not expressed. (Dr. L. L. Moldawer of the University of Florida School of Medicine, an outstanding investigator in the field of cytokines, has been a very important colleague in these studies.) With the finding that Ad5 induced elaboration of cytokines, experiments were done to test the effect of cortisone on the inflammatory response to WtAd5 infection. As predicted, cortisone markedly reduced the inflammatory response to WtAd5, and the cortisone therapy essentially eradicated cytokine production (unpublished data).

We hypothesied that the E3 19-kDa gene product normally suppressed expression of the cellular major histocompatibility complex (MHC) antigen and that deletion of the E3 19-kDa protein gene would permit extensive MHC expression on the surface of the mutant-infected cells, thus promoting an extensive lymphoid inflammatory response. Unfortunately, the reagents to test this hypothesis were not available. Several years later, however, Burgert *et al.* (16) did demonstrate that this hypothesis was correct. This finding was critical in elucidating the mechanism by which adenovirus can establish and maintain a latent

infection, which had been exposed in the seminal discovery by Rowe, Huebner, and their colleagues of adenoviruses in adenoids (3). Furthermore, in inbred nude (Nu/Nu) mice, pulmonary infection did not induce any inflammatory response (unpublished data).

At this time in the history of adenoviruses, the attention of many investigators was focused on the use of adenoviruses as a vector for gene therapy. Of particular importance in this regard was the fact that Ad5 could be replicated to very high titers in cell cultures. In one of the earliest vectors developed, the entire E3 region had been deleted, as well as the E1A region in which the cystic fibrosis gene had been placed for expression. Expression of the cystic fibrosis gene was very satisfactory, but the vector produced serious pneumonia in some of the recipients because the E3 region had been deleted. We then turned our attention to the development of an entirely different adenovirus vector because we had demonstrated that a mutant in which the E1B 55-kDa protein was deleted induced an infection in which there was a marked reduction in the inflammatory response in both cotton rats and mice (publication in preparation). In addition, a mutant in which the entire E4 region was deleted (obtained from Dr. Thomas Shenk) did not affect the viral inflammatory response in cotton rats or mice (unpublished data). A vector in which the E1B 55-kDa protein gene and the entire E4 region were deleted should provide ample space for insertion of the gene to be expressed; in addition, this vector should not produce any pulmonary pathology. One serious problem still remains, however, in the use of adenoviruses as vectors: they produce a prominent immunological response inducing neutralizing antibodies so that the vector is not expressed for long periods, and it cannot be readministered. These facts markedly diminish the value of adenoviruses as vectors unless a vector can be developed in which the hexon is altered so that the virion is still formed but does not induce neutralizing antibodies and cellular immunity—wishful thinking, but a product for which it is worth working.

Summary

With Wallace Rowe *et al.'s* and Hilleman and Werner's isolations of viruses, subsequently termed "adenoviruses," a new area of research opened for me and gradually for many others. I was quickly able to associate the viruses with diseases in humans, and then our attention turned to the structure of the virion and how it replicated. Many virologists entered these areas of adenovirus research, for they were the central themes for most virologists at that time. We obtained more and

more knowledge of the structure of the virion, its genome, and how it replicated and killed cells in culture so that they could no longer divide, although the virus infection did not lyse the infected cells, but we did not have the slightest idea how Ad5 produced disease *in vivo.* Then Wallace Clyde's timely note appeared, and we entered an exciting and profitable new field: an investigation of the mechanism by which Ad5 produces pneumonia. It must again be emphasized that the pneumonia that WtAd5 produces in cotton rats is pathologically very similar to that induced in humans. One of our earliest sets of experiments in the cotton rats was designed to determine whether region E3 was really nonessential even though the genes contained therein were not required for viral replication. We soon demonstrated that deletion of the E3 region produced a mutant that induced a highly pathogenic viral pneumonia. The potential role in pathogenesis of each of the genes within the E3 region was then investigated. Of maximum importance was the finding that deletion of the 19-kDa gene near the 5′ end of the region produced a severe inflammatory response. This result led to the discovery that the E3 19-kDa protein regulated expression of the MHC factor on the surface of infected cells, and deletion of this gene produced a marked increase in MHC on the surfaces of infected cells and, therefore, a marked increase in the response of cytotoxic T cells. In addition, deletion of the gene encoding the 14.7-kDa protein, which was situated at the 3′ end of the E3 region, resulted in an increase in polymorphonuclear leukocytes in the inflammatory response.

A number of these findings led to hypotheses that could not be tested in the cotton rat since the necessary reagents were not available. Fortunately, our findings that only early viral genes are required to produce full pathogenesis led us to test mice because we had shown in a culture of mouse cells that all of the early viral genes are expressed. The C57BL/6N mouse proved to be an excellent host in which Ad5 produced full pulmonary inflammation. Thus, it was possible to test our hypotheses and to demonstrate their validity, showing that the virus induces cytokine elaboration, as well as to demonstrate the role of cytotoxic T cells in permitting Ad5 to produce persistent infections in lymphoid cells of organs such as the adenoid, from which the first adenovirus was isolated, and which had immediately led to my interest in investigating it and helping to develop the story of adenoviruses.

Acknowledgments

In addition to my excellent graduate students and postdoctoral fellows, I want to express special thanks to the two superb technicians who assisted in making my research

productive: Ms. Mary Dixon worked with me during my entire period at Western Reserve and the University of Pennsylvania. When I moved to the College of Physicians and Surgeons of Columbia University, Mrs. Ulla Lundholm-Beuchamp worked with me during most of my career there.

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ROLE OF OUTER CAPSID PROTEINS IN TRANSMISSION OF *Phytoreovirus* BY INSECT VECTORS

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I. Introduction

The plant viruses that cause serious damage to economically important crops are transmitted predominantly by vectors in the field. Details of the viruses can be found in "Descriptions of Plant Viruses"; one of these viruses is cucumber mosaic virus (Gibbs and Harrison, 1970). Typical vectors involved in the transmission of plant viruses are aphids, leafhoppers, planthoppers, white flies, thrips, mealybugs, mites, nematodes, and fungi (Matthews, 1991). Viruses can be transmitted in a persistent, semipersistent, or nonpersistent manner. Some of the viruses that are transmitted persistently are able to multiply in vectors as well as in their plant hosts.

In the field, many plant viruses can be dispersed only by transmission via vectors. Therefore, the vector transmission of viruses has been studied extensively by traditional methods, as well as by current molecular biological techniques (Hull, 1994). Factors that assist in the nonpersistent transmission of potyviruses and caulimoviruses have been well characterized in investigation of the molecular mechanism of viral transmission by vector insects (Pirone and Blanc, 1996). An explanation of the involvement of a symbiont microorganism in transmission of potato leafroll virus by vector aphids has been presented (Hogenhout *et al.,* 1998). Furthermore, existence of a potential cellular receptor in the midgut of a thrips that serves as a vector for tomato spotted wilt

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virus has been suggested in an analytical study of the vector (Bandla *et al.,* 1998).

An understanding of the intimate nature of virus–vector, vector–plant host, and plant host–virus interactions is indispensable for the development of measures to control plant viruses that are transmitted by vectors. Thus, qualified research systems suitable for various investigations are required for studies of the fundamental events that determine the respective affinities of the various organisms mentioned above. The systems developed for such studies include transmission tests using vectors and vector cell monolayers (VCM), as well as serological methods for detection of viral antigens in individual vectors, in association with contemporary biochemical and molecular biological techniques.

One of the major problems in plant virus research is that a long period of time is required to obtain results of infectivity tests, in particular in vector transmission studies, for example, a couple of weeks in the case of nonpersistent transmission and as much as two months in the case of viruses that propagate in a vector insect (Inoue and Omura, 1982; Omura and Kimura, 1994). Furthermore, cell biology systems for studies of viral activities in the host are limited to viruses for which protoplasts are available (Takebe, 1977).

VCM provide efficient experimental systems for bioassay, as well as for cell biology studies of plant viruses. However, the VCM established to date for use in plant virus research have been derived from only a few species of leafhoppers. Viruses that can potentialy be studied in these systems are wound tumor phytoreovirus (WTV) (Black, 1970), potato yellow dwarf virus, rice dwarf phytoreovirus (RDV; Iida *et al.,* 1972), and rice gall dwarf phytoreovirus (RGDV; Omura and Inoue, 1985) (Kimura and Omura, 1988). Lines of leafhopper cells and the details of cell culture and of studies of viruses using VCM were reviewed by Omura and Kimura (1994). The advantages and disadvantages of VCM and traditional experimental systems for virus research were summarized in the same review.

Rice, one of the most important food crops, together with wheat and maize, is infected by 15 major viruses (Hibino, 1996), all of which, with the exception of rice necrosis mosaic virus and rice stripe necrosis virus, whose vector is a fungus, are transmitted by insects. Among these viruses, RDV and RGDV, which threaten rice production in Asian countries, are transmitted in a persistent manner by leafhoppers, the *Nephotettix* species. Furthermore, the viruses can propagate both in plants and in invertebrates. These properties are shared by viruses that belong to other groups: *Fijivirus* and *Oryzavirus,* transmitted by planthoppers; *Marafivirus,* transmitted by leafhoppers; *Rhabdovirus,* transmitted by

leafhoppers, planthoppers, and aphids; *Tenuivirus,* transmitted by planthoppers (Matthews, 1991); and *Tospovirus,* transmitted by thrips (Ullman *et al.,* 1993; Wijkamp *et al.,* 1993). The mechanism that allows these viruses to infect and multiply both in plants and in animals is of interest from a scientific as well as a practical perspective as attempts are made to interfere with the viral multiplication cycle.

An understanding of the molecular organization of virus components (Nuss and Dall, 1990; Omura, 1995; Suzuki, 1995), as well as of the cell biology of virus-infected hosts, is required for the development of methods to control these viruses by finding a way to interrupt viral infection or multiplication processes in leafhopper vectors. If we could understand how viruses affect host cell metabolism, this information might help us to attenuate or block viral functions in infected cells. Using current research systems combined with reliable traditional experimental systems, we have studied the viral proteins responsible for infection by *Phytoreovirus* of vector cells, with the goal of defining the mechanism of vector transmission of such viruses.

II. Localization of Structural Proteins in Particles of *Phytoreovirus*

It is likely that component proteins with specific functions are located at specific sites in viral particles for the efficient functioning of the respective proteins during the course of viral adsorption to cells, penetration of cells, and viral multiplication in host cells. Therefore, it is important to determine the relationships between the biochemical or biological functions of component proteins and their locations in viral particles.

Phytoreovirus, one of the nine genera in the family *Reoviridae* (Holmes *et al.,* 1995), includes WTV, RDV, and RGDV. These viruses are icosahedral double-shelled particles approximately 65–70 nm in diameter that contain 12 segments of double-stranded RNA (dsRNA) as their genome and six or seven component proteins (Figs. 1 and 2). In RDV, the core is composed of P1, an RNA-dependent RNA polymerase (Suzuki *et al.,* 1992); P3, the core capsid protein; P5, a guanylyltransferase (Suzuki *et al.,* 1996); and P7, an RNA-binding protein (Ueda and Uyeda, 1997). These proteins are required for RNA replication and are basically similar to those of viruses that belong to other genera, such as reoviruses (Dryden *et al.,* 1993), rotaviruses (Patton, 1994), and bluetongue virus (BTV) (Roy, 1996b). The P2 and P8 proteins of RDV, which were shown to be required for viral infection of insect vector

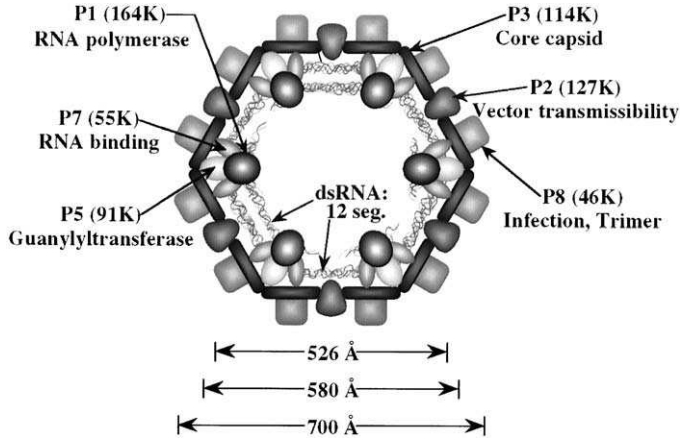

FIG 1. Schematic representation of the morphology and disposition of viral components in particles of RDV (data summarized from Inoue and Timmins, 1985; Mizuno *et al.,* 1991; Yan *et al.,* 1996; Zhu *et al.,* 1997; Naitow *et al.,* 1999).

cells and, therefore, to be associated with viral transmission by vector insects (see below), are part of the outer capsid of the virus (Yan *et al.,* 1996). It seems that the P2 protein is not located at the outermost region of the virus because antiserum raised against P2, purified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), did not aggregate purified RDV particles that included P2 (T. Omura, unpublished data, 1997). RGDV has a similar protein composition, and the respective sites of analogous proteins in particles are also similar (Omura *et al.,* 1985). The P2 protein of RGDV (Section IV) is also considered to be located in the outer capsid layer but not to be exposed on the surface of viral particles, in view of results similar to those described above for RDV, and purified virus that contained P2 did not generate an antibody that reacted with the P2 in immunoblotting (Omura *et al.,* 1985).

III. Role of P2 Protein in Viral Infection of Insect Vector Cells

Assays of viral infectivity with the use of cultured cells of the vector *Nephotettix cincticeps* (Omura and Kimura, 1994) revealed that viral infectivity was lost during the purification of the virus from sap of RDV-infected plants that contained a high titer of infectious virus. Virus particles in the purified preparations and in the crude sap appeared similar in morphology under the electron microscope. These

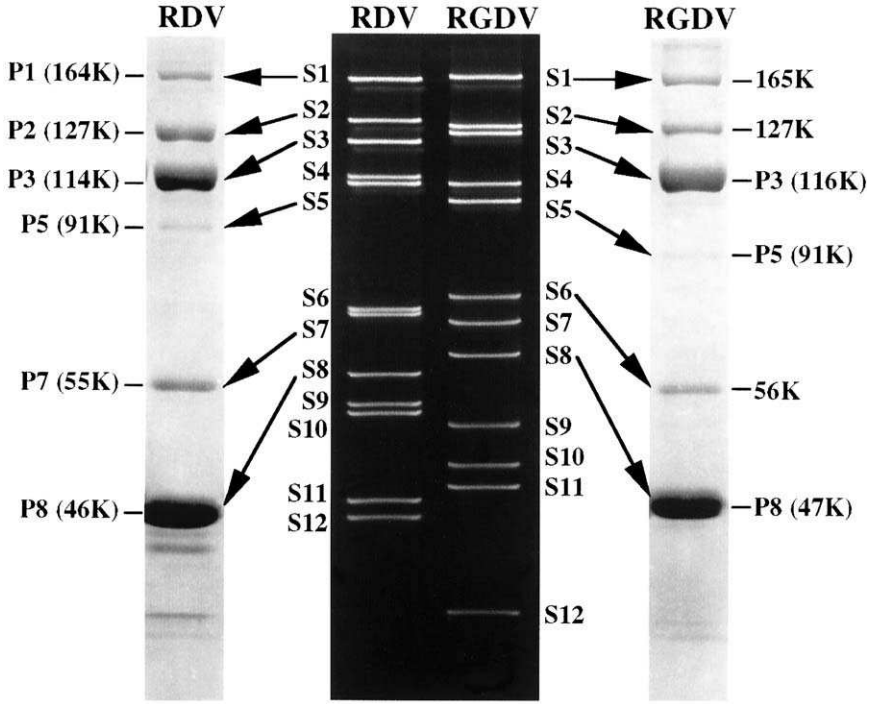

FIG 2. Assignment of genome segments to the structural proteins of RDV and RGDV (data summarized from Omura, 1995; Suzuki, 1995; Yan *et al.,* 1996).

observations prompted us to look for the molecular entity that is associated with the ability of the virus to infect vector cells (Yan *et al.,* 1996; Omura *et al.,* 1997).

First, the effects of each chemical and physical treatment during the purification procedure (Omura *et al.,* 1982) on viral infectivity were studied. No substantial loss of infectivity of viruses in the sap of RDV-infected plants was found after treatment with Triton X-100 or polyethylene glycol (6%) or after high-speed centrifugation or sucrose density gradient centrifugation. However, the infectivity fell below 0.1% on treatment with CCl_4 and was entirely lost after two cycles of such treatment (Fig. 3, upper panel, B). Therefore, the original purification procedure for RDV was modified to exclude treatments with CCl_4 and to add density gradient centrifugation in 10–40% (w/v) sucrose as a final step. As anticipated, preparations of virus purified without CCl_4 were able to infect VCM (Fig. 3, upper panel, A).

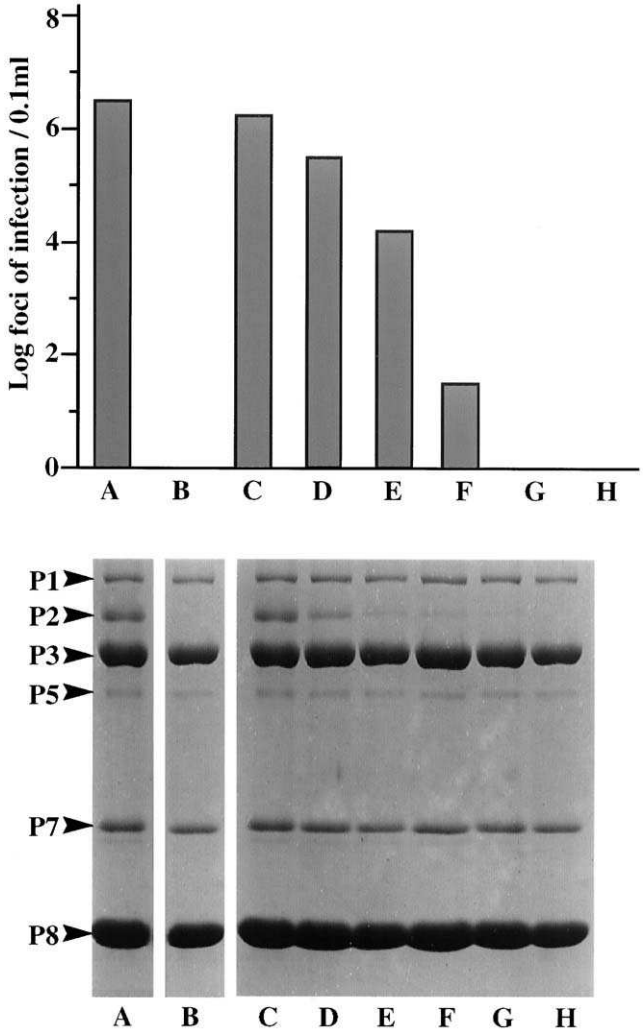

FIG 3. Ability of preparations of RDV to infect VCM (upper panel) and the protein components of various preparations of RDV (SDS–PAGE; 10% polyacrylamide; lower panel). A, RDV purified without CCl_4 treatment; B, RDV purified with CCl_4 treatment; C, RDV purified without CCl_4 and then treated without CCl_4 (control); D, E, F, G, and H, RDV purified without CCl_4 and then treated with CCl_4 for 1 second, 5 seconds, 1 minute, 5 minutes, and 10 minutes, respectively. Bands of the viral structural proteins (P1, P2, P3, P5, P7, P8) in the lower panel are identified on the left. Reproduced with permission from Yan *et al.* (1996).

Under the electron microscope, virus particles purified with or without CCl_4 appeared to be isometric, with a diameter of approximately 70 nm (Omura *et al.,* 1989), and they appeared similar in morphology. Because RDV particles lost their outer layer to yield core particles in CsCl (T. Omura, unpublished data, 1986), the density of RDV purified with or without CCl_4 was not analyzed. After linear sucrose density gradient centrifugation in 10–40% sucrose, no significant difference was found in sedimentation rates between RDV purified with or without CCl_4 (J. Yan and T. Omura, unpublished data, 1996).

These results suggest that the loss of infectivity on treatment with CCl_4 was due to loss of a minor viral component and/or to some damage to the structure of the virus that was undetectable by electron microscopy. Analysis of nucleic acids by SDS–PAGE revealed the presence of all 12 genome segments of dsRNA in RDV purified with or without treatment with CCl_4, and the relative respective levels of each of the 12 segments appeared similar (T. Omura and J. Yan, unpublished data, 1996).

Analysis of protein components by SDS–PAGE (10% polyacrylamide) revealed that RDV purified without CCl_4 treatment contained five proteins that were also found in RDV purified with CCl_4, as well as an additional protein that migrated between P1 (164 kDa; Suzuki *et al.,* 1992) and P3 (114 kDa; Kano *et al.,* 1990; Suzuki *et al.,* 1990; Yamada *et al.,* 1990) (Fig. 3, lanes A and B). This result suggested that treatment with CCl_4 removed one protein from RDV particles but did not affect particle morphology and, moreover, that the removal of this protein might possibly have abolished the infectivity of RDV.

Loss of a viral component protein that accounts for approximately 5% of the total component proteins, as measured by densitometric analysis of Coomassie Brilliant Blue-stained (CBB-stained) proteins (Fig. 2; Fig. 3, lower panel, A) (T. Omura, unpublished data, 1996) did not alter the images of viral particles under the electron microscope. Removal of the minor protein σ1 from reovirus by treatment at pH 11 also does not alter the morphology and buoyant density of viral particles (Drayna and Fields, 1982). It might be possible to identify morphological changes due to loss of a minor component protein that is associated with the ability to infect insect vector cells by cryoelectron microscopy (Lu *et al.,* 1998) or X-ray diffraction studies (Grimes *et al.,* 1998; Naitow *et al.,* 1999) in the future. These techniques might allow a detailed correlation between the biological function of the protein and its location in viral particles.

Partial amino acid sequences of the protein that was specific to particles of RDV purified without CCl_4 coincided with those of the 123-kDa protein that has been deduced from the nucleotide sequence of

genome segment S2 of the H strain of RDV (Uyeda *et al.,* 1994). This protein was therefore designated the P2 protein.

To correlate the presence of P2 protein in virus particles with viral infectivity, RDV that purified without CCl_4 was subjected to different types of treatment with CCl_4, followed by an infectivity assay with VCM and analysis by SDS–PAGE of the level of P2 protein. As shown in Fig. 3 (upper panel, C–H), infectivity decreased with increases in CCl_4 treatment time. Preparation without CCl_4 produced about 10^6 foci per 0.1 ml, whereas samples treated with CCl_4 for 5 minutes or more were not infectious. The analysis of protein components showed that the amount of P2 protein, relative to the amounts of other structural proteins, decreased with increases in the duration of treatment with CCl_4, whereas relative levels of other proteins showed no significant changes (Fig. 3, lower panel, C–H). These results suggested that a decrease in infectivity was correlated with a decrease in the relative amount of P2 protein in virus particles.

The inability of RDV particles that lacked P2 protein to infect VCM suggested that such defective particles might also be unable to infect the intact vector insect. To examine this possibility, infectivity of particles that lacked P2 protein to the insect was examined by feeding insects with viral preparations through a membrane (referred to below as "membrane feeding"). RDV purified without CCl_4 infected insects, and the insects gave positive results in an enzyme-linked immunosorbent assay (ELISA) (Takahashi *et al.,* 1991) and transmitted virus (Table I). By contrast, RDV purified with CCl_4 did not infect any of the

TABLE I

NUMBER OF INDIVIDUAL LEAFHOPPERS THAT HARBORED OR TRANSMITTED RDV AFTER MEMBRANE-FEEDING ON RDV PURIFIED WITH OR WITHOUT TREATMENT WITH CCl_4[a]

Test[b]	Exp. no.	Feeding on RDV		Feeding on His-Mg buffer
		Without CCL_4	With CCl_4	
Propagation[c]	I	53	0	0
	II	72	0	0
Transmission[d]	I	28	0	0
	II	54	0	0

[a] From Yan *et al.* (1996) with permission.
[b] One hundred specimens of *N. cincticeps* were used in each experiment.
[c] Percentage of insects that gave a positive result in an ELISA.
[d] Percentage of insects that transmitted RDV to rice seedlings.

insects tested and consequently was not transmitted to any plants, indicating that the RDV particles lost their ability to infect intact insects when the P2 protein was removed.

It is not known why only the P2 protein is removed from RDV particles on treatment with CCl_4. The mechanism of binding of P2 protein to RDV particles might be different from that of other proteins. Among the solvents chloroform, dichloromethane, isoamyl alcohol, acetone, methanol, and 2-propanol, the former three specifically removed the P2 protein from virus particles and decreased the infectivity of RDV (J. Yan and T. Omura, unpublished data, 1996).

All the above results together suggest that P2 protein is required for infection of RDV of vector insects. Thus, this protein seems likely to play an important role in some of the step(s) from adsorption of virus onto insect cells to initial onset of multiplication of the virus within the cells.

IV. Correlation between Infectivity and Presence of P2 Protein in RGDV

The results reported in the previous section indicate that P2, a minor outer capsid protein of RDV, is essential for infection of vector cells. However, the cited experiments failed to eliminate the possibility that CCl_4 caused a change in the conformation of the viral particles that was undetectable by electron microscopy or that it removed some organic material from the surface of virus particles that was not detectable by SDS–PAGE. To confirm the results described above, infection by RGDV was examined. RGDV and RDV have distinct biological and biochemical properties but have many common characteristics and are grouped together in the same genus, *Phytoreovirus* (Omura, 1995). One major biological difference is that RGDV that has been purified with CCl_4 is able to infect VCM (Omura *et al.,* 1988), whereas RDV is not (Fig. 3). RDV has no protein that corresponds to the 143K protein of RGDV in preparations purified with the use of CCl_4 (Omura *et al.,* 1985). In this context, it is noteworthy that RGDV purified with CCl_4 retains the 143K protein and can infect VCM. These results prompted us to compare the 143K protein of RGDV with the P2 protein of RDV in greater detail. Accordingly, the sequence of S2 of RGDV was analyzed because genome segment S2 has approximately the appropriate length to encode the 143K protein.

The amino acid sequence deduced from the open reading frame (ORF) of RGDV S2 consisted of 1148 amino acids (127 kDa), and it was identi-

cal to that of the 127-kDa protein of the O strain of RDV (RDV-O; Kimura *et al.,* 1987; Maruyama *et al.,* 1997). In this review, all analyzed data for S2 of RDV-O are used as those of S2 from RDV rather than those of the H strain of RDV (Uyeda *et al.,* 1994), described in the previous section.

To verify the coding assignments of RGDV S2, the partial amino acid sequence of a polypeptide fragment of the 143K protein was analyzed. It was apparent that the protein was encoded by S2; therefore, this protein was renamed RGDV P2 (Maruyama *et al.,* 1997).

RGDV P2 was not removed from the virus particles by treatment with CCl_4, in contrast to the specific removal of the P2 protein of RDV by the same procedure (Fig. 4, lower panel). CCl_4-treated RGDV particles that contained P2 retained the ability to infect VCM (Fig. 4, upper panel). By contrast, RDV particles treated with CCl_4 lost both infectivity and P2 (Fig. 4), as discussed above.

The stability of RGDV particles to treatment with CCl_4 suggested that vector insects might transmit CCl_4-treated particles to rice plants. As shown in Table II, RGDV purified with or without CCl_4 treatment was able to infect insects, which in turn transmitted the virus to plants. Thus, the biological activity was correlated with retention of P2, irrespective of treatment with CCl_4.

The amino acid sequence of the P2 protein of RGDV showed 38% identity to that of RDV P2. No other proteins registered in the Swiss-Prot, GenPept, PIR, and RDB databases exhibited significant homology to P2, suggesting that the P2 protein of RGDV has a function similar to that of RDV P2 (Maruyama *et al.,* 1997). Stretches of amino acids with complete sequence identity were found, in particular in the carboxyl-terminal region (amino acids 795 to 1148). The extent of amino acid sequence homology identity in this entire region was 60%, in contrast to 29% in the amino-terminal region. Amino acid sequence homology is higher among structural proteins of the viruses in the genus *Phytoreovirus* (Nuss and Dall, 1990; Omura, 1995; Suzuki, 1995); there is 32–55% homology among structural proteins and 20–31% homology among nonstructural proteins. Furthermore, the extent of homology is uniform among respective regions of corresponding proteins in structural proteins. The significant differences in homology in the different regions of P2 proteins suggest that the region with lower homology might have a function that is specifically associated with insect vector cells, whereas the region with higher homology might play a role in structural organization that allows the protein to fit appropriately into the three-dimensional structure of the virus particle.

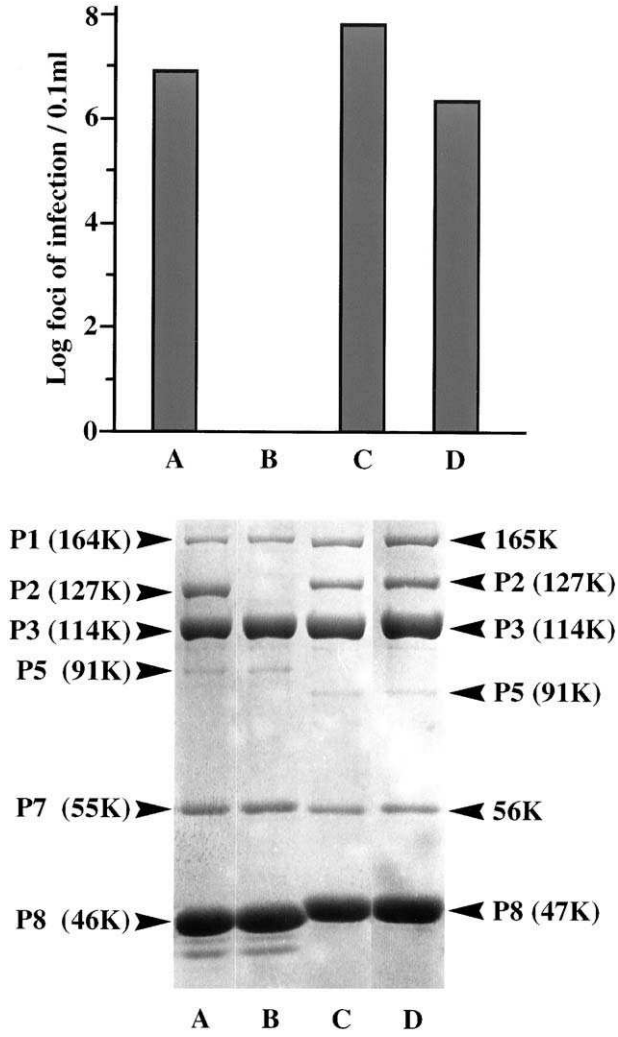

FIG 4. Ability of preparations of RGDV and RDV to infect VCM (upper panel) and protein components of RGDV and RDV (SDS–PAGE; 10% polyacrylamide; lower panel). A, RDV prepared without CCl_4 treatment; B, RDV prepared with CCl_4 treatment; C, RGDV prepared without CCl_4 treatment; D, RGDV prepared with CCl_4 treatment. Reproduced with permission from Maruyama *et al.* (1997).

TABLE II

NUMBER OF INDIVIDUAL LEAFHOPPERS THAT HARBORED OR TRANSMITTED RGDV AFTER MEMBRANE-FEEDING ON RGDV PURIFIED WITH OR WITHOUT TREATMENT WITH CCl_4[a]

Test[b]	Exp. no.	Feeding on RGDV		Feeding on His-Mg buffer
		Without CCl_4	With CCl_4	
Propagation[c]	I	27	29	0
	II	33	42	0
Transmission[d]	I	24	18	0
	II	31	34	0

[a] From Maruyama *et al.* (1997) with permission.
[b] One hundred specimens of *N. nigropictus* were used in each experiment.
[c] Percentage of insects that gave a positive result in an ELISA.
[d] Percentage of insects that transmitted RGDV to rice seedlings.

As summarized in the previous section, RDV requires the P2 protein for infection of vector insects. The P2 proteins of RGDV and RDV are similar in size, and both are located in the outer capsid (Omura *et al.,* 1985; Yan *et al.,* 1996). Furthermore, the proteins have similar predicted amino acid sequences, which suggests that RGDV P2 is a counterpart of RDV P2 and that the proteins have similar functions.

The ready removal of RDV P2 by CCl_4 and the contrasting stability in virus particles of RGDV P2 might reflect differences in interactions of the proteins with core particles. RGDV P2 and RDV P2 are similar in both amino acid sequence and component hydrophobic amino acids, but the secondary structures predicted by the methods of Chou and Fasman (1978) suggest that the central portion of RGDV P2 has a greater propensity for formation of a β turn than does that of RDV P2. This feature of RGDV P2 might contribute to local differences in conformation, resulting in differences in resistance to removal by CCl_4. Differences have been reported among strains of reovirus with respect to the sensitivity of virions to removal of σ1 protein, a cell-attachment protein, by treatment of the virions at pH 11 (Drayna and Fields, 1982). Comparative conformational studies on the differences in the adhesive nature of structural proteins and of proteins that function specifically, for example, in cell attachment might provide some insight into the stable or unstable organization of supermolecules, such as reovirus particles.

RDV lost the ability to infect VCM, as well as P2 protein, on treatment with CCl_4. Loss of infectivity was correlated with loss of P2 pro-

tein. By contrast, RGDV retained both P2 protein and infectivity irrespective of treatment with CCl_4. Furthermore, transmission by the vector of RGDV was insensitive to treatment of RGDV with CCl_4. The infection by CCl_4 treated RGDV particles of VCM (*in vitro*) and the transmissibility by vector insects (*in vivo*) probably require the presence of P2 in the virus particle. Although it is still necessary to prove that RGDV particles that lack the P2 protein do not infect insect vector cells, it is inferred that RGDV P2 protein is also essential for infection of vector cells and for acquisition of virus by vector insects. Experiments designed specifically to remove P2 from RGDV with the use of various organic solvents, such as chloroform and dichloromethane, did not yield informative results.

V. P2 Protein Allowing RDV to Adsorb to Insect Vector Cells

The results described in the previous sections indicate that the P2 protein is one of the factors that is essential for infection of vector cells by RDV and RGDV and, thus, that the P2 protein influences transmissibility by vector insects. Further details on the function of the P2 protein during the infection of insect vector cells are summarized below (Omura *et al.,* 1998).

To determine whether the failure of particles that lacked P2 to infect VCM was due to the inability of the particles to interact with the insect cells, the ability of viral particles to adsorb to VCM was examined. As shown in Fig. 5, high titers of viral antigen were detected in cells that had been exposed to intact RDV. By contrast, no viral antigen was detected in cells after exposure to treatment with P2-free RDV. These results suggest that P2 plays an important role in the interaction of RDV with VCM and, together with the observation that P2-free RDV did not interfere with infection by intact RDV (J. Yan and T. Omura, unpublished result, 1997), may suggest that P2 protein plays a role in the attachment of virus particles to cells of the insect vector.

To confirm that viral particles had penetrated the vector cells, thin sections of VCM that had been exposed to intact RDV were examined by electron microscopy. Characteristic intact virus particles of the expected size were observed on the surface of all cell membranes and in vesicles of VCM cells that had been exposed to intact RDV (Fig. 6, left). No such particles were found in VCM that had been incubated with P2-free RDV (Fig. 6, right). RDV particles that had penetrated into cells seemed to be in the double-layered form, as judged from the size and appearance of the particles. These observations were supported

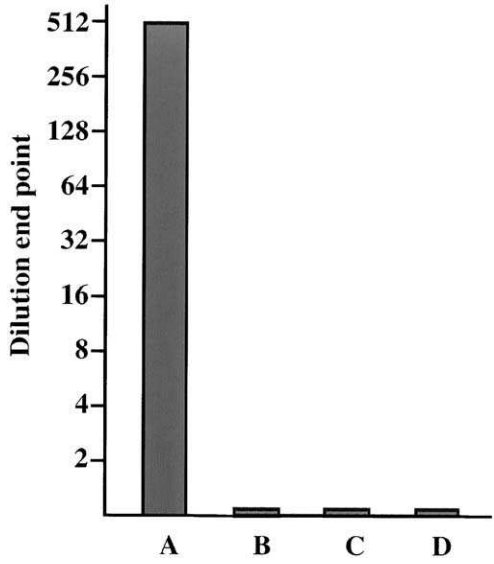

FIG 5. Adsorption of RDV particles to insect cells, as indicated by the reciprocal values of dilution end points that gave positive results in an ELISA. Results are shown for VCM that incubated with intact RDV (A), for VCM incubated with P2-free RDV (B), for High Five cells that originated from the nonhost insect *Trichoplusia ni* incubated with intact RDV (C), and for VCM incubated with His-Mg buffer (D). Reproduced with permission from Omura *et al.* (1998).

by the results of electron microscopic analysis of dipped preparations, stained with uranyl acetate, of the material that had been used for the ELISA. The particles were double-layered and appeared to be intact (Omura *et al.,* 1989), and they reacted with an antiserum against the P8 outer capsid protein that had been purified as described by Zhu *et al.* (1997), by the method developed by Milne and Luisoni (1977) (T. Omura, unpublished data, 1997).

The inability of particles that lacked P2 to infect VCM (Fig. 3) appeared to be due to their inability to attach to the insect cells (Fig. 5) rather than to failure to initiate infection after entry of virus particles into cells. The same phenomenon might also occur at the surface of cells in the intestinal tracts of insects that are allowed to feed on virus particles through a membrane. Thus, the possibility that P2-free particles might be infectious if they were introduced into the insect vector by injection into the abdomen was examined next.

As shown in Table III, injected P2-free RDV, as well as injected intact RDV, was able to infect and proliferate in insects, as determined

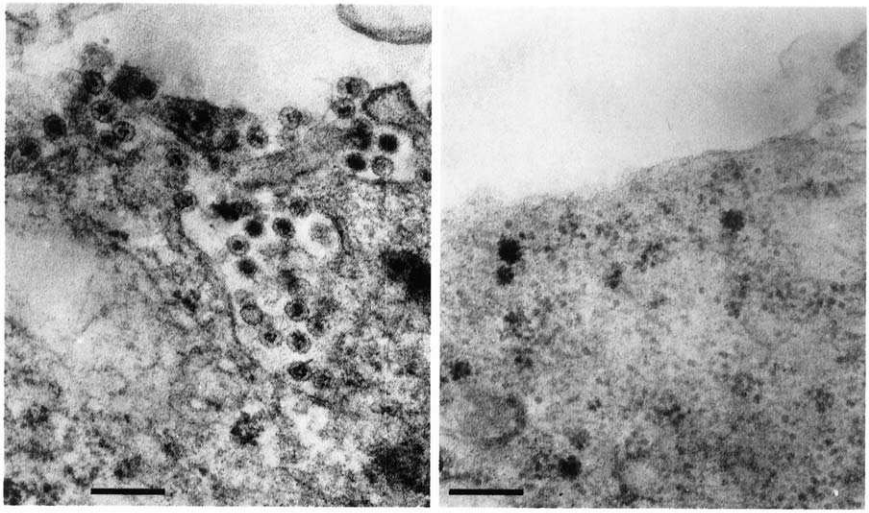

FIG 6. Electron microscopy of thin sections of VCM derived from the vector insect *N. cincticeps* after incubation with intact (left) and with P2-free (right) TC-RDV. Bars represent 200 nm. Reproduced with permission from Omura *et al.* (1998).

TABLE III

NUMBER OF LEAFHOPPERS THAT HARBORED OR TRANSMITTED RDV AFTER INJECTION WITH OR MEMBRANE-FEEDING ON EITHER INTACT OR P2-FREE RDV[a]

Method	Test[b]	Exp. no.	Intact RDV	P2-free RDV	His-Mg buffer
Injection	Propagation[c]	I	51	54	0
		II	80	69	0
	Transmission[d]	I	36	44	0
		II	68	57	0
Membrane-feeding	Propagation	I	42	0	0
		II	61	0	0
	Transmission	I	29	0	0
		II	56	0	0

[a] From Omura *et al.* (1998) with permission.
[b] One hundred specimens of *N. cincticeps* were used in each experiment.
[c] Percentage of insects that gave a positive result in an ELISA.
[d] Percentage of insects that transmitted RDV to rice seedlings.

by ELISA of individual insects. These infected insects were also able to transmit the virus to rice seedlings, as judged by the appearance of symptoms on rice plants. By contrast, in membrane-feeding tests, only intact RDV was able to infect insects, which were then able to transmit the virus to rice seedings. These results demonstrated that P2-free RDV retained infectivity when introduced directly into the abdomen of the vector insect through injection.

Injection via glass capillary tubes allowed particles that lacked P2 to infect and multiply in the vector insects. This result implies that P2-free particles cannot normally enter the cells of the intestinal tract, the site at which virus particles are believed to infect the vector (Black, 1984), but P2-free particles can infect and multiply once the particles have been introduced into the hemolymph, bypassing the wall of the gut, which forms a barrier to infection. This hypothesis is supported by earlier results showing that insects of nontransmitting races of the leafhopper *Cicadulina mbila* became infective when they were allowed to feed on maize streak virus-infected plants and then punctured with a fine needle to allow materials in the digestive system to be introduced into the insect's body (Storey, 1933). Clearly, there are cells in the vector insect that are sensitive to RDV particles that lack P2. In other words, once P2-free RDV has been introduced physically either into the hemolymph and then to into sensitive cells or directly into cells through openings in cell membranes, infection can occur in the absence of P2. After the onset of the initial infection, all the viral proteins, including P2, can be expressed and the normal multiplication cycle can proceed. RDV produced in cells that have been initially infected with P2-free RDV can infect neighboring cells, as can intact RDV. When RDV was purified from plants infected by insects that had been injected with P2-free RDV, the virus was found to contain P2 and other protein components at levels similar to those of the respective components of native RDV (J. Yan and T. Omura, unpublished data, 1997). These results, together with the observation that P2-free particles did not make intimate contact with insect cells and were unable to enter insect cells (Fig. 5), demonstrate that the P2 protein of RDV allows virus particles to adsorb to cells of the insect vector. Thus, P2 seems to be one of the molecules that is essential for infection of insect cells by RDV.

Among the structural proteins of viruses that belong to the family *Reoviridae,* σ1 of reovirus (Nibert *et al.,* 1996), VP4 of rotavirus (Chen and Ramig, 1993; Estes, 1996), and VP2 protein of BTV (Roy, 1996a) correspond to RDV P2 in terms of their location in the outer capsid of virus particles and in terms of their role in attachment to host cells and, thus, in terms of an association with viral infectivity. However,

no significant homology was found between these proteins and P2. The results suggesting that P2 might not be exposed on the surface of virus particles (Section II) run counter to the observation that all the cell-attachment proteins of animal-infecting viruses are exposed on the surface of virus particles (Dryden *et al.,* 1993; Patton, 1994; Roy, 1996b).

VI. Absence of P2 Protein Expression in Transmission-Defective Isolate of RDV

Transmission-defective (TD) isolates of phytoreoviruses have been reported for both WTV (Reddy and Black, 1974, 1977) and RDV (Kimura, 1976). Because transmissibility via the insect vector was lost by virus particles from which the P2 protein had been removed by chemical treatment (Fig. 3), it was of interest to determine whether the P2 protein might be present in a TD isolate of RDV that had been derived from a transmission-competent (TC) isolate. In this way, it was hoped, it might be possible to identify the molecular entity responsible for the transmissibility of phytoreoviruses with the use of a mutant with a defect in virus transmission. The TC isolate used was the RDV-O isolate, used in the experiments described in earlier sections.

RDV from plants that had been vegetatively maintained for 12 years without passage through an insect vector was neither acquired by insects nor transmitted to healthy rice plants, by contrast to the high frequency of transmission of an isolate of virus maintained in plants with the use of viruliferous insects (Tomaru *et al.,* 1997). Thus, it appeared that the former isolate had become TD, as reported by Kimura (1976), who noted that a TD isolate was obtained after vegetative propagations for more than three or four years of TC-RDV in infected plants. This virus was used as the TD isolate in the following studies.

The ability of the TD isolate to infect VCM was examined in an effort to characterize the TD isolate in further detail. Crude sap prepared from TD-infected plants failed to infect VCM, by contrast to the high infectivity of the TC isolate (Fig. 7, left). Furthermore, virions purified from the TD isolate without the use of CCl_4 (Section III) were not infectious, whereas the TC isolate was highly infectious. These results suggested that the inability of the TD isolate to infect vector cells was responsible for the defect in transmission.

Artificial removal of P2 in the TC isolate was shown to be associated with loss of the ability to infect vector cells (Section III). Therefore, the loss of infectivity of the TD isolate (Fig. 7, left) might have been due to the lack of some structural protein(s) of RDV. Among the six structural

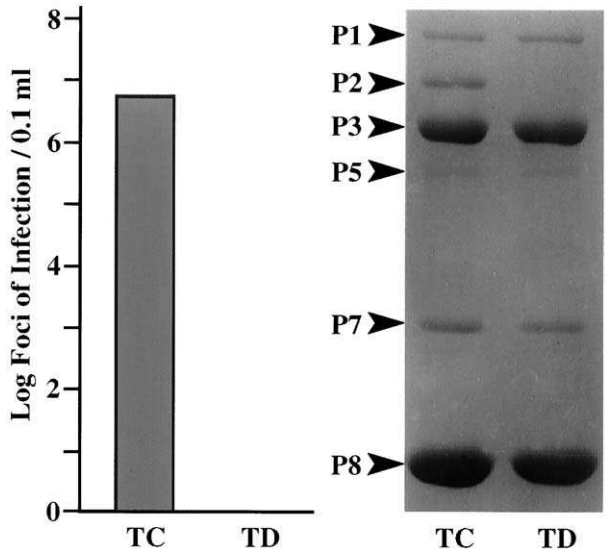

FIG 7. *Left:* Infection of VCM by preparations of RDV. Crude sap prepared from rice plants infected with a TC or a TD isolate was diluted appropriately and incubated with VCM. *Right*: Analysis by SDS–PAGE (10% polyacrylamide) of protein components of the TC and TD isolates of purified RDV. The various proteins are indicated on the left side (see also Fig. 3). Reproduced with permission from Tomaru *et al.* (1997).

proteins, only the P2 protein was undetectable after fractionation by SDS–PAGE of the proteins in the purified TD isolate (Fig. 7, right). These results demonstrated again that lack of the P2 protein was associated with loss of the ability to infect vector cells.

In certain isolates (TD isolates) of WTV, genome segment S2 or S5 was missing (Reddy and Black, 1977; Nuss, 1984). The product of S2 is the 130-kDa outer capsid protein P2 (Anzola *et al.,* 1987). Thus, it seems likely that WTV P2 is also associated with infectivity of the virus to insect vector cells.

To clarify whether the absence of P2 protein from the purified TD isolate was due to failure to synthesize the P2 protein or to failure of the P2 protein to become integrated into viral particles, the presence of five major structural proteins (P1, P2, P3, P7, and P8) of RDV in rice plants that had been infected with TD or TC isolates was examined by immunoblotting. Of these proteins, only P2 was not detected in the TD isolate, whereas all five proteins were detected in the TC isolate (Table IV). All five viral proteins were also detected in vector insects that carried the TC isolate and in VCM inoculated with TC. These results suggest that

TABLE IV

IMMUNODETECTION OF MAJOR STRUCTURAL PROTEINS OF RDV SYNTHESIZED *in Vivo*[a]

Viral protein	RDV-infected plants[b]			Vector insect (five-fold dilution)		VCM (five-fold dilution)	
	TD[c]	TC[d]	Healthy	Virus infected	Healthy	Virus infected	Mock infected
P1	80	320	<5	+	−	+	−
P2	<5	640	<5	+	−	+	−
P3	160	640	<5	+	−	+	−
P7	40	640	<5	+	−	+	−
P8	320	320	<5	+	−	+	−

[a] From Tomaru *et al.* (1997) with permission.
[b] Data are the reciprocal values of dilution endpoints (grams per milliliter) that gave positive reactions.
[c] TD, transmission-defective isolate.
[d] TC, transmission-competent isolate.

P2 was not synthesized in rice plants that had been infected with the TD isolate, thus explaining that absence of the P2 protein in the purified virus particles was due to failure to synthesize P2 and not to failure of the P2 protein to be integrated into viral particles.

One possible reason for failure to synthesize the P2 protein is an internal deletion in the genome segment S2, as reported in the case of S5 of WTV (Anzola *et al.,* 1987). Then genome segments of purified TD and TC isolates were examined by SDS–PAGE. As shown in Fig. 8, all 12 anticipated segments were detected in equimolar amounts in the purified TD and TC isolates. No significant difference in the size of the S2 segment was detected between the isolates, suggesting that the absence of the P2 protein was due to a defect in either transcription or translation. Apparent differences in mobility were observed in the case of genome segments S4 and S11, but the biological implications of this observation remain to be clarified.

The reduced efficiency of S2 transcription might explain the failure to synthesize the P2 protein. Therefore, the positive- and negative-sense RNAs transcribed from S2 *in vivo* were studied to explore this hypothesis. As shown in Fig. 9, RNA blotting analysis revealed the presence of both positive- (+) and negative-sense (−) S2 RNAs in rice plants that had been infected with the TC and TD isolates *in vivo,* and the respective sizes did not differ from those of the RNAs in the viral genome. It was then assumed that if S2 were transcribed efficiently,

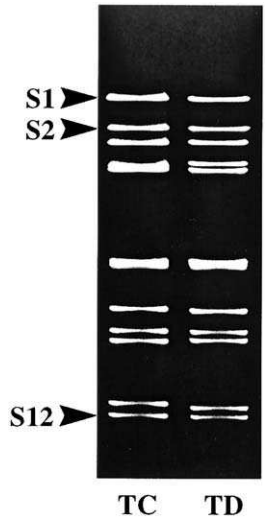

FIG 8. Analysis of genomic dsRNAs stained with ethidium bromide from TC and TD isolates of RDV by SDS–PAGE (10% polyacrylamide). Positions of some segments of the genome are indicated on the left side. Reproduced with permission from Tomaru *et al.* (1997).

more single-stranded (ss)(+)RNA should be detected than ss(−)RNA. The intensities of signals due to the ss(+) and ss(−) RNAs were quantitated relative to the levels of purified dsRNAs as controls, and the ratio of the ss(+)RNA to the ss(−)RNA was calculated for both isolates. The ratios were 4.9 and 2.5 for the TC and TD isolates, respectively, indicating that the transcripts of S2 were present in both isolates. Because the multiplication cycles of viruses in the genus *Phytoreovirus* appear to be similar to those of other viruses that belong to the *Reoviridae* (Nuss and Dall, 1990; Suzuki, 1995; Uyeda and Milne, 1995), the ss(+)RNAs, transcripts of RDV genes, are likely to function as mRNAs in the cytoplasm of host cells and to be translated into polypeptides (Eaton *et al.,* 1990; McCrae, 1986; Patton, 1994; Zarbl and Milward, 1983). Therefore, the presence of the ss(+)RNA transcript of S2 in TD-infected rice suggests that failure of translation was responsible for the absence of P2 protein. Mutations within the ORF of S2, if any, seem not to affect the efficiency of transcription and replication of this segment.

A mutation in the genome segment S2 that affects the efficiency of translation, as shown for WTV (Xu *et al.,* 1989), and, alternatively, inability of the P2 protein to function due to a very small deletion or

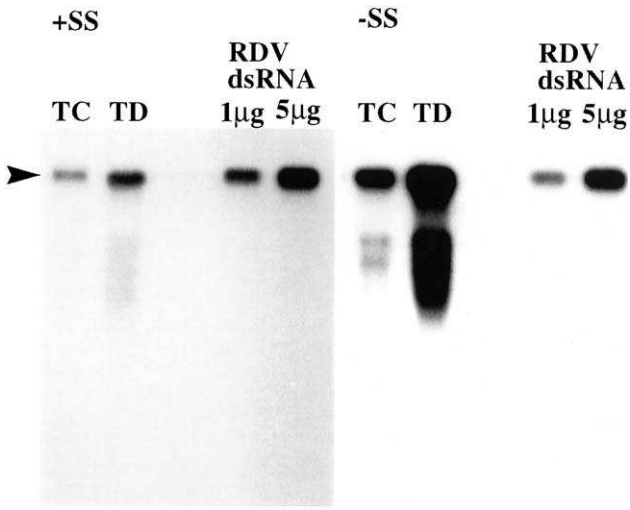

FIG 9. Northern blotting analysis of RDV RNAs. Total RNA from TD and TC isolates was subjected to electrophoresis on a 1% agarose gel, blotted onto a membrane, and allowed to hybridize with a ^{32}P-labeled positive-sense RNA (+ss) or negative-sense RNA (−ss) that corresponded to genome segment S2 of RDV, expressed after cloning of the cDNA into pBluescriptII. One and five micrograms of genomic dsRNA from RDV were processed similarly as controls. The arrowhead indicates the position of the full-sized transcript (3.5 kb). Reproduced with permission from Tomaru *et al.* (1997).

a point mutation are further possibilities that might explain the failure to detect the P2 protein in the TD isolate. The sizes of the S2 segment in the TD and TC isolates were exactly the same, but approximately 0.6% of the nucleotides in the S2 segment from the TD isolate had point mutations. A change in nucleotide (nt) at position 47 in the TD isolate generated a termination codon. Thus, the ORF ended at the codon for the 11th amino acid residue of the predicted 127-kDa polypeptide encoded by the S2 segment in the TC isolate of the O strain of RDV (Fig. 10). Accordingly, the major ORF (nt 141–3461) of the S2 segment of the TD isolate, which is 142 bp shorter than the ORF of authentic S2, is preceded by a minicistron (nt 15–44 that encode 11 amino acids). The large ORF of such an mRNA has sometimes been found to be translated (Kozak, 1986). However, this is not considered to be the case for the S2 segment of the TD isolate. If the downstream major ORF were to be translated, the AUG codon of the major ORF would have to be recognized by directly binding ribosomes that are guided by an internal binding sequence for a ribosome (Pelletier and Sonenberg, 1988), or by ribosomes that bypass the first AUG codon and

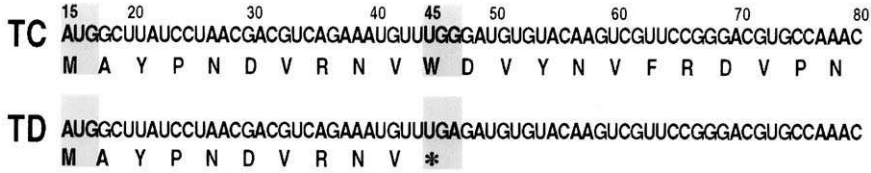

FIG 10. Part of the ORF of the plus strand of genome segment S2 and the predicted amino acid sequences for the TC and TD isolates. Nucleotides are numbered from the 5′ end. The amino acids encoded by each segment are indicated below the sequence. The termination codon is indicated by the asterisk. Reproduced with permission from Tomaru *et al.* (1997).

search for a downstream AUG triplet (Kozak, 1991), or by reinitiation-competent ribosomes (Kozak, 1987, 1991). No internal binding sequence for a ribosome was found in the 5′-terminal region of the major ORF of S2 of the TD isolate. The first AUG (nt 15–17) is unlikely to be leaky because it is a strong initiator, according to Kozak (1989). Although reinitiation might be possible in view of Kozak's observations with a mammalian system (Kozak, 1987), reinitiation is unlikely to be the case for the expression of S2 of the TD isolate in plants, as shown in Fig. 10. No protein band of the expected mobility was detected by immunoblotting with antiserum raised against P2 protein (T. Omura, unpublished data, 1996).

The results related to infection described in this section resemble those obtained with a TC isolate from which the P2 protein had been eliminated by chemical (CCl_4) treatment (Fig. 3, Table I). This coincidence suggests that a similar mechanism caused the loss of virus infectivity and failure to transmit the virus in both cases. Thus, a correlation between the presence of the P2 protein in viral particles and the ability of the virus to infect vector cells was apparent for two distinct materials: a TD isolate generated from a living plant and a TC isolate from which the P2 protein had been removed by chemical treatment. These results suggest that the virus in the TD isolate was not transmissible by the vector because it had lost the ability to infect insect vector cells as a result of the absence of the P2 protein in virus particles. All these results demonstrate that the P2 protein is one of the factors that is essential for infection of insect vector cells by RDV.

The virus in infected rice plants is taken up orally by insects through a stylet. The virus probably enters cells in the intestinal tract, multiplies there in intestinal cells, and is then distributed to various organs (Black, 1984). The P2 protein probably plays an important role in

adsorption of the virus to the insect cells, which is discussed in Section V. The insect starts to transmit the virus to plants after a latent period of 10–20 days (Black, 1984; Boccardo and Milne, 1984). The absence of the P2 protein in the TD isolate might result in the failure of the virus to infect cells in the intestinal tract of the vector insect, with a consequent defect in the transmission of the isolate.

TD-RDV, devoid of the P2, did not proliferate in intact insects, nor was it transmitted to plants by either the membrane-feeding or the injection method (Omura *et al.,* 1998), in contrast to successful transmission to plants after proliferation that followed injection of P2-free TC-RDV (Table III). Thus, it appears that even if initial infection by P2-free TD-RDV can be achieved by injection, secondary infection of neighboring cells might not occur because of failure to express a functional P2 protein as a consequence of a mutation in the ORF in S2 (Fig. 10).

All the results described above confirm that P2 is one of the factors essential for infection of vector cells by the virus and thus influences transmissibility by vector insects.

VII. P8 Outer Capsid Protein of RDV Involved in Viral Infection of Insect Vector Cells

There are six structural proteins in RDV (Figs. 1 and 2). However, neutralizing antibodies that prevent infection of VCM were generated only when the P8 outer capsid protein was used as immunogen (J. Yan and T. Omura, unpublished data, 1997). Thus, it is likely that the P8 protein, located in the outermost region of RDV, plays an important role in the initial stage of infection of insect vector cells. Indeed, it is possible that adsorption to and entry into cells by the virus require both P2 and P8 proteins.

The P8 protein used as antigen for the generation of neutralizing antibodies was isolated from gels after SDS–PAGE. By contrast, the VP2 protein of BTV failed to induce the formation of neutralizing antibodies when the antigen was purified from gels after SDS–PAGE (White and Eaton, 1990; Hwang and Li, 1993). These results might be due to the dependence on conformation of the exposure of relevant epitopes of RDV P8 protein.

The P8 protein of RDV corresponds to the μ1 protein of reovirus (Nibert, 1996), the VP6 protein of rotavirus (Estes, 1996), and the VP7 protein of BTV (Roy, 1996a) based on the facts that each of these proteins is the most abundant in their respective virus particles; each

is located in the second layer of the virus particle; and each has a trimeric unit structure (Dryden *et al.,* 1993; Patton, 1994; Prasad and Chiu, 1994; Roy, 1996b; Lu *et al.,* 1998, Zhu *et al.,* 1997). It is of interest that three animal-infecting reoviruses have proteins that are located in the outermost region of the virus particle, beyond the second layer, namely, $\sigma 3$ in reovirus, VP7 in rotavirus, and VP2 and VP5 in BTV. By contrast, RDV is a strictly double-layered particle (Fig. 1). This difference in particle organization might be one of the factors that determine host specificity.

VIII. Concluding Remarks

The viruses in the TD isolate of RDV propagate vigorously in newly emerged leaves. This fact implies that TD-RDV has the ability to multiply in plant cells without the aid of P2 protein. Thus, P2 protein might not be indispensable for propagation of RDV in plant tissue once a plant has been infected.

In contrast to the replication of P2-free RDV in plants, functional P2 is indispensable for propagation of the virus in vector insects. P2 might function in the secondary infection of neighboring cells or of cells in organs beyond the intestinal tract after transport in the hemolymph throughout the insect's body. Thus, the insect becomes a host and a vector of the virus when P2 protein is present in the virus. Proteins such as P2 might be host determinants in nature.

Several factors probably determine host specificity during the initial intimate contact between pathogen and host. Characteristics of the pathogen and the host must influence adsorption of the pathogen to host cells, propagation, and, finally, release of the virus from infected cells. In the case of some plant or animal viruses, the host range can be expanded when protoplasts (Furusawa and Okuno, 1978) or cultured cells are used for infection; for example, Colorado tick fever virus proliferates in a line of mosquito cells (Yunker and Cory, 1969). In such situations, one or several factors, such as P2, that are required for binding of the virus to cells of the intact host might be lacking, in spite of the fact that the virus has machinery sufficient for its multiplication once it has been introduced into cultured cells or protoplasts of a nonhost intact plant or animal. The protein-oriented host specificity described in this article can be categorized as one of the mechanisms that determines host specificity. The actions of helper factors in the case of potyviruses and caulimoviruses (Pirone and Blanc, 1996), the combination of luteoviruses with a specific protein of a symbiont microorganism in aphids (Hogenhout *et al.,* 1998), and adjustment of the envelope

proteins of tomato spotted wilt virus to the putative cellular receptor in thrips (Bandla *et al.,* 1998) provide other examples of such mechanisms.

All the results described in Section VI suggest that genome segment S2, which encodes the P2 protein that is not required for propagation of RDV in plants, might be freed from the pressures of natural selection in such an environment. Moreover, S2 with a mutation might have an advantage over normal S2 in replication because it is not involved in translation. Such P2-free TD-RDV would never be able to infect insects as a host, except when the virus acquires a gene for functional P2 by chance. It is not clear whether a small number of TD mutant viruses was present in the original TC population at the onset of selection for TD-RDV as a quasi-species with a heterologous population of variants due to the high error rate of RNA polymerase (Holland *et al.,* 1992) or whether the nucleotide at position 47 in genome segment S2 is a hot spot for mutations.

Interkingdom differences in the expression of specific proteins, such as P2 in *Phytoreovirus,* have been reported for some plant viruses that use invertebrates as vectors. After repeated mechanical transfers of an aphid-transmissible isolate of pea enation mosaic virus in *Pisum sativum* L, the virus was no longer transmissible by aphids and had also lost a 56-kDa protein (Adam *et al.,* 1979). By contrast, continuous passage of sowthistle yellow vein rhabdovirus in the vector aphid by serial mechanical inoculation resulted in the generation of the isolates that had lost the ability to infect the plant host (Sylvester and Richardson, 1971). In the case of maize stripe tenuivirus, a difference in protein expression was observed after one passage. A major 16.5-kDa virally encoded nonstructural protein was readily detected in infected maize but could not be detected in the vector insect, the planthopper *Peregrinus* (Falk *et al.,* 1987). Clarification of the molecular mechanisms of viral multiplication in distinct hosts and of interkingdom differences in the expression of specific proteins, as mentioned above, should provide new insights into the roles of proteins that function in the virus–host relationship. Furthermore, such investigations of the viral molecules that are essential for virus transmission by vectors and of the ways in which these molecules function should help us develop measures to control plant viruses that are transmitted by vectors. It might even be possible to generate transgenic plants that express proteins that interfere with the normal interactions between a virus and its vector.

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ADVANCES IN VIRUS RESEARCH, VOL. 54

REPLICATION OF HUMAN HEPATITIS DELTA VIRUS: INFLUENCE OF STUDIES ON SUBVIRAL PLANT PATHOGENS

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I. Introduction and Scope

Studies of the plant viroids and the plant satellite RNAs and viruses have had major effects, both direct and indirect, on how we have sought an understanding of human hepatitis delta virus (HDV). This review is intended to give not only a current picture of HDV studies but also an historical perspective. The purpose throughout will be to show how

0065-3527/99 $30.00

studies of the subviral pathogens of plants have influenced and continue to influence the nature of HDV research.

II. Early History

A. Discovery

The origin of delta research dates back to 1977. Mario Rizzetto, in Italy, discovered an unusual protein in patients with a more damaging form of hepatitis B virus (HBV) infection (Rizzetto *et al.,* 1977). He was studying liver biopsy specimens taken from these patients at the peak of the disease. He found that if he used the serum of the convalescent patient in an immunostaining assay, he detected a novel signal in the nuclei of the hepatocytes. He called this reactivity the "delta antigen." He thought he had discovered a novel protein made by a variant of HBV that was more pathogenic. But he was wrong.

B. Passage in Chimpanzees

Over the next three years, he and others worked to define this antigen and, contrary to their expectations, they found that it was part of a novel infectious agent, one that replicated only in the presence of the HBV. From experimental infections of chimpanzees, they demonstrated that HBV was acting as a helper virus (Rizzetto *et al.,* 1980a).

C. Particles

HBV replicates in the liver and releases its DNA-containing particles into the bloodstream. In a serum sample from an infected individual, as many as 10^9 infectious particles per milliliter can be found. However, if one looks more closely, this replication by HBV is wasteful because what is also released from the infected hepatocytes is a great excess of empty noninfectious particles. These are made in the form of 22-n*M*-diameter spheres and filaments. These empty particles represent 100 to 10,000 times more than the number of infectious particles. HDV, as a successful parasite, actually a parasite of a parasite, makes use of such defective assembly. It packages both its RNA genome and the delta antigen into HBV envelopes. Such infectious hepatitis delta particles can number up to 10^{12} per milliliter of serum (Ponzetto *et al.,* 1987).

D. Passage in Woodchucks

In 1984, Ponzetto and colleagues showed that HDV, as it was now called, could be experimentally transmitted to the eastern woodchuck (Ponzetto *et al.,* 1984). Under such conditions the helper virus was changed from HBV to the very similar virus of woodchucks, called "woodchuck hepatitis virus" (WHV). At this time, it was becoming clear that the role of the helper virus, be it HBV or WHV, is no more than to provide the envelope proteins needed for assembly and release, and for subsequent entry into a new susceptible cell.

III. Transmission, Epidemiology, and Pathogenesis

This research led to the development and application of commercial tests for the delta antigen and especially for delta antibody. Initially, most of the cases studied were from southern Italy. The highest risk groups for transmission were identified as intravenous drug users and persons who received multiple blood transfusions, such as hemophiliacs. In some groups of intravenous drug users, the percentage of HBV-positive individuals who were also HDV positive was greater than 70%. As these tests were applied around the world, it became clearer that the fraction of people with chronic or acute HBV that also had HDV was quite variable. The percentage was sometimes less than 5%, but in other situations it was more than 60%. In 1996, it was estimated by the Centers for Disease Control and Prevention that, worldwide, there might be about 25 million cases of chronic HDV infection (Alter and Hadler, 1993).

The pathogenicity associated with HDV replication can be discussed at different levels. At the outset, HDV infection was considered to be a major risk factor for increased levels of liver damage. It seemed to be an agent that, in collaboration with HBV, could make HBV infections much more likely to lead to a fulminant hepatitis and to death. There are also data indicating that infection by certain genotypes of HDV can be especially pathogenic (Casey and Gerin, 1995a). However, in the absence of a host immune response, HDV, like HBV, may be at most only mildly pathogenic. Replication of the HDV genome or expression of the delta proteins has minor or no detectable deleterious effects (Gowans and Bonino, 1993). Mice transgenic for either a replicating HDV genome or either of the two forms of the delta protein demonstrate no ill effects (Guilhot *et al.,* 1994; Polo *et al.,* 1995).

In an infected primate or woodchuck, HDV replication is typically associated with that of the helper hepadnavirus. It is believed that

such a combination can be pathogenic. Certainly the presence of a host immune response to either or both of these viruses can be associated with pathogenesis.

IV. Initial Application of Molecular Virology

A. Small RNA

In the 1980s the emerging techniques of molecular biology were brought to bear on HDV. In a 1980 paper by Rizzetto and co-workers, the particles in serum were shown to contain not only the delta antigen and the envelope proteins of the helper virus HBV but also an RNA (Rizzetto *et al.,* 1980b). This was a surprise because it was different from the helper virus, which has a DNA genome. Furthermore, even though the sizing of this RNA was quite imprecise, the RNA itself was clearly very small.

From 1980 to 1986, not much more was discovered about this RNA. The exception was that John Gerin and colleagues at Georgetown University (Washington, DC) obtained a cDNA clone of a 166-nucleotide (nt) region of the RNA (Denniston *et al.,* 1986). With help from John Gerin, several laboratories, including my own, began to try to understand the molecular biology of HDV.

B. Circular RNA

In 1986 three groups, for different reasons, came to the same intriguing conclusion about the RNA genome of HDV. The studies of Michael Houghton at Chiron (Wang *et al.,* 1986), a Dutch group led by Ton Kos (Kos *et al.,* 1986), and my own laboratory (Chen *et al.,* 1986) were first presented that year at an exciting hepatitis delta virus meeting held in northern Italy, near Turin, the early home of HDV discovery and research. Michael Houghton and his group had attempted complete cDNA cloning. To their surprise, they found that their collection of subgenomic cDNA clones was consistent with a circular rather than a linear genome.

This was the first example of an infectious agent of animals with an RNA genome that was circular in conformation, and today it remains the only example. However, in the plant world, the first discovery of a circular RNA had been made earlier, in 1971, in the work of Diener with the RNA of the potato spindle tuber viroid (Diener, 1971). Moreover, there were now other examples of such plant agents.

The existence of viroid and HDV RNA circles is probably linked to evidence that RNAs with a circular conformation are significantly more stable within a cell relative to the corresponding linear RNAs (Puttaraju and Been, 1995). A primary reason is that most of the cellular RNase activities are exo- rather than endonucleolytic.

C. Rodlike Folding

In addition to being circular, this HDV RNA was able to fold into an unbranched rod-like structure. Again, this was like some of the plant viroids, but this rod was about four times longer. It could be seen by electron microscopy in the study of Kos *et al.* (1986). Also, from a computer analysis of the complete nucleotide sequence, it was predicted by Houghton and co-workers that about 70% of the nucleotides would be paired (Wang *et al.,* 1986).

D. Three RNAs

It was promptly shown that HDV was replicating without a detectable DNA intermediate (Chen *et al.,* 1986). This meant that HDV was not replicating in the same manner as its helper virus, HBV, which was known to utilize reverse transcription to make a double-stranded DNA intermediate (Summers and Mason, 1982). In contrast, HDV was replicating via RNA synthesis that was RNA directed. There is now evidence that this is achieved by the redirection of the host RNA polymerase II, as discussed below.

Northern analyses of RNA from infected liver showed that there were three main HDV-related RNAs (Chen *et al.,* 1986). First, there was the genomic RNA, also found within the virus particles. Second, there was an exact complement, which we called the "antigenome," that was also circular. And third, there was a less abundant species that was less than full length and polyadenylated. This was the likely candidate for the mRNA for the delta protein. In an infected liver there are about 300,000 copies of the genomic RNA per average hepatocyte. The antigenome is 5–10 times less abundant, and the mRNA is perhaps 500 times less abundant (Chen *et al.,* 1986).

V. Initial Similarities to Plant Agents

Diener was present at the 1986 HDV meeting mentioned earlier. He reviewed the viroids, the agents he had discovered in plants, and also

the plant satellite RNAs and viruses. He set the stage for research to determine whether HDV had roots in the plant kingdom. There was a rush to determine the extent of the similarities of HDV to what was already known about the plant agents. Some attempts were more successful than others.

As an example of an unsuccessful attempt, at least two laboratories tried to grow HDV in plants. Earlier, in 1973, it had been reported that HBV would grow in plants, specifically in the leaves of the tobacco plant (Banatvala and Payne, 1973). By the early 1980s, this claim seemed more plausible because the structure and replication of HBV were found to be similar to those of cauliflower mosaic virus (Mason *et al.,* 1987). In 1987, this claim was put to rest when another laboratory reported that they had tried, without success, to repeat the "HBV in plants" experiment. This group also failed to replicate HDV (Faust *et al.,* 1987).

However, there were also successful attempts to extrapolate from the plant agents to HDV. For example, certain viroids and satellite RNAs were already known to undergo self-cleavage in the presence of heat and magnesium ions (Bruening, 1989). Therefore, we looked for such a site on HDV. We found that on both the antigenomic and genomic RNA there was such a ribozyme (Sharmeen *et al.,* 1988). Because the plant agents were already known to cleave by a transesterification mechanism to generate a 5′-OH and a cyclic 2′,3′-monophosphate, we asked whether the same was true for HDV, and again, it was (Kuo *et al.,* 1988).

VI. Subsequent Molecular Virology

A. Ribozymes

Michael Been and co-workers have shown that the two HDV ribozymes need only 85 nucleotides to self-cleave (Perrotta and Been, 1991). The folding includes a pseudoknot structure and some unusual pairings, such as a G-G pair. It is already clear that these HDV ribozymes have a structure quite different from what is known of the ribozymes of the plant agents. One of the HDV ribozymes has been crystallized (Ferre-D'Amare *et al.,* 1998b), and a detailed structure has been published (Ferre-D'Amare *et al.,* 1998a).

Partly on the basis of the plant work, we were able to reverse the HDV self-cleavage reaction and achieve ligation in the test tube in the absence of any protein (Sharmeen *et al.,* 1989). However, it is still controversial whether, *in vivo,* the circles are formed via such a self-

ligation or via the action of a host RNA ligase (Neel and Robertson, 1997).

B. Rolling Circles

For the plant viroids it had already been proposed, by Branch and Robertson in 1984, that the genomes were replicated by what is known as a "rolling-circle mechanism." Given that we knew that three RNAs were detected in HDV-infected cells, and had evidence for self-cleavage and ligation, it was not too difficult to adapt such a scheme to HDV. The model suggests that RNA-directed transcripts that were longer than the genome could be processed to make either polyadenylated mRNA or new circular RNAs. Such a model does explain the known RNA species, but one must remain aware that it contains many untested speculations. For example, as will be considered later, it still has to be established that the 5′ end of the mRNA species, near one end of the rodlike structure, actually corresponds to an initiation site. Furthermore, we do not know the site or sites for initiation for the transcription of the antigenome into genomic RNA.

C. Initiation on Circles

If circular RNAs act as templates, where does this transcription initiate? In 1989, Diener pointed out that for both the viroids and HDV, multimeric transcripts that are going to be processed to form unit-length species do not need to have a specific initiation site. He also considered in this paper whether such circular RNAs, HDV, and viroids might have been precursors in the evolution of introns. Actually, the reverse is also possible because of a phenomenon known as "missplicing," which is now known to occur for a number of host mRNA precursors. What happens in missplicing is that a splice donor acts on an inappropriate splice acceptor, one that is located 5′ of the splice donor, rather than at the normal 3′ acceptor site (Nigro *et al.,* 1991). This leads to the production of a small circular RNA. Such RNAs can and do accumulate because circular RNAs are much more stable than linear RNAs (Puttaraju and Been, 1995).

D. Infectious Clones

After the initial reports of a circular genome and an antigenome, it was important to put the HDV cDNA pieces together to obtain an

infectious cDNA construct. As will be explained, we were successful for a reason we did not immediately understand (Kuo *et al.,* 1989). It relates to a heterogeneity between the genomic RNA sequences present in the serum virus (Wang *et al.,* 1986). One sequence leads to the production of a 195-amino acid (aa) delta protein. The other differs in the middle of the termination codon and therefore encodes a protein that is 19 (aa) longer at the C terminus, making it 214 aa total (Weiner *et al.,* 1988).

We were fortunate to assemble a construct with the small protein because this protein was essential for genome replication (Kuo *et al.,* 1989), whereas the large form of the delta protein was subsequently shown to be a potent dominant negative inhibitor of replication (Chao *et al.,* 1990).

E. RNA Editing

Another surprise occurred when, in a collaboration with Camille Sureau, we transfected our infectious clone into the liver of an HBV-infected chimpanzee. In addition to detecting HDV replication, we found that even though the cDNA that was used encoded the small protein, there rapidly appeared an almost equal amount of the large protein (Sureau *et al.,* 1989).

Next, we checked and found that the same phenomenon occurred in transfected cells. When we looked at the RNA sequence, we found that as many as 50% of the RNAs were changed at the nucleotide corresponding to the middle of the termination codon (Luo *et al.,* 1990). This led to the realization that during HDV replication some of the RNAs were undergoing what is referred to as "posttranscriptional RNA editing."

We now know, largely from the work of Casey and Gerin (1995b), that this editing is achieved by the action of a class of nuclear enzymes currently referred to as "adenosine deaminase activated by double-stranded RNA" (ADAR) (Bass *et al.,* 1997).

F. Two Proteins

As mentioned above, the 195-aa small delta protein was found to be essential for genome replication, whereas the large protein acted as a potent dominant negative inhibitor (Kuo *et al.,* 1989). The next surprise came when Chen and co-workers showed that the large protein was

not just an inhibitor of replication but was actually needed for assembly of new virus particles (Chang *et al.,* 1991).

Later, Glenn *et al.* (1992) predicted and showed that this large delta protein can undergo a posttranslational modification that is essential for this protein to be active in virus assembly. There is a unique cysteine four amino acids from the C terminus of the large protein that becomes farnesylated. The delta protein is the only viral protein known to be so modified, although there are many important host proteins that become modified in this way (Casey and Seabra, 1996). Examples include the two nuclear lamins and many ras-related proteins. The process involves three enzymatic steps: the addition of the 15-carbon farnesyl group to the cysteine, the removal of the three terminal amino acids, and the blocking of the new C terminus with a methyl group. We now know that more than 60% of the large protein gets modified this way and that the modification is needed for efficient assembly of virus particles (Moraleda *et al.,* 1999).

The large and small forms of the delta antigen share certain properties. Lai and colleagues made the first major progress in determining domains on these two proteins to which functions could be ascribed (Lai, 1995). We now know of the following three domains.

The first domain is located near the N-terminus, spanning amino acids 12–60. Zuccola and colleagues (1998) have now determined the crystal structure of this domain. It contains an extended alpha-helical region and will dimerize through an antiparallel leucine zipper structure. Both this region and the entire delta protein are able to form higher multimers, especially octamers (Rozzelle *et al.,* 1995; Zuccola *et al.,* 1998). With advice based on the structure of the dimerization domain, we have designed critical single amino acid changes. We are now in a position to obtain information on the functional importance of dimerization and multimerization in different aspects of the HDV life cycle, such as genome replication and particle assembly (G. Moraleda, *et al.,* unpublished observations, 1999).

The second domain shared by the two delta proteins is a nuclear localization signal, which Lai and co-workers have shown to be made up of two almost adjacent elements (Lai, 1995). The third domain, which is involved in RNA binding, is also bipartite, that is, made up of two almost adjacent elements (Lai, 1995).

Overall, the small delta protein is highly basic, with a predicted charge of plus 12. *In vitro,* the delta protein binds RNA and DNA, but it has a more stable and specific interaction with the rod-like RNAs of

HDV (Chao *et al.,* 1991), consistent with the importance of this protein in genome replication.

VII. Subsequent Similarities to Plant Agents

A. Viroid-like Domain

Section V considered some of the initial similarities seen between HDV and the plant agents. Section VI,F considered the two delta proteins and their roles in the HDV life cycle. These proteins represent a fundamental difference between HDV and the plant viroids, which do not encode any proteins. However, in order to maintain the concept of a fundamental similarity between HDV and the plant viroids, some authors have proposed that the larger HDV genome is made up of two domains, a delta protein encoding region and a viroid-like domain (Elena *et al.,* 1991). Branch *et al.* (1990) have attempted to make such a division. From a study of different HDV sequences, they believe that there is less nucleotide variation in this viroid-like region relative to that corresponding to the coding region for the delta protein.

The concept of a viroid-like domain on HDV returned again in 1996, when Brazas and Ganem used the yeast two-hybrid system and found a mammalian protein that interacted with the delta protein. The protein they found, which they called "delta interacting protein A" (DIPA), A" (DIPA), was 202 aa in length, and for several reasons, including its size and interaction ability, they considered it to be a cellular homolog of the delta protein. They even suggested that HDV might have arisen via some hypothetical human equivalent of a plant viroid making a transcription-linked, recombination-like interaction with the mRNA for DIPA. Their model is plausible, but at this time it is still quite controversial (Brazas and Ganem, 1997; Long *et al.,* 1997).

B. Homology to 7S L RNA

There have been other attempts to determine whether there are host sequences related to the viroids or to HDV. Haas *et al.* (1988) showed that most viroids contain a patch of homology to an abundant cellular RNA, the 300-nt 7S L RNA, that is the backbone of the signal recognition particle. In addition, Negro and co-workers (1991) reported a similar region of homology on the genome of HDV. Intriguingly, this region is in the putative viroid-like domain. However, for both the viroids and HDV, we have yet to see data establishing the significance of this similarity.

C. Sequence Homology to Viroids

There have also been direct homology comparisons between the plant agents and HDV RNA. In 1991, computer analyses were used to compare the putative viroid-like domain of HDV with sequences of many of the available plant viroids and satellite agents (Elena *et al.,* 1991). It was found that many of the plant agents could thus be organized into groups, but no convincing relationship of HDV to any one of these was detected.

D. Mini-HDV

There have been experiments designed to test the hypothesis that HDV does have such a viroid-like domain that could support replication. We made a series of deletions while maintaining the predicted rodlike folding (Lazinski and Taylor, 1994). At 348 nt, the smallest construct tested was almost the size of a large viroid. Of course, these size changes interfered with the expression of the delta proteins. Therefore, we contransfected the cells with a plasmid that expressed an abundance of the small delta protein. We found that DNA-directed multimeric RNA transcripts were processed into circles if the delta antigen was present. However, these RNAs did not act as templates for RNA-directed RNA synthesis. That is, we were unable to separate out a functional viroid-like domain.

In addition to this negative result, we found that larger RNA circles were not stable unless the delta protein was also present. However, as the circles got smaller, down to the size of a viroid, they no longer needed the delta antigen for stabilization.

E. Transcription by Pol II and Possible Promoter Elements

Perhaps the most interesting problem shared by the plant viroids and HDV is how to copy RNA into RNA in the absence of a virus-encoded RNA polymerase. In this area, HDV research was again foreshadowed by studies of viroids. Several investigators using plant cell extracts have reported that the host RNA pol II could be the enzyme for the transcription of potato spindle tuber viroid RNA (Rackwitz *et al.,* 1981; Spiesmacher *et al.,* 1985). With HDV comparable *in vitro* studies have been done with nuclear extracts (Macnaughton *et al.,* 1991; Fu and Taylor, 1993; Beard *et al.,* 1996) and with purified enzymes. Such studies indicate that pol II can transcribe HDV RNA

templates *in vitro*. However, these studies need to be confirmed and extended, for example, to determine if there are specific sites of initiation.

A better understanding of HDV RNA-directed transcription will need both *in vitro* and *in vivo* studies. As mentioned earlier, we know that *in vivo* during HDV replication a polyadenylated mRNA-like species can be detected. The 5′ end of this RNA maps near one end of the predicted rodlike structure of the RNA. More specifically, it is in the middle of a predicted 3-nt external bulge. This finding has tempted many of us to say that there is a preferred site for the initiation of RNA-directed RNA transcription. Others have been tempted to propose that there is a feature analogous to a DNA promoter for pol II in this region of the genome. Indeed, Macnaughton *et al.* (1993) made double-stranded cDNAs of this region and reported that a 30-nt region of HDV, as a double-stranded DNA, will act as a modest promoter for DNA-directed transcription in transfected cells.

Attempts have been made to determine what defines this 5′-end of the polyadenylated mRNA. Our approach has been to apply a 5′-RACE procedure, coupled with cloning and sequencing to the polyadenylated species synthesized in cells undergoing HDV genome replication. With infected liver we found that all the clones had a 5′-end at nucleotide 1630 in the middle of the predicted 3-nt bulge (Gudima *et al.,* 1999).

Such data support but do not yet prove that the 5′-end corresponds to a specific site of initiation of transcription. For example, it is equally possible that there are multiple sites on both the genome and antigenome at which initiation occurs but that in terms of stability, the only species as stable as the processed RNA circles are the linear antigenomic RNAs with a 5′-end located at or near nucleotide 1630.

It may be very difficult to determine just what structural features present in the HDV RNA are being used to redirect the host RNA pol II to copy an RNA template. We have predicted structures, but the biologically relevant ones, like those for the self-splicing tetrahymena self-splicing intron, might be quite different (Wu and Tinoco, 1998). Also, as shown for certain viroid RNAs, there can be multiple structures, all of which are relevant for replication (Loss *et al.,* 1991). Without such correct structures, we may not be able to identify HDV promoters or carry out correct transcription *in vitro*.

We have developed a transfection procedure that allows us to look at the early events of RNA-directed RNA synthesis as they occur inside a transfected cell (Dingle *et al.,* 1998). Our strategy is to preassemble purified recombinant delta protein with *in vitro* synthesized HDV RNA. When these ribonucleoprotein (RNP) complexes are transfected into

cells, we can subsequently detect genome replication by Northern analyses, by immunoblot, or by immunofluorescence. (Without delta protein, the transfected RNA is not functional.) However, with a 5′-RACE procedure we can detect initiation of transcription at nucleotide 1630 as early as 24 hours after transfection. With this new assay, we can now alter the nature and stoichiometry of both the protein and RNA components of the RNP and follow the consequences. However, one still needs to make the qualification that the 5′ ends detected have yet to be established as sites for the initiation of transcription.

VIII. Conclusions and Outlook

It is important to point out two more things possibly related to the origin and replication of HDV. The first concerns the newly achieved realization of the diversity of the plant viroids. It is no longer believed that all viroids replicate in the nucleus using the host RNA pol II. For example, there are three viroids that accumulate their RNAs in the plant chloroplast rather than in the nucleus. The prototype is avocado sunblotch viroid. These viroids might use a chloroplast RNA polymerase for their transcription (Lima *et al.,* 1994).

The second item concerns some data on the origin of certain viroid-like RNAs. We are now hearing of some unusual RNAs that are replicated via the reverse transcriptase of a helper virus to produce a DNA intermediate. Daros and Flores (1995) have reported one intriguing example of such an RNA. Carnation small viroid-like RNA is an agent that replicates with the help of a virus, similar to cauliflower mosaic virus, that also replicates through reverse transcription. It is hard not to notice that this association of two agents appears quite a bit like that between HDV and HBV, and prompts the speculation that HDV might once have been similarly dependent on the reverse transcriptase of HBV, its helper virus.

This article is largely a personal historical overview of HDV research, with an emphasis on how studies with the plant agents have in many ways impacted on the questions we have asked and the approaches we have used. It is based on a talk presented as part of small symposium held April 24, 1998, and organized by the University of Maryland Biotechnology Center to honor the retirement of Professor Theodor O. Diener. Thomas Kuhn (1996) wrote in his book “The Structure of Scientific Revolutions” that most scientists carry out “normal science” in which they work within the current paradigm. However, for a very small number of scientists, the opportunity comes through a combina-

tion of intelligence, hard work, and often a touch of serendipity, to propose and achieve, usually in the face of militant contrary forces, the acceptance of a new paradigm. Dr. Diener is one such person. Such a contribution in itself should give great satisfaction. However, for an even rarer individual, and again Dr. Diener is an example, there is an additional unexpected satisfaction. This is what I have tried to make clear in this article. That is, to see one's work have an unexpected relevance and impact in what might seem at first glance to be a quite separate field of scientific investigation.

Acknowledgments

The author was supported by Grants AI26522 and CA-06927 from the NIH and by an appropriation from the Commonwealth of Pennsylvania. As indicated in the text, the unpublished observations of several colleagues have been cited. Also, thanks are offered to William Mason, Glenn Rall, and Severin Gudima for constructive comments on the manuscript.

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ADVANCES IN VIRUS RESEARCH, VOL. 54

REGULATION OF HUMAN CYTOMEGALOVIRUS GENE EXPRESSION

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I. Introduction

The cytomegaloviruses (CMV) are large enveloped viruses in the herpesvirus group. The double-stranded DNA genome of human cytomegalovirus (HCMV) is approximately 230 kilobase pairs (kbp) and consists of two covalently linked segments, UL (unique long) and US

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(unique short). Each of these segments is bounded by inverted repeats, designated TRL and IRL (terminal [T] and interior [I] repeat [R] bounding the long [L] segment) and TRS and IRS. When the sequencing of the HCMV genome was completed, predicted open reading frames (ORFs) were numbered sequentially according to their position on the genome (TRL, UL, IRL, IRS, US, and TRS), and these designations, as well as common names for the known proteins, will be used throughout this review.

The various strains of CMV are species-specific and produce both persistent and latent infections in their respective hosts (for review, see Britt and Alford, 1996). HCMV is a common pathogen in humans, capable of causing disease that affects all age groups. It is efficiently transmitted to the fetus during pregnancy, with 0.5–2.5% of all newborns showing evidence of congenital infection. Although infection in the normal child and adult is usually asymptomatic, the *in utero* infection is not benign, and 5–10% of congenitally infected infants will be symptomatic at birth, with serious neurological defects. Even the infected newborns who initially appear asymptomatic are at high risk, as 10–15% of these children will show varying degrees of neurological damage later in life. Although maternal infection prior to pregnancy confers some protection to the fetus from symptomatic congenital infection, the protection is not complete. HCMV also causes significant morbidity and mortality in immunosuppressed individuals, notably transplant patients and persons infected with human immunodeficiency virus. The serious medical problems associated with HCMV infections, particularly in newborn and immunocompromised individuals, have provided a major impetus for understanding the molecular biology of this virus and the regulatory mechanisms governing its gene expression and replication. It is hoped that this information will then serve as a foundation for the development of rational strategies directed at the prevention and treatment of disease.

The growth cycle of HCMV is relatively slow; although viral DNA synthesis begins in the nucleus at 16 to 24 hours postinfection (hpi), virus is not released until 72 to 96 hpi (reviewed in Mocarski, 1996). Viral gene expression proceeds in a defined temporal order, which, for the permissive infection, has been divided into three phases: immediate early (IE), early, and late. It should be noted, however, that the boundaries between these phases are somewhat arbitrary and that the assignment of a gene product to a particular class may be significantly influenced by the multiplicity of infection, strain of virus, and host cell. The IE gene products are synthesized initially after infection and rely primarily on host factors for their expression. As described below, sev-

eral of the viral IE proteins serve as essential transactivators of the next class of gene products, the early genes. Although induction of early gene transcription occurs prior to viral DNA replication, the pattern of expression of individual early gene products, as will be described below, can be quite different as the infection progresses. Included in the early class are those viral proteins required to "activate" the cell to a metabolic state most conducive for viral DNA synthesis and those proteins involved in the replication process itself. Finally, late genes, which constitute the majority of the viral genome and encode primarily structural and maturation proteins, are transcribed in abundance only after the onset of viral DNA replication.

Since the mid-1980s, we have accumulated a wealth of information regarding HCMV gene expression. Taken together, the results of many studies suggest that, analogous to their cellular gene counterparts, viral genes are subject to multiple regulatory controls operating at the level of initiation of transcription, RNA processing and transport, translation, and mRNA and protein stability. This review will focus on the level of control about which we know the most: the regulation of viral gene expression at the level of transcription.

II. Viral Entry

HCMV has a wide range of permissivity *in vivo.* The major targets of infection are ubiquitously distributed cell types such as epithelial and endothelial cells and fibroblasts, but peripheral blood leukocytes, specialized parenchymal cells such as neurons in the brain and retina, smooth muscle cells, and hepatocytes can also be infected with the virus (Sinzger and Jahn, 1996). The range of permissivity is restricted, however, when the virus is grown in tissue culture cells. Although there are reports regarding the ability of most of the above-mentioned cell types to support growth of HCMV in tissue culture, primary fibroblasts are the cell type of choice and produce the highest yield of virus. Undifferentiated, transformed, or aneuploid cells, including most tested cell lines, are generally nonpermissive or yield greatly reduced titers compared to primary fibroblasts (Mocarski, 1996). The key to this restriction in many of the cell types tested may be differentiation, as downregulation of the promoter for the major IE genes in some undifferentiated cells can reportedly be relieved on differentiation (Mocarski, 1996; Sinzger and Jahn, 1996; Spiller *et al.,* 1997).

For most cell types, entry of HCMV occurs efficiently. The initial interaction with the host cell involves two distinct events: the first is

adsorption, which can occur at 4°C, and the second is penetration, which follows fusion of the viral envelope with the cell membrane and can occur only when the temperature is raised to 37°C (Topilko and Michelson, 1994). Early studies by Taylor and Cooper (1989) showed that ^{35}S-labeled virions from strain AD169 could bind covalently to cells at 4°C and this binding could be inhibited by protease treatment, suggesting that a cell surface molecule was involved. The initial hypothesis was that this was a single protein species. However, it has since become apparent that binding and entry are complex processes involving heparin sulfate proteoglycans on the cell surface (Compton *et al.,* 1992), at least two or three different cellular receptors (Keay *et al.,* 1989; Taylor and Cooper, 1990; Wright *et al.,* 1994, 1995; Pietropaolo and Compton, 1997), and several glycoprotein complexes in the virion envelope (Compton *et al.,* 1992; Kari and Gehrz, 1993).

III. Immediate Early Events Mediated by Initial Virus–Host Interaction

To date, four major regions on the HCMV genome have been identified as encoding IE gene products with demonstrated regulatory activities: IE1/IE2 (UL123/UL122), UL36-38, IRS1-TRS1, and US3 (Everett, 1984; Colberg-Poley *et al.,* 1992; Stasiak and Mocarski, 1992; Iskenderian *et al.,* 1996; Kerry *et al.,* 1996). The results of many studies from our laboratory and others have shown that HCMV early promoters, as well as many heterologous viral promoters, can be activated by the region of the genome specifying the IE1 72 and IE2 86-kDa proteins (Everett, 1984; Davis *et al.,* 1987; Hermiston *et al.,* 1987; Tevethia *et al.,* 1987; Pizzorno *et al.,* 1988; Staprans *et al.,* 1988; Chang *et al.,* 1989a; Klucher *et al.,* 1989; Barry *et al.,* 1990; Malone *et al.,* 1990; Rando *et al.,* 1990; Biegalke and Geballe, 1991; Ghazal *et al.,* 1991; Paya *et al.,* 1991; Colberg-Poley *et al.,* 1992; Hagemeier *et al.,* 1992; Stasiak and Mocarski, 1992; Walker *et al.,* 1992). As will be described later, the other IE gene products appear to play an accessory role in achieving full activation (Colberg-Poley *et al.,* 1992; Stasiak and Mocarski, 1992; Winkler *et al.,* 1994; Iskenderian *et al.,* 1996; Kerry *et al.,* 1996). Due to the extremely important role of the IE gene products in establishing a productive infection, the virus has devised methods of activating the cell to expedite their expression. Several of these early events will be described.

A. Physiological Events Triggered by Viral Binding

The binding and fusion of the virion envelope with the cell membrane initiates a cascade of physiological responses and distinct virus–cellular

protein interactions that together set the stage for a productive HCMV infection. The physiological response of the host cell to HCMV binding occurs rapidly and is characterized by an immediate Ca^{2+} influx and hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to produce the signaling molecules inositol trisphosphate (IP_3) and diglyceride (DG). This is followed by an increase in the intracellular stores of Ca^{2+} and in cyclic AMP and GMP levels. Blocking of these events with the drug papaverine has a marked effect on the progression of infection, and cells remain small and rounded, with relatively little DNA replication observed (Albrecht *et al.,* 1990). It is noteworthy that not only intact infectious virions but also noninfectious enveloped particles and dense bodies can penetrate the cell membrane, indicating that they too may contribute to the triggering of this initial signal transduction cascade. It is also conceivable that some of these effects may be due to the transient dephosphorylation of cellular proteins that occurs several minutes after contact of the virus with the host cell, possibly mediated by the cellular serine/threonine phosphatases protein phosphatase 1 and 2A (PP1 and PP2A, respectively) carried within the virions (Michelson *et al.,* 1996).

B. Induction of Cellular mRNAs

In addition to the early physiological responses, there is a rapid, albeit transient, induction of c-*fos,* c-*jun,* and c-*myc* mRNAs following infection of fibroblasts (Boldogh *et al.,* 1991). This increase in transcription occurs in the absence of serum and with ultraviolet (UV)-inactivated virus, indicating that no viral gene expression is required and that a virion-associated protein is probably involved (Albrecht *et al.,* 1991). Another class of mRNAs that are upregulated at an early point in the viral infection is a subset of the family of genes that are normally induced by alpha (α) interferon in uninfected cells (Zhu *et al.,* 1997; Navarro *et al.,* 1998). The HCMV-associated induction, however, occurs in the absence of any detectable α interferon and, as described above for c-*fos,* c-*jun,* and c-*myc* mRNAs, appears to require simply the exposure of the cell membrane to virions, noninfectious enveloped particles, or dense bodies. A study by Zhu and colleagues (1998) with the use of DNA array technology has further identified 258 cellular mRNAs whose levels of expression were changed by a factor of 4 or more before the onset of viral DNA synthesis. Although protein levels were not analyzed in this preliminary study, several mRNAs identified could potentially play key roles in HCMV-induced pathogenesis.

C. Modification of Transcription Factors

1. IRF-3 Activity

Related to the above observation that HCMV induces the expression of α interferon-responsive genes is the finding that there is a concomitant appearance of a novel DNA binding complex that binds to the interferon-stimulated response element (Navarro *et al.,* 1998). Two of the components of this complex have been identified as the interferon-stimulated gene factor 3 (IRF3) and the CREB binding protein (CBP). It has become apparent that this complex is not unique to HCMV infection and is in fact similar to the inducible factor (sometimes referred to as VAF) that binds to a subset of interferon-inducible genes that are activated by other viruses as well as by double-stranded RNA (Lin *et al.,* 1998; Schafer *et al.,* 1998; Wathelet *et al.,* 1998; Weaver *et al.,* 1998). The data indicate that in response to viral infection, IRF-3 is phosphorylated and translocated from the cytoplasm to the nucleus. It then associates with CBP and probably with other proteins (cellular or viral) that may be specific for a given viral infection or cell type (Lin *et al.,* 1998; Wathelet *et al.,* 1998). Phosphorylation also appears to be a signal for proteasome-mediated degradation of IRF-3. Although it might seem counter-intuitive for the virus to activate the host cell's interferon-inducible antiviral defense genes, it is possible that some benefit to viral replication is derived from the induction of only a subset of the interferon-inducible cellular genes. In addition, the induction of this IRF-3-containing complex by HCMV may facilitate the activation of its own IE genes.

2. NF-κB and Sp1 Activation

Nuclear factor-κB (NF-κB) was one of the first transcription factors found to be influenced by HCMV infection (Sambucetti *et al.,* 1989). Under physiologically normal conditions, the heterodimeric complex (50- and 65-kDa subunits) is sequestered in an inactive form in the cytoplasm by its inhibitor, I-κB. However, in response to a variety of signals at the cell surface, a cascade of events leading to the degradation of I-κB and the transit of free NF-κB into the nucleus is activated (Thanos and Maniatis, 1995). In the case of the HCMV infection, it appears that the NF-κB complex is regulated at the level of both DNA binding and transcription of the mRNA encoding its two components.

Although the initial studies indicated that there was a fairly rapid induction of nuclear NF-κB DNA binding early after infection (between 3 and 12 hpi) (Sambucetti *et al.,* 1989; Kowalik *et al.,* 1993), it has now been defined as occurring on contact with the virus (Yurochko *et al.,*

1995). This almost instantaneous increase in NF-κB binding has been attributed to the release of cytoplasmic stores of the complex due to a signaling cascade brought about by binding of the virus particle to the cell membrane. Not only the intact infectious virus, but also UV-irradiated virus, purified viral membranes, and purified gB protein can elicit this response (Yurochko *et al.,* 1997).

One argument holds that the sustained binding activity observed in infected cells is due not only to the release of cytoplasmic stores of NF-κB but also to upregulation of NF-κB transcription (Yurochko *et al.,* 1995). A key question is, what precipitates this increase in NF-κB mRNA? It has been suggested (Yurochko *et al.,* 1995) that upregulation of Sp1 may be responsible, and in support of this hypothesis, an increase in Sp1 DNA binding activity is observed on contact with the virus (Yurochko *et al.,* 1997). Other evidence is that, at least in transient assays, the Sp1 sites within the p65 promoter (and, to a lesser extent, within the p50 promoter) are important for HCMV- induced activation. However, the connection between the increased Sp1 activity and the sustained higher levels of NF-κB mRNA seems tenuous at best. One problem with these studies is that appropriate mock controls were not performed in parallel for the entire time course; therefore, serum stimulation and/or factors other than viral infection could play a role in activating these transcription factors. In addition, the steady-state concentration of the various factors within the infected cells has not been determined, and without this information the relative importance of the increased mRNA levels cannot be fully assessed.

D. Activation by HCMV Tegument Proteins

The HCMV virion particle consists of an inner nucleocapsid and an outer envelope that are separated by the tegument. The tegument (or matrix) is composed of multiple proteins, some of which appear to enter the cell and travel to the nucleus following adsorption of the virus to the host cell. In addition to the effects mediated by interaction of the viral envelope with the cell membrane, several viral matrix proteins entering the cell also appear to regulate early events in the infection. At present, only UL69, UL82 (also known as pp71 or upper matrix protein), and IRS1/TRS1 have been shown to have transcriptional regulatory activities. However, it is very likely that similar functions will be ascribed to other proteins present in the viral particles as their various properties begin to be elucidated.

The UL69 gene specifies at least three subforms of a protein that can activate several viral and cellular promoters in transient transfection

assays (Winkler *et al.,* 1994). Two of the subforms are phosphorylated, and one of these appears to be incorporated into the tegument of the virus particle (Winkler and Stamminger, 1996). In transient transfection studies, expression from a plasmid construct containing the major IE promoter is activated by the viral tegument proteins UL69 and UL82 (Liu and Stinski, 1992; Winkler and Stamminger, 1996). The UL69 gene product can also activate the UL112/113 early gene promoter and the promoter of Rous sarcoma virus. In addition, as discussed below, both IRS1 and TRS1 can cooperate with UL69 in transient expression assays to activate the IRS1/TRS1 promoter and further enhance transcription directed by the major IE promoter (Romanowski *et al.,* 1997).

In a separate study, Baldick and colleagues (1997) have examined the effects of the UL69 and UL82 gene products on the infectivity of HCMV DNA introduced into the cell by transfection. The results of their studies showed that only UL82 was able to enhance the infectivity of the transfected HCMV DNA and increase expression of the major IE1 and IE2 gene products. Although the finding that UL69 activated the major IE promoter when present on a plasmid construct but did not activate it on the transfected DNA is somewhat surprising, it nevertheless highlights the difficulty of extrapolating the results of transient transfection assays to what occurs when the viral DNA is introduced into the cell as a consequence of the normal infection process. The actual contribution of any of these virion tegument proteins, either alone or in combination, to activation of viral or cellular gene expression at the beginning of the infection remains to be determined. However, the question is an important one to address, as the answer may provide insight into the absence of viral gene expression in some cells types, as well as the conditions favoring the establishment of latency.

One additional tegument protein that may contribute, albeit indirectly, to viral (or possibly cellular) gene expression early in the infection is UL83 (also known as pp65 or lower matrix protein), which is a major structural component of the virus and is responsible for generating most of the protective T-cell response in humans (McLaughlin-Taylor *et al.,* 1994; Walter *et al.,* 1995; Boppana and Britt, 1996; Wills *et al.,* 1996). Following infection, UL83 rapidly transits to the nucleus, and can serve as a marker for successful adsorption and penetration of the virus. Although transient expression assays have failed to reveal a direct activator function for UL83, it has been associated with a kinase that phosphorylates the IE protein IE1 72 (Somogyi *et al.,* 1990; Gilbert *et al.,* 1996), which plays a role in regulating viral gene expression. Interestingly, UL83-mediated phosphorylation of IE1 72 prevents the protein from being processed and presented in the context of major

histocompatibility complex (MHC) class I molecules, thus accounting for one of the mechanisms whereby the virus can evade the host immune response (Gilbert *et al.,* 1996).

IV. Utilization of Preexisting Nuclear Architecture by HCMV

It is well established for most DNA viruses (an exception being the pox virus group) that following penetration of the virus into the host cell, the viral genome travels to the nucleus, where transcription and replication take place. However, the molecular events occurring between the penetration of the virus and the appearance of the first viral transcripts have largely remained a black box. An important clue has been provided by the observation that the input viral DNAs of simian virus 40 (SV40), adenovirus 5 (Ad5), and herpes simplex virus type 1 (HSV-1) target nuclear structures known as nuclear domain 10 (ND10) and that the periphery of these structures serves as a preferred site for early viral transcription and DNA replication (Ishov and Maul, 1996). At least five cellular proteins, including the autoantigen Sp100 and the growth suppressor protein PML (promyelocytic leukemia protein), localize to these structures. All five of these proteins are also interferon inducible, suggesting a role for them in antiviral defense. As will be discussed later, several proteins expressed by these viruses at early times postinfection can disperse these ND10-associated proteins.

On the basis of the above findings, it seemed very likely that the input HCMV genome would also localize at these ND10 sites, and in an elegant study, Ishov and colleagues (1997) showed that at 2 hpi, a certain proportion of the input HCMV DNA was associated with ND10 sites. Moreover, when the early mRNA transcripts were localized, all were positioned at ND10 sites (but not all ND10 sites had transcripts), indicating that only the genomes deposited at ND10 were engaged in early transcription. It was further noted that the transcripts were juxtaposed to the spliceosome assembly factor SC35 domains. Because a similar relationship between SC35 and ND10 exists at approximately 80% of the ND10 sites in mock cells, it seems likely that these structural characteristics of the ND10 domains preexist in uninfected cells and that this nuclear architecture is therefore utilized by the virus for rapid expression of IE gene products.

V. Influence of Host Cell Cycle Phase on IE Gene Expression

In the past four years, our laboratory and others have shown that infection of primary fibroblasts with HCMV inhibits cell cycle progression

and alters the expression of several key regulatory proteins including cyclins A, B, and E and the tumor suppressor proteins p53 and Rb (Jault *et al.,* 1995; Bresnahan *et al.,* 1996; Lu and Shenk, 1996; Dittmer and Mocarski, 1997). In order to further understand the regulation of the key cyclins during infection, we took the approach of infecting cells at different stages of the cell cycle without the use of cycle-arresting drugs (Salvant *et al.,* 1998). Contact inhibition was utilized to arrest the cells in a G_0 state, and infections were then carried out on release of the cells from contact inhibition or 24 hours later [when approximately 50% of the cells were in S phase as analyzed by fluorescence-activated cell sorting (FACS)]. Although the regulation of the cyclins was not dramatically affected by altering the cell cycle phase at the initiation of the infection, there was an effect on the ability of the virus to begin IE gene expression. During a G_0 infection, all cells exhibited cytopathic effect (CPE) and expressed IE1/IE2 proteins by 12 hpi. However, this was not the case with the cells infected 24 hours after release from confluence. At the 12 hpi time point, approximately 50% of the cells exhibited CPE, and approximately 40% expressed IE1/IE2 proteins. Moreover, when the cultures were pulsed with bromodeoxyuridine (BrdU) to label the cells actively replicating at the time of infection and then harvested at 12 hpi, the staining pattern of the cells for IE1/IE2 and BrdU showed little overlap. This suggested that the predominant proportion of cells in S at the time of infection could not synthesize IE1/IE2 proteins within the first 12 hours after labeling and infection. Interestingly, there was a small fraction of double-positive cells appearing at the 12 hpi time point, which we hypothesize are cells that were infected in the later stages of S and had then cycled back to the next G_1 phase before expressing IE1/IE2 proteins. Our experiments have additionally revealed a population of cells (presumably on the cusp of the restriction point of commitment to S phase and division) that have the potential to first express IE1/IE2 proteins and then proceed through cellular DNA synthesis and division (E. A. Fortunato and D. H. Spector, unpublished, 1998). We are particularly interested in this group of cells because they could potentially be precursors to the cells that sustain DNA damage during development of the infected fetus.

VI. Major Immediate Early Gene Expression: IE1/IE2 (UL123/122)

The four main regions of the HCMV genome encoding IE gene products are delineated in Fig. 1. Of these, the IE1/IE2 region has been most extensively analyzed with respect to regulation of gene expression and function of the gene products, and as such will be the main focus of this section of the review.

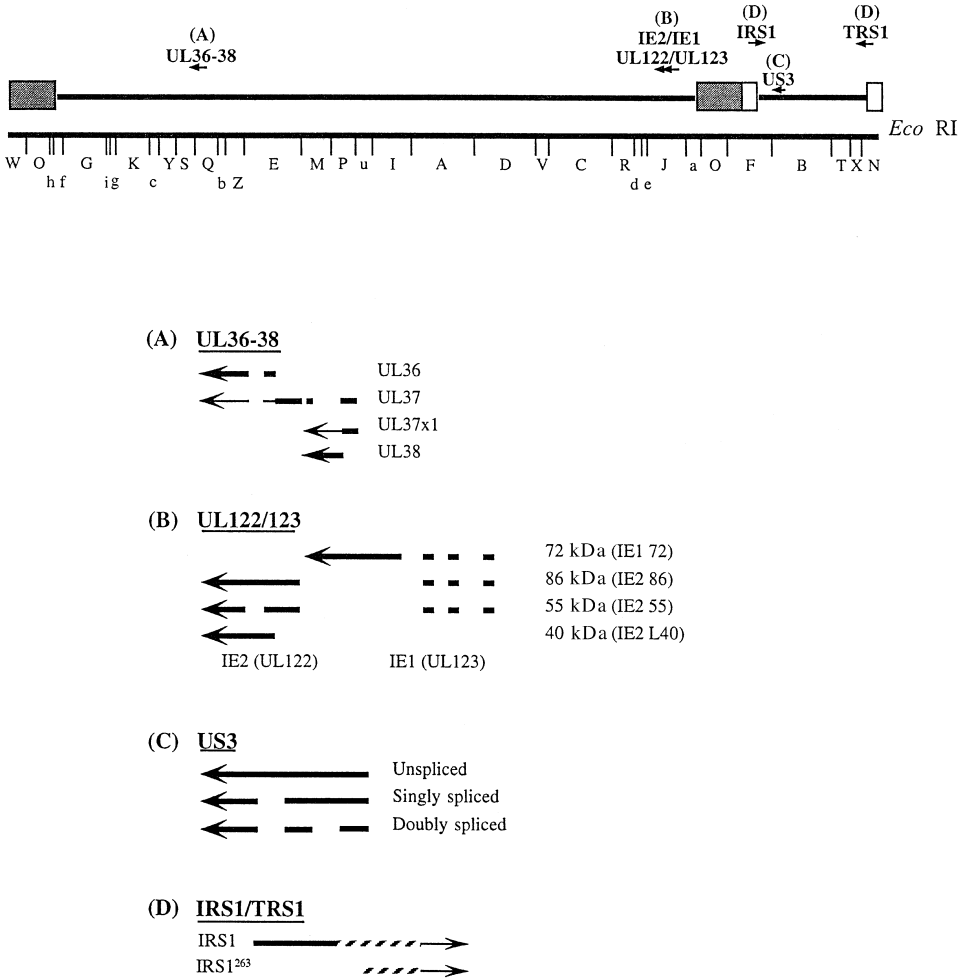


FIG 1. The regulatory immediate early genes of HCMV. The upper part of the figure represents the HCMV genome, with unique long and unique short regions in solid lines. The terminal and inverted repeat regions are shown as boxed areas. Arrows above the diagram indicate the direction of transcription for the four sets of IE genes described in the text. The *Eco*RI restriction map of strain AD169 is provided for reference. The lower portion of the figure provides the pattern of splicing for the individual transcripts encoded by these ORFs. Thick lines indicate exons, and thin lines indicate 3′ untranslated regions. The IRS1 and TRS1 transcripts have identical 5′ regions encoded within the repeat regions flanking unique short regions. Their coding regions diverge, however, when the transcripts enter the unique short region, as indicated by the differently patterned lines in the diagram.

Early RNA mapping studies showed that the majority of the IE transcripts were synthesized from one region in the UL segment that contained two genetic units, referred to as IE1 and IE2 (Wathen *et al.,* 1981; Wathen and Stinski, 1982; McDonough and Spector, 1983; Stinski *et al.,* 1983; Jahn *et al.,* 1984a,b; Stenberg *et al.,* 1984; Wilkinson *et al.,* 1984; Hermiston *et al.,* 1987; Plachter *et al.,* 1988). The predominant IE RNA (IE1) is 1.9 kb in length and consists of four exons; an ORF (UL123) initiates in exon 2 and encodes a 72-kDa (491-aa) nuclear phosphoprotein designated IE1 72 (Gibson, 1981, 1984; Gibson *et al.,* 1981; Stinski *et al.,* 1983; Jahn *et al.,* 1984a,b; Stenberg *et al.,* 1984; Akrigg *et al.,* 1985). IE region 2 encodes two IE RNAs of 2.25 and 1.7 kb that, through differential splicing mechanisms, contain the three 5′ exons of IE1 fused to the IE2 region (Stenberg *et al.,* 1985). The major IE2 gene product, IE2 86, is an 86-kDa phosphoprotein (ORF UL122), 579 amino acids (aa) in length, that is translated from the larger transcript. It shares 85 aa at its amino terminus with the IE1 72 protein. The smaller transcript is derived from an in-frame splice in the IE2 region, generating a 55-kDa protein that is missing aa 365–519 from IE2 86. Its biological relevance is unclear because it is observed only when cells are released from cycloheximide inhibition. There is also another low-abundance splice variant generated from the IE2 region (not shown in Fig. 1) that has been detected in infected human monocyte-derived macrophages and is predicted to encode a 164-aa protein (Kerry *et al.,* 1995). In addition, late in the infection, a late promoter in the fourth exon of IE2 appears to direct the synthesis of an unspliced transcript that encodes a 40-kDa protein, colinear with the carboxy-terminal half of IE2 86 (Stenberg *et al.,* 1989; Puchtler and Stamminger, 1991; Jenkins *et al.,* 1994).

A. Vast Array of Cis-Acting Sequences Defining IE1/IE2 Promoter

Expression of the IE1/IE2 genes is controlled by a complex upstream regulatory region that consists of a modulator, an NF-1 domain, an enhancer, and a minimal promoter (see Fig. 2). In reviewing the proposed functions of these regions, it is important to keep in mind that for the most part, these domains have been studied only in transient expression or in *in vitro* DNA–protein binding assays that may not accurately portray the relevance of that region within the context of the viral infection. In fact, there are now several examples in which the results of earlier assays have not accurately reflected the function observed when viral recombinants were used to study the element in the context of the replicating virus.

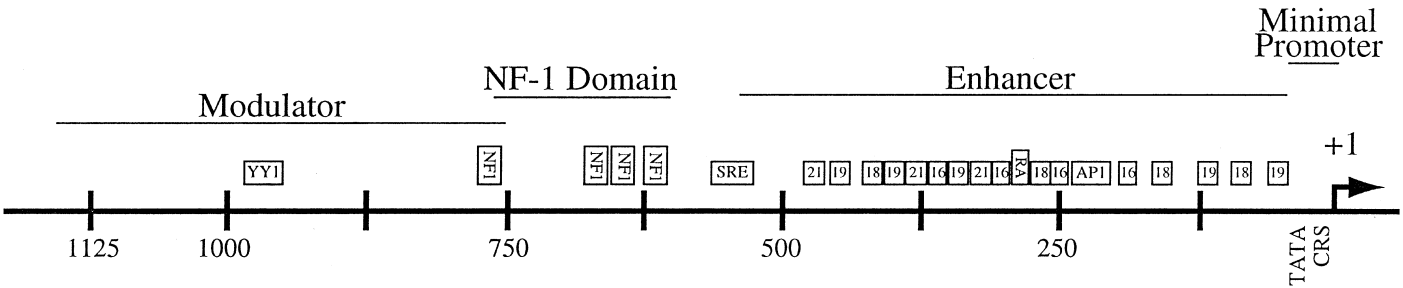


FIG 2. The upstream regulatory region of the major immediate early transcript UL122–123 (IE1/IE2). Potentially important binding sites for transcription factors are indicated above the nucleotides where they are located. This is not an exhaustive list of factor binding sites. See text for a description of various regions. The start of transcription is indicated by +1. *Key:* SRE, serum response element; RA, retinoic acid site; 21, 21-bp repeat containing Sp1 and YY1 sites; 19, 19-bp repeat containing ATF/CREB site; 18, 18-bp repeat containing NF-κB/rel site; 16, 16-bp repeat.

The modulator region of the major IE promoter, which is located between nucleotides (nt) −750 and −1150 relative to the transcription start site, has been shown in transient expression assays to affect the rate of transcription both positively and negatively, depending on the differentiation state of the cell. In these assays, this region appears to upregulate transcription in permissive cells such as fibroblasts. However, the modulator has a negative inhibitory effect on transcription in undifferentiated Tera-2 cells and monocytic THP-1 cells (Nelson *et al.,* 1987; Lubon *et al.,* 1989; Shelbourn *et al.,* 1989; Huang *et al.,* 1996), which may be due to the binding of cellular factors to specific repeat elements within this region (Shelbourn *et al.,* 1989; Kothari *et al.,* 1991; Sinclair *et al.,* 1992; Liu *et al.,* 1994; Thrower *et al.,* 1996). The biological relevance of any of these observations with respect to the infection, however, is questionable because it has been shown that deletion of the modulator from the major IE promoter in the context of the replicating viral genome does not affect transcription from this promoter in fibroblasts or in undifferentiated cells (Meier and Stinski, 1997).

Downstream of the modulator is a segment between nt −780 and −610 that contains four consensus recognition sites for the cellular factor NF-1 (Hennighausen and Fleckenstein, 1986; Jeang *et al.,* 1987). An NF-1 site has also been identified in the first intron of the major IE genes. The evidence that the modulator region may serve a regulatory role is derived primarily from the observation that DNase I hypersensitive sites are present in this region during the productive infection in fibroblasts and differentiated Tera-2 cells but not during the nonproductive infection in undifferentiated Tera-2 cells (Nelson and Groudine, 1986).

Between nt −550 and −50 lies the major IE enhancer, which is one of the strongest and most complex regulatory regions for RNA pol II-directed transcription. This complexity may be related to the cell- and tissue-specific requirements for viral replication and to the central role the IE1/IE2 gene products play in the initiation of infection. Although earlier studies with transgenic mice suggested that the major IE enhancer might be universally active in all tissues and cells (Schmidt *et al.,* 1990; Furth *et al.,* 1991), more recent analyses indicate that activity is more restricted, with a preference for expression in cell types and target tissues normally infected by HCMV (Koedood *et al.,* 1995; Baskar *et al.,* 1996a,b). Dissection of the enhancer reveals arrays of 19-, 18-, and 21-bp repeats containing consensus binding sites for ATF/CREB, NF-κB/rel, Sp1, and YY1 sites, respectively (Thomsen *et al.,* 1984; Boshart *et al.,* 1985). On the basis of the results of transient expression assays, it seems that the ATF/CREB site plays an important role in

the basal activity of the major IE promoter (Hunnighake *et al.,* 1989; Chang *et al.,* 1990; Niller and Hennighausen, 1990), whereas the NF-κB/rel sites respond to factors induced by the HCMV virions, cytokines, and the IE1 protein itself (Cherrington and Mocarski, 1989; Sambucetti *et al.,* 1989; Boldogh *et al.,* 1990).

In addition to the above sites, a number of consensus and nonconsensus Sp1 sites and binding sites for the serum response element (SRE), CCAAT/enhancer binding protein (C/EBP), ETS, and the retinoic acid receptor RAR-RXR family members contribute to activity of the major IE promoter (LaFemina and Hayward, 1986; Ghazal *et al.,* 1992; Lang *et al.,* 1992; Angulo *et al.,* 1996). Although a functional role for the retinoic acid receptor binding site is still speculative, it is interesting to note that retinoic acid treatment of the human embryonal carcinoma cell line NT-2/D1 induces differentiation of the cells so that they are permissive for HCMV replication (Ghazal *et al.,* 1992; Angulo *et al.,* 1995). In addition, Angulo and co-workers (1998) have reported that a retinoic acid receptor-mediated pathway enhances both *in vivo* and *in vitro* infection by murine CMV, which also has retinoic acid-responsive elements in its major IE enhancer/promoter.

The minimal promoter for the IE1/IE2 genes is between nt -50 and $+1$ and contains a canonical TATAA box. Early studies involving transient expression and *in vitro* transcription assays indicated that IE2 86 was able to repress its own promoter through a sequence [referred to as the "*cis*-repression signal" (CRS)] located between the TATAA box and the transcription start site (Pizzorno and Hayward, 1990; Cherrington *et al.,* 1991; Liu *et al.,* 1991). Subsequently, it was shown that IE2 86 mediated this repression by binding directly to the CRS through contacts with the minor groove (Chiou *et al.,* 1993, Lang and Stamminger, 1993; 1994; Macias and Stinski, 1993). One puzzling feature of this repression was that although the binding of IE2 86 to the DNA interfered with RNA polymerase II preinitiation complex formation (Wu *et al.,* 1993), it did not prevent the TATA-box-binding protein (TBP) from binding to the TATAA box (Jupp *et al.,* 1993a). It now appears that IE2 86 does not interfere with the establishment of the preinitiation complex by blocking binding of transcription factor IID (TFIID) and transcription factor IIB (TFIIB) to the promoter, but rather prevents the recruitment of RNA polymerase II to the assembled complex (Lee *et al.,* 1996). Another complicating feature of this region is that a 150-kDa cellular protein binds a characterisitic initiator-like sequence located at the start site of transcription (Macias *et al.,* 1996). Experiments utilizing *in vitro* transcription assays with uninfected nuclear extracts indicate that the binding of this cellular protein has

a positive effect on transcription. Therefore competition between the cellular protein and IE2 86 for binding near the transcription start site *in vivo* may be another mechanism for regulation of RNA synthesis and steady-state levels of the IE1 and IE2 gene products. Other sequences that appear to affect transcription positively have been localized within the leader segment of exon 1 and the intron between exon 1 and 2 (Chapman *et al.,* 1991; Ghazal and Nelson, 1991), but little is known about their mechanism of action.

B. Physical Properties of IE1/IE2 Proteins

High-level activation of promoters by the IE1/IE2 proteins requires sequences upstream of the TATAA box. However, the inability to identify any common element in the promoters other than a TATAA box makes it appear unlikely that the IE1/IE2 proteins function as specific transcription factors composed of sequence-specific DNA-binding and activator domains. Although the molecular mechanisms underlying the various observed activities of these proteins are still uncertain, the evidence suggests that interaction with host cellular factors, as well as binding to DNA, play roles in their functions.

1. Protein–Protein Interactions Involving the IE1/IE2 Proteins

a. IE2 86. Studies from our laboratory and others have shown that the IE2 86 protein can interact with itself, as well as with the components of the basal transcription complex TBP, TFIIB, and several TBP-associated factors (TAFs); the products of the tumor suppressor genes Rb and p53; an HCMV 75-kDa early protein (UL84); and a number of transcription factors including Sp1, Tef-1, c-Jun, JunB, ATF-2, NF-κB, protein kinase A-phosphorylated delta CREB, p300, CBP, Nil-2A, CHD-1, Egr-1, and UBF (Hagemeier *et al.,* 1992, 1994; Caswell *et al.,* 1993; Chiou *et al.,* 1993; Furnari *et al.,* 1993; Jupp *et al.,* 1993b; Lukac *et al.,* 1994, 1997; Sommer *et al.,* 1994; Spector and Tevethia, 1994; Speir *et al.,* 1994; Choi *et al.,* 1995; Lang *et al.,* 1995; Scully *et al.,* 1995; Schwartz *et al.,* 1996; Yoo *et al.,* 1996; F. Ruchti and D. J. Spector, unpublished, 1996). One concern with the above findings is that most of the interactions were observed either in cells where the proteins were overexpressed or in *in vitro* binding assays. Therefore any connection drawn between the protein–protein interactions and the transactivation properties of IE2 86 should still be regarded as informed guesswork.

b. IE1 72. IE1 72 has also been found to form a complex with multiple regulatory proteins including several TAFs, the transcription factor

CTF-1, and the Rb-related protein p107 (Hayhurst *et al.,* 1995; Poma *et al.,* 1996; Lukac *et al.,* 1997). As discussed below, IE1 72 can localize to PML-containing nuclear bodies (ND10 sites) and promote the dispersal of PML (Ahn and Hayward, 1997; Ishov *et al.,* 1997). It appears that the N-terminal region between aa 1 and 346 can mediate targeting to the ND10 sites and interaction with PML, but the C terminus is also required for PML displacement (Ahn *et al.,* 1998a).

2. Protein–DNA Interactions

Binding to DNA has been demonstrated for IE2 86 but not for IE1 72. As described above, early studies indicated that IE2 86 was able to repress its own promoter through the CRS element located between the TATAA box and the transcription start site on the major IE promoters (Pizzorno and Hayward, 1990; Cherrington *et al.,* 1991; Liu *et al.,* 1991). In addition to the CRS element, other binding sites for the IE2 86 protein have been identified just upstream of the TATAA box on three HCMV early promoters—one for the UL112/113 gene, a second for a gene in the long repeat that encodes an abundant 1.2-kb RNA, and a third for the gene encoding an early viral envelope glycoprotein of 48 kDa (ORF UL4) (Chang *et al.,* 1989a,b; Arlt *et al.,* 1994; Schwartz *et al.,* 1994; Huang and Stinski, 1995; Scully *et al.,* 1995). In the case of the UL112/113 promoter, there are three strong binding regions and a fourth weaker site (Arlt *et al.,* 1994; Schwartz *et al.,* 1994); the 1.2-kb RNA promoter contains one strong and one weak binding site (Scully *et al.,* 1995); and the UL4 promoter contains one strong IE2 86 binding site (Huang *et al.,* 1994). Transcription directed by the UL4 promoter in transient expression assays requires IE2 86 for its activation, and IE2 86 appears to function by counteracting the effect of a *cis*-acting negative element at this same site (nt −168 to −134) (Huang *et al.,* 1994). This is another example in which IE2 86 and a cellular factor may compete for binding to the same site, but in contrast to what may occur at the CRS element in the major IE promoter, IE2 86 may activate the UL4 promoter by displacing or competing for the binding of a negative cellular factor. One of the interesting features of the known IE2 86 binding sites is that there is no clear consensus sequence, although there is some similarity that is defined by the presence of CG residues at both ends of a 14-nt sequence that is AT-rich internally. For the weaker sites, the CG residues are present only at one end. These observations, coupled with the finding that IE2 86 binds to the minor groove of the DNA, raise the question of whether the IE2 86 protein may be site specific but sequence tolerant and recognize structural features of the DNA rather than specific sequences. It is

important to recognize, however, that a definitive role (or lack thereof) for the binding of IE2 86 to any of the above promoters in transcriptional regulation will require a careful analysis of both promoter and IE2 86 mutants in the context of the viral genome.

C. Delineation of Transacting Functions of IE1/IE2 Gene Products

A variety of *in vitro* and *in vivo* assays have been used to identify the functional activities of the IE1 and IE2 proteins. The most common approach has utilized the transient expression assay, whereby a plasmid containing a reporter gene under control of a viral or cellular promoter is transfected into cells along with an expression plasmid encoding one or more of the HCMV regulatory proteins. With these and other assays described below, defined mutations in the IE1/IE2 gene products have been used to identify the functional domains on the proteins. Although these experimental approaches have provided some useful information, they are limited in that they do not necessarily present a complete or even an accurate picture of the regulatory events that occur during a normal infection. Most agree that the best approach is to test mutants of these genes in the context of the viral genome. However, two major obstacles have precluded such studies: the difficulty of producing HCMV recombinants and the lack of complementing cell lines to propagate null mutants. As described below, there has been some initial progress in using this approach to study the function of the IE1 72 protein. However, many laboratories, including our own, have tried for several years to generate a complementing cell line expressing functional IE2 gene products. The uniform lack of success has led to the suspicion that the wild-type (wt) IE2 proteins may have a deleterious effect on the host cell. In support of this, a cell line expressing high levels of IE2 86 contains a protein that has minimal ability to activate an HCMV early promoter (Bonin and McDougall, 1997; our unpublished results, 1998).

1. In Vivo Transient Transfection Assays Used to Define the Transactivating Functions of Specific IE1/IE2 Proteins

With the above caveat in mind, the major strategy used to determine the role of each of the major HCMV IE proteins in early promoter activation has involved transient expression assays with specific effector and target plasmids. To illustrate the types of experiments performed and the results obtained, we will describe experiments carried out in our laboratory, in which eukaryotic expression vectors consisting

of individual cDNAs for each of the IE1/2 proteins under the control of the SV40 early promoter functioned as the effectors, and hybrid HCMV early promoter-CAT vectors that contained the promoter for the 2.2-kb family of RNAs (UL112/113) served as the target (Staprans *et al.,* 1988). We used the SV40 promoter to drive expression of the cDNAs, rather than the major IE promoter, to avoid the potential difficulty in interpreting the data as a result of downregulation of this promoter by IE2 86 and the ability of IE1 72 to alleviate this repression (Pizzorno *et al.,* 1988; Stenberg *et al.,* 1990; Cherrington *et al.,* 1991; Liu *et al.,* 1991). However, this has not proven to be a major complication, as Colberg-Poley and colleagues (1992) used genomic constructs engineered to express either the IE1 72 or the IE2 86 gene products under the direction of the major IE promoter and observed results similar to those described below.

For most studies, human fibroblasts or U373 cells, both of which are fully permissive for the HCMV infection, have been used as the host cells to measure the activation of a given viral promoter by a specific IE1/IE2 protein or combination of IE1/IE2 proteins following cotransfection (Klucher *et al.,* 1993; Schwartz *et al.,* 1994; Scully *et al.,* 1995). These experiments showed that IE2 86 was the major transactivator of the UL112/113 promoter, with levels of CAT gene expression stimulated 40- to 80-fold over the background observed in the absence of IE2 86. In contrast, a cDNA construct expressing the IE1 72 protein did not significantly activate transcription from this promoter. When both the IE1 72 and IE2 86 cDNA constructs were transfected together, only a small further increase in activation of the promoter was seen relative to the IE2 86 cDNA construct alone. By itself, the 55-kDa IE protein was unable to activate transcription from this early promoter. Moreover, when both the IE2 55 and IE2 86 cDNA constructs were transfected together, the level of early promoter activation was reduced three- to fourfold relative to that achieved by the IE2 86 cDNA construct alone.

Although the IE2 86 protein appears to be the major viral transactivator of the UL112/113 promoter, there is evidence that other viral proteins may also participate in its induction. This was first reported by Colberg-Poley and co-workers (1992), who found that in nonpermissive HeLa cells but not in permissive fibroblasts, cotransfection of constructs expressing the IE2 86 gene product and the UL36–38 gene products enhanced the level of activation seen with IE2 86 alone by approximately twofold. By themselves, however, the UL36–38 gene products had no measurable effect on this promoter in either cell type. Subsequently, Iskenderian and colleagues (1996) extended this observation

to show that expression from this promoter was enhanced in fibroblasts when a genomic construct encoding both the IE1 and IE2 proteins was cotransfected with constructs specifying the UL36–38 and IRS1 gene products (see below). Another HCMV early gene product, UL69, also appeared to be able to activate this promoter independently, albeit at a level that was significantly lower (10- to 15-fold) than that observed following transfection of the genomic construct expressing the IE1 and IE2 proteins (Winkler *et al.,* 1994). Although the ability of several different HCMV gene products to activate this promoter may simply represent functional redundancy, it seems more likely that it reflects the essential role of the proteins encoded by the UL112/113 gene (see below) and the importance of ensuring their expression in all cell types infected by HCMV.

2. Functional Similarity of IE1/IE2 Proteins to TAFs

Recently, Lukac and colleagues (1997) proposed the interesting hypothesis that the function of the major IE1/IE2 proteins might be similar to that of the TAFs. The basis of this hypothesis is derived from their observation that these IE proteins copurify with TFIID isolated from infected cell nuclei, coimmunoprecipitate with purified TFIID, and interact *in vitro* with a number of TAFs. IE1 72 interacts with *Drosophila* $TAF_{II}40$, and both IE2 86 and IE1 72 can form a complex with *Drosophila* $TAF_{II}110$ and human $TAF_{II}130$. Although there were background problems, it also appeared that IE1 72 and IE2 86 could bind simultaneously to this latter protein *in vitro.* Additional support for this hypothesis was suggested by the finding that IE2 86, with help provided by IE1 72, could rescue a temperature-sensitive transcriptional defect in $TAF_{II}250$ in the BHK-21 cell line *ts*13. The authors propose that IE1 72 may be able to augment the activation provided by IE2 86 through its simultaneous binding to the TAFs. One intriguing possibility, in view of the observed interaction of IE2 86 with CBP and p300 (both of which are histone transacetylases), is that IE1/IE2 and their associated proteins are providing the histone transacetylase activity normally provided by $TAF_{II}250$. An interaction between IE1/IE2 proteins and the TAFs could also account for negative effects on transcription when an IE2 86 DNA binding site is also present at the transcription start site because the combined interaction of IE2 86 with the TAFs and the DNA might provide significant steric hindrance for the binding of RNA polymerase II to the preinitiation complex (as in the case of binding to the CRS). Although a model in which the IE1/IE2 proteins are functionally similar to TAFs is an interesting one, several problems arise in the interpretation of some of the data. First,

the rescue of this temperature-sensitive defect in the BHK-21 cell line *ts*13 is based on examination of the cyclin A promoter in transient expression assays for restored transcriptional activity. Yet, we have found that transcription of cyclin A mRNA is inhibited in the context of the viral infection (Salvant *et al.,* 1998). Second, this BHK-21 cell line is generally nonpermissive, and therefore, the major IE promoter may not act as it normally does under infection conditions. Finally, because other viral proteins that may interact with the IE1/IE2 proteins are not present in these experiments, it is difficult to extrapolate the findings to what may actually take place in the context of an infected cell.

3. Interactions of IE1 72 with p107; Disruption of E2F Complexes

One potential pathway that may aid in establishing the activated state necessary for HCMV replication involves the transcription factor E2F, whose binding sites are present in the promoters of many genes essential for cellular DNA synthesis and entrance into S phase. The results of several studies show that this pathway is affected, but it is still not clear which players interact directly with viral proteins. The early transient transfection assays in fibroblasts with the DHFR promoter as the target suggested that the E2F sites in this promoter were important for activation by the immediate early gene products, particularly IE1 72 (Wade *et al.,* 1992). In addition, it was subsequently shown that IE1 72 could be coprecipitated with E2F1 from infected cell lysates and could bind to GST-E2F1 (Margolis *et al.,* 1995). Interestingly, the formation of the complex between E2F1 and IE1 72 *in vitro* occurred only in the presence of an uninfected cell nuclear extract, indicating that the interaction of IE1 72 with E2F1 might be indirect and involve some other cellular protein(s) that served as a bridge by binding to both IE1 72 and E2F1.

Two of the cellular proteins that may interact directly with IE1 72 and function in the activation of promoters through their E2F sites are the retinoblastoma family members p107 and p130. Poma and colleagues (1996) have shown that p107 and IE1 72 form a complex *in vitro* and can be coprecipitated from infected cells at 24 hpi (the interaction appears to be maximal at this point). They have proposed that phosphorylation of IE1 72 may drive this interaction and that it is the job of IE1 72 to interact with p107 to relieve the repression of promoters containing E2F sites. The notion that HCMV infection leads to phosphorylation of pocket proteins and the associated relief of repression of E2F-containing promoters is supported by the following findings. First, it has been shown that *in vitro* His-tagged IE1 72 can phosphory-

late itself in addition to phosphorylating GST-E2F-1, -2, or -3, p107, and p130, but not GST-Rb or GST-E2F-4 or -5. Second, a mutant IE1 72 lacking the ATP-binding domain and kinase activity cannot transactivate the DHFR promoter in transient assays. Third, the *in vitro* interaction between E2F4 (which cannot be phosphorylated by IE1 72) and either GST-107 or -130 can take place only in the presence of mutant IE1 72. Moreover, electrophoretic mobility shift assays using purified proteins and the DHFR promoter show a complex with E2F4/DP1 and a more slowly migrating complex when p107 or p130 is added (Pajovic *et al.,* 1997). This latter complex is dissociated, at least in part, in the presence of prephosphorylated IE1 72 protein. As is the case for many transient assay situations, these experiments using IE1 72 alone must be interpreted with caution, as the complex interplay between this protein and other viral gene products may enhance or preclude these reported interactions during the actual infection.

4. Interaction of IE1/IE2 Proteins with ND10 Sites; Dispersal of PML

As discussed in Section IV, the input HCMV virion DNA enters the nucleus and associates with ND10 sites where IE RNAs are transcribed. At approximately 2 hpi, newly synthesized IE1 and IE2 proteins can be detected in the nucleus, where they colocalize with the preexisting ND10 sites. Earlier work by Maul and co-workers (1993) described a dispersal of the ND10-associated proteins Sp100 and PML by the HSV-1 IE protein ICP0. Relocalization of these proteins was also shown to occur during an Ad5 infection (Maul *et al.,* 1996). Two groups have shown that, true to form, HCMV also disperses these proteins. Although IE2 86 maintained the punctate staining pattern characteristic of ND10 sites for the duration of their experiments (6–12 hpi) (Ahn and Hayward, 1997; Ishov *et al.,* 1997), beginning at the 2- to 3-hpi period, 50% of the cells showed a diffuse nuclear staining pattern for the IE1 72 protein, and by 4 hpi, dispersal was complete for IE1 72 (Ahn and Hayward, 1997). PML-containing ND10 sites also appeared to undergo disruption, resulting in a diffuse pattern of staining for PML that paralleled that of IE1 (Kelly *et al.,* 1995; Korioth *et al.,* 1996; Ahn and Hayward, 1997). A close examination of the punctate sites prior to disruption revealed that IE2 was localized to a very small number of the much more abundant IE1/ND10 spots. From these observations, it has been proposed that at early times both IE1 and IE2 localize to the ND10 sites (IE2 to a subset). IE1 begins disrupting the ND10-associated proteins at about 3 hpi, and by 6 hpi both ND10-associated proteins and IE1 are completely dispersed throughout the nucleus.

However, the IE2 stays associated with the initial ND10 sites until at least 6–12 hpi. Transfection of either IE1 or IE2 alone produced an effect similar to that seen during infection, with IE2 localizing to the ND10 sites but unable to disrupt them, and IE1 capable of both colocalizing with and dispersing the ND10-associated proteins (Ahn and Hayward, 1997; Ishov *et al.,* 1997). Interestingly, it has been found that following a high multiplicity infection with a mutant recombinant virus unable to express IE1 72, IE2 86 still localizes to the ND10 sites but is unable to disperse the associated proteins. As noted below, under these conditions, the mutant virus replicates with wild-type efficiency. Therefore, although the localization of IE2 86 to the ND10 sites may play a role in establishing the optimal environment for the HCMV infection to prosper, the localization of IE1 72 to these sites and the ensuing dispersal of the ND10 associated proteins does not appear to be essential (Ahn *et al.,* 1998a).

5. Interactions of IE2 86 with Tumor Suppressor Proteins

a. Interactions with Rb. The tumor suppressor protein Rb plays a pivotal role in regulating cell cycle progression and differentiation (for review, see Weinberg, 1995). A key to Rb's growth regulatory function is its ability to form a complex with and inhibit the activity of the transcription factor E2F (Qian *et al.,* 1992; Qin *et al.,* 1992; Hiebert, 1993). Rb, like IE2 86, has at least two independent protein binding domains (for review, see Wang *et al.,* 1994; Taya, 1997; Wang, 1997). The first is the A/B pocket (aa 379–772), identified as the domain involved in complex formation with several viral oncoproteins and cellular proteins containing an LXCXE binding motif. Expression of this A/B pocket domain, however, is not sufficient for Rb to exert growth suppression; this also requires the adjacent C-terminal residues up to aa 869. This entire region (aa 379–869) is required for binding to D-type cyclins and the E2F family members. Another protein binding domain (the C pocket) (aa 768–869) interacts with c-abl and mdm2. Although it is still unclear how these various protein binding domains interact *in vivo,* Wang (1997) presents evidence that the minimal domain for growth suppression can potentially accommodate three separate Rb binding proteins—an LXCXE protein, E2F, and a C-pocket binding protein.

Several studies have shown an interaction *in vitro* between IE2 86 and Rb (Hagemeier *et al.,* 1994; Sommer *et al.,* 1994; Choi *et al.,* 1995; Fortunato *et al.,* 1997). The interacting domains on both proteins have been mapped. Three independent domains on IE2 86 can interact with Rb when expressed as GST fusion proteins (aa 86–135, 136–290 and

291–364) (Sommer *et al.,* 1994). We have also shown that IE2 86 can interact independently with both the A/B pocket and the C pocket of Rb (Fortunato *et al.,* 1997).

One approach to address the functional significance of the Rb/IE2 86 interaction has involved cotransfection experiments. In this way, Hagemeier and colleagues (1994) showed that Rb-mediated repression of an E2F site-containing reporter construct could be ameliorated by the addition of IE2 86 and that the Rb binding site on IE2 86 was needed for this derepression. Rb could also affect the functioning of IE2 86, as Rb was able to abolish IE2 86-mediated repression of a major IE promoter-driven reporter construct. In addition, using heterologous promoter constructs (driven by SV40, MMTV, and LTR promoters) as targets, Choi and colleagues (1995) showed that the transactivating ability of IE2 86 could also be downregulated when Rb was coexpressed.

Our laboratory has used a different approach to assess the biological consequences of the interaction between Rb and IE2 86. In our studies, we have used the human osteosarcoma cell line Saos-2, which lacks functional Rb and undergoes cell cycle arrest in G_1 (as measured by FACS analysis) and an increase in cell size when wild-type Rb function is restored. One common assay for determining the functional interaction between Rb and another protein involves cotransfection of an expression vector for Rb, a puromycin resistance plasmid, and a construct expressing the protein to be tested (Huang *et al.,* 1988; Templeton *et al.,* 1991; Hinds *et al.,* 1992). Cells that express wild-type Rb (and the selectable puromycin resistance gene) do not divide but maintain protein synthesis, thereby remaining resistant to puromycin and appearing as large, flat single cells. When a protein to be tested is cotransfected along with Rb, one of two phenotypes is observed. If the protein cannot counter the growth-suppression block imposed by Rb, the cells remain flat and comparable in number to cells transfected with Rb alone. However, if the cotransfected protein can somehow interfere with Rb function, the cells divide (or die), thus reducing the number of flat cells.

We demonstrated that both IE2 86 and IE1 72 could counter the enlarged flat cell phenotype that results from expression of wild-type Rb in Saos-2 cells. Mutational analysis revealed that there were two domains on IE2 86 that could independently affect Rb function. One region (aa 241–369) included the major Rb binding domain, and the second mapped to the amino terminal region (aa 1–85) common to both IE2 86 and IE1 72. Interestingly, neither of the IE1/IE2 proteins could overcome the G_1 block brought about by transient expression of Rb in these cells.

All of the above *in vivo* studies point to a variety of roles for the IE2 86–Rb interaction during infection. Given the *in vitro* evidence suggesting a preference of IE2 86 for unphosphorylated Rb (Hagemeier *et al.,* 1994), any interactions between the proteins in the course of infection would have to occur very early, as the Rb protein is hyperphosphorylated by 12 hpi (Jault *et al.,* 1995). Analogous to interactions of other DNA virus proteins, the interaction of IE2 86 with Rb may be necessary for the accumulation of free E2F, followed by activation of E2F-regulated promoters, including those directing expression of cell cycle-related factors required for infection. The experiments showing that IE2 86 can relieve Rb-mediated repression of an E2F site reporter construct in transient expression assays certainly supports this theory (Hagemeier *et al.,* 1994). In addition, the ability of IE2 86 to bind independently to both the A/B pocket and the C-terminal domain of Rb may allow a greater number of cellular regulatory factors to dissociate from Rb and become functionally active. Alternatively, the IE2 86–Rb interaction may block Rb-mediated complex formation. Interaction between Rb and IE2 86 may also compromise IE2 86 function, as was seen in transient expression assays using IE2 86, Rb, and either the major IE promoter or several heterologous promoters normally induced by IE2 86 (Hagemeier *et al.,* 1994; Choi *et al.,* 1995). The ability of IE2 86 to associate with either region of Rb may also facilitate interactions of the HCMV protein with other Rb binding proteins. One potential reason for the disparity in the ability of the IE1/IE2 proteins to overcome the flat cell but not the G_1 arrest phenotypes may in fact reflect the ability of the IE1/IE2 proteins to interrupt certain complexes involving Rb (or Rb family members) but not others. Although each of these mechanisms is possible, there are still no definitive data to distinguish between these or other models.

b. p53/IE2 86 Interactions. The tumor suppressor protein p53 has been called the "guardian of the genome" because of its involvement in the regulation of the G_1 and G_2 checkpoints (reviewed in Gottlieb and Oren, 1996). Loss of p53 function is associated with a broad spectrum of human cancers (Hollstein *et al.,* 1991), and although mice lacking the *p53* gene are viable, they are susceptible to multiple types of tumors at a young age (Donehower *et al.,* 1992). Levels of p53 protein are elevated in response to exposure to UV or gamma ray irradiation, extreme heat, hypoxia, and stress (reviewed in Ko and Prives, 1996). Increased levels of p53 usually result in either cell cycle arrest, presumably to allow repair of damaged DNA, or apoptosis. Control of which outcome occurs hinges on p53-dependent sequence-specific binding and transactivation of particular target genes, including *p21*(El-Deiry *et*

al., 1993), *GADD45* (Kastan *et al.,* 1992), *cyclin G* (Okamoto and Beach, 1994), *IGF-BP3* (Buckbinder *et al.,* 1995), *bax* (Miyashita and Reed, 1995), and *mdm2* (Juven *et al.,* 1993). In addition to p53's regulation of the DNA damage response through transcriptional activation of particular target genes, there is increasing evidence that it participates in this response by binding to damaged regions with its C-terminal domain and recruiting the repair machinery to the site via protein–protein interactions. To this end, p53 interacts directly with several proteins involved in nucleotide excision repair and/or homologous recombination, including the two TFIIH components XPB and XPD (Wang *et al.,* 1995), CSB (Wang *et al.,* 1995), RAD51 (Sturzbecher *et al.,* 1996), and RPA (Dutta *et al.,* 1993; Sturzbecher *et al.,* 1996; Miller *et al.,* 1997).

Several lines of evidence point to interactions between HCMV and p53. Steady-state levels of the protein increase dramatically by 24 hpi when cells of different origins (fibroblasts and smooth muscle cells) are infected at multiplicities of infection (MOIs) as low as 0.5 (Muganda *et al.,* 1994; Speir *et al.,* 1994; Jault *et al.,* 1995); however, activation of particular target proteins, namely, p21 (Jault *et al.,* 1995; Bresnahan *et al.,* 1996) and mdm2 (our unpublished results, 1998), does not occur in infected cells. Like several other viruses, HCMV encodes a protein, IE2 86, that has the ability to bind p53 both *in vitro* (Speir *et al.,* 1994) and in coprecipitation experiments performed in IE2 86-overexpressing cells (Bonin and McDougall, 1997). Muganda and co-workers (1994) have also argued that expression of IE1/IE2 is enough to increase the steady-state levels of p53 in transiently transfected fibroblasts.

The functional significance of the interaction between p53 and IE2 86, however, is under debate. Transient cotransfection assays show the ability of IE2 86 to downregulate p53 activation of a reporter construct (Speir *et al.,* 1994); however, the arrest in G_1 brought about by treatment of cells with actinomycin D is intact within the overexpressing cells mentioned above, complete with upregulation of both p53 and its target proteins' steady-state levels. The functionality of the IE2 86 within these overexpressing cells is questionable, as the ability of this protein to either repress the major IE promoter or activate an HCMV early promoter is only twofold at best, which is greatly reduced from what is seen within the context of the infection. However, it may be, as the authors suggest, that the full effects on p53 cannot be achieved without the expression of other viral proteins present during a permissive infection.

6. Potential Role for UL84 Early Protein in Regulation of IE2 86

As mentioned above, IE2 86 can interact with the early protein UL84 (Gebert *et al.,* 1997). The two proteins are coimmunoprecipitated from

infected cell lysates, regardless of the phosphorylation state of IE2 86, and can form a complex *in vitro* and in cotransfection experiments in the absence of other viral proteins, indicating a direct interaction (Spector and Tevethia, 1994). Like the cellular factor Rb, UL84 appears to be able to interfere with the ability of IE2 86 to transactivate heterologous promoters. However, in contrast to Rb, UL84 augments IE2 86-mediated repression of the major IE promoter in a dose-dependent manner in cotransfection assays. In experiments designed to assess the importance of this effect of UL84 binding, UL84-overexpressing cell lines were derived and subsequently infected with AD169 (Gebert *et al.,* 1997). IE expression was observed, but infection did not progress further; no early gene expression or production of virus was obtained, suggesting to the authors that UL84 interaction with IE2 86 was important for regulation of IE function within the infected cell. Although this possibility is intriguing, a few things are troubling about these experiments. UL84 is being temporally expressed at an earlier time than it would be observed in a normal infection, and thus proteins that might normally prevent an interaction between UL84 and IE2 86 may not yet be expressed within these cells. Moreover, the coprecipitation experiments in the wild-type infected cells suggest that the greatest interaction between these two proteins occurs at around 48 hpi, much later than the proteins interact in the UL84-overexpressing cells. The studies therefore use an artificial environment at early times postinfection to show that the interaction between the two proteins could have deleterious effects on the infection.

7. *Potential Mutagenic Effects of IE1/IE2*

Studies of HCMV infection in nonpermissive cells have indicated that HCMV can act as a mutagen. Boldogh and co-workers (1992) isolated high molecular weight DNA from human embryonic lung fibroblasts infected with a partially UV-irradiated HCMV strain and found that transfection of infected cellular DNA (isolated prior to the onset of viral replication) into hamster embryo fibroblasts resulted in transformation of the recipient cells, as assayed by an increase in focus formation. This appeared to be contingent on expression of the IE1/IE2 proteins within the initially infected cells, but no viral sequences were found within the transformed foci. Similar results were obtained by Shen and co-workers (1997), who found that coexpression of the IE1/IE2 proteins with the adenovirus E1A gene products could transform primary BRK cells. However, the IE1/IE2 proteins could not form foci alone or complement an activated Ras protein in BRKs. As described above, cell lines derived from foci showed no signs of either IE1/IE2 protein or viral DNA. In addition, the authors found that expression

of the IE1/IE2 genes in Chinese hamster cells hemizygous at the *hprt* and *aprt* loci resulted in an increased frequency of mutations at these loci. Moreover, they found mutations in the p53 protein in 60% of the E1A/IE transformants, a result not usually seen in transformants produced by E1A alone. Others have also found that infection of Chinese hamster cells with HCMV can cause an increase in mutation frequency at the *hprt* locus (Albrecht *et al.,* 1997). This required active viral particles, and again, colonies formed from these cells had no viral DNA or proteins. Taken together, these results suggest that the IE1/IE2 proteins of HCMV possess the potential for "hit and run" mutagenesis.

8. Functional Domains of the IE2 86 Protein

To delineate the functional domains of the IE2 86 protein, we and others have constructed a large number of single-site and deletion mutants of this protein and have tested them with respect to autoregulation, DNA binding, promoter activation, and protein–protein interaction. The general consensus is that the carboxy-terminal half of the IE2 86 protein (aa 290–579) is responsible for autoregulation and DNA binding to its own promoter and to HCMV early promoters (Pizzorno *et al.,* 1988; Hermiston *et al.,* 1990; Pizzorno and Hayward, 1990; Stenberg *et al.,* 1990; Chiou *et al.,* 1993; Furnari *et al.,* 1993; Jupp *et al.,* 1993a,b; Lang and Stamminger, 1993; Macias and Stinski, 1993; Arlt *et al.,* 1994; Jenkins *et al.,* 1994; Schwartz *et al.,* 1994; Sommer *et al.,* 1994; Scully *et al.,* 1995; Ahn *et al.,* 1998b). The IE2 55 protein (missing aa 365–519) and site-specific mutations in the putative zinc finger domain of the IE2 86 protein (aa 428–452) fail to autorepress or bind to DNA and in some assays can even stimulate transcription from the major IE promoter (Pizzorno *et al.,* 1991; Baracchini *et al.,* 1992; Jupp *et al.,* 1993b; Macias and Stinski, 1993; Wu *et al.,* 1993; Schwartz *et al.,* 1994).

The studies directed at mapping the activation domains of the IE2 86 protein have yielded results that are somewhat variable and depend on the target promoter used and, to a lesser extent, on the host cell. Some have found that only sequences between aa 170 and 579 are needed to activate heterologous viral promoters (Hermiston *et al.,* 1987; Yeung *et al.,* 1993). In contrast, for some heterologous viral promoters and HCMV early promoters, it appears that both this region and the first 98 aa are required for activation (Malone *et al.,* 1990; Stenberg *et al.,* 1990; Pizzorno *et al.,* 1991; Sommer *et al.,* 1994; Scully *et al.,* 1995). In our studies, HCMV early promoters driving expression of the UL112/113 gene products and the 1.2-kb RNA were used as targets, and primary fibroblasts and U373 cells served as the host cells. These experi-

ments showed that activation of both promoters by IE2 86 required the sequences within aa 26–85 and 290–579 and that stimulation of the 1.2-kb RNA promoter also required aa 86–135 (Sommer *et al.,* 1994; Scully *et al.,* 1995).

More precise mapping studies have shown that the regions around aa 359 and 540 within the carboxy-terminal half of the IE2 86 protein play a particularly important role in activation, as evidenced by the demonstration that insertion of four aa at either site significantly reduces stimulation of the early promoter for the viral DNA polymerase (UL54) (Stenberg *et al.,* 1990). Preliminary studies in our laboratory also indicate that the amino acid substitutions R356A, R357A, R359A, I493E, C509M, C509D, or M533I render the IE2 86 protein nonfunctional with respect to activation of the UL112/113 promoter. In addition, the results of domain swap experiments with Gal4 fusion constructs have further delineated two activation domains on the IE2 86 protein, one within aa 544–579 and the other within aa 25–85 (Pizzorno *et al.,* 1991).

As discussed above, the IE2 86 protein homodimerizes and binds to a number of cellular regulatory proteins that are important for RNA transcription. To date, all of these interactions appear to map to the middle of the protein (aa 86–542). Dimerization of the IE2 86 protein requires aa 388–542, which includes the region containing the putative zinc finger (aa 428–452) and a helix–loop–helix motif (aa 463–513) (Chiou *et al.,* 1993). In our studies, to determine the domains of the IE2 86 protein involved in interacting with TBP, Rb, TFIIB, c-Jun and JunB, we used an approach that involved measuring not only the binding of *in vitro* translated segments of IE2 86 to the various glutathione *S*-transferase (GST)–cell factor fusion proteins, but also the binding of the *in vitro* translated cellular factors to the GST-IE2 86 deletion mutants (Sommer *et al.,* 1994; Scully *et al.,* 1995; M. H. Sommer and D. H. Spector, unpublished). In this way, it was possible to identify three distinct regions on the IE2 86 protein (aa 85–135, aa 136–290, and aa 291–364) that, when present as a GST–fusion protein, could mediate interaction with the *in vitro* translated cellular factors. However, only the domain in IE2 86 between aa 291 and 364 was functional when IE2 86 was *in vitro* translated; aa 85–135 were nonfunctional, and aa 136–290 inhibited binding to the cellular factors. Preliminary evidence suggests that the phosphorylation of aa 136–290 in the *in vitro* translation extracts (probably by casein kinase II) is responsible for this inhibition. These results indicate that the phosphorylation state of the IE2 86 protein may influence its ability to engage in protein–protein interactions. In this regard, Harel and Alwine (1998)

presented data suggesting that mutation of several potential ERK2 phosphorylation sites in IE2 86 affects its transactivating ability in transient transfections.

Others have also found that the region of IE2 86 from aa 290 to aa 360 is essential for protein–protein interactions, but they have observed some differential requirements for sequences carboxy-terminal to aa 365 for binding of the *in vitro* translated IE2 86 deletion mutants to GST-TBP, GST-Rb, or GST-TFIIB (Caswell *et al.,* 1993; Hagemeier *et al.,* 1994). In related studies, it has also been shown that IE2 86 protein can both stabilize TBP binding to the major IE promoter (nt −43 to −19) and bind to the TBP–DNA complex (Jupp *et al.,* 1993a). From deletion analysis, it appears that the region between aa 142 and aa 153 is required for both functions, but other amino- and carboxy-terminal domains can also independently stabilize the binding of TBP to the TATAA box. It is possible that this stabilization is the result of IE2 86–TBP interactions that are occurring in solution.

9. In Vitro Transcription Assays

In order to study the function of the IE1/IE2 gene products in *in vitro* transcription assays, it was necessary to obtain large quantities of relatively pure protein. This was accomplished by cloning the cDNA constructs expressing the IE1 72, IE2 86, and IE2 55 proteins into vectors allowing their expression in *Escherichia coli* as fusion proteins containing GST at their amino terminus. The fusion proteins or GST alone were purified by affinity chromatography and added to nuclear protein extracts from U373 or HeLa cells along with a plasmid construct consisting of the UL112/113 promoter linked to a reporter gene. The results obtained from these *in vitro* transcription studies have correlated well with those obtained *in vivo* (Klucher *et al.,* 1993). The basal activity of the UL112/113 promoter was minimal in both the HeLa and U373 extracts but was stimulated 6- to 10-fold by the IE2 86 protein. Additional evidence of specificity was provided by the demonstration that a 37-aa carboxy-terminal deletion mutant of the IE2 86 protein was unable to activate the promoter in either *in vivo* transient expression assays or *in vitro* transcription assays. In these experiments, the IE1 72 protein by itself was unable to activate transcription from this promoter, and the presence of both the IE2 86 and IE1 72 proteins gave no significant further increase in transcription relative to that with the IE2 86 protein alone.

D. Importance of IE1 72 in Growth and Replication of HCMV

Greaves and Mocarski (1998) were the first to directly address the function of IE1 72 in the context of the infection. With the use of a

complementing cell line expressing the IE1 72 protein, they were able to derive a mutant virus that lacked exon 4 of the IE1/IE2 region and therefore produced no IE1 72 protein. When this virus was used at low MOI to infect fetal lung fibroblasts, there was a marked decrease in replication. Nine days postinfection, titers were reduced by 3 orders of magnitude relative to wt titers, and the cells exhibited significantly less CPE. In addition, the ability of the mutant virus to form normal-sized plaques and the number of infected cells within these plaques decreased. All of these phenotypes were rescued by restoration of exon 4 to the virus. A key finding of this study was that fibroblasts infected with mutant virus at a low MOI did not express UL44 (a processivity factor for the HCMV DNA polymerase), establish viral replication centers, or incorporate BrdU into these centers to any significant extent. Thus it appeared that IE1 72 played a key role in transactivating at least one early gene that is responsible for establishing viral replication, namely, UL44. Whether the effect of IE1 72 is promoter specific is still unknown and is an important question to address. It may be that IE1 72 only activates promoters driving genes involved in establishing replication centers. Alternatively, IE1 72 may function as a more general activator of a wide range of early viral genes. Regardless of its precise function, IE1 72 is clearly a key factor in promoting the early events of infection. However, the IE1 72 gene product is not absolutely essential for the viral infection, and its absence can be compensated for by infecting cells with mutant virus at a high MOI. Under these conditions, the infection proceeds normally and the mutant virus replicates to wild-type levels. Whether the compensating function is provided by virally encoded or virally induced cellular proteins remains to be determined. However, it is unlikely that the viral transactivator IE2 86 is providing this compensation, as this protein is present at wild-type levels when the cells are infected at either low or high MOI.

VII. Other IE Gene Products

A. IRS1/TRS1

At the beginning of the infection, the *irs*1 and *trs*1 genes give rise to transcripts of 3.5 kb and 2.7 kb that initiate from within the internal and terminal repeats bounding the short segment of the viral genome (see Fig. 1). The transcripts extend into both ends of the unique region of this segment (Weston and Barrell, 1986; Rasmussen, 1988; Stasiak and Mocarski, 1992) and specify proteins, 846 aa and 788 aa in size, that are present in the virions (Romanowski *et al.,* 1997). These proteins are identical for the first 549 aa and have significant similarity in their

carboxy-terminal region. It has been found that the *irs*1 unique region can also give rise to a smaller RNA that encodes a 263-aa protein (Romanowski and Shenk, 1997). It appears that the two larger proteins localize to both the nucleus and the cytoplasm at IE and early times in the infection but reside primarily in the cytoplasm at late times, becoming part of the tegument of the virion. In contrast, the smaller protein remains in the nucleus throughout the infection.

Studies using transient expression assays have shown that either the IRS1 or the TRS1 gene product is required for complementation of HCMV origin-dependent (*ori*Lyt-dependent) replication (Pari and Anders, 1993; Pari *et al.,* 1993). This complementation assay was developed by Pari and Anders (1993) and was used to define 11 loci that encode factors required for the replication of a plasmid containing the HCMV origin of DNA replication. Included in this set were the genes for the viral DNA polymerase (UL54), a polymerase accessory protein (UL44 or ICP36), a single-stranded DNA binding protein (UL57), three proteins homologous to the HSV-1 helicase–primase subunits (UL70, UL102, UL105), the UL112/113 gene products, the early protein UL84, and the IE transactivator proteins IE1/IE2 (UL122–123), UL36–38 and IRS1/TRS1.

The actual role of the IRS1/TRS1 proteins in viral DNA replication remains to be elucidated, but one hypothesis stems from their observed ability to cooperate with the IE1 and IE2 gene products to activate the early gene promoters required in a DNA replication complementation assay (Stasiak and Mocarski, 1992; Iskenderian *et al.,* 1996; Kerry *et al.,* 1996). It is also possible that the larger IRS1 or TRS1 gene products present in the virion may function as activators at IE times. Either protein can modestly activate the IE1/IE2 promoter in transient expression assays, and this activity is significantly augmented by another virion protein, the UL69 gene product (Romanowski *et al.,* 1997). Cotransfection of the genes expressing UL69 and either IRS1 or TRS1 can also stimulate the IRS1/TRS1 promoter, although neither the IRS1 nor the TRS1 protein alone has any effect on its own promoter. Somewhat surprisingly, the smaller 263-aa protein does not have an activator function, but rather appears to counter the transactivating functions of IE1 and IE2 when they are acting alone or in conjunction with the IRS1 and TRS1 gene products (Romanowski and Shenk, 1997). However, the fact that the entire IRS1 region can be deleted with no apparent effect on the replication of the virus in tissue culture (Jones and Muzithras, 1992) raises the question of whether the smaller protein actually plays a significant role during the infection. Alternatively, this smaller protein may be another example of a gene product whose

regulatory role is either redundant or restricted to a specific target cell *in vivo.*

B. UL36–38

The UL36–38 genes specify at least four IE transcripts from three promoters (see Fig. 1). One of the promoters directs the synthesis of a spliced 3.4-kb RNA (UL37 ORF) that is present only at IE times, as well as a 1.7-kb unspliced RNA (UL37exon1) that is present throughout the infection. The second IE promoter is responsible for the synthesis of a 1.65-kb spliced RNA (UL36 ORF) that increases in abundance at early times and is 3′ coterminal with the 3.4-kb RNA (Kouzarides *et al.,* 1988; Tenney and Colberg-Poley, 1991a,b). In addition, there is an early transcript of 1.35 kb (UL38 ORF) specified by this region. In contrast to the major IE and US3 promoters (see below), the promoter for the UL37 and UL37exon1 ORF does not seem to be negatively regulated. The evidence for this is derived from the observation that when protein synthesis is inhibited, only the level of UL37 RNA, and not that of UL37exon1 RNA, increases. However, it is also possible that the relative levels of these two RNAs may be controlled posttranscriptionally by an unstable protein.

The UL37exon1, UL37, and UL36 proteins all have regulatory activity, and the locus encoding these proteins is required for HCMV DNA replication in the transient complementation assay described above (Colberg-Poley *et al.,* 1992; Pari *et al.,* 1993; Tenney *et al.,* 1993; Smith and Pari, 1995; Iskenderian *et al.,* 1996; Zhang *et al.,* 1996). Further supporting data show that antisense oligonucleotides to either the UL36 or UL37 RNAs inhibit the replication of HCMV (Pari *et al.,* 1995; Smith and Pari, 1995). The UL37exon1 protein and the UL37 *N*-glycoprotein are encoded by overlapping transcripts and have a common amino terminus of 162 amino acids. Within this common domain is a hydrophobic signal sequence (aa 1–22) and an acidic domain (aa 81–108). The UL37 glycoprotein also has 17 *N*-glycosylation sites, a basic domain, a transmembrane region, and a cytosolic tail. Experiments by Colberg-Poley and co-workers (1998) indicate that the acidic domain common to both proteins plays a role in the activation of HCMV early gene promoters directing the synthesis of viral gene products required for viral DNA replication.

Interestingly, the regulatory properties of the UL36–38 gene products were first identified using the hsp70 promoter. Yet, it appears that this promoter is uniquely activated in transient expression assays by

the UL37 glycoprotein, a membrane-bound protein that does not appear to localize to the nucleus. As discussed below, further activation of the hsp70 promoter is also observed when UL37 is cotransfected with either IE1 72 or the US3 gene product (another glycoprotein resident in the endoplasmic reticulum) (Zhang *et al.,* 1996). Surprisingly, the acidic domain is not required for transactivation of the hsp70 promoter by gpUL37 alone, but is required for synergistic activation by UL37 and US3 in cotransfection experiments. A possible explanation for these divergent and interesting results is presented below.

C. US3

The US3 gene gives rise to a 0.9-kb unspliced RNA and several multiply spliced transcripts that encode glycoproteins residing in the endoplasmic reticulum (Weston, 1988; Tenney *et al.,* 1993; Ahn *et al.,* 1996). Although the US3 gene products do not seem to be essential for replication of the virus in tissue culture, they may play an important role in the ability of the virus to evade the immune response (Kollert-Jons *et al.,* 1991; Jones and Muzithras, 1992; Greaves *et al.,* 1995). The evidence for this is derived from the observation that the US3 protein product binds and retains MHC class I chains in the endoplasmic reticulum (Ahn *et al.,* 1996; Jones *et al.,* 1996).

It has also been shown that the US3 protein can function as a weak transactivator, alone or in conjunction with the UL37 gene product, but this effect appears to be restricted to the hsp70 promoter (Colberg-Poley *et al.,* 1992; Tenney *et al.,* 1993). As noted above, the UL37 protein has an acidic domain that is necessary for its synergism with US3 (Zhang *et al.,* 1996). It is possible that UL37 and US3 function as part of a signal tranduction pathway, with one domain of the UL37 protein interacting with US3 and another region interacting with a cellular protein that leads to activation of the hsp70 promoter. In fact, it has been suggested (Ahn *et al.,* 1996) that this induction might be the result of a cellular stress response initiated by accumulation of US3, and possibly UL37, in the endoplasmic reticulum. This stress response might then lead to activation of transcription factors specific for the hsp70 promoter.

Upstream of the US3 gene is a complex regulatory region that is subject to both negative and positive controls (Weston and Barrell, 1986; Weston, 1988). Between nt -259 and -84 relative to the start site of transcription are five copies of an 18-bp element (Thrower *et al.,* 1996). In transient transfection assays this region positively regulates

transcription and is responsive to NF-κB (Weston, 1988; Chan *et al.,* 1996a; Thrower *et al.,* 1996). Further upstream between nt −560 and −340 are seven copies of a 10-bp palindromic sequence (R1) and three copies of a 7-bp degenerate interrupted core of R1 that together serve to repress transcription (Thrower *et al.,* 1996). Gel shift analysis has revealed that cellular proteins bind to a fragment of DNA containing half of the palindromic R1 sequence (the pentanucleotide 5′ TCTGC 3′), as well as to a fragment containing two of these pentanucleotides arranged 5′ to 5′ but not arranged 3′ to 3′ (the wt configuration). Interestingly, the formation of the 5′ to 5′ specific DNA–protein complex was sensitive to dephosphorylation, and this complex was lost when extracts were prepared from infected cells at the time of maximal transcription of the US3 gene. It has also been noted that this pentanucleotide sequence is clustered in the modulator of the HCMV IE1/IE2 gene promoter between nt −1145 and −760, but no functional activity has been ascribed to it in this position.

In addition to the 18- and 10-bp repeat elements, transcription from the US3 gene is affected negatively by a sequence (designated the tre) between nt −18 and +1 that functions in a position-dependent but orientation-independent fashion (Biegalke, 1995; 1998). Because this *cis*-acting element has no negative effect on viral RNA levels when protein synthesis inhibitors are present from the beginning of the infection, it has been suggested that a viral IE protein or a cellular factor that is either unstable or induced by the infection is required for the observed inhibition. Biegalke (1998) used *in vivo* footprinting to examine the nature of protein–DNA interactions in the region containing the *cis*-repressive element and found that when the promoter is transcriptionally active, a large region including the TATA box and tre are protected from methylation. In contrast, under conditions of transcriptional repression, only the tre is partially protected from methylation. Based on these results, Biegalke proposes that a repressor protein bound to the tre interferes with the formation of the large initiation complex required for high levels of transcription of the promoter. The identity of the repressor has engendered some controversy. Biegalke (1997) argues that it is unlikely that the IE1 and IE2 proteins play a direct role in mediating this effect because cotransfection experiments in primary fibroblasts show that IE1 and IE2, either alone or together, activate the US3 promoter, and this activation is mediated by a domain on the DNA that is independent of this *cis*-repressive region. In contrast, Lashmit and colleagues (1998) show that IE2 86 can repress the US3 promoter through the *cis*-repressive element, although it should be noted that their cotransfection experiments were performed in

293-T cells, which are nonpermissive for the HCMV infection and which express high levels of the adenovirus E1A and E1B proteins.

VIII. Activation of Early Genes

A. Signal Transduction Events Important for Early Gene Expression

As described above, transcription of the IE genes after infection does not require *de novo* protein synthesis, whereas the early RNAs depend on the IE gene products for their expression. Both IE and early gene expression also require the host cell transcriptional machinery, including binding of key transcription factors to promoter sites. Examples of this include regulation of the UL112/113 promoter by an ATF/CREB site (Schwartz *et al.,* 1996; Rodems *et al.,* 1998) and the promoter for the 1.2-kb RNA by an AP-1 site (Scully *et al.,* 1995). In addition, the major IE promoter contains sites for NF-κB, CREB, SRF, and the Ets family of transcription factors, as described above (Chan *et al.,* 1996b; Mocarski, 1996).

Modulation of the phosphorylation state is a common mechanism for regulating many of the host transcription factors involved in controlling HCMV gene expression. The prototypical mitogen-activated protein kinase (MAPK) pathway, the extracellular signal-regulated kinase (ERK) pathway, can regulate the activity of SRF/Ets, AP-1, and CREB in this way (Boldogh *et al.,* 1991; Karin, 1994; Hill and Treisman, 1995; Xing *et al.,* 1996, 1998; Pende *et al.,* 1997). In this pathway, mitogen receptor interaction leads to Ras-dependent sequential activation (via phosphorylation) of the Raf, MEK, ERK, and $p90^{RSK}$ kinases. This activation is transient, and cellular phosphatases begin the inactivation of these proteins within minutes after stimulation (Hunter, 1995; Marshall, 1995).

We have found that the ERK1 and ERK2 kinases activated by serum stimulation of cells remain in their active phosphorylated form (as evidenced by $p90^{RSK}$ phosphorylation) for extended periods of time (8 hours versus 1 hour in mock-infected cells) on infection with HCMV. HCMV infection does not appear to activate these kinases initially but seems to act by inhibiting a phosphatase involved in inactivating these signal-transducing proteins. Sustained ERK activation appears to be important for early viral gene expression, as evidenced by a decrease in the transcriptional activity of the HCMV UL112–113 early promoter when infection is carried out in the presence of the MEK inhibitor PD98059. At this point, it is not clear how the ERKs are involved in regulating HCMV early promoter activation. Among the many possibili-

ties are that the activities of IE2 86 and IE1 72 may be affected by phosphorylation, in turn affecting those promoters responsive to these IE proteins. The report (Harel and Alwine, 1998) that ERK2 phosphorylates several domains of IE2 86 *in vitro* and *in vivo,* and our own preliminary data showing the *in vitro* phosphorylation of both IE1 72 and IE2 86 by ERK, certainly support this hypothesis. Taken together, these data indicate a potential means of IE and subsequent early gene activation via cellular signal transduction pathways.

B. Transcriptional Control of HCMV Early Promoters

Two well-studied genes, UL112/113 and UL54 (DNA polymerase), exemplify the basic principles of transcriptional control observed at the early time in the infection. These genes are also transcribed at late times and serve as excellent examples of the differences in transcriptional control mechanisms observed as the infection progresses.

1. Regulation of 2.2-kb Class of Transcripts (UL112–113)

The studies on the regulatory controls governing the expression of the 2.2-kb class of transcripts (ORFs UL112–113) and the family of DNA binding proteins encoded by them have provided a useful model for formulating and testing hypotheses regarding the activation of HCMV early genes (Staprans and Spector, 1986; Staprans *et al.,* 1988; Wright *et al.,* 1988; Wright and Spector, 1989). Gene expression from this region of the genome is regulated by both transcriptional and posttranscriptional mechanisms. By 8 hpi, RNA transcription is fully underway, generating two multiply spliced transcripts 2.1 kb and 2.2 kb in length. The RNAs are 5′ and 3′ coterminal and consist of invariable 5′ and internal exons and 3′ exons that utilize alternative splice acceptor sites (Staprans and Spector, 1986). Beginning at 48 hpi, transcription from the early (+1) start site declines and transcription initiates from a new start site at −62 (Staprans and Spector, 1986). The splicing pattern also changes as the infection progresses, resulting in the appearance of two additional transcripts, one of 2.5 kb that has spliced out only the first intron and a second completely unspliced transcript of 2.65 kb (Staprans and Spector, 1986; Wright *et al.,* 1988; Wright and Spector, 1989). However, the overall abundance of transcripts derived from this locus does not significantly increase.

a. Localization of Essential Cis-Acting Sequences on Promoter. The initial analysis of this promoter utilized transient transfection assays with 5′ and internal deletion mutants of the promoter driving the

expression of the CAT gene. From these studies, it was determined that sequences residing between nt −113 and −59 relative to the transcription start site were required for high-level activation by the HCMV infection, although upstream sequences did contribute to the overall level of induction (Staprans *et al.,* 1988). This region was also essential for specific activation by the IE2 86 protein (Schwartz *et al.,* 1994).

Staprans and co-workers (1988) initially noted that within the major regulatory domain between nt −71 and −66 was the consensus binding site for the cellular transcription factor ATF/CREB, and they hypothesized that activation of this promoter might involve this family of transcription factors. As described above, the domain bounded by nt −113 and −59 also contained a weak binding site for the IE2 86 protein (Schwartz *et al.,* 1994). To assess the relative importance of the region between nt −84 and −59 and that of the weak IE2 86 binding site between nt −113 and −85, additional mutants in this region were tested in transient transfection assays. Results of both our group and Arlt and colleagues (1994) demonstrated that the sequences between nt −84 and −59 were absolutely required for high-level activation of this promoter by IE2 86. In contrast, the IE2 86 DNA binding sites were not essential but rather seemed to serve an accessory function. Additional site-specific mutational analysis localized the sequences critical for transactivation by IE2 86 to the region between nt −72 and −61. These results, coupled with our previous observation that a consensus ATF/CREB binding site was located between nt −71 and −66, further supported our earlier hypothesis that activation of this promoter might involve the ATF/CREB family of transcription factors (Staprans *et al.,* 1988). Although the ATF/CREB site contribution to the transactivation of this promoter seems to be dominant, mutational analysis of nt −84 to −59 indicates that there may be other cooperative interactions between the various subregions of the promoter.

b. Identification of Cellular Factors Required for Activation of Promoter by IE2 86: A Key Role for CREB and CREB Binding Proteins. A multifaceted approach was used to identify the transacting cellular factors important for activation of the UL112/113 promoter. This approach involved a variety of gel retardation assays using wild-type promoter-, mutant promoter-, and ATF/CREB consensus-DNA probes; proteins from nuclear extracts or synthesized by *in vitro* translation; and antibodies specific for individual ATF/CREB transcription factors. Gel retardation analysis with a probe containing the wild-type promoter sequences (nt −84 to −59) and U373 nuclear extracts generated three specific bands, of which bands 2 and possibly 3 appeared to contain

ATF/CREB transcription factor(s). Evidence for this included the following: (1) the wt promoter formed a complex with *in vitro* translated ATF-2 or CREB, (2) a DNA probe consisting of the consensus ATF/CREB binding site generated a band that comigrated with the above described band 2 formed with the wild-type promoter, (3) excess unlabeled wt promoter competed well with the formation of the complex formed with the consensus ATF/CREB probe, and (4) supershift analysis with antibodies directed against specific members of the ATF/CREB family documented that one of the binding factors in the U373 cells was clearly CREB, and with the wild-type UL112/113 promoter probe, a fraction of band 2 complexes was supershifted. There was complete or nearly complete abolition of complex supershifting when the probe contained mutations in the sequences between nt −72 and −67 or between nt −66 and −61, respectively, indicating that sequences between nt −72 and −61 are critical for IE2 86-mediated transactivation of this promoter. However, there is still the possibility that other factors that bind to this domain, particularly those that form the complex corresponding to band 3, also contribute to activation of this promoter by IE2 86.

A major question prompted by the above findings was, how does CREB transactivate the 2.2-kb RNA promoter in conjunction with IE2 86? This question was particularly troublesome because we had found that although IE2 86 was able to form complexes with multiple cellular transcription factors and regulatory proteins, it was not able to bind to *in vitro* translated CREB using the same experimental conditions that allow efficient complex formation between IE2 86 and TBP, TFIIB, Rb, c-Jun, and JunB (Sommer *et al.,* 1994; Scully *et al.,* 1995). In view of these results, we considered the possibility that CREB and IE2 86 do not bind to each other directly to activate this promoter. Because there was evidence that two cellular factors, p300 and CBP, bind to CREB after it has been phosphorylated at serine 133 by protein kinase A (Chrivia *et al.,* 1993; Kwok *et al.,* 1994; Lundblad *et al.,* 1995), and that both p300 and CBP function as coactivators for CREB (Arany *et al.,* 1995; Lundblad *et al.,* 1995), it seemed reasonable that IE2 86 could be interacting with CREB through one of these proteins. In fact, this appears to be the case, as IE2 86 is capable of binding to both CBP and p300 *in vitro.* These results suggest that activation of the 2.2-kb RNA promoter might involve a multiprotein complex including CREB, p300 or CBP, and IE2 86 (Schwartz *et al.,* 1996). At this point, however, we cannot exclude the possibility that the binding of IE2 86 to CBP/p300 serves to modulate the transcriptional adapter properties of these proteins. There is precedent for such a mechanism, as the adenovirus

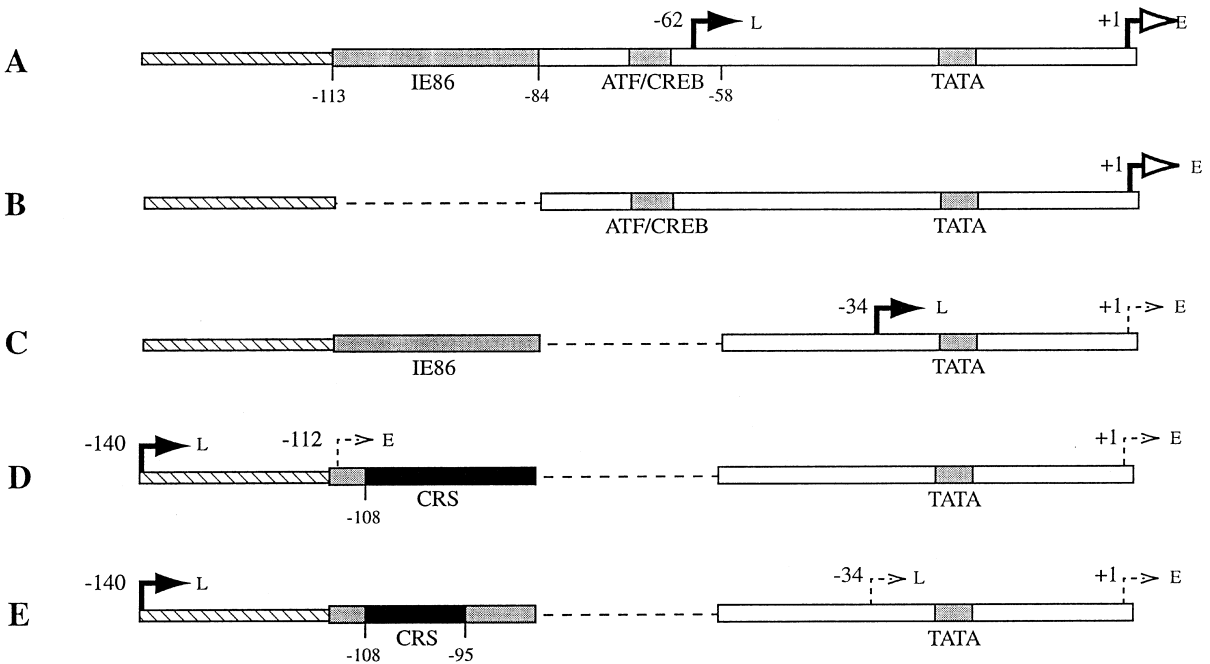
E1A protein, through its binding to CBP or p300, interferes with the ability of these proteins to serve as coactivators for CREB (Arany *et al.,* 1995; Lundblad *et al.,* 1995). This is probably not the case, as we have found that IE2 86 can significantly stimulate transcription mediated by a GAL4-CBP fusion protein brought to a promoter by GAL4 DNA binding sites (Schwartz *et al.,* 1996). Alternatively, the activation through CREB at early times in the infection may be indirect and may involve the regulation of protein kinase A and hence the phosphorylation state of CREB.

Experiments similar to those described above have also been reported by Lang and co-workers (1995). In their studies they used delta CREB, a protein that contains a deletion of the region between aa 88 and 101 (Yamamoto *et al.,* 1990). Both forms of CREB do exist in cells, and thus some of the observed differences described below may be biologically relevant. Lang and colleagues demonstrated that prokaryotically expressed delta CREB protects a region on the 2.2-kb RNA promoter between nt −78 and −56. However, when they exchanged two nucleotides within this site and abolished binding of delta CREB in DNase I protection experiments, activation of the promoter by IE2 86 was reduced less than twofold relative to that of the wt. In contrast, the mutations introduced into the ATF/CREB site in our experiments reduced the level of activation to a much greater extent and significantly affected the binding of the U373 nuclear factors to the promoter. Interestingly, they did show that IE2 86 could mediate activation through the ATF/CREB site using engineered constructs that contained oligonucleotides corresponding to either genuine or mutated ATF/CREB sites cloned as single copies or multimers just upstream of the TATAA box of this promoter. Additionally, the results of their transient expression assays indicated that there was stimulation of transcription from a reporter construct containing five GAL4 binding sites upstream of the β-globin TATAA box when it was cotransfected with expression plasmids for both a GAL4-delta CREB fusion protein and IE2 86. However, in our studies, the stimulation by IE2 86 is far greater with the GAL4-CBP fusion protein. Experiments have also been performed in both our laboratory and that of Lang *et al.* to determine whether IE2 86 can bind to delta CREB. Although there is a weak interaction between GST-IE2 86 and *in vitro* translated delta CREB, it is significantly less than that between IE2 86 and CBP or p300. However, Lang and colleagues did find that delta CREB phosphorylated by protein kinase A *in vitro* binds more strongly to IE2 86 than does the unphosphorylated protein. Because p300 and CBP bind to protein kinase A-phosphorylated CREB (Chrivia *et al.,* 1993; Kwok *et al.,* 1994; Lund-

blad *et al.,* 1995), the combined results from both laboratories suggest that strong activation of this HCMV early promoter through its CREB site may result from the ability of IE2 86 to interact with both proteins in the phosphorylated CREB–p300/CBP complexes.

c. Separate DNA Elements Containing ATF/CREB and IE2 86 Binding Sites Differentially Regulating HCMV UL112/113 Promoter at Early and Late Times in Infection. To determine the functions of the various sequence elements within the UL112/113 promoter at different times during the HCMV infection without the potential inaccuracies associated with transfection assays, we used a gene replacement strategy developed by Kohler and colleagues (1994) that was based on the finding that a portion of the unique short region of the HCMV genome is dispensable for viral growth in tissue culture. Briefly, the approach involves the construction of recombinant viruses in which a viral promoter linked to a reporter gene is inserted between the US9 and US10 genes. In this position, expression from the reporter gene linked to a wt viral promoter shows kinetics similar to those of the endogenous viral gene.

For our studies, we constructed recombinant viruses in which various mutations of the UL112/113 promoter, linked to the CAT gene, were inserted between the US9 and US10 genes in the viral genome, and then analyzed for UL112–113 promoter-CAT expression at different times during the infection (Rodems *et al.,* 1998) (Fig. 3). We found that transcription from the inserted wt UL112–113 promoter (nt −113 to +35) (Fig. 3A) showed the same temporal pattern as the endogenous promoter, including the switch to an upstream RNA start site late in infection (Staprans and Spector, 1986). Consistent with the transient expression data, the results demonstrated that the sequences containing the ATF/CREB site play a major role in UL112/113 promoter activity early during the viral infection, whereas the region containing the weak IE2 86 binding site has a moderate effect on transcription. However, at late times in the infection, it was found that the ATF/CREB site plays little if any role in expression, but the sequences between −113 and −85, which include the weak IE2 86 binding site, are required for transcription from the late start site within the UL112/113 promoter (Fig. 3B). When the region containing the ATF/CREB site (−84 to −59) was deleted (Fig. 3C), early RNA synthesis was almost completely abolished, but late gene transcription was comparable to that of wt, with repositioning of the late RNA start site downstream by the number of nucleotides deleted (from −62 to −34). These results suggest that the ATF/CREB site is functional only at early times, whereas the sequences containing the IE2 86 DNA binding site modulate the level of early


A
-62
L
+1
E
-113
IE86
-84
ATF/CREB
-58
TATA
B
+1
E
ATF/CREB
TATA
C
-34
L
+1
E
IE86
TATA
D
-140
L
-112
E
+1
E
CRS
-108
TATA
E
-140
L
-34
L
+1
E
CRS
-108
-95
TATA

RNAs and are essential for activating late gene transcription in a distance-dependent manner.

To further assess which sequences between −113 and −85 were involved in directing late transcription initiation, two additional recombinant viruses were constructed. In one virus (II CRS), the two AT-rich sequences that overlap the IE2 86 binding site were deleted and the CRS (plus some additional sequences) from the HCMV major IE promoter (which is a strong binding site for IE2 86) was inserted (Fig. 3D). The second virus (CRS) contained the CRS but left intact one set of AT-rich sequences between −94 and −85 (Fig. 3E). Both mutants showed the same pattern of CAT protein accumulation as the parent construct, indicating that the mutations, which were actually different IE2 86 binding sites, had no effect on late transcription. Surprisingly, transcription from the normal late start site was abolished in the II CRS and was barely detectable in the CRS virus. In addition, a new strong RNA start site with identical late kinetics was detected upstream. Of particular interest was the observation that this new late start site was located the same distance from the center of the CRS as the distance between the normal strong late start site and the wt IE2 86 binding site (Fig. 3A). Taken together, these results suggest that the sequences containing the IE2 86 binding site play a role in directing late transcription from the UL112/113 promoter in a distance-dependent and possibly an orientation-dependent manner. Because IE2 86 binds to the minor groove of the DNA, the orientation of the IE2 86 binding site relative to other surrounding sequences may result in structural effects on IE2 86 binding that determine the position of late transcription initiation.

2. Regulation of HCMV DNA Polymerase Gene UL54

Six of the 11 HCMV loci originally defined by Pari and Anders (1993) in their reconstitution experiments on *ori*Lyt-dependent viral DNA

FIG 3. Summary of recombinant UL112–113 viruses, as described in the text. (A) The wild-type UL112–113 promoter, indicating IE2 86 and ATF/CREB binding sites important for early and late transcription. The major early (open arrowhead) and late (closed arrowhead) start sites are indicated above the construct. (B) Deletion of the IE2 86 binding site has a moderate effect on early transcription but completely abolishes late transcription (as indicated by the absence of a closed arrowhead). (C) Deletion of the ATF/CREB binding site greatly diminishes early transcription (indicated by a dashed arrow) and moves the major late start site downstream. (D and E) An additional change in the IE2 86 binding site does not change the kinetics of late transcription accumulation in either the II CRS (D) or CRS (E) virus, but it does change the strong late start site to a site further upstream. The CRS virus also maintains a low level of transcription from the −34 site. Adapted from Rodems *et al.* (1998).

replication encode early proteins homologous to known HSV-1 replication proteins. Of these, regulation of the expression of the UL54 gene has been most closely studied. In the initial experiments using transient expression assays and the CAT reporter gene, an 8-bp element located between nt −61 and −43 (designated IR1) was shown to be important for activation by both the virus and major immediate early proteins IE1 72 and IE2 86 (Kerry *et al.,* 1994). Furthermore, it was demonstrated that cellular proteins bound to this region. It was also shown that the gene products encoded by ORFs UL112/113, UL36-38, and IRS1/TRS1 cooperated with each other and with the major IE1/IE2 proteins to stimulate expression from the UL54 promoter, and that mutation of the IR1 element between nt −53 and −45 not only reduced the activation of this promoter by the IE1/IE2 gene products but also affected the increased stimulation provided by the other viral proteins (Kerry *et al.,* 1994, 1996). The functional relevance of this finding was supported by experiments showing that mutation of IR1 in the context of the viral genome significantly reduced activation of the promoter at early times in the infection (Kohler *et al.,* 1994; Kerry *et al.,* 1996).

In one study, it was confirmed by extensive mutational analysis that an element that overlapped the IR1 between nt −54 and −43 was important for activation (Luu and Flores, 1997). Moreover, a protein was isolated from uninfected HeLa cell lysates that could bind to this region and was identified it as Sp1. Wu and colleagues (1998) published the results of similar studies, but the findings differ somewhat. There is agreement that in permissive cells IE2 86 is the major transactivator of the UL54 promoter, and that the region between nt −54 and −43 is required for this activation. Using nuclear extracts from U373-IE2 86-overexpressing cells, they saw a specific complex formed on the IR1 site, but in contrast to the other investigators, they did not see a specific complex in the parental U373 cells, in the HeLa cells, or in a line of HeLa cells overexpressing IE2 86. IE2 86 appeared to be associated with the complex, but its DNA binding domain was not needed, as the CRS element could not compete. An Sp1 oligonucleotide could compete, and an Sp1 antibody was able to supershift the complex. Because Wu and colleagues (1998) did not detect this complex in HeLa cells, they proposed that there might be some inhibitory factor present in the HeLa cells that prevented Sp1 from binding to this site. One possible explanation for the divergent results of these studies may relate to the specific probes used. In the studies by Luu and Flores (1997) and Kerry and co-workers (1996) the probe was at least 30 bp in length, whereas Wu and co-workers (1998) used a small probe of only 18 bp. Because surrounding sequences are important for the stability of the DNA–

protein complex, it may be that the binding of Sp1 to the 18-bp probe was at the borderline of stability. Alternatively, there might have been some modification of Sp1 and/or IE2 86 or induction of an associated cellular protein that stabilized the complex in the U373 cells overexpressing IE2 86. Unfortunately, the studies by both Wu and co-workers (1998) and Luu and Flores (1997) were not performed in the context of viral infection, and thus their biological relevance remains uncertain.

Another region in the UL54 promoter that appears to be important for its regulation contains a binding site for the ATF/CREB family of transcription factors. In the initial transient transfection assays, this site was found to play a small but consistent role in activation (Kerry *et al.,* 1994). It was shown by gel-shift analysis that infected cell lysates produced increased binding to the site beginning at 48 hpi and continuing until 72 hpi. Binding to the site was greatly diminished by the addition of phosphonoaceticacid (PAA), indicating the need for some late viral gene product for increased binding. A mutant virus was constructed by inserting between US9 and US10 a construct containing the CAT gene driven by the UL54 promoter with an ATF site mutation. Infection with this virus revealed a decrease in CAT gene expression at all times in the infection, with early transcription being most severely inhibited. The authors concluded that ATF-1 was able to regulate the promoter at both early and late times, but as discussed below, it is likely that this site plays a significant role only at early times and that a region further upstream is primarily responsible for transcription of this gene at late times.

Northern blot data presented by Kerry and co-workers (1996, 1997) provide some important information regarding the elements required for early versus late transcription directed by the UL54 promoter. These data show transcription of a reporter CAT gene driven by various promoters containing putative wt and mutant sequences. In the case of the CAT gene driven by the full-length wt promoter (-425 to $+20$), there appears to be one transcript present at early and late times and a second, larger transcript that is present only late in the infection. In contrast, for the CAT gene driven by the putative minimal-length wt promoter (-128 to $+20$), only the smaller transcript is observed at early and late times. The levels of transcripts remain relatively constant throughout the infection, although the amount of the smaller transcript is greater when the minimal promoter is used versus the larger wt promoter due to a potential negative element in the region upstream of nt -128. When the CAT gene is driven by the full-length wt promoter containing a mutation in either the IR1 or the ATF-1 sites, there is little transcription at early times, and at late times the smaller transcript is

undetectable but the larger transcript is present at wt levels. The simplest interpretation of these data is that both the IR1 (Sp1) and ATF elements are required for synthesis of the smaller transcript that first appears at early times, whereas a sequence between −128 and −425 is responsible for specific synthesis of the larger transcript at late times. Thus, analogous to the promoter for the UL112/113 gene, it appears that a different start site and different regulatory sequences may be used for transcription at early and late times.

C. Establishment of Replication Centers by Early Genes

One hallmark of a productive HCMV infection is the appearance of the large, amorphous structures within the nucleus of an infected cell termed "replication centers." An interesting study by Sarisky and Hayward (1996) emphasized the importance of some of the early proteins for the proper establishment of these centers. These authors were able to establish functional replication centers in Vero cells when they transfected an *ori*Lyt-containing plasmid and the 11 loci determined by Pari and Anders (1993) to be essential for HCMV replication. Establishment of these functional centers was dependent on IE2 86, UL112–113, and UL84, as omission of any one of these loci restricted the centers to their prereplicative size. The role of these and other early proteins in the initial establishment of replication centers in the early hours postinfection was examined by Penfold and Mocarski (1997). Their data suggest that the early UL112–113 gene products may establish the eventual sites of replication centers, as these proteins localized to three to six small globular sites as early as 6 hpi. By 12 hpi, only a small amount of UL57 and no UL44 colocalized to these sites. By 24 hpi, however, all three proteins were in bipolar spots (which they concluded were derived from the original three to six UL112 spots) that merged to form a single center by 48–72 hpi. An interesting question that needs to be addressed relates to the relationship of these centers to the original ND10 sites targeted by the input virion DNA.

Although the above centers are clearly sites of viral DNA synthesis at later times in the infection, it has been difficult to resolve their precise relationship to early sites of viral DNA replication. Penfold and Mocarski (1997) found that when infected cells were labeled with BrdU at 12 hpi, they exhibited a punctate pattern of staining similar to that observed for UL57, but the BrdU-staining foci were more numerous. However, there was partial overlap of BrdU and UL57. When the cells were labeled at 24 hpi, there was BrdU staining in the bipolar spots,

but again, there was a good deal of staining in punctate structures throughout the nucleus. The authors claimed that when this BrdU-labeled DNA was chased, it entered the bipolar structures and then eventually moved into the cytoplasm and out of the cell. From these observations, they concluded that this punctate labeling was viral DNA replication occurring outside of the replication centers. In view of the absence of viral proteins in these spots, this conclusion must be interpreted with caution, as these cells were asynchronous at the time of infection. Thus it is very likely that at least some of the cells were in S phase at that time and were therefore replicating their cellular DNA. It should be possible to determine whether viral or cellular DNA is located at these foci by *in situ* hybridization with a labeled viral DNA probe.

D. Sequestration of Several Cellular Proteins into Replication Centers

As mentioned earlier, steady-state levels of p53 are increased by HCMV infection (Muganda *et al.,* 1994; Speir *et al.,* 1994; Jault *et al.,* 1995). We have shown that in infected fibroblasts, this elevation is due not to an increase in protein synthesis but rather to a decrease in protein degradation (Fortunato and Spector, 1998). There is evidence that IE2 86 may interact with the p53 protein, potentially shielding it from degradation and affecting its ability to downregulate its target genes (Speir *et al.,* 1994; Bonin and McDougall, 1997). Our data also point to another event, the sequestration of p53 into the viral replication centers (starting at about 30 hpi), as a potential mechanism for not only stabilization of the protein but also perturbation of its normal protein–protein and protein–DNA interactions. The sequestration of several other proteins, including RPA, PCNA (Dittmer and Mocarski, 1997), and two xeroderma pigmentosum proteins, (XPB and XPD) (E. A. Fortunato and D. H. Spector, unpublished) has led us to propose two hypotheses regarding the functionality of sequestration into these replication centers: either the virus sequesters these proteins in order to prevent them from interacting with their normal cellular partners or these proteins play an active role in viral replication and repair. We favor the latter hypothesis, as all of these proteins have been implicated in both DNA replication and repair, and could potentially aid in the fidelity of both or either of these processes with respect to viral DNA. This sequestration could also lead to a potential preference for repair of the viral versus the host cell genome. This is an important question

to address, particularly because it may be relevant to the birth defects caused by *in utero* infection.

IX. Regulation of True Late Genes

The late genes are the last class of gene products to be expressed temporally in the HCMV life cycle. These genes are generally expressed after 48 hpi and require viral DNA replication for their expression. At least 12 different RNA species were identified as being true late genes by virtue of their expression at late times in cells infected with wild-type but not temperature-sensitive, nonreplicating virus (Depto and Stenberg, 1992). Although little is known about the regulation of these late genes with respect to what viral and/or cellular gene products might be required for expression, three late gene loci were fairly well characterized during the 1990s. These analyses have shed some light on the complexity of regulation of these gene products and will be described in the sections below.

A. UL94 Locus

The UL94 locus is located within a family of 3′ coterminal transcripts that range in size from 1.3 to 10.5 kb and encompass ORFs UL93–UL99. The transcripts are arranged in a linear fashion, with the largest (10.5 kb) encoding ORF 93 and the two smallest (1.3 and 1.6 kb) encoding ORF 99. Northern blot analysis at different times postinfection and in the presence of the viral DNA inhibitor ganciclovir revealed that the two largest transcripts of 9.1 and 10.5 kb (corresponding to ORFs UL94 and UL93, respectively) exhibited true late kinetics of accumulation (Wing and Huang, 1995). Exogenous expression of the UL94 ORF *in vitro* produced a 36-kDa protein, the same size as the protein detected in infected cell lysates at late times of infection by an antibody made to the predicted amino acid sequence. Fractionation of viral particles revealed that the 36-kDa protein was associated with the capsid/tegument fraction (Wing *et al.,* 1996).

Several cellular transcription factor binding sites have been delineated within the putative UL94 promoter, including sites for p53, NF-κB, ATF, and Sp1 (Wing and Huang, 1995). Using the UL94 promoter to drive the CAT gene in transient transfection/infection experiments, Wing and co-workers (1998) saw an approximately 20-fold increase in expression of the construct at late times postinfection (72 hpi). Regions upstream (the negative regulatory element or NRE) and downstream

(the positive regulatory element or PRE) of the TATA both appeared to exert two- to threefold effects at late times (72 hpi). It was also noted that in the presence of viral DNA inhibitors, the NRE exerted a more marked repression of the transcript. Results of studies attempting to investigate the importance of a p53 binding site within the NRE are very difficult to interpret, as the experiments were performed in a nonpermissive cell line (Saos 2) and not in the context of a viral infection. In addition, analysis of the promoter under transient conditions, although potentially informative, must be followed by analysis of the promoter within the context of the virus itself.

B. UL99 (pp28) Gene

The UL99 ORF is encoded on two different 3′ coterminal RNAs of 1.3 and 1.6 kb (Martinez *et al.,* 1989). These two transcripts are both capable of producing the 28-kDa protein product, but because the kinetics of accumulation of the 1.6-kb transcript more closely resemble those of a late gene, this transcript has been used for further characterization. The 1.6-kb transcript also encodes the C-terminal region of the UL98 gene, but only the pp28 protein appears to be translated from this mRNA species (Adam *et al.,* 1995). Analysis of the 1.6-kb promoter from −609 to +106 in transient transfection/infection experiments revealed that regions between −609 and −40 (d24/26) could be deleted with only a twofold drop in activation by wild-type virus (Depto and Stenberg, 1992). The region from −40 to −32, which contains an inverted repeat motif, could not be further deleted without a large drop in activation. Problems arose, however, when essentially the same results were obtained when cells were infected with a nonreplicating temperature-sensitive mutant virus at the nonpermissive temperature, indicating that the activation of the promoter in a transient assay did not accurately portray the *in vivo* situation. In addition, in the transient assays, activation occurred earlier (at about 24 hpi) and to higher levels than was observed *in vivo* (Kohler *et al.,* 1994). To solve these problems, recombinant viruses were constructed that encoded a CAT construct driven by either the full-length UL94 promoter (−609 to +106) or the deleted promoter (d24/26) inserted within the nonessential US9/10 region of the virus. These viruses exhibited CAT activity with true late kinetics (starting at 48 hpi, high at 72 hpi and sensitive to PAA) and produced essentially equivalent CAT activities at late times.

To further define regions that were potentially important in regulating pp28 expression, a deletion of two other inverted repeats directly

downstream of the TATA was made (deletion of −6 to +46). When tested in transient assays, this deletion increased CAT activity by threefold over d24/26. Deletion of these repeats within the viral genome did not change the kinetics of expression of the CAT enzyme but did reveal a novel type of regulation by this element. When this element was removed, there was an increase in CAT activity but a decrease in the level of CAT RNA present, indicating a role for this region in translational control of the pp28 gene product under normal infection conditions (Kerry *et al.,* 1997). This conclusion could be reached only after the amount of CAT RNA was normalized to the amount of endogenous UL99 expressed in the infected cells, again highlighting the importance of defining the true parameters of expression with the use of this recombinant virus system.

C. 1.2-kb RNA Transcriptional Unit

The 1.2-kb RNA transcriptional unit exhibits a biphasic pattern of expression; it is clearly activated with early gene kinetics, but it also undergoes a second period of induction after the onset of viral DNA replication (McDonough *et al.,* 1985). Because the initial reporter plasmid constructs with the use of this promoter displayed the early- but not the characteristic late-phase induction, the studies in our laboratory first focused on the nature of the sequences and factors involved in the regulation of this gene at early times with the use of either transfection/infection or cotransfection with IE2 86 protein alone (Wade *et al.,* 1992; Scully *et al.,* 1995). Deletion and point mutational analyses revealed the importance of the TATAA-like sequence CATAAA at −30 and an AP-1 binding site at −75. Gel shift analysis revealed a specific cellular factor bound to this AP-1 site and this binding could be eliminated by preincubation with a Fos antibody broadly reactive with Fos family members. This provided support for a complex containing one or more heterodimeric Fos-Jun AP-1 factors. *In vitro* binding experiments also defined the ability of IE2 86 to bind to the Jun but not the Fos members of the AP-1 family of transcription factors. In addition, IE2 86-jun coimmunoprecipitations could be performed *in vivo* (Scully *et al.,* 1995). An additional site upstream of the AP-1 site that shares sequence homology with the CRS element on the major IE promoter and that is a strong binding site for IE2 86 was also defined. However, from the mutational analysis performed, it appeared that this site played only an accessory role in the IE2 86-mediated activation of the 1.2-kb RNA promoter, at least in transient expression assays.

Because numerous attempts to identify elements either upstream, downstream, or within the gene that would confer late induction of this promoter in transient assays had failed (Wade *et al.,* 1992), we hypothesized that inclusion of the HCMV origin of replication (*ori*Lyt) on the plasmid construct might be helpful in transient transfection/infection experiments. This approach proved to be successful. DNA analysis confirmed that the construct was able to replicate, and RNase protection experiments showed that there was late induction of the CAT RNA encoded by the plasmid that paralleled the late induction of the viral 1.2-kb RNA. In addition, the late induction was prevented by the presence of ganciclovir. Surprisingly, site-specific mutations in the AP-1 binding site and the CATAAA site indicated that these sites continued to contribute to full promoter activity at late times but that the replication-dependent late induction acted independently of these sites.

Preliminary analysis of the 1.2-kb promoter in the context of recombinant viruses confirmed our earlier transient assay results with the plasmid containing *ori*Lyt. Recombinant virus containing the full promoter shows both early and late mRNA induction and CAT activity, with the largest CAT activity : RNA ratio occurring at late times in infection. Mutation of the AP-1 site within the promoter markedly reduces the activity and RNA accumulation at all times, indicating the importance of this site in regulation throughout the infection. Deletion of the IE2 86 binding site further upstream of the AP-1 site appears to play only an ancillary role in the regulation of this promoter at both early and late times postinfection, as was also observed in our transient assays. Therefore, although there is both early and late transcription from both the 2.2 and 1.2-kb promoters of the virus, it appears that the IE2 86 binding sites do not play the same role in the control of the two promoters. Potential explanations for this variation include usage of different regions of IE2 86 for interaction with different DNA segments, variation of IE2 86 binding sites with respect to transactivating ability, and the influence of other cellular factors and their respective binding sites within the two promoters. Experiments are currently underway to try to distinguish between these various possibilities.

X. Conclusions

In this review, we have tried to emphasize that HCMV infection does not operate within a vacuum; viral replication proceeds only through a set of intricate interactions of the virus with the host cellular machin-

ery. These interactions begin at the point of contact of the virus with the cell membrane, triggering not only physiological changes but also important signaling cascades, and proceed to include interactions with the nuclear subarchitecture, transcription and replication machinery, cell cycle proteins, and probably several other factors not yet defined. In addition, it appears that the cell cycle phase at the time of infection is critical for the initiation of IE gene expression. Whether this restriction is due to effects on viral DNA localization or expression of IE genes at the transcriptional or translational level is yet to be determined. This blockade to initiation may also play a role in the restricted replication of the virus within undifferentiated cells. Although not addressed in this review, the ability of the virus to establish latency or semipermissive infection in some cell types may depend on an entirely different set of interactions with the host cell than that occurring within the permissive cell type. Nevertheless, the information derived from the analysis of viral gene expression in the permissive cell has provided a strong foundation for formulating hypotheses to be tested in the context of the restricted viral infection in cells that are clinically relevant but far more difficult to study. Only when we understand the functioning of the virus in both fully permissive and semipermissive cells will we be able to develop strategies to combat the debilitating effects of the HCMV infection in neonates and immunocompromised individuals.

Acknowledgments

Research conducted in the authors' laboratory was supported by NIH Grants CA34729, CA73490 and T32 AI07036. We thank the members of our laboratory for their tireless efforts and many helpful discussions.

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ADVANCES IN VIRUS RESEARCH, VOL. 54

DNA VACCINES: A REVIEW

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I. DNA-Based Vaccines

Immunization of animals with naked nucleic acid encoding antigens under the control of a variety of gene regulatory elements has been described as "polynucleotide immunization," "DNA-based vaccination,"

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0065-3527/99 $30.00

"somatic transgene vaccination," "genetic vaccination," and "nucleic acid vaccination" (Gerloni *et al.,* 1997; Robinson, 1997). Regardless of the terminology, and despite the need for technical correctness, most researchers have settled on "DNA-based" or simply "DNA vaccines" to describe this novel method of immunization. DNA-based immunization was first described in 1992 despite the fact that the evidence for delivery, uptake, and expression had been described much earlier (Chattergoon *et al.,* 1997). Since that time, detractors have described DNA-based vaccination as "biological cold fusion" (Fomsgaard, 1995), whereas proponents have termed this unique form of immunization the "third revolution in vaccinology" (Waine and McManus, 1995). DNA-based vaccination is, without question, well beyond the less than flattering comparison to "cold fusion." To date, well over 400 publications have appeared in respected scientific journals since the first publication coined the phrase "genetic immunization" in 1992 (Tang *et al.,* 1992). The inherent simplicity of the technology is unquestionably revolutionary, and it is this simplicity that has, in part, driven detractors to distraction. Certainly the simple eloquence of DNA vaccine technology is in stark contrast to such inherently complex forms of vaccine construction as attenuated and attenuated recombinant pathogens, recombinant subunit vaccines, and formulations involving novel adjuvants. Despite the technical simplicity of DNA-based vaccines, it has been over 30 years from the time researchers first demonstrated immune responses following injection of naked DNA to the first published description of "genetic immunization" (Atanasiu, 1962; Tang *et al.,* 1992). This simple leap of logic from *in vivo* introduction of naked genetic material to immunogen synthesis and immune response was as surprising as it was profound.

A. Injection-Grade Plasmid Preparation

From a purely technical standpoint, systemic immunization of animals with naked nucleic acid is eloquent in its simplicity. Plasmid DNA containing any of a variety of eukaryotic gene regulatory sequences, in the appropriate context of a gene, or genes, encoding antigen, is injected intramuscularly or intradermally. Purification of amplified plasmid is typically carried out with the use of cation exchange affinity columns, with or without removal of endotoxin (Davis *et al.,* 1996). Traditional techniques such as cesium chloride (CsCl) centrifugation may also be used; however, injection-quality DNA typically requires a double CsCl centrifugation, usually followed by dialysis. This centrifu-

gation and dialysis procedure is time-consuming and hazardous and results in substantial plasmid loss. At present, issues regarding plasmid preparation protocols and correlation with immunization efficacy remain unresolved and may simply reflect differences in such things as plasmid sequences, bacterial host cell types, and/or growth media. Undoubtedly, as interest in immunostimulatory sequences (ISSs) encoded within bacterially derived DNA, as well as inclusion of immune modulatory genes, grows, researchers will begin moving toward greater standardization of plasmid purification protocols. This move will ensure that experiment reproduction and accurate interlaboratory data comparisons are made possible and reliable.

B. Delivery of DNA Vaccines

Regardless of purification protocol differences, injection-grade supercoiled plasmid DNA may be stored for long periods of time or resuspended immediately in sterile saline or phosphate-buffered saline (PBS). Intramuscular or intradermal delivery may be carried out with the use of standard 0.3- to 0.5-mL sterile syringes and small-gauge hypodermic needles to inject the vaccines. Several other methods have been described for delivery of plasmid-based vaccines, including ballistic delivery (Pertmer *et al.,* 1995; Tang *et al.,* 1992), cationic liposomal or microsphere encapsulation (Gregoriadis *et al.,* 1997; Huang and Wang, 1989; Jones *et al.,* 1997; Keller *et al.,* 1996; O'Hagan *et al.,* 1993), and the use of bacterial or host cell carriers (Gerloni *et al.,* 1997; Manickan *et al.,* 1997a; Sizemore *et al.,* 1997). Ballistic delivery of DNA-based vaccines involves coprecipitation of plasmid DNA with gold or tungsten microparticles (0.95–2.6 μm) such that DNA adsorbs to the surface of the beads. Beads are then "shot" into the epidermis, resulting in *in vivo* transfection of a variety of dermal cell types including keratinocytes, fibroblasts, and Langerhans cells (Raz *et al.,* 1994). Aerosol or droplet instillation of naked DNA on mucosal surfaces, in the absence of any facilitators, has also elicited immune responses. Mucosal and transdermal (Li and Hoffman, 1995) delivery of DNA vaccines may be facilitated by incorporating DNA-based vaccines into a variety of cationic liposome preparations or biodegradable microspheres (Alpar *et al.,* 1997; Barnfield *et al.,* 1997). Finally, attenuated bacterial vectors, such as *Shigella flexneri* or *Listeria monocytogenes,* which normally infect via intestinal mucosa and also penetrate and persist within host cells, have been specifically mutated such that lysis occurs within host cells. This intracellular lysis releases plasmid DNA

carried by these bacteria into the cytoplasm of the host cell, which then sets the stage for nuclear translocation followed by expression of plasmid-encoded sequences. Thus, these DNA-vaccine-carrying bacteria provide a mechanism to immunize against the delivery vehicle, as well as a potentially efficient method of immunizing against a variety of antigens encoded within plasmid DNAs used to transform these bacteria. All of these methods have been demonstrated to deliver intact plasmid DNA to sites of interest, with expression of encoded reporter genes or immunogens. Ultimately, the purpose of these facilitated delivery systems is to protect and enhance cellular uptake of encapsulated DNA sequences.

II. Serum Kinetics and Uptake Mechanisms

From the few pharmacokinetic studies done, we know that intravenously injected plasmid DNA (naked or packaged in liposomes) has a serum (extracellular) half-life of $<5–10$ minutes and that the majority of injected DNA is degraded by serum nucleases and cleared from the body through the kidneys (Kawabata *et al.,* 1995). It is also known that intravenously injected DNA tends to distribute to selected tissues such as the liver, spleen, and skeletal muscle and that muscle-associated intracellular DNA appears to persist longest *in vivo* (Lew *et al.,* 1995). We have demonstrated that DNA injected intramuscularly travels from the injection site to the draining lymph nodes within minutes of injection (unpublished data, 1996). It is not known what percentage of DNA, or indeed if any DNA, that enters the draining lymph node actually passes out of the node and spreads systemically. Despite evidence derived using the polymerase chain reaction (PCR), showing efflux of DNA from injection sites, we do not know if this DNA is cell-associated (intracellular or cell surface) or free in the serum. However, we can safely predict that the vast majority of injected DNA is rapidly degraded and probably cleared through the kidneys.

When Wolff *et al.* (1992a) first demonstrated *in vivo* transient transfection of myocytes, it was proposed that these cells were unique in their ability to take up plasmid DNA. This was believed to occur as a result of the substantial network of T tubules that penetrated deeply into myocytes. It was also postulated, primarily on the basis of electron microscopic studies, that uptake may be facilitated by non-clathrin-coated pits called "caveolae" (Anderson *et al.,* 1992; Danko and Wolff, 1994; Wolff *et al.,* 1992a). However, in both instances, no discussion was presented as to how a relatively large, polyanionic, and hydrophilic

molecule could successfully penetrate the charged lipid bilayer into the largely anionic cell cytoplasm. Further explanations for this mystery were not immediately forthcoming and, indeed, with the demonstration that nonmyocytic cells such as keratinocytes, fibroblasts, and epithelial Langerhans cells were also able to take up naked plasmid DNA, the mystery of membrane association and translocation seemed even greater than before (Casares *et al.,* 1997; Condon *et al.,* 1996; Raz *et al.,* 1994). Indeed, there has been little research, in the context of DNA-based vaccines, to try to determine how plasmid DNA is perceived at the cell surface, let alone translocated into the cytoplasmic and nuclear compartments. In the absence of direct data, we must rely on several unrelated studies that allow us to theorize on several additional possible mechanisms of plasmid DNA uptake.

Naked DNA uptake by cells *in vivo* may be occurring either by a nonspecific method akin to a phagocytic or pinocytotic process or by a specific receptor-mediated mechanism. Certainly it has been demonstrated that DNA can access the cytoplasm through "leaky" phagosomal membranes within the cytoplasm (Kovacsovics-Bankowski, and Rock, 1995). Receptor-mediated uptake is also plausible, although the specific mechanism by which bound plasmid DNA is translocated into the cytoplasm is still unknown (Bennett *et al.,* 1985). There is preliminary evidence that polyguanylate sequences may facilitate receptor-mediated uptake (Kimura *et al.,* 1994; Pisetsky, 1996a). Candidate DNA-binding cell-surface receptors include the macrophage scavenger receptors and a 30-kDa surface receptor found to bind 4500-bp fragments of genomic DNA (Bennett *et al.,* 1988; Kimura *et al.,* 1994; Krieger *et al.,* 1985). Macrophage scavenger receptors are capable of binding a variety of macromolecules including polyribonucleotides such as poly(I) and poly(G). A novel 30-kDa DNA-binding receptor, first identified on cancerous cells, appears to be very specific and also appears to be restricted to professional antigen-presenting cells and T cells. Complexes composed of this 30-kDa receptor and DNA have been shown to internalize, and there is evidence that some of this internalized DNA is degraded.

Packaging and protection of plasmid-based vaccines within cationic liposomes, cochleates, dendrimers, or ballistic delivery systems may seem to make further research into mechanisms of naked DNA redundant; however, a clear understanding of the modality of cell association and translocation may result in a reduced need for cytofectins and ultimately may increase the cost-effectiveness of this novel form of immunization. Indeed, cost is uniquely important in the development of practical and efficacious vaccines for the food animals industry.

III. Mechanism of Antigen Presentation

Despite the fact that intramuscular injection has been the most prevalent route of delivery for DNA-based vaccines, the details of the mechanism of immunity are, at least hypothetically, more clearly understood with dermal and intradermal delivery of DNA. Initially it appeared that intramuscular delivery was the most efficacious mode of administration due to the presence of the unique T-tubular network, which presumably allowed deep penetration of the injected DNA into the cells and conceivably facilitated cytoplasmic access of injected DNA. Certainly intramuscularly injected, plasmid-encoded reporter genes such as β-galactosidase clearly demonstrated that myocytes were effectively transfected and, depending on the protocol and construct, could be transfected, at least transiently, with efficiencies approaching 30% (Hartikka *et al.,* 1996). Despite the demonstration that major histocompatibility complex (MHC) molecules could be upregulated on myoblasts, and to varying degrees on myocytes, and that these cells were capable of producing the cytokine interleukin (IL)-15, it was shown that these cells were incapable of synthesis of important costimulatory molecules such as B7 or CD40 ligand. This information, in conjunction with the inability of myocytes to travel to draining lymph nodes, led researchers to predict that transfected, antigen-producing myocytes were highly unlikely to be directly involved in the priming and expansion of the antigen-specific T- and B-cell receptor repertoires. Indeed, it has been demonstrated by several authors that myocytes transfected with plasmids "hand off" antigen to professional antigen-presenting cells such as dendritic cells or macrophages. An important experiment confirmed the suspicions that myocytes transfected *in vivo* were indirectly involved in T-cell priming and that direct *in vivo* transfection of professional antigen-presenting cells was not an absolute requirement for priming responses (Ulmer *et al.,* 1996a). Ulmer *et al.* injected myoblasts of the H-2^k haplotype, transfected *in vitro* with plasmid-encoded influenza virus nucleoprotein antigen, into F_1 hybrid mice derived from a parental cross of H-2^k and H-2^d haplotypes and found that $CD8^+$ cytotoxic T-cell (CTL) responses developed to both haplotypes. Similar independent studies confirmed the theory that the transfected myocyte was primarily a source of antigen that could be accessed through the normal release of secreted protein or the release of cell-associated antigen through antigen leakiness, overproduction-related cytotoxicity, or antigen-specific CTL activity (Corr *et al.,* 1996; Iwasaki *et al.,* 1997). Although this theory is still plausible and may explain the longevity of humoral and cell-mediated immune (CMI) responses associated with DNA-based vaccination, it has come under fire (Feltquate *et al.,* 1997).

We have demonstrated, with the use of PCR, that within minutes after an intramuscular injection of plasmid-encoded antigen, the gene sequence can be detected within the draining lymph node (unpublished data, 1996). However, to date, no one has demonstrated the presence of plasmid DNA within draining lymph nodes 20 hours or more after intramuscular injection, which suggests that intracellular localization within this compartment is limited. Also, "Van Gogh" experiments in which tissues, including muscles, receiving plasmids have been amputated at various time points following injection suggest that excision of DNA-immunized muscle within 1 minute of DNA delivery had no effect on the initial immune response (Torres *et al.,* 1997). This work suggests that transfection of myocytes is irrelevant to the developing immune response and that critical transfection of cells distal (lymphatics and draining lymph nodes) to the injection site, or possibly the extremely rapid exit of transfected resident interstitial dendritic cells, must occur. The latter explanation seems unlikely because it assumes that unfacilitated transfection (or at least a stable receptor-mediated preuptake association with cells) occurs extremely rapidly and that maturation and/or mobilization signaling must occur almost simultaneously with DNA binding. These studies remain controversial. For example, some studies have demonstrated that excision of skin sites within minutes of ballistic delivery of plasmid had little impact on the developing immune response (Taubes, 1997). These initial observations contradict more recent information and leave considerable ambiguity with regard to plausible explanations for the mechanisms of the immune response to naked DNA delivered intramuscularly or intradermally (Torres *et al.,* 1997). Thus, from a mechanistic standpoint, the contribution of transfected myocytes, or any nonprofessional antigen-presenting cell at the injection site, to the character of immunity is very much in question. Although it is not surprising that myocytes do not play a direct role in priming naive T and B cells, it will be surprising if they are found to play no role whatsoever in some aspect of the immune response. Perhaps longer-term studies will demonstrate that myocytes, by virtue of their relatively long period of plasmid-encoded gene expression ($\geq$30–60 days) or "bolus" antigen release following antigen-specific CTL activity, do contribute to the durability of the immune response by providing a reservoir of antigen that serves to sustain B- and T-cell responses (Bachman *et al.,* 1994, 1996; Neuberger, 1997; Robinson and Torres, 1997).

The demonstration that a multiplicity of cell types including keratinocytes, fibroblasts, and cells with Langerhans cell morphology were able to take up, in the absence of any cytofectins, intradermally injected

plasmid DNA and express an encoded reporter gene was perhaps pivotal in allowing a greater understanding of the mechanistic possibilities of immune responses to DNA-based vaccines (Fynan *et al.,* 1993; Raz *et al.,* 1994). This work, in the context of maturation and mobilization studies on peripheral dendritic cells, allowed the prediction that injection site transfection of Langerhans cells, and subsequent maturation and migration to draining lymph nodes, was a likely mechanism for priming of T- and B-cell responses (Austyn, 1996; Lutz *et al.,* 1996). Several articles have established that *in vitro* and *in vivo* transfected Langerhans cells are able to localize within the draining lymph node and are of critical importance in the initiation and expansion of the developing immune response (Condon *et al.,* 1996; Manickan *et al.,* 1997a). Thus research into dermal/epidermal delivery of plasmids encoding reporter genes has allowed investigators studying intramuscular delivery of DNA vaccines to develop a more accurate picture of the underlying mechanism of immunity following immunization with plasmid DNA.

Apart from the issue of the role transfected myocytes play in the overall character of immunity, a significant question remains. It concerns the possibility that a lymph node resident population of relevant professional antigen-presenting cells is transfected by the "wave" of antigen-encoding DNA that washes into the node following injection. If this occurs, then one must ask what, if any, role these node resident cells play in the overall immune response. Certainly, the literature suggests that many interdigitating and possibly follicular dendritic cells within the lymph node represent a mature population that have arrived via afferent lymphatics (Steinman *et al.,* 1997). Theoretically, transfected, interdigitating, phagocytically inactive antigen-presenting cells within the paracortical regions would, in the absence of an efficient endogenous to exogenous antigen-processing pathway, present plasmid-encoded antigen primarily in the context of MHC I. If this is the case, then distal transfection of a dendritic cell population may preferentially expand a $CD8^+$ CTL population. Certainly expansion of CTL populations is an important feature of DNA-based vaccines. Conversely, one might predict that transfection of peripheral dendritic cells found at the site of injection and expressing cell surface or secreted antigens may, on migration to the draining lymph node, facilitate more efficient priming and expansion of $CD4^+$ T-helper populations and ultimately B-cell responses. Indeed, studies utilizing Langerhans cells transfected *in vitro* and *in vivo* with plasmid expressing the marker gene for green fluorescent protein (GFP) have clearly shown trafficking of transfected Langerhans cells to the paracortical regions of the drain-

ing lymph node (Condon *et al.,* 1996). Alternatively, lymph node resident interdigitating dendritic cells, although hypothetically exhibiting greatly diminished phagocytic capacity, on transfection with plasmid-encoding, membrane-anchored antigens may result in significant MHC II loading and subsequent $CD4^+$ T-cell priming as a result of normal cellular "housekeeping" and membrane turnover (Guery and Adorini, 1995).

Although histological and immunocytochemical information abounds with regard to skin immunology and resident cell populations, this information is largely absent for tissues such as skeletal muscle. Although an interstitial population of dendritic cells has been identified within cardiac muscle, it is not known if a similar population occurs within skeletal muscle. Despite this paucity of information, predictions can be made regarding the mechanism of immune priming based on what is known to occur following intramuscular introduction of plasmid-encoded genes. First, myocytes are transfected relatively efficaciously following intramuscular injection (Hartikka *et al.,* 1996; Wolff *et al.,* 1990, 1991). Muscles injected with plasmid encoding the reporter gene β-galactosidase clearly show considerable ability to express this protein to a very high degree following uptake and nuclear localization of plasmid. Second, myocytes are immunologically incapable of priming T-cell responses directly and must "hand off" antigen to professional antigen-presenting cells in order to initiate the immune response (Hohlfeld and Engel, 1994; Torres *et al.,* 1997; Ulmer *et al.,* 1996a). Third, this priming population of professional antigen-presenting cells is likely to consist of dendritic-like cells because studies utilizing *in vitro* transfected antigen-presenting cells subsequently introduced into naive animals described immunity only if the transfected cells were dendritic cells and not macrophages (Manickan *et al.,* 1997a). It has been demonstrated that *in vivo* transfection of splenic B cells with plasmid encoding a heavy chain immunoglobulin displaying a B-cell epitope within the complementarity determining region 3 resulted in the development of a moderate humoral response after boosting with antigen peptide in adjuvant (Gerloni *et al.,* 1997). These researchers limited expression to B cells by utilizing immunoglobulin promoter enhancer elements and demonstrated that transgene integration into the B-cell genome occurred with high frequency (Xiong *et al.,* 1997). In this instance, the extent to which transfected B cells were contributing directly to the ensuing immune response is not known. It seems likely that FcR-mediated uptake of antigen by dendritic ells was largely responsible for priming responses; however, this remains to be demonstrated. It is also not known why coligation of B-cell receptors and FcγRIIB1 did

not inhibit the humoral response (D'Ambrosio *et al.,* 1995). Fourth, a wave of injected plasmid DNA washes into the draining lymph node within minutes of injection, supporting the possibility that extramuscular transfection may occur. Finally, studies involving Van Gogh experiments, in which the injected muscle bundle was excised within minutes of injection, had little impact on immunological priming or the early anamnestic response. This final piece of evidence is very compelling and suggests that a population of skeletal muscle resident dendritic cells, even if it exists, is of questionable relevance to the developing immune response. It also suggests that recruitment of immature dendritic cells to the skeletal muscle injection site, even if it did occur, would be of questionable use to the development of the priming immune response. On the basis of this information, we must conclude that the relatively high doses of intramuscularly injected DNA required to induce immunity are needed to effectively transfect critical immune surveillance cells distal to the site of injection (within draining secondary lymphoid tissues).

As stated previously, a variety of cell types can be transfected *in vivo* in the absence of any uptake-facilitating cytofectins. However, given that antigen-presenting cell acquisition of antigen occurs indirectly (Ulmer *et al.,* 1996a), and that normal resident and recent immigrant dendritic cells, in the course of normal maturation, undergo significant phagocytic downregulation, one might predict that transfection of virtually any cell type (including afferent lymphatic endothelial cells, subcapsular lining macrophages, or fibroblastic reticular cells) would require the presence of an immature, phagocytically active population of dendritic cells. Although it has been demonstrated that B cells and macrophages have the capacity to prime naive T cells, these antigen presenting cells are generally less efficient than dendritic cells in this regard (Cassell and Schwartz, 1994; Guery *et al.,* 1996; Romani *et al.,* 1989; Steinman, 1991; Ziegler and Unanue, 1981). This may, in part, be a result of the normal architectural constraints within the lymph node that are designed to bring together T cells (and possibly naive B cells) in the context of interstitial dendritic cells within the paracortical corridors (Steinman *et al.,* 1997). The larger doses typically utilized with DNA-based vaccines injected intramuscularly or intradermally may reflect the diminished efficacy of macrophage or B-cell priming of T cells and the need to transfect antigen-presenting cells, including dendritic cells, at some threshold level of efficacy. In light of data describing the potent immunostimulating, or adjuvant, capacity of bacterially derived DNA (Pisetsky, 1996a), it does not seem unreasonable to hypothesize recruitment of immature dendritic cells to the distal

afferent lymphatic, subcapsular sinus, or cortical regions of the draining lymph, followed by uptake and processing of antigen-expressed by resident nonprofessional antigen-presenting cells. For the moment, it appears likely that transfection of cell populations distal to the intramuscular injection site occurs; however, the exact role these populations play in development and maintenance of immune responses remains unclear.

IV. Adjuvancy Effects of Bacterially Derived DNA

Bacterially derived DNA (genomic and extrachromosomal) has been described as a member of bacterial, innate immune, activating substances that include lipopolysaccharide (LPS), teichoic acid, or peptidoglycans (Klinman *et al.,* 1997; Krieg, 1996; Pardoll and Beckerleg, 1995; Pisetsky, 1996a,b; Sparwasser *et al.,* 1997). The intrinsic adjuvant capacity of bacteria had been described as early as 1984 (Tokunaga *et al.,* 1984); however, it is only within the last several years, coincident with the first publications describing immune responses to DNA-based vaccines, that details of nucleic acid adjuvant effect have emerged (Sato *et al.,* 1996; Sun *et al.,* 1997). At present, three distinct elements of ISSs have been determined and/or postulated to play pivotal roles in the activation of the innate immune response, as well as in deviation of immunity toward a Th1-type phenotype. First, eukaryotes and plants exhibit what is termed "CpG suppression," characterized by a 20-fold lower than expected occurrence of the CpG dinucleotide pairs within genome nucleotide sequences. Conversely, bacterial, certain viral, and some nonvertebrate DNA sequences have been demonstrated to exhibit the expected number of CpG dinucleotide pairs within the coding and noncoding regions (Yamamoto *et al.,* 1992). Second, the CpG motifs that do occur in eukaryotic DNA are all methylated at the cytosine nucleotide, whereas the equivalent motif within bacterial chromosomal or extrachromosomal DNA is hypomethylated (Bird, 1987; Hergersberg, 1991; Pisetsky, 1996a). Finally, 5′ and 3′ DNA sequences immediately adjacent to the CpG motifs can determine both the efficacy of the target cell association of the DNA fragment and stimulatory capacity of the core sequence. Currently it is believed that a PuPu-CpG-PyPy hexamer is the most efficacious stimulatory hexamer, although this information was originally obtained by assessing blastogenesis and IgM production in DBA/2 and C3H/HeJ mice (Krieg *et al.,* 1995; Sato *et al.,* 1996). It has also been demonstrated that short synthetic 5′-NPu-CpG-PuN-3′ oligonucleotides with no additional flanking sequences are

also stimulatory, but only when prepackaged in cationic liposomes prior to delivery to murine splenocytes (Sonehara *et al.,* 1996). Further, has been demonstrated that flanking sequences outside of this hexamer core must include a guanine-rich region to ensure binding and subsequently uptake to target cells (Kimura *et al.,* 1994). These observations suggest that the hexameric ISSs must penetrate the cytosol of target cells to elicit responses. The exact mechanism of DNA association, and of uptake and cytoplasmic delivery, is unknown; however, there is evidence that the potential quadruplex polyanionic structure that arises due to the polyguanylate sequence domains binds with high affinity to the polycationic regions of the collagen-like domain of macrophage scavenger receptor I (Kimura *et al.,* 1994). It has been suggested that on entry into the cytosol, CpG motifs bind to the cAMP responsive element-binding protein (Sonehara *et al.,* 1996). Indeed, addition of the cAMP agonist forskolin results in the inhibition of ISS-induced upregulation of interferon (IFN) γ. The responses of professional antigen-presenting cells, T cells, and natural killer (NK) cells following stimulation with hexameric ISSs is wide ranging and profound, and includes blastogenesis, immunoglobulin M (IgM) secretion, and upregulation of cytokine expression and secretion.

Cytokines induced by ISSs include tumor necrosis factor (TNF) α, IFN α and β, IFN γ, IL-6, IL-12 and IL-18 (Halpern *et al.,* 1996; Klinman *et al.,* 1996; Roman *et al.,* 1997; Sparwasser *et al.,* 1997; Yi *et al.,* 1996). All but one of these cytokines have been shown to be derived from ISS stimulated macrophages, whereas B cells appear to be the major source of IL-6 and some IL-12. Inbred mouse strains including BALB/c, C3H/HeN, C3H/HeJ (LPS insensitive), CBA, C57BL/6, and SCID Beige, representing a variety of genetic and immunologic backgrounds, have been demonstrated to respond to ISS sequences displaying the 5′-PuPu-CpG-PyPy-3′ motif (Sparwasser *et al.,* 1997). It has been clearly shown that inclusion of these CpG motifs in plasmid-encoded vaccines not only ensures the success of the immune responses to the plasmid-encoded antigen but also specifically drives the immune response toward the Th1 phenotype (Carson and Raz, 1997; Chu *et al.,* 1997; Raz *et al.,* 1996; Roman *et al.,* 1997; Sato *et al.,* 1996). Theoretically, direct induction of IFN α and β, IL-12, IL-18, and TNF α, in conjunction with indirect NK cell-derived IFN γ, could deviate the ensuing immune response to a plasmid-encoded antigen toward the Th1 phenotype and/or enhance the serum levels of IgG_{2a} (Snapper and Paul, 1987). It has been demonstrated for one protein antigen and a commercially available subunit human influenza vaccine delivered intradermally that codelivery of 50 μg of plasmids containing ISSs not only increases

total IgG serum levels, but also leads to increases in serum IgG_{2a}, splenic IFN γ, and inhibition of serum IgE in BALB/c mice (Roman *et al.,* 1997). Surprisingly, Roman *et al.* (1997) also demonstrated that secondary immunization with a DNA-based vaccine could redirect a preexisting, Ag-specific Th2 response toward a Th1-type immune response. It has been established that some cytokines characteristically associated with early innate immune responses (IL-12, IL-18, IFN α and β) are all inducers of IFN γ and promote the development of Th1 lymphocytes (Roman *et al.,* 1997). Thus the ability of the innate immune system to instruct the adaptive immune response not only to mount an immune response but also to polarize the developing immunity is potentially useful when protecting against pathogens that require Th1-type immunity for clearance (Mosmann and Sad, 1996).

It must be pointed out that virtually all studies on ISSs of bacterial origin, or on synthetic oligodeoxynucleotides (ODNs), have taken place in mice. Caution, as always, should be exercised when attempting to extrapolate from ISS data gathered in mice to other species. For example, the defined, highly efficacious ISS 5′-PuPu-CpG-PyPy-3′ motif described for mice may require unique alternate flanking sequences to induce immune stimulation in other species. Indeed, it has been shown that binding properties of scavenger receptors can differ from species to species (Krieger *et al.,* 1985). For that matter, ruminant species, which exhibit a huge gastrointestinal microbial load, may have evolved an innate response that, through necessity, is relatively insensitive to putative ISSs. There is no doubt that ISSs have the potential to play an intriguing role in ensuring that rapid immune responses are evoked against invading pathogens by upregulating many aspects of the innate and, consequently, adaptive immune responses. It may even be possible that this "third genetic code" may define the preeminent example of initial early distinction between self and nonself (prokaryote and eukaryote) that drives the decision to mount an adaptive response (Janeway, 1992; Matzinger, 1994; Pisetsky, 1997).

V. Immune Response to DNA-Based Vaccines

A. Intramuscular Delivery

Table I shows a compilation of immune outcomes to a variety of DNA-based vaccines delivered intramuscularly in mice. This is by no means a complete list of references describing immune responses to plasmid-encoded antigen injected intramuscularly. However, it is a relatively comprehensive collection of citations describing data that

TABLE I

IMMUNE RESPONSES IN MICE IMMUNIZED INTRAMUSCULARLY WITH DNA VACCINES[a]

Pathogen or disease	Antigen and location	Mouse strain	Promoter	Method dose (boost)	Antibody response	CMI	References
Bovine herpesvirus-1	AgD p.m. SgD e.c CgD i.c.	C3H/HeN	RSV	100 μg (Y)	$IgG_{2a} > IgG_1$ $IgG_1 > IgG_{2a}$ $IgG_{2a} > IgG_1$	Splenic IFN $\gamma \gg$ IL-4 Splenic IFN $\gamma \gg$ IL-4 Splenic IFN $\gamma \gg$ IL-4	Lewis *et al.* (1997)
Herpes simplex virus-1	gB p.m.	BALB/c	HCMV	90 μg (Y)	$IgG_{2a} > IgG_1$	Splenic IFN $\gamma \gg$ IL-4	Manickan *et al.* (1995) Kuklin *et al.* (1997)
Hepatitis B virus	pre-S_2 + S (?)	C57BL/6	HCMV	100 μg (cardiotoxin pretreatment)	$IgG_1 > IgG_{2a}$	Splenic IFN $\gamma \gg$ IL-4	Mancini *et al.* (1996)
Mycobacterium tuberculosis	Ag85A e.c.	BALB/c C57BL/6	HCMV IntronA	100 μg (Y)	$IgG_1 > IgG_{2a}$ $IgG_{2a} > IgG_1$	Splenic IFN $\gamma \gg$ IL-4	Huygen *et al.* (1996)
Influenza virus	NP i.c.	BALB/c	HCMV	100 μg (Y)	$IgG_{2a} > IgG_1$	Splenic IFN $\gamma \gg$ IL-4 CTL	Pertmer *et al.* (1996)
Influenza virus	HA p.m.	BALB/c	HCMV	100 μg (Y/N)	$IgG_{2a} > IgG_1$	n.d.	Deck *et al.* (1997)
Hepatitis C virus	(Nucleocapsid)/ i.c. C2N (HBV Sag : C fusion)	BALB/c	HCMV	100 μg (Y/N) Cardiotoxin	IgG_{2a} IgG_{2a}	Splenic IFN γ + IL-2 Splenic IFN γ	Inchauspe *et al.* (1997)
Hepatitis B virus	S (major) pre-S2 (middle)	BALB/c C3H/HeN C57BL/6	HCMV	100 μg (Y/N)	n.d.	Splenic IFN γ + IL-2 > IL-4	Chow *et al.* (1997)
Bovine rotavirus	VP4 i.c.	C57BL/6	HCMV	200 μg (*Y)	IgG after viral boost	Splenic IFN $\gamma >$ IL-4 CTL	Suradhat *et al.* (1997)

Influenza A virus	H1 p.m.	BALB/c C57BL/6	?	1, 10, 25, 100 (Y/N)	$IgG_{2a} > IgG_1$	Splenic IFN $\gamma >$ IL-4	Feltquate *et al.* (1997)
Experimental autoimmune encephalitis	Vβ8.2 TCR i.c.	PL/J	HCMV	100 μg (Y) tibialis and cardiotoxin pretreatment	$IgG_1 > IgG_{2a}$	Splenic IL-4 > IFN γ CTL	Waisman *et al.* (1996)
Plasmodium yoelii	Circumsporozoite (CSP)	BALB/c	HCMV	40 μg (Y)	$IgG_1 > IgG_{2a}$ preboost $IgG_{2a} > IgG_1$ postboost	Splenic IL-4 > IFN γ (preboost) Splenic IFN $\gamma >$ IL-4 (postboost**)	Mor *et al.* (1995)
Herpes simplex virus-1	gB p.m.	BALB/c	HCMV	90 μg (Y)	$IgG_{2a} > IgG_1$	Splenic IFN $\gamma >$ IL-4 $\pm$CTL	Manickan *et al.* (1995)
Measles virus	HA p.m. sHA e.c.	BALB/c	HCMV	100 μg (Y)	$IgG_{2a} > IgG_1$ but $\uparrow\uparrow$ IgG_1 with sHA	CTL	Cardoso *et al.* (1996)
Clostridium tetani	Fragment C	BALB/c	HCMV	100 μg (Y)	$IgG_{2a} > IgG_1$	Splenic IFN $\gamma \gg$ IL-5	Anderson *et al.* (1996)
Murine B-cell lymphoma	Idiotype	C3H/HeN	HCMV	100 μg (Y)	$IgG_{2a} > IgG_1$	n.d.	Syrengelas *et al.* (1996)
Influenza virus	NP i.c.	BALB/c	HCMV	100 μg (Y/N)	$IgG_{2a} > IgG_1$***	Splenic IFN $\gamma >$ IL-4 CTL	Pertmer *et al.* (1996)
Hepatitis B virus	S ?	BALB/c	HCMV	5 μg (Y)	$IgG_1 > IgG_{2a}$	Splenic IFN $\gamma >$ IL-4	Gregoriadis *et al.* (1997)

[a] i.c., intracellular; p.m., plasma membrane; e.c., extracellular; (?), unknown; (Y), yes; (N), no; n.d., not done; >, greater than; $\gg$ much greater than; *, boost with DNA at 3 weeks, followed by boost with live BRV at 5 weeks; **, no *in vitro* restimulation of cytokine secreting cells; ***, three immunizations with plasmid DNA resulted in decreases in IFN γ and CTL levels and increases in IL-4 and IgG_{2a}.

include serum IgG isotype and/or splenic or lymph node cytokine profiles in immune mice. These data confirm that mice immunized intramuscularly with plasmid-encoded antigen typically, but not exclusively, respond with a Th1-type immune response, characterized by a predominance of IFN γ, in stimulated splenocytes, and of serum IgG_{2a}. However, Table I also shows that there is a significant number of instances in which splenic cytokine profiles characteristic of a Th1-type immune responses do not support the predominance of IgG_1 occurring in the serum (Lewis *et al.,* 1997; Mancini *et al.,* 1996; Mor *et al.,* 1995; Sallberg *et al.,* 1997). Indeed, there is at least one article that shows a profound Th2-type immune response with a predominance of serum IgG_1 and splenic IL-4 (Waisman *et al.,* 1996). These exceptions to the rule that "intramuscular delivery of plasmid-encoded antigens = Th1-type of immune response" clearly illustrate that parameters such as antigen, strain of mouse, and compartment in which the antigen occurs (Cardoso *et al.,* 1996; Lewis *et al.,* 1997) can affect the immunological outcome. They also suggest that timing of analysis, presence or absence of secondary immunizations, source of immune cells for ELISPOT (spleen versus draining lymph node versus bone marrow), inclusion or exclusion of an *in vitro* stimulation step, and use of ELISPOT or bioassay techniques should be considered when assessing and interpreting immunological outcomes following immunization.

There is growing evidence that intradermally delivered DNA is a more efficacious mode of immunization when using DNA-based vaccines. However, there is also evidence that incorporation of plasmids encoding antigen(s) within cationic lipids formulated with neutral lipids to form CLDC (cationic liposomes:DNA complexes) enhances humoral and T-helper cell responses following intramuscular delivery (Gregoriadis *et al.,* 1997). Innovations such as these will continue to push the dose and efficacy boundaries until DNA-based vaccines become legitimate alternatives to a variety of conventional vaccines.

B. Intradermal Delivery

Intradermally injected and ballistically delivered, DNA-based vaccines target the immunologically rich dermal and epidermal tissues of the skin. These regions of the murine skin are composed of a varied population of cell types, including keratinocytes, endothelial cells, fibroblasts, dendritic epidermal T cells ($\gamma\delta$ T cells), and Langerhans cells (Williams and Kupper, 1996). Epidermal Langerhans cells, which display highly proficient mechanisms for antigen capture and presenta-

tion in the context of MHC antigens and the ability to mobilize to regional lymph nodes, are necessary and sufficient to initiate immune responses to foreign proteins encoded within plasmids delivered to the skin. Although epidermal Langerhans cells account for only 1% of skin cells, the surface area available to this relatively small population of these vital cells is in the range of 25% of the epidermal surface area (Abbas *et al.,* 1997). One must also consider the microenvironmental milieu in the context of injection or ballistic delivery trauma and DNA adjuvancy effects. Keratinocytes , endothelial cells, $\gamma\delta$ T cells, fibroblasts, and mast cells are all capable of responding to trauma or inflammatory molecules by upregulating expression of a variety of cytokines (IL-1α and -β, IL-3, IL-6, IL-7, IL-10, IL-12, IL-15, and TNF α), chemokines (IL-8, MIP 2, IP-10, and RANTES), major histocompatibility antigens (MHC I and II), and costimulatory molecules (B7-2) (Bos and Kapsenberg, 1993; Gerritsen and Bloor, 1993; Schroder, 1995; Tigelaar and Lewis, 1995; Williams and Kupper, 1996). This microenvironmental milieu may contribute significantly to the resulting character of immunity, depending on the method of delivery and the "adjuvant dose" of DNA. Thus, method of delivery and immunological outcome must be considered in the context of the route (skin versus muscle) of delivery.

There has been some controversy as to whether intradermally injected DNA elicits a predominantly Th1/IgG_{2a} response or a Th2/IgG_1 response (Barry and Johnston, 1997; Feltquate *et al.,* 1997). In the few published articles describing splenic cytokine profiles and serum IgG isotypes, there appears to be little consistency in the character of the immune response, with antigen, mouse strain, and possibly variations in DNA adjuvancy (dose) playing a greater role than the method or route of administration (Table II). Splenic cytokine responses typically suggest that the predominant T-helper response is of the Th1 phenotype, although, once again, caution must be exercised to avoid overinterpreting this information in the absence of draining lymph node or peripheral blood lymphocyte (PBL) $CD4^+$ T-cell cytokine profiles. Typically, potent CTL responses are also achieved following intradermal injection of DNA-based vaccines (Raz *et al.,* 1994). However, none of the authors cited in Table II assessed this parameter of the immune response.

As with intramuscularly delivered DNA vaccines, there does not appear to be a hard-and-fast rule that describes the anticipated serological outcomes to intradermally injected DNA-based vaccines. There does appear to be a tendency toward diminished levels of serum IgG_{2a} and suggestive increases in serum IgG_1 compared to serum responses

TABLE II

IMMUNE RESPONSES IN MICE IMMUNIZED INTRADERMALLY WITH DNA VACCINES[a]

Pathogen	Antigen	Mouse strain	Promoter	Dose (boost) (site)	Antibody response	CMI (splenic)	References
Influenza virus	NP i.c.	BALB/c	HCMV	100 μg (Y) (tailbase)	$IgG_1 > IgG_{2a}$ $IgG_{2a} \uparrow\uparrow$ with boost but still $< IgG_1$	n.d.	Pertmer *et al.* (1996)
Influenza A	H1 p.m.	BALB/c	?	100 μg (Y/N) (tailbase)	$IgG_{2a} > IgG_1$ preboost $IgG_{2a} > IgG_1$ postboost	IFN $\gamma >$ IL-4	Feltquate *et al.* (1997)
Reporter gene	β-Gal i.c	BALB/c	HCMV	100 μg (Y) (tailbase)	$IgG_{2a} > IgG_1$	IFN $\gamma >$ IL-4	Raz *et al.* (1996)
B-cell lymphoma	Idiotype e.c.	C3H/HeN	HCMV	100 μg (Y) (tailbase)	$IgG_1 > IgG_{2a}$	n.d.	Syrengelas *et al.* (1996)
Bovine herpesvirus-1	gD p.m. tgD e.c.	C57BL/6	HCMV intronA	10 μg (Y) (tailbase)	$IgG_1 > IgG_{2a}$ (gD) $IgG_1 > IgG_{2a}$ (tgD)	IFN $\gamma >$ IL-4	Braun *et al.* (1997)

[a] i.c., intracellular; p.m., plasma membrane; e.c., extracellular; (?), unknown; (Y), yes; (N), no; n.d., not done; $>$, greater than; $\gg$, much greater than; $\uparrow\uparrow$, 15-fold increase in serum IgG_{2a} levels following boost antigens and a larger selection of mouse haplotypes or in outbred species.

following intramuscular injection of plasmids of the same dose. These serological trends tend to support the argument that the immunological composition of target sites (route) determines, to some extent, the character of immunity following delivery of plasmid-encoded antigen. Feltquate *et al.* (1997) have argued that the method (gene gun versus injection) of DNA delivery is solely responsible for the immunological outcome and that the cellular composition of the target site is essentially irrelevant to the resulting character of immunity. These data are compelling, and it will be of considerable interest and importance to see if this hypothesis holds up with a variety of antigens and a larger selection of mouse haplotypes or outbred species.

C. Ballistic Delivery

Ballistic delivery of DNA-based vaccines involves adsorption of small amounts (typically 1.0 μg or less) of plasmid to gold particles and acceleration of the coated particles into the epidermal layer and dermal tissues (Haynes *et al.,* 1996). Originally Mylar discs, coated with gold particles onto which plasmid DNA was adsorbed, were accelerated onto a stop screen that retained the mylar disc while allowing the gold particles and DNA to continue through the screen and into the skin surface. More recently, the gold particles with adsorbed DNA have been coated on the internal surface of Teflon tubing (Pertmer *et al.,* 1996). One end of the coated tube is placed on the pressure nozzle of the device used to provide propulsion to the particles, and the other is placed against the skin surface to be "immunized." Pressurized gas, typically helium, is utilized to accelerate the particles from the sides of the tubing and into the skin. This method of particle delivery minimizes the side spray of the vaccine. Obviously a significant drawback of Mylar disc delivery is the potential for waste of vaccine, as well as the distinct possibility of immunizing not only vaccinees but also workers delivering vaccines. Serological responses to ballistically delivered DNA appear to show less variation in isotype than observed in mice immunized intradermally or intramuscularly (Feltquate *et al.,* 1997; Pertmer *et al.,* 1996). However, the number of published articles describing serum antibody isotype and lymph node/spleen/bone marrow B-cell and cytokine profiles is small (Table III). More studies must be published before any dogmatic statements can be made regarding the predilection toward one Th type or another following ballistic delivery of DNA-based vaccines. Inclusion or exclusion of secondary immunization appears to be an important consideration when assessing cytokine

TABLE III

IMMUNE RESPONSES IN MICE IMMUNIZED WITH DNA VACCINES DELIVERED BALLISTICALLY[a]

Pathogen	Antigen	Mouse strain	Promoter	Dose (boost) (site)	Antibody response	CMI[A] (splenic)	References
Influenza virus	NP	BALB/c	HCMV	100 (yes) (abdominal epidermis)	$IgG_1 > IgG_{2a}$	*IFN γ > IL-4 **IL-4 > IFN γ CTL	Pertmer *et al.* (1996)
Influenza A	H1	BALB/c	HCMV	0.04–0.4 μg (Y/N) (skin)	$IgG_1 > IgG_{2a}$	IL-4 > IFN γ	Feltquate *et al.* (1997)
Influenza A	H1	BALB/c	HCMV	0.04–0.4 μg (?) (quadriceps)	$IgG_1 > IgG_{2a}$	n.d.	Feltquate *et al.* (1997)
Influenza A	H1	C57BL/6	HCMV	0.04–0.4 μg (Y/N) (skin)	$IgG_1 > IgG_{2a}$	n.d.	Feltquate *et al.* (1997)

[a] i.c., intracellular; p.m., plasma membrane; e.c., extracellular; (?), unknown; (Y), yes; (N), no; >, greater than; $\gg$, much greater than; [A]cytokine levels are determined from splenocytes unless otherwise indicated; $\uparrow\uparrow$, 15-fold increase in serum IgG_{2a} levels following boost; *, after one or two immunizations; **, significant decrease in IFN γ and increase in IL-4 after the third immunization.

or serum isotype data following ballistic immunization with plasmids encoding antigen. Indeed, it has been shown that splenic cytokine profiles may initially show a predominance of either IL-4 or IFN γ and that boosting can significantly increase the splenic levels of IL-4, with an accompanying reduction in levels of IFN γ (Pertmer *et al.,* 1996). Conversely, Feltquate *et al.* (1997) demonstrated that splenic cytokines characteristic of the Th2 phenotype, and accompanying high levels of serum IgG_1, were not altered by subsequent booster immunizations with intradermally or intramuscularly delivered vaccine. In this instance, the priming Th2 response could not be deviated toward the expected Th1 response (with increased levels of IgG_{2a}) when DNA was delivered via intradermal or intramuscular routes. One might predict that the initial Th2-type response is a direct result of the greatly diminished dose of hypomethylated CpG motif-mediated adjuvancy as a result of the dose limits associated with ballistically delivered DNA. Furthermore, one might argue that the trauma experienced by keratinocytes, fibroblasts, Langerhans cells, and endothelial cells may create a unique local cytokine and chemokine profile that deviates the developing cognate immune response toward a Th2-type response. However, the contribution of this postulated cellular trauma and associated inflammatory response may be minimal when one considers that transdermal delivery of liposome-encapsulated recombinant antigen leads to potent Th2 immune responses with the virtually exclusive production of serum IgG_1 (Dr. M. Baca-Estrada, personal communication, 1997). Immune responses to transdermally delivered antigen appear to be restricted to the local draining lymph node and argues that delivery site trauma plays little or no role in the ensuing character of immunity. It also suggests that a "preset" immune response within lymph nodes (or due to the entry of skin-derived dendritic cells) draining skin drives the response toward a potent Th2/IgG_1 immunity in the absence of an immune polarizing adjuvant such as LPS or hypomethylated CpG motifs (Everson *et al.,* 1996; Raz *et al.,* 1996). It would appear that the dose, in terms of diminished adjuvancy, and perhaps immune compartmentalization within the draining lymph node microenvironment may be primarily responsible for the Th2-type character of the immune response to ballistically delivered, plasmid-encoded antigen. Thus, the compelling argument made by Feltquate *et al.* (1997) that the method of DNA delivery determines the immunological outcome should be considered in the context of DNA dose as it relates to adjuvancy and immune compartment restriction. Furthermore, the cytokine/chemokine profiles *in vitro, ex vivo,* and *in situ* following intradermal and ballistic delivery of DNA vaccines must be assessed to ascertain what

roles, if any, the route, site, or method of DNA delivery has in the developing immune response.

VI. Mucosal and Epidermal Delivery

A. Unfacilitated Mucosal Delivery

Administration of DNA-based vaccines to mucosal and skin sites represents an exciting extension of more conventional systemic immunization studies currently underway (Ulmer *et al.,* 1996b). Surprisingly, mucosal delivery of naked DNA, without the aid cytofectins, results in substantial levels of transfection both local and distal to the inoculation site (Etchart *et al.,* 1997; Fynan *et al.,* 1993; Kuklin *et al.,* 1997). Fynan *et al.* (1993) demonstrated a 75% survival rate against lethal influenza virus in mice immunized intranasally with 100 μg of a plasmid-encoding a hemagglutinin glycoprotein (subtype H1). While mucosal levels of IgA were not assessed, it was clearly shown that intranasal immunization primed for systemic IgG responses. Kuklin *et al.* (1997) immunized BALB/c mice three times with 100 μg of plasmid-encoding glycoprotein B from herpes simplex virus (HSV)-1 and demonstrated expression of gB in the lungs and cervical lymph nodes. Although these mice developed delayed-type hypersensitivity responses and moderate to high levels of IgA in the vagina and feces, they were not protected following intravaginal challenge. Coadministration of 2 μg of cholera toxin (a potent adjuvant) with the DNA vaccine significantly increased the levels of gB-specific IgA occurring at the vaginal mucosa and offered some protection against a low dose of HSV-1 after vaginal challenge. Thus, the potential for using DNA vaccines at mucosal surfaces has been clearly established. However, researchers recognize the need to investigate methodologies whereby naked DNA may be delivered with greater efficiency to relevant cells associated with mucosal surfaces. Encapsulation of naked DNA within cationic liposomes or microspheres, and utilization of attenuated intracellular bacteria carrying plasmid-encoded antigens, represent novel approaches for enhancing delivery of DNA to cells of the mucosa and associated lymphoid tissues.

B. Liposome-Facilitated Delivery

It has been demonstrated that packaging naked DNA vaccines in cationic liposomes or microparticles can greatly increase the efficiency of delivery to certain mucosal surfaces (Ban *et al.,* 1997; Etchart *et al.,*

1997; Gregoriadis *et al.,* 1997; Klavinskis *et al.,* 1997; Puyal *et al.,* 1995; B. Wang *et al.,* 1997; Wheeler *et al.,* 1996). Despite preliminary evidence suggesting delivery limitations of liposomes, it now seems apparent that modification of existing cationic and neutral lipids, in conjunction with extensive testing of various combinations of these lipids, can greatly enhance delivery and expression of genes encoded within plasmids. It has been demonstrated that novel cationic lipids such as GAP-DLRIE, and existing cationic lipids such as DOTIM or DOTAP formulated with neutral lipids [DOPE (dioleoylphosphatidylethanolamine) or cholesterol], can enhance *in vivo* uptake and expression of intranasally or intravenously administered CLDCs 1000- to 2000-fold (Liu *et al.,* 1997; Templeton *et al.,* 1997; Wheeler *et al.,* 1996).

Immune responses to liposome-encapsulated, DNA-based vaccines delivered to mucosal surfaces are broad and include disseminated mucosal antibody and CTL activity, as well as significant serum levels of IgG and systemic $CD8^+$ CTL activity (Etchart *et al.,* 1997; Gao *et al.,* 1995). Further advances in lipid structural modification and formulation, in conjunction with a greater understanding of how the three-dimensional structure of cochleates, multilamellar vesicles (MLVs), and unilamellar vesicles (ULVs), alter the effectiveness of gene delivery, and the potential for targeting to specific cell types predict a significant increase in the success and use of DNA-based immunization (Gould-Fogerite and Mannino, 1996).

C. Microsphere-Facilitated Delivery

Encapsulation or adsorption of plasmids encoding reporter genes or antigens within or on the surface of biodegradable microparticles such as poly(DL-lactide-co-glycolide) can elicit antigen-specific mucosal IgA responses, as well as serum IgG titers, when delivered to mucosal surfaces (Alpar *et al.,* 1997; Jones *et al.,* 1997). These microparticles may provide a mechanism whereby adsorbed DNA vaccines gain access to immunologically critical dendritic cells (Lomotan *et al.,* 1997). Mice immunized with DNA complexed with microparticles displayed antigen-specific IgA responses at and distal to the oral and gastric mucosal administration sites (Jones *et al.,* 1997). Furthermore, oral immunization alone was able to stimulate significant levels of mucosal IgA and serum IgG. Alpar *et al.* (1997) demonstrated that intranasal administration of DNA adsorbed to biodegradable pseudo-polyamino acid microspheres [poly(DTH carbonate)] induced systemic IgG responses that equaled or exceeded antibody responses to Lipofectin (Life Technologies Inc., Gaithersburg, MD)-encapsulated DNA despite the use of four to eight times less DNA.

D. Adenovirus-Facilitated Delivery

Cointernalization of replication-deficient adenovirus with plasmids encoding genes can increase reporter gene expression in cultured cell lines with efficiencies equal to those achieved with cationic lipid-mediated transfection (Yoshimura *et al.,* 1993). Adenovirus-facilitated uptake of plasmids encoding gene sequences occurs as a result of receptor-mediated binding of virus to ubiquitously expressed receptors on host cells and subsequent endosomolytic activity of viral capsid proteins during acidification of the endosomal compartment (Allgood *et al.,* 1997). Adenovirus-mediated escape from the endosomal vesicles leads to the relatively efficient release of other macromolecules within the endosome. A number of modifications to the coinoculation protocol have been utilized, including cationic polymer-facilitated noncovalent association of plasmids to adenovirus and cationic lipid- and ligand-based enhancement of adenovirus–plasmid–DNA complex uptake (Fasbender *et al.,* 1997; Ferkol *et al.,* 1995; Schwarzenberger *et al.,* 1997; Wagner *et al.,* 1992). Covalent or noncovalent linkage of cationic polymer–plasmid DNA complexes to adenovirus to augment uptake of genes of interest has been reported (Allgood *et al.,* 1997). Typically, the cationic polymers have a very high affinity for the polyanionic phosphate groups of DNA and usually include poly(L-lysine) or polyethyleneimine moieties (Baker and Cotten, 1997). It has been hypothesized that poly(L-lysine) sequences may facilitate nuclear targeting of associated DNA sequences (Ferkol *et al.,* 1995). Although receptors for adenovirus are relatively ubiquitous on host cells, researchers have found that *in vivo* transfection of airway mucosa proceeds poorly due to inefficient binding of adenoviruses to the apical surface of epithelial cells (Ferkol *et al.,* 1995). One approach utilized to rectify deficiencies in mucosal epithelial delivery included formulating recombinant adenovirus vectors, expressing β-galactosidase, with polycationic lipids such as Lipofectamine (Life Technologies Inc., Gaithersburg, MD), GL-67, or a combination of GL-67/DOPE (Fasbender *et al.,* 1997). Although recombinant viral vectors alone were utilized in this instance, it is conceivable that polylysine–adenovirus–DNA conglomerates may be complexed with polycationic lipids to facilitate augmented mucosal delivery and uptake of plasmids expressing foreign antigens or immune modulating genes. A second approach utilized to circumvent problems associated with mucosal delivery of DNA vaccines involved covalent linkage of Fab fragments, directed against the IgA receptor at the basolateral surface of epithelial cells, to poly(L-lysine) and subsequent condensation with plasmid-encoding genes of interest (Ferkol *et al.,*

1995). Intravenous introduction of the ligand–poly(L-lysine)–DNA complexes leads to specific targeting of plasmids encoding genes to the airway epithelium. Although adenoviruses were not a component of these Fab–polylysine–DNA complexes, they have been utilized as ligand–adenovirus–poly(L-lysine) complexes and do appear to facilitate receptor-mediated augmentation of plasmid delivery to the cytoplasm of specific cell types (Curiel *et al.,* 1991).

E. Bacteria-Facilitated Delivery

In 1995 researchers utilized *Shigella flexneri,* an intracellular pathogen that normally invades hosts at intestinal mucosal surfaces, to deliver plasmids containing eukaryotic gene regulatory elements controlling the expression of β-galactosidase (Sizemore *et al.,* 1995). *Shigella* has the ability to enter mucosal epithelial cells and subsequently to escape the phagocytic vacuole in order to exist within the cytosol of the host cell. Researchers created an attenuated version of *Shigella* by deleting the *asd* gene, which encodes an enzyme essential for synthesis of a bacterial cell wall constituent. These deletion mutants retained the ability to penetrate host cells; however, cytosolic bacteria eventually lysed in the absence of a functional cell wall. Transformation of these attenuated bacteria with a eukaryotic expression plasmid containing the gene for expression of β-galactosidase, and delivery of bacteria to the eye surface and intranasally, demonstrated expression of plasmid-encoded reporter gene and cell-mediated immune responses, respectively. Researchers have demonstrated that attenuated *Salmonella typhimurium,* another intracellular pathogen, facilitated substantial levels of protection in mice after oral immunization with *Salmonella* transformed with plasmids encoding two virulence-associated antigens from *Listeria monocytogenes* (Darji *et al.,* 1998). This observation was somewhat surprising, considering that *S. typhimurium* does not access the host cell cytosol as easily as *Shigella* or *Listeria* species do and tends to persist within endocytic vacuoles. Despite this, transfer of plasmids encoding antigen or reporter proceeded efficiently. Finally, the intracellular pathogen *L. monocytogenes,* transformed with plasmids containing reporter genes or encoding antigen, has been demonstrated to facilitate the delivery of plasmids to the nucleus of several macrophage cell lines with great efficiency. In this instance, *Listeria* was attenuated by deletion of the virulence-associated gene *actA* and transformation with a plasmid encoding a bacteriophage lysin under the control of the promoter for *actA.* Following infection of a macrophage cell line, these attenuated mutants were unable to spread to neighbor-

ing cells and displayed >90% bacterial killing in macrophages. Subsequent transformation of these attenuated bacteria with plasmids containing genes encoding reporter proteins or antigens, under the control of a eukaryotic promoter, resulted in nuclear translocation of expression cassettes following infection of a P388D1 macrophage cell line (Dietrich *et al.* 1998).

Bacterial delivery systems may offer a unique opportunity to enhance delivery of DNA vaccines to mucosal surfaces of a variety of animal species. Wild-type *L. monocytogenes* has the capacity to infect over 40 different animal species; however, the lack of details on the pathogenesis of the neurological form of disease caused by this pathogen may limit its use at present.

F. Future Prospects

The future of liposomal and microsphere-facilitated, DNA-based immunization appears to lie in the ability to increase the serum or mucosal half-life of encapsulated DNA and to increase the efficiency of delivery to the cytoplasm of cells. These delivery vehicles also have the potential to target specific cells of the immune system more effectively than naked DNA alone (Eldridge *et al.,* 1989; O'Hagan *et al.,* 1993). There is also preliminary evidence that specific cell targeting may be further enhanced by inclusion of appropriate ligands in the outer lipid envelope of liposomal vesicles (Puyal *et al.,* 1995). It is not known whether targeting will offer any advantages at this point or if this is a possibility with biodegradable microspheres.

VII. Transdermal Delivery

The potential exists to immunize animals through topical application of DNA vaccines to the epidermis. The potential of this concept was first demonstrated in 1995, when researchers showed that reporter genes, packaged in liposomes, transfected hair follicular cells with high efficiency (Li and Hoffman, 1995). Although there was little evidence of transfection outside of hair follicles, transfection of cells within the base of the hair bulb was clearly evident. Transfection of this cell population, in conjunction with the vascularization and likelihood of lymphatic drainage of the dermal papillar region, allows one to hypothesize that plasmid-encoded antigen would quickly gain access to secondary lymphoid tissue. It has been demonstrated in several independent studies that immunization in mice can be carried out successfully by

topical application of virus (adenovirus) or protein to the skin surface (di Pellegrino and Ladavas, 1997; Wang *et al.,* 1996). This information, in conjunction with advances in transdermal delivery of drugs and proteins, clearly suggests that transdermal immunization with DNA vaccines is a plausible and novel approach to immunization.

VIII. Cytokine Modulation of Immunity to DNA-Based Vaccines

One of the primary goals of immunologists involved in vaccine development is to ensure that vaccination results in an immune response that best protects animals or humans against a given pathogen or disease (Mosmann and Sad, 1996). In order to accomplish this, we must be able to polarize $CD4^+$ T helper cells toward Th2 or Th1 phenotypes; ensure that the magnitude of humoral immunity, when required, is adequate; and elicit $CD8^+$ T-cell-mediated cytotoxicity when protection from infection requires this type of immunity. DNA-based vaccines currently display the potential to polarize immune responses by controlling several parameters somewhat unique to DNA vaccines. These parameters include the form of the antigen encoded within the plasmid, the method and route of delivery, and the dose of plasmids delivered (Cardoso *et al.,* 1996; Feltquate *et al.,* 1997; Lewis *et al.,* 1997; Pertmer *et al.,* 1996; Roman *et al.,* 1997; Sato *et al.,* 1996). A further advantage of DNA-based vaccines is the relative ease with which immunomodulating cytokines can be coadministered as plasmid-encoded gene(s) (Xiang and Ertl, 1995). The advantages of this approach include low cost, simplicity of codelivery, elimination of the need for multiple dosing of recombinant cytokines with short serum half-lives, and avoidance of toxicities and regulatory issues plaguing many current adjuvants (Gupta *et al.,* 1993; Hughes *et al.,* 1992). Disadvantages of this approach revolve around issues of potential toxicities associated with relatively long-term expression (Cohen, 1995). There is also the absolute requirement for identification and isolation of many cytokine genes in most economically important veterinary species and the need to characterize immune responses to antigen following coadministration of plasmids encoding antigen and cytokine.

Coadministration of plasmid-encoded cytokines can be carried out either by delivering a mixture of two separate plasmids, one of which encodes the antigen and one of which encodes the cytokine, or by delivering a single bi- or polycistronic vector with internal ribosomal entry sites separating genes representing antigen and cytokine (Clarke *et al.,* 1997). Cytokines may also be delivered as plasmid-encoded molecular

chimeras composed of antigen and cytokine usually separated by a domain-preserving spacer region (Maecker *et al.,* 1997; Syrengelas *et al.,* 1996). To date, a variety of cytokines and growth factors have been coadministered with plasmid-encoded antigen, including GM-CSF, IL-12, IL-2, IL-4, IL-6, IL-7, IL-1α and IL-β (Table IV). The data included in Table IV are not meant to be complete; however, the information presented summarizes a relatively comprehensive list of publications that have included data describing serum isotypes, splenic cytokine profiles, and CTL data.

Mice immunized with plasmids encoding antigens coadministered with plasmids encoding GM-CSF show substantial augmentation of serum IgG titer, seroconversion efficiency, and cell-mediated responses that include expansion of both $CD4^+$ and $CD8^+$ T cell populations. The mechanisms involved in these responses appear to involve enhancement of antigen immunogenicity (Iwasaki *et al.,* 1997), significant upregulation of IL-4 in spleens and draining lymph nodes (P. J. Lewis, unpublished data, 1997), and enhancement and maintenance of antigen capture and processing functions of local or recruited dendritic cell populations (Lutz *et al.,* 1996; Sallusto and Lanzavecchia, 1994). The dose of plasmids encoding GM-CSF and the number of secondary immunizations can have very significant, and surprising, effects on the immune outcome to coadministered, plasmid-encoded antigen. We demonstrated that a single dose (50 μg) of plasmids encoding GM-CSF, codelivered with a plasmid encoding a secreted version of bovine herpesvirus-1 glycoprotein D (BHV-1 gD) (which normally gave us very high serum titers of IgG_1), resulted in suppression of the humoral response (unpublished results, 1997). However, postboost titers reflected a potent anamnestic humoral response and a significant trend toward increased serum IgG_{2a} Conversely, moderate doses (10 μg) of plasmids encoding GM-CSF showed serum immunoglobulin isotypes that were predominantly IgG_1, regardless of whether mice received secondary immunizations. In some instances, coadministration of plasmids encoding GM-CSF enhanced antigen-specific cytotoxic immunity. Geissler *et al.* (1997) and Iwasaki *et al.* (1997) demonstrated enhancement of T-cell-mediated cytotoxicity to hepatitis C virus p21 core antigen and influenza virus nucleoprotein when plasmids encoding these antigens were coadministered with plasmids encoding GM-CSF (see Table IV).

Coadministered plasmids encoding the p35 and p40 subunits of IL-12 also demonstrated the capacity to enhance T-cell-mediated cytotoxicity responses, confer protection against challenge, and deviate immunity toward a Th1-type response (Irvine *et al.,* 1996; Iwasaki *et al.,* 1997; Kim *et al.,* 1997; Tsuji *et al.,* 1997). In these instances, a decrease in

TABLE IV

IMMUNE RESPONSES TO PLASMIDS ENCODING ANTIGEN COADMINISTERED WITH PLASMIDS ENCODING CYTOKINES[a]

Antigen	Mouse strain	Cytokine	Dose (boost) route/method	Antibody response	CMI	References
BHV-1 SgD	C3H/HeN	GM-CSF	10.0 μg (N) i.m.	↑ Ab kinetics ↑ seroconversion ↑ magnitude	n.d.	Lewis *et al.* (1997)
BHV-1 SgD 50 μg	C3H/HeN	GM-CSF	2.0 μg (Y) i.m.	small ↑ Ab kinetics ↑ magnitude (at 2–4 weeks)	↑ IL-4 (splenic/DLN)	P. J. Lewis, unpublished data, 1997
			10.0 μg (Y) i.m.	↑ mean serum IgG (at 2 weeks) ↑ serum IgG_1	↑ ↑ IL-4 (splenic/DLN) ↑ IFN γ (splenic/DLN)	
			50.0 μg (Y) i.m.	↓ mean serum IgG preboost ↑ ↑ mean serum IgG postboost ↑ ↑ serum IgG_{2a} postboost	↑ ↑ IL-4 (splenic/DLN) ↑ IFN γ (splenic/DLN)	
Rabies virus G protein 50 μg	C3H/He	GM-CSF	*10.0 μg i.m. *50.0 μg i.m. *250.0 μg i.m.	Direct correlation between dose of GM CSF and mean serum IgG titer	n.d. n.d. n.d.	Xiang and Ertl (1995)
20 μg	C3H/He	GM-CSF	100.0 μg (N) i.m.	↑ VNA after prime with ↑ ↑ after succesive boosts No change in survival postchallenge	n.d.	Xiang and Ertl (1995)

continues

TABLE IV (*Continued*)

Antigen	Mouse strain	Cytokine	Dose (boost) route/method	Antibody response	CMI	References
50 μg	C3H/He	GM-CSF	100.0 μg (N) i.m.	n.d.	↑ IL-2, GM-CSF	Xiang and Ertl (1995)
HIV-1 pcEnv	BALB/c	GM-CSF	50 μg (N) i.m.	↑ serum IgG	↑ blastogenesis n.c in CTL	Kim *et al.* (1997)
pcGag/pol		GM-CSF	50 μg (N) i.m.	↑ serum IgG	↑ ↑ Blastogenesis, n.c. in CTL	
HCV p21 core protein	BALB/c	GM-CSF	50 μg (Y) i.m.	** ↑ seroconversion ** ↑ serum IgG titer	n.c. in splenic IFN γ/IL-4 ↑ CTL IL-2 + GM-CSF = ↑ ↑ CTL	Geissler *et al.* (1997)
Influenza nonimmunogenic NP_0	BALB/c	GM-CSF	100 μg (Y) i.m.	n.d.	↑ ↑ in CTL after boost IL-12 and/or GM-CS	Iwasaki *et al.* (1997)
Carcinoembryonic antigen 2 μg	C57BL/6	GM-CSF	2.0 μg (Y) ballistic (abd.)	*** ↑ serum titer	*** ↑ blastogenesis	Conry *et al.* (1996)
B-cell tumor idiotype Ag p38c13	C3H/HeN	Id:GM-CSF chimaera	100 μg (Y) i.m.	$IgG_{2a} > IgG_1$	n.d.	Syrengelas *et al.* (1996)
			100 μg (Y) i.d. (tailbase)	$IgG_1 > IgG_{2a}$	↓ survival compared to that achieved with rId:GM-CSF conventional vaccine	
BHV-1 SgD 10.0 μg	C3H/HeN	IL-4	10.0 μg (N) i.m.	↑ mean serum IgG enhanced early humoral kinetics	n.d.	P. J. Lewis, unpublished data, 1997

50.0 μg	C3H/HeN	IFN γ	2.0 μg (Y) i.m.	↓ mean serum IgG ↑ mean serum IgG_{2a}	n.c in splenic IFN γ or IL-4 small ↑ in l.n. IFN γ or IL-4	P. J. Lewis, unpublished data, 1997
			10.0 μg (Y/N) i.m.	↓ mean serum IgG ↑ mean serum IgG_{2a}	n.c in splenic IFN γ or IL-4 ↓ in l.n. IFN γ or IL-4	Lewis *et al.* (1997)
			50.0 μg (Y) i.m.	↑ mean serum IgG ↑ mean serum IgG_{2a}	n.c in splenic IFN γ or IL-4	Lewis *et al.* (1997)
10.0 μg	C3H/HeN	TNF α	10.0 μg (N) i.m.	↑ 2 weeks; mean serum titers ↑ mean serum IgG_{2a}	n.d.	Lewis *et al.* (1997)
10.0 μg	C3H/HeN	IL-6	10.0 μg (N) i.m.	↑ mean serum IgG enhanced early humoral immunity; no impact on early kinetics	n.d.	Lewis *et al.* (1997)
****KLH or transferrin	BALB/c	IL-2	100 μg (Y) i.m.	↑ serum titer	↑ DTH	Raz *et al.* (1993)
		IL-4	100 μg (Y) i.m.	↑ serum IgG ↑ serum IgG_1		
Plasmid-encoded β-Gal	BALB/c	IL-2 IL-6 IL-7	1.0 μg (Y) ballistic (abdominal epidermis)	n.d.	↑ protection against murine colon adenocarcinoma cell line CT26 transfected with plasmid-encoded β-gal IL-2 > IL-6 > IL-7	Irvine *et al.* (1996)
	BALB/c	IL-12	0.1–1.0 μg (Y) ballistic (abdominal epidermis)	n.d.	↑ ↑ protection IL-2 > IL-6 > IL-7	

continues

TABLE IV (*Continued*)

Antigen	Mouse strain	Cytokine	Dose (boost) route/method	Antibody response	CMI	References
HIV-1 Gag/pol pcENv	BALB/c	IL-12	50 μg (N) i.m. myotoxin	$\downarrow$ mean serum IgG	$\uparrow\uparrow$ blastogenesis $\uparrow\uparrow$ spleen size and splenocyte numbers $\uparrow\uparrow$ CTL	Kim *et al.* (1997)
HIV-1 gp160 rev	BALB/c	IL-12	2–200 μg (N) i.m. (sucrose) (gastrocnemius)	No change in serum IgG or isotype profiles ($IgG_1 > IgG_{2a}$)	$\uparrow\uparrow$ DTH $\uparrow\uparrow$ IFN γ, $\downarrow$ IL-4 $\uparrow\uparrow$ CTL	Tsuji *et al.* (1997)
Influenza NP_0	BALB/c	IL-12	100 μg (Y) i.m.	n.d.	$\uparrow\uparrow$ CTL postboost; coadministration of plasmid-encoded B7-2 $\uparrow$'s CTL at first boost	Iwasaki *et al.* (1997)

Rabies virus G protein	C3H/He	IFN γ	100 μg (?) i.m.	$\downarrow$ VNA	boost $\downarrow$ IL-2	Xiang and Ertl (1995)
Hepatitis B virus S or $preS_2$ + S	BALB/c	IL-2	10–100 μg (N) i.m. Quads	$\uparrow$ serum IgG	$\uparrow\uparrow$ blastogenesis $\uparrow\uparrow$ IFN γ, IL-2 $\uparrow$ IL-4	Chow *et al.* (1997)
HCV p21 core protein	BALB/c	IL-2	50 μg (N) i.m.	$\uparrow$ serum, IgG, $\uparrow$ seroconversion, efficiency	$\uparrow$ blastogenesis, $\uparrow$ CTL IL-2, IFN γ (Th1 phenotype)	Geissler *et al.* (1997)
		IL-4	Quads—opposite leg from p21 plasmid injection	$\uparrow$ serum IgG, $\uparrow$ seroconversion efficiency	$\uparrow$ blastogenesis IL-2, IL-4 (Th0 phenotype)	

[a] (?), unknown; (Y), yes; (N), no; n.d., not done; n.c., no change; VNA, virus-neutralizing antibody; $>$, greater than; $\gg$, much greater than; $\uparrow$, increase; $\uparrow\uparrow$, large increase; *, cytokine coadministered at first immunization only; **, not statistically significant; ***, only if plasmid-encoded GM-CSF administered 3 days prior to G protein-encoding plasmid; ****, antigen delivered as a recombinant purified protein.

antigen-specific serum IgG and splenic IL-4 levels was consistent with the apparent polarization of the immune response toward a Th1-type immune phenotype (Tsuji *et al.,* 1997).

IFN γ is known to be involved in deviation of the immune response toward a Th1 phenotype and an immunoglobulin switch to an IgG_{2a} isotype (a surrogate marker for Th1 responses) (Snapper and Mond, 1993). Early studies suggested that codelivery of plasmids encoding IFN γ resulted in suppression of both humoral and cell-mediated immune responses (Xiang and Ertl, 1995). We also found clear evidence for a suppressive humoral effect following coadministration of 10 μg of this cytokine (Lewis *et al.,* 1997). However, we also noted that coadministration of plasmids encoding IFN γ resulted in increases in relative amounts of IgG_{2a}, although this increase was not consistent in all mice. More recently, we observed that following coadministration of 50-μg doses of IFN γ, with plasmids encoding a secreted form of BHV-1 gD, the suppressive humoral effect observed with the use of a lower dose was not evident and the tendency toward increased levels of serum IgG_{2a} was maintained (unpublished data, 1997). Interestingly, we found that coadministration of plasmids encoding TNFα with plasmids encoding a secreted form of BHV-1 gD not only increased the magnitude of serum IgG responses at 2 weeks but also increased the mean serum IgG_{2a} levels (Lewis *et al.,* 1997). However, despite the tendency toward higher levels of serum IgG_{2a}, we have not been able to demonstrate consistent isotype switching in individual animals with the use of a dose range of plasmids encoding IFN γ (unpublished data, 1997). Historically, immunoglobulin isotypes that fixed complement efficiently were believed to be critical in the efficient clearance of pathogens. More recently, the issue of isotype contribution to protection was raised by Bachmann *et al.* (1997), who demonstrated that beyond a threshold level of serum antibody, isotype becomes an irrelevant factor in clearance of a viral pathogen in a mouse model. Although this article is worth noting, it does not diminish the potential importance of efficient complement-fixing isotypes, such as murine IgG_{2a}, in other disease models (Ishizaka *et al.,* 1995) or the protective advantages a predominance of complement-fixing isotypes may offer in poor vaccine responders in outbred populations. Thus, it will be of considerable interest to assess other cytokines, such as IFNs α and β and IL-18, which are known to play roles in deviating the immune responses toward a Th1-type phenotype and potentially modulate the serum antibody profile, as well as facilitate the development of cytotoxic $CD8^+$ T-cell responses (Roman *et al.,* 1997).

Coadministration of plasmids encoding IL-2 or IL-4 with plasmids encoding antigens has been demonstrated to enhance humoral and cell-

mediated responses (Chow *et al.,* 1997; Geissler *et al.,* 1997; Raz *et al.,* 1993). Mice immunized with plasmids encoding IL-2 and antigen display increased serum IgG levels, increased seroconversion efficacy, and increased protection following challenge (Irvine *et al.,* 1996). Plasmids encoding IL-2 have also been demonstrated to enhance antigen-specific $CD4^+$ T-cell levels, cytotoxic T-cell levels, both IFN γ and IL-4 cytokine expression levels within splenic T-cell populations, and delayed-type hypersensitivity reactions (Chow *et al.,* 1997; Geissler *et al.,* 1997; Raz *et al.,* 1993). We were not able to show any augmentation of immunity following coadministration of IL-2 with a suboptimal dose of plasmids encoding a secreted form of BHV-1 gD. However, we assessed humoral responses only to a single moderate dose of IL-2 that may not have been optimized for C3H/HeN mice (Lewis *et al.,* 1997). Mice immunized with plasmids encoding IL-4 displayed increases in serum IgG in seropositive animals, increases in IgG_1, and seroconversion efficiency (Geissler *et al.,* 1997; Raz *et al.,* 1993). Mice receiving plasmid-encoded IL-4 also displayed a substantial increase in splenic T-cell blastogenic responses following *in vitro* stimulation with antigen. We demonstrated increases in mean serum IgG titers using a 10-μg dose of plasmids encoding IL-4 coadministered with plasmids encoding a secreted form of BHV-1 gD (Lewis *et al.,* 1997).

Several other cytokines, including IL-1α and β, IL-6, and IL-7, have shown a varying impact on the immune responses in mice immunized with DNA-based vaccines (see Table IV). It would appear, at first glance, that these cytokines are not worth pursuing as vaccine modulators; however, it must be pointed out that in many instances, responses to a dose range of these plasmids encoding cytokines have not been determined. In these cases, and with cytokines that do show modulatory effects, the dose range impact on the immunological outcome must be assessed before any generalizations can be made. Indeed, the dose range with each antigen, particularly if the expression compartment varies (intracellular, extracellular, etc.) in many different murine haplotypic backgrounds and outbred target species, must be determined before researchers can begin to appreciate the possibilities for immune augmentation or deviation through coadministration of plasmid-encoded cytokines.

Finally, several articles have assessed immunity to DNA-based vaccines encoding antigen:cytokine fusion proteins (Maecker *et al.,* 1997; Syrengelas *et al.,* 1996). Syrengelas *et al.* (1996) demonstrated that intramuscular delivery of plasmids encoding a B-cell lymphoma idiotype : GM-CSF fusion protein resulted in significant increases in serum IgG_1 levels that coincided with moderate increases in serum IgG_{2a}.

Intradermal delivery of this plasmid resulted in suppression of serum IgG_1 compared to plasmid encoding the B-cell lymphoma idiotype alone. Maecker *et al.* (1997) created plasmids encoding fusions between ovalbumin (OVA) and GM-CSF, IFN γ, IL-2, IL-4, IL-12, and a 9-amino acid fragment of IL-1β known to possess adjuvant activity. Serum antibody levels were minimal with all constructs except for the OVA:IL-4 chimera. Immunization with plasmids encoding OVA:IL-1β and OVA:IL-12 fusions appeared to deviate splenic cytokine responses toward a Th1 phenotype and appeared to elicit the strongest T-cell-mediated cytotoxic responses. In light of the molecular intricacies involved in creating and expressing stable, novel, chimeric proteins, these preliminary data should be compared directly to those derived from simpler immunization models in which modulatory proteins are coadministered as separate plasmids or coexpressed from the same plasmid that encodes antigen.

IX. Safety

A. Autoaggression and Autoimmunity

Several safety issues are of concern when addressing possible deleterious outcomes to the use of DNA-based vaccines. These issues include the potential for chronic autoaggression with autoimmune sequelae and the development of anti-DNA antibodies or a lupus-like syndrome. The technology exists, at least in mouse models, to achieve 25–30% *in vivo* transfection efficiencies following intramuscular immunization (Hartikka *et al.,* 1996). Several articles demonstrating the existence of CTL activity within the muscle mass (Davis *et al.,* 1997; Yokoyama *et al.,* 1997) clearly illustrate the potential for significant levels of immune-mediated myositis and potentially the development of a true autoimmune disorder through epitope spreading (Vanderlugt and Miller, 1996). Although the development of autoimmunity through epitope spreading remains a possibility, there has been at least one published report in which assessment of immunity toward the self-antigen myosin failed to detect any evidence of autoimmune responses following intramuscular DNA immunization (Mor *et al.,* 1997).

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by substantial levels of serum IgG that are typically reactive to double-stranded (ds) mammalian DNA (Gilkeson *et al.,* 1989; Steinberg *et al.,* 1990). Gilkeson *et al.* (1989) demonstrated that immunization of normal mice with bacterially derived (*Escherichia coli*) DNA, complexed to methylated bovine serum albumin (mBSA) in Freund's

complete adjuvant (CFA), elicits very high levels of antibacterial DNA antibodies. However, these mice showed no evidence of cross-reactivity with mammalian (calf thymus) dsDNA. More recently, it was demonstrated that immunization of BALB/c mice with single-stranded (ss) DNA complexed with mBSA in CFA also induced significant renal pathology (Gilkeson *et al.,* 1993). Most recently, cross-reactive anti-mammalian dsDNA antibodies developed in preautoimmune NZB/NZW mice immunized with bacterial DNA in mBSA and CFA (Gilkeson *et al.,* 1995). Thus it is possible, with the use of heavily adjuvanted bacterial DNA, to elicit antiDNA antibodies. However, it is important to note that these responses are highly dependent on formulation with potent adjuvants, demonstration in susceptible strains of mice, and the frequent requirement (particularly when inducing renal pathology) for ssDNA of bacterial origin as the immunizing antigen. Despite the apparent unlikelihood of lupus-like autoimmune disorders developing following immunization with DNA vaccines, the possibility remains that the potent immunostimulatory sequences encoded within injected plasmids may provide sufficient costimulatory signaling to activate B cells simultaneously recognizing plasmid DNA through engagement at the B-cell receptor (Krieg *et al.,* 1998). Certainly it has been clearly demonstrated that immunostimulatory sequences within plasmid DNA can induce proliferation, MHC class II antigen expression, costimulatory signal upregulation, and immunoglobulin synthesis in naive B cells.

Although it is still early, there has been no evidence for the sustained development of anti-DNA antibodies in animals immunized with naked DNA-based vaccines (Mor *et al.,* 1997). There has been some suggestion of a transient, predominantly IgM, antibody response. However, this response did not develop into a clinically relevant problem. Researchers investigating the potential of DNA-based vaccines to cause a lupus-like syndrome have focused on identifying serum antibodies that recognize DNA. Information indicates that autoimmune antibodies characteristic of this disorder are not directed solely against nucleic acids (Speciale, 1993). In over 95% of persons with SLE, antibodies binding a 30-kDa cell surface protein that normally binds cell membrane DNA were identified (Bennett *et al.,* 1987, 1988). The role these anti-DNA receptor antibodies might play in the development or maintenance of SLE is unknown. Indeed, these antibodies may simply be a diagnostic indicator for an increased risk of developing SLE or they may be involved in the conversion of a transient, T-cell-independent IgM response to a T-cell-dependent response. In either case, it will be important to screen for seroconversion to this neoantigenic complex (plasmid DNA:30-kDa re-

ceptor) when studying the potential for development of autoimmune disorders resembling SLE following immunizations with DNA-based vaccines.

B. Integration

Integration of injected plasmid DNA into host genomic sequences remains an issue of contention between researchers and regulatory agencies (Robertson, 1994). Initially, when it was believed that myocytes were unique in the ability to take up plasmid DNA and translocate it to the nucleus, the argument was made that the absence of mitotic activity in myocytes would virtually eliminate the occurrence of hazardous mutational integration (Danko and Wolff, 1994; Wolff *et al.,* 1992b). Of course, the frequent use of myotoxic agents such as cardiotoxin or bupivicaine to upregulate myoblastic mitotic activity diminished the importance of this argument. Similarly, the demonstration that uptake and nuclear localization can occur in a variety of cell types, and that injected DNA does not remain immediately adjacent to the injection site, has raised the specter of genomic integration once again (Condon *et al.,* 1996; Raz *et al.,* 1994; Torres *et al.,* 1997). More recently, following intramuscular injection of plasmids at doses of 100 μg per quadriceps, researchers were unable to demonstrate any evidence of integration in the genomic DNA harvested from 11 tissues (Nichols *et al.,* 1995). Indeed, these authors calculated that a mutation event arising from random integration of plasmid DNA into a host gene is 1.3×10^{-9} per cell. However, deleterious mutational events do not necessarily require integration into the coding region host cell gene. Also, with a spontaneous random integration frequency calculated at 2×10^{-6}, one may assume that the mutational frequency might actually be higher than the 1.3×10^{-9} per cell value given. Nichols *et al.* (1995) did envision a "worst case scenario" and assumed that each "gene" was composed of 1.0×10^{6} base pairs, an excessive length that would allow for integration into intron, regulatory, or other sequences not immediately adjacent to the coding region of the "gene." Indeed, the calculated value for a random mutational event occurring within the host cell genome may actually be artificially high.

Integration into the genomic DNA of stem cells, particularly those of the reproductive organs, is a matter of concern, particularly in light of the growing interest in liposomal or microsphere-mediated delivery that allows the circumvention of many uptake limitations that occur when delivering naked DNA. However, liposome-packaged, intrave-

nously administered plasmid DNA has been demonstrated to partition poorly to the testes or ovaries of mice (Lew *et al.,* 1995). This evidence, although speculative to some extent, suggests that the injection of DNA as a systemic or mucosal vaccine in animals and humans, does not provide great risk to recipients. Conversely, there is evidence demonstrating unequivocally that integration can occur following intrasplenic or oral administration of naked plasmid DNA (Gerloni *et al.,* 1997; Schubbert *et al.,* 1997). These results suggest that integration is a very real possibility and, more important, describes a procedure in which high levels of integration can occur. These data will allow researchers to begin to determine what the integration potential is for a given plasmid delivered in a specific manner, and perhaps provide the tools and information to establish assessment criteria that will determine the true risk of DNA vaccine integration into the genomes of relevant and irrelevant host cells. It would be unfortunate to have to assess the integration potential of DNA-based vaccines on a plasmid-by-plasmid basis as sequences within DNA-based vaccines are altered to accommodate changes in regulatory elements or new antigens. This "overkill" approach for assessing the risk of DNA-based vaccines would be unfortunate and, in all likelihood, unnecessary, particularly in light of the fact that a nonprescription form of genomic DNA (from salmon sperm) has been taken by injection, orally, and by topical administration for decades without any apparent adverse effects (Whalen and Davis, 1995). Indeed, the oral doses of this DNA-based supplement are 1000- to 100,000-fold greater (125–250 mg) than the highest doses of DNA vaccines currently injected into mice. Of course, fundamental differences remain between intrinsically immunosuppressive eukaryotic DNA and profoundly immunostimulatory bacterial DNA that must not be overlooked. Indeed, short linear fragments of self-DNA have been identified that are normally bound to the outer cell membrane of antigen-presenting cells (Lerner *et al.,* 1971). The exact function of this cell surface population of nucleic acid is unknown; however, it is possible that these sequences suppress effector T-cell functions that may be directed against antigen-presenting cells presenting antigen in the context of MHC antigens (Russell and Golub, 1978). This information, in the context of mucosal bacterial loads and the resulting exposure of host tissues to both genomic and extrachromosomal DNA, suggests that the risk of deleterious mutation events due to integration of nonself DNA at mucosal surfaces is minimal. Finally, the question of integration may be moot for the food animal industry given the relatively short time during which these animals would be exposed to any potentially

deleterious effects arising from an integration/mutation event following DNA immunization.

X. Future Directions

A. Mechanistic Studies

Poor understanding of the exact mechanism of immune induction by DNA-based vaccines necessitates the need to identify relevant cell populations involved in immune responses and to determine if naked DNA uptake by cells is a receptor-mediated phenomenon. Identification of critical cell populations and an uptake mechanism will potentially allow scientists to direct plasmids encoding antigens or immunomodulatory genes to cell populations in a more efficient and specific manner.

Immunostimulatory sequences or hypomethylated CpG motifs have a profound impact on the immune response to DNA vaccines and should prove to be a very interesting area of study. There are several issues with regard to immunostimulatory sequences that remain to be resolved, including the question of adjacent high-G sequences and their putative involvement in surface receptor binding and uptake. One may predict that motifs such as the high-G sequences may play a fundamental role in facilitating uptake of injected plasmid DNA, not only for adjuvant effects but also for translocation to the nucleus. Identification and mutation of these putative receptor sequences could possibly enhance natural transfection efficiency, allow targeting to specific APC populations, and enhance the efficacy of encoded adjuvant effects.

B. Target Species

Tables V summarizes delivery, dose, immunological, and challenge outcomes for a variety of target species. Generally speaking, the preliminary data summarized in Table V suggest that there is a very real potential for DNA-based vaccines to offer protection against diseases in outbred populations of veterinary importance. The efficacy of DNA vaccines has been a concern, particularly when one is faced with "scale-up" to species, such as cattle, that are 2000 times heavier at birth than the typical adult laboratory mouse.

Immunization of cattle with plasmids encoding BHV-1 gD required substantial doses of DNA with multiple boosting when administered intramuscularly (Cox *et al.,* 1993). Resulting serum antibody titers were relatively low when vaccine was delivered by this route, although

moderate levels of protection did occur. Subsequently it was demonstrated that intradermal administration of plasmids encoding a secreted version of BHV-1 gD (tgD) in the ear of cattle elicited moderate levels of serum antibody and offered greater levels of protection than immunization with plasmids encoding a membrane-anchored, authentic version of BHV-1 gD (van Drunen Littel-van den Hurk *et al.,* 1998). Additionally, immunization in the dermis of the ear in these cattle focuses immune responses with the parotid lymph node, which also drains the external nares and eyes in cattle (Sisson and Grossman, 1963). Localizing immunity within this lymph node will theoretically contribute to the level of local protective immunity at the normal mucosal route of entry for BHV-1 (Gao *et al.,* 1995). In this instance, it appears that form of antigen, choice of route, and method of immunization had a profound impact on the immunological outcome. Although there is a suggestion that delivery to dermal tissue results in greater immunological efficacy, the potential of intramuscular immunization must not be ignored. Indeed, it has been demonstrated that DNA immunization of different muscle groups in mice can result in substantial differences in the magnitude of the immune responses (Yokoyama *et al.,* 1997). It is also important to recognize that humoral immunity (levels of serum antibodies) is not always a good indicator of protection against a given disease. Intramuscular immunization of calves with plasmids encoding protective antigens from the parasite *Theileria annulata* failed to elicit detectable serum antibodies and yet provided significant levels of protection in 66% of animals following lethal challenge (d'Oliveira *et al.,* 1997). Similarly, dogs immunized with plasmids encoding canine parvovirus antigens did not develop any detectable serum antibody responses but were still protected from parvovirus infection (R. Schultz, personal communication, 1997).

Table V shows that in two instances there was either no evidence of protection (Monteil *et al.,* 1996) or disease progression was actually exacerbated following immunization (Richardson *et al.,* 1997). In one case, 1-day-old pigs from nonimmune mothers immunized intramuscularly with a DNA vaccine encoding pseudorabies virus gD failed to develop detectable levels of serum antibodies and were not protected following challenge (Monteil *et al.,* 1996). In this instance, immaturity of the neonatal immune system in piglets was probably a complicating factor. Immunization of young cats with plasmids encoding the feline immunodeficiency virus envelope protein resulted in enhancement of the early stages of infection (Richardson *et al.,* 1997). In this instance, the authors believed that low serum antibody titers or low-affinity antibodies may have played a role in dissemination of challenge virus.

TABLE V

DNA-Based Vaccination in Target Species[a]

Target species	Pathogen/antigen	Method/dose boost	Immune response	Challenge outcome	References
Cattle	BHV-1 gD	i.m./500 μg or 125 μg 4 boosts	Moderate serum ELISA and s.n. Ab at 20 weeks in high-dose animals Low Ab in low-dose animal	Decreased clinical signs Decreased viral shedding	Cox *et al.* (1993)
Cattle	BHV-1 gD and tgD[b]	i.m./1 mg 2 boosts	Low ELISA and s.n. Ab	n.d. with i.m. vaccinees	van Drunen Littel-van den Hurk *et al.* (1998)
		i.d. (ear)/500 μg 1 boost	Moderate ELISA and s.n. Ab	Decreased clinical signs Decreased viral shedding tgD offered greater protection	
Cattle	BRSV G protein	i.d. + i.m. total of 1.0 mg multiple boosts	Moderate to high serum ELISA Ab	Decreased viral shedding at day 7 but not at day 5	Schrijver *et al.* (1997)
Cattle	*Theileria annulata* Tams 1-1, 1-2	i.m./500 μg/of each antigen 2 boosts	No serum ELISA Ab	50–60% decrease in mortality following lethal challenge	d'Oliveira *et al.* (1997)
Sheep	*Taenia ovis* 45W	i.m./200 μg 2 boosts	Low serum ELISA Ab with DNA only	n.d.	Rothel *et al.* (1997)
		i.d./200 μg 1 boost	Moderate serum ELISA Ab with DNA followed by rec45W/QuilA boost		
Pigs	PRV gD	i.m./400 μg 1 boost	No serum ELISA or s.n. Ab preboost Low serum ELISA and s.n. Ab post boost	No significant clinical protection	Monteil *et al.* (1996)
Pigs	PRV gD	i.m./370 mg 1 boost with commercial vaccine	No s.n. Ab preboost Substantial increase in s.n. Ab postboost	Highest level of clinical protection compared to that achieved with a variety of vaccination regimens	Le Potier (1997)

Poultry	Influenza H1, H7	i.m., i.v., i.n., i.t./ 100–200 μg 1 boost	n.d.	Increased protection in 25–63% of chickens	Fynan *et al.* (1993)
Ducks	DHBV pre s/s and S protein	i.m./250 or 750 μg 2 boosts	High serum ELISA Ab after third immunization	Increased rate of systemic viral clearance in animals immunized with S protein Reduced viral replication in hepatocytes Increased *in vivo* neutralization with anti-S sera but not with anti-pre-S/S	Triyatini *et al.* (1998)
Fish	IHNV NP and G protein	i.m./10 μg	Increased serum ELISA Ab at 8 weeks with G protein and at 4 weeks with G protein + NP Increased s.n. Ab at 6 weeks with G protein and G protein + NP	50–60% decrease in mortality following lethal challenge	Anderson *et al.* (1996)
Cats	FIV env	i.m./400 μg 2 boosts	Low to undetectable serum ELISA Ab	Enhancement of infection	Richardson *et al.* (1997)
Dogs	IL-2, IL-6, GM-CSF	ballistic/0.5 μg buccal mucosa or epidermis	Increased infiltration of neutrophils at epidermal injection site with GM-CSF No change at buccal mucosa	n.d.	Keller *et al.* (1996)
Rabbits	CRPV major capsid protein (L1)	ballistic/1.0 μg over 30 sites 3 boosts	High serum ELISA and s.n. Ab Antigen-specific proliferation of PBMCs	90–100% protection following challenge	Sundaram *et al.* (1997)

[a] i.m., intramuscular; i.d., intradermal; i.n., intranasal; i.t., intratracheal; n.d., not done; s.n., serum neutralization.
[b] Truncated, secreted version of bovine herpesvirus-1 glycoprotein D.

They also hypothesized that plasmid-derived immunostimulatory sequences may have facilitated activation of T or B cells and subsequently augmented early levels of viral replication.

Despite the apparent absence of immune-mediated protection in certain models, preliminary data support the potential efficacy of DNA-based vaccines in target species. Enhancement of DNA vaccine efficacy in these species will necessitate a closer look at the method of immunization, targeting of appropriate immunization sites, the appropriateness of antigen form, expression cassette choice, and identification of the most effective cellular compartment in which antigen expression occurs. Also, coadministration of plasmids encoding cytokines is an area that has been largely unexplored in target species, as has enhancement of plasmid uptake by microparticle, adenoviral, or liposome facilitated delivery. These methods, and others, offer mechanisms whereby immune responses to antigens encoded in DNA-based vaccines and delivered to target species can be made more efficacious and practical.

C. Neonatal Vaccines

The presence of maternal antibody in the sera of neonates specifically inhibits the development of humoral responses in vaccinated animals (Ravetch, 1997). Conventional neonatal vaccination typically involves multiple high-dose immunization regimens designed to minimize the time between waning passive immunity and development of active immunity during which young animals are susceptible to disease (MacDonald, 1992). For many species of food animals this approach may not be cost effective, or achievable, with current management practices.

Use of DNA-based vaccines may allow certain advantages over existing licensed conventional vaccines when immunizing neonatal animals of immune mothers. First, longevity of expression of DNA vaccines may provide the means to ensure that all animals of similar ages (litters, posthatch) will develop active titers as quickly as possible after suppressive maternal-derived serum titers are no longer protective (Wolff *et al.,* 1992b). Second, DNA-based vaccines almost invariably induce potent CTL and frequently Th1-type immune responses, and may prove to be a more practical alternative than current vaccination approaches that typically demand high levels of serum antibodies following immunization of neonatal animals.

At this point it is presumptuous to assume that longevity of expression afforded by DNA-based vaccines will outperform existing or candidate adjuvants currently available (Cox and Coulter, 1997; Dalsgaard

et al., 1990; Davis *et al.,* 1997; Yokoyama *et al.,* 1997). This may certainly be the case when protection to a given pathogen requires a potent neutralizing antibody response. Consideration must also be given to the issue of immune stimulatory sequences within DNA-based vaccines and the contribution these sequences are expected to make to an immune outcome (Roman *et al.,* 1997). It is logical to assume that passively acquired maternal antibodies may functionally separate the benefits of the adjuvant effect of CpG motifs within delivered plasmids and the time point at which plasmid-encoded antigen can interact with naive B cells without the simultaneous inhibitory coligation of the FcγRIIB1 receptor. Temporal segregation of CpG-mediated immunostimulation and BCR interaction with antigen is further complicated when neonatal mice are used as models. Evidence demonstrates that the naiveté of the immune system in this species is such that high zone tolerance or a profound deviation toward a Th2 phenotype is the reaction to standard adult doses of vaccine (Forsthuber *et al.,* 1996; Ridge *et al.,* 1996). Although intradermal delivery typically utilizes 10- to 100-fold less DNA vaccine than intramuscular administration, these reduced doses further complicate the problem of CpG-mediated enhancement of immunity in neonates. With regard to tolerance induction, at least one article demonstrated B- and T-cell tolerance in neonatal mice following intramuscular (gluteal) immunization with a low-dose (10-μg) plasmid encoding the circumsporozoite protein (Mor *et al.,* 1996). These problems, and others, will challenge the ability of any vaccine to elicit strong B-cell-mediated immunity in passively immune neonatal animals. Fortunately, as mentioned earlier, DNA-based vaccines have the unique capacity to activate the cell-mediated and cytotoxic arms of the immune response, which may represent a more appropriate and achievable goal for protection in neonates. Obviously, the presence of a primed, expanded CTL population would convey a significant degree of protection to a variety of pathogens (Mosmann and Sad, 1996). Furthermore, a primed population of Th cells would narrow the window of susceptibility to disease by shortening the time to anamnestic immune responses. To date, no published article has described the development of humoral immunity in neonatal models following immunization with DNA vaccines. However, clear evidence of $CD4^+$ T-cell priming and development of $CD8^+$ cytotoxic T cells has been described (Bot *et al.,* 1996; Hassett *et al.,* 1997; Y. Wang *et al.,* 1997). Obviously, the duration of antigen expression is also a determining factor and will depend, to a large extent, on the species studied, the antigenic strength of plasmid-encoded immunogens, and the method and route of immunization. These aspects of DNA-based vaccines, and the growing awareness that

plasmid uptake and expression are occurring within critical lymphoid compartments distal to the site of vaccination, make the largely unexplored area of DNA–vaccine applications to neonatal immunity an exciting area of research.

D. Identification and Manipulation of Novel Antigens

In 1995 a group of researchers described a technique, termed "expression library immunization," that provided new possibilities for rapid, yet exhaustive, identification of protective immunogens from a variety of pathogens (Barry *et al.,* 1995). Researchers created a genomic expression library that represented most of the antigens (B- and T-cell epitopes) from the lung pathogen *Mycoplasma pulmonis.* Immunization of groups of mice with expression sublibraries followed by challenge with *M. pulmonis* allowed the identification of specific fragments of genomic DNA that encoded protective epitopes. This approach established that the specific identification of antigens was not necessarily required for creation of a protective immune response. It also provided the basis for an unconventional, yet highly efficient, procedure to screen entire genomes of pathogens for protective epitopes. Researchers involved in these studies have projected that expression library creation and initial screening for immunogens would take between 6 and 12 months for most pathogens (Taubes, 1997). Indeed, they have currently accomplished this task for several bacteria, including *M. pulmonas* and *Mycobacterium tuberculosis* (Taubes, 1997). There are several potential caveats inherent in this technique, including the absence of two possible reading frames from a single expression library, inherent costs of screening for protective epitopes in species other than mice, antigenic competition when immunizing with sublibraries, and partial protein expression resulting in the loss of conformational epitopes (Ulmer and Liu, 1996).

Ultimately, DNA-based vaccines are faced with many of the problems that have historically plagued immunologists involved in conventional vaccine development. Perhaps the most relevant problem involves poor antigenicity. Ultimately, if a given antigen tends to elicit poor immune responses as a conventional vaccine, it may also work poorly as a DNA-based vaccine. This leaves researchers with no choice but to spend money, time, and effort dissecting the structural and functional character of the antigen such that an informed, logical approach can be developed to enhance the antigenicity of the protein in question.

Bovine, human, and murine rotavirus VP7 antigens are a case in point. This antigen has been notoriously difficult to elicit immune re-

sponses against despite evidence that antibody responses directed against VP7 are protective (Andrew *et al.,* 1990; Hermann *et al.,* 1996). Researchers have utilized a variety of different approaches to enhance the immunogenicity of these antigens, with varying degrees of success. We have demonstrated that plasmids encoding bovine rotavirus VP7, delivered by a variety of different routes and methods, fail to elicit either humoral or cell-mediated immunity (unpublished data, 1997). *In vitro* expression of plasmid-encoded VP7 was achieved by altering the cell compartment in which VP7 normally occurs or by anchoring the antigen within the host cell membrane by the addition of a transmembrane anchoring domain from BHV-1 gD. However, these molecular modifications to VP7 failed to elicit immune responses following immunization with plasmids encoding these constructs. Coadministration of plasmids encoding GM-CSF and IL-4 also failed to facilitate immunity to this antigen (unpublished data, 1997). Finally, fusion of VP7 to multiple copies of C3d fragment, previously demonstrated to be a potent adjuvant for B-cell responses, did nothing to enhance immune responses to VP7 despite evidence showing significant increases in *in vitro* expression and greater stability of expression product (Dempsey *et al.,* 1996). We are faced with the necessity to dissect this antigen in an effort to determine if peptide sequences exist that specifically inhibit the responses to this antigen (Hengel and Koszinowski, 1997; Ijaz *et al.,* 1991; Volchkov *et al.,* 1992). Fortunately, DNA-based vaccine technology gives us the methodology to significantly shorten the time and cost required for preliminary testing of VP7 subfragments. In this instance, the obvious value of expression library immunization, even for small viral genomes, for the rapid identification of alternative immunogens becomes self-evident.

XI. Summary

Therapeutic and prophylactic DNA vaccine clinical trials for a variety of pathogens and cancers are underway (Chattergoon *et al.,* 1997; Taubes, 1997). The speed with which initiation of these trials occurred is no less than astounding; clinical trials for a human immunodeficiency virus (HIV) gp160 DNA-based vaccine were underway within 36 months of the first description of "genetic immunization" (Tang *et al.,* 1992) and within 24 months of publication of the first article describing intramuscular delivery of a DNA vaccine (Ulmer *et al.,* 1993).

Despite the relative fervor with which clinical trials have progressed, it can be safely stated that DNA-based vaccines will not be an immuno-

logical "silver bullet." In this regard, it was satisfying to see a publication entitled "DNA Vaccines—A Modern Gimmick or a Boon to Vaccinology?" (Manickan *et al.,* 1997b). There is no doubt that this technology is well beyond the phenomenology phase of study. Research niches and models have been established and will allow the truly difficult questions of mechanism and application to target species to be studied. These two aspects of future studies are intricately interwoven and will ultimately determine the necessity for mechanistic understanding and the evolution of target species studies. The basic science of DNA vaccines has yet to be clearly defined and will ultimately determine the success or failure of this technology to find a place in the immunological arsenal against disease.

In a commentary on a published study describing DNA vaccine-mediated protection against heterologous challenge with HIV-1 in chimpanzees, Ronald Kennedy (1997) states, "As someone who has been in the trenches of AIDS vaccine research for over a decade and who, together with collaborators, has attempted a number of different vaccine approaches that have not panned out, I have a relatively pessimistic view of new AIDS vaccine approaches." Kennedy then goes on to summarize a DNA-based multigene vaccine approach and the subsequent development of neutralizing titers and potent CTL activity in immunized chimpanzees (Boyer *et al.,* 1997). Dr. Kennedy closes his commentary by stating, "The most exciting aspect of this report is the experimental challenge studies. . . . Viraemia was extremely transient and present at low levels during a single time point. These animals remained seronegative . . . for one year after challenge" and "Overall, these observations engender some excitement" (Kennedy, 1997). Although this may seem a less than rousing cheer for DNA vaccine technology, it is a refreshingly hopeful outlook for a pathogen to which experience has taught humility. It has also been suggested that DNA vaccine technology may find its true worth as a novel alternative option for the development of vaccines against diseases that conventional vaccines have been unsuccessful in controlling (Manickan *et al.,* 1997b). This is a difficult task for any vaccine, let alone a novel technology.

DNA-based vaccine technology represents a powerful and novel entry into the field of immunological control of disease. The spinoff research has also been dramatic, and includes the rediscovery of potent bacterially derived immunomodulatory DNA sequences (Gilkeson *et al.,* 1989), as well as availability of a methodology that allows extremely rapid assessment and dissection of both antigens and immunity. The benefits of potent Th1-type immune responses to DNA vaccines must not be overlooked, particularly in the light of suggestions that Western culture

immunization practices may be responsible for the rapid increases in adult allergic and possibly autoimmune disorders (Rook and Stanford, 1998). The full utility of this technology has not yet been realized, and yet its broad potential is clearly evident. Future investigations of this technology must not be hindered by impatience, misunderstanding, and lack of funding or failure of an informed collective and collaborative effort.

Acknowledgments

The authors thank Ms. Michelle Balaski for excellent secretarial support. Work in the authors' laboratory is supported by the Medical Research Council and the Natural Sciences and Engineering Council of Canada. Dr. Jeff Lewis was supported by an Interprovincial Scholarship.

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EPIDEMIOLOGICAL RISKS FROM MIXED VIRUS INFECTIONS AND TRANSGENIC PLANTS EXPRESSING VIRAL GENES

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I. Introduction

Risk has been defined as Hazard × Probability. Risk assessment must be considered against the baseline of the natural situation. In the case of potential epidemiological risks from virus-resistant transgenic crops, the appropriate baseline is current farming practices and the

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A LIST OF VIRUS ACRONYMS USED

Acronym	Definition	Acronym	Definition
ACMV	African cassava mosaic	MSpV	Maize stripe
AMV	Alfalfa mosaic	MSV	Maize streak
ASGV	Apple stem grooving	PAMV	Potato aucuba mosaic
AYV	Anthriscus yellows	PCV	Peanut clump
BCTV	Beet curly top	PEBV	Pea early browning
BMV	Brome mosaic	PeLRV	Pea leaf roll
BMYV	Beet mild yellowing	PEMV	Pea enation mosaic
BNYVV	Beet necrotic yellow vein	PLRV	Potato leaf roll
BSBV	Beet soilborne	PMV	Panicum mosaic
BSMV	Barley stripe mosaic	PMWaV	Pineapple mealybug wilt-associated
BtMV	Beet mosaic		
BWYV	Beet western yellows	PNRSV	Prunus necrotic ringspot
BYDV	Barley yellow dwarf	PPV	Plum pox
BYMV	Bean yellow mosaic	PRSV	Papaya ringspot
BYV	Beet yellows	PSbMV	Pea seed-borne mosaic
CABYV	Cucurbit aphid-borne yellows	PStV	Peanut stripe
CaMV	Cauliflower mosaic	PVA	Potato virus A
CCMV	Cowpea chlorotic mottle	PVC	Potato virus C
CGMMV	Cucumber green mottle mosaic	PVV	Potato virus V
		PVX	Potato virus X
CLCuV	Cotton leaf curl	PVY	Potato virus Y
CLRV	Cherry leaf roll	PYFV	Parsnip yellow fleck
CMoV	Carrot mottle	RCNMV	Red clover necrotic mosaic
CMV	Cucumber mosaic	RTBV	Rice tungro bacilliform
CPMV	Cowpea mosaic	RTSV	Rice tungro spherical
CSSV	Cacao swollen shoot	SCMV	Sugarcane mosaic
CtRLV	Carrot red leaf	SHMV	Sunn-hemp mosaic
CTV	Citrus tristeza	SMV	Soybean mosaic
CVB	Chrysanthemum virus B	SPMMV	Sweet potato mild mosaic
CymRSV	Cymbidium ringspot	SPMV	Satellite panicum mosaic
EACMV	East African cassava mosaic	SqMV	Squash mosaic
GCMV	Grapevine chrome mosaic	STMV	Satellite tobacco mosaic
GLRaV-3	Grapevine leafroll-associated virus 3	TAV	Tomato aspermy
		TBRV	Tomato black ring
GRV	Groundnut rosette	TBSV	Tomato bushy stunt
HIV-1	Human immunodeficiency virus type 1	TCV	Turnip crinkle
		TEV	Tobacco etch
LIYV	Lettuce infectious yellows	TICV	Tomato infectious chlorosis
LMV	Lettuce mosaic	TmRSV	Tomato ringspot
LSMV	Lettuce speckles mottle	TMV	Tobacco mosaic
MCDV	Maize chlorotic dwarf	TNV	Tobacco necrosis
MCMV	Maize chlorotic mosaic	ToCV	Tomato chlorosis
MDMV	Maize dwarf mosaic	ToMoV	Tomato mottle
MNSV	Melon necrotic spot	ToMV	Tomato mosaic

(*continues*)

A LIST OF VIRUS ACRONYMS USED (*continued*)

Acronym	Definition	Acronym	Definition
TRV	Tobacco rattle	TYMV	Turnip yellow mosaic
TRSV	Tobacco ringspot	UgV	Ugandan cassava mosaic
TStV	Tobacco stunt	VNPV	Vanilla necrosis potyvirus
TSWV	Tomato spotted wilt	WMV2	Watermelon mosaic 2
TuMV	Turnip mosaic	WSMV	Wheat streak mosaic
TVMV	Tobacco vein mottling	ZYMV	Zucchini yellow mosaic
TYLCV	Tomato yellow leaf curl		

interactions that occur between viruses in mixed infections in nontransgenic crops. Hull (1994b) has pointed out that widespread deployment of transgenic plants may cause a significant increase in the probability of any of the interactions thought to have the potential for risk. The approach of refining transgenes to effect biological containment and thus reduce or eliminate the possibility of risk is therefore recommended (Hull, 1994b). Detailed understanding of the interactions that contribute to possible adverse effects is therefore desirable. One factor that must be considered in calculating both potential risks and the value of measures taken to reduce such risks is the relative levels of expression of transgenes in a plant infected by a different virus compared to the levels of both viral genomes in a natural mixed infection. In this article we will review the interactions between viruses in mixed infections and relate these interactions to the situation in virus-infected transgenic plants expressing viral sequences intended to confer viral resistance. Such comparisons are necessary to put perceived risks into perspective and to balance perceived risk against the potential benefits of effective viral resistance.

There are several types of potential interactions in such transgenic plants that have been posited to introduce the risk of new viral diseases or to increase the severity of diseases caused by extant viruses. The major areas of concern are recombination between viruses and viral transgenes to create new viruses (e.g., de Zoeten, 1991; Rissler and Mellon, 1993; Tepfer, 1993; Greene and Allison, 1994; Falk and Bruening, 1994; Miller *et al.,* 1997; Rubio *et al.,* 1999a) and the possible effects of either recombination or nonrecombinatory virus–transgene interactions to affect vectored transmission or viral ecology (e.g., Hull, 1990; de Zoeten, 1991; Rissler and Mellon, 1993; Tepfer, 1993; Falk and Bruening, 1994; Miller *et al.,* 1997). Another concern is possible synergistic reactions in transgenic plants (Hull, 1990; Miller *et al.,* 1997).

We will start by considering the occurrence of interactions in mixed infections, followed by examination of potential interactions in transgenic plants expressing viral sequences. We will also briefly review general aspects of vector specificity in different virus groups, as this could be altered either by recombination or by virus–transgene interactions. We will then discuss means of minimizing the perceived risks of interactions between viruses and transgenically expressed viral sequences. Finally, we will offer our opinion on the potential benefits to be gained from virus-resistant transgenic plants in comparison to the perceived risks.

II. Mixed Infections

A. Natural Occurrence of Mixed Infections

"Mixed infection" may be defined as the situation in which two or more strains of the same virus, or two or more distinct viruses, coinfect the same host. Many hosts are found to be susceptible to natural infection by more than one virus (e.g., Alper *et al.,* 1984; Brunt and Phillips, 1980; Davis and Mizuki, 1987; Duffus, 1963; Falk and Bruening, 1994; Hammond, 1981; Rochow, 1972). Mixed infections are common in many wild plants (e.g., Falk and Bruening, 1994). For example, 17% of randomly selected plants of *Plantago* spp. collected in the United Kingdom were found to be carrying mixed infections of as many as four viruses (Hammond, 1981, 1982), and Dodds (1993) reported multiple infections of five to seven viruses to be common in natural populations of *Nicotiana glauca.* Mixed infections are also common in many crop plants, especially those that are vegetatively propagated or that are perennial and thus have greater potential for exposure to multiple viruses. As many as nine distinct viruses have been isolated from individual spinach plants (Duffus, unpublished data, 1979, cited in Falk and Duffus, 1981). Plants may become coinfected by multiple inoculation by insects or other vectors. Many plant species carry viruses that are seed-borne or are propagated vegetatively (e.g., by tubers or cuttings), thus perpetuating virus infection; the plants may then be infected with additional viruses by vectors. There is a greater tendency for mixed infections to build up in perennial crops and in crops that are frequently handled. For example, Kassanis (1955) noted that stocks of most carnation varieties were virus-infected, and even carnation plants lacking apparent symptoms were readily shown to be infected by inoculation to indicator species. Kassanis observed that "most crops that are propagated vegetatively without rigorous selection for health become increasingly infected

with viruses." Similarly, Hollings (1957) wrote that "it is difficult to find a virus-free plant of many chrysanthemum varieties and normal-looking stocks are often carrying at least one virus"; no healthy plants could be found in several varieties from widely different sources. Many of the plants infected with chrysanthemum virus B (CVB) were also infected with other chrysanthemum viruses (Hollings, 1957).

Mixed infections can sometimes be identified on the basis of symptomatology if both of the two viruses produce different symptoms or if the syndrome occurring when the two viruses are present differs from the symptoms of each individual virus. However, mixed infections probably occur much more frequently than reported, either because certain infections are symptomless or because one virus dominates and masks the infection by another virus (Cheo, 1970; Sulzinsky and Zaitlin, 1982). Many multiply infected plants of *Plantago lanceolata* did not show any obvious symptoms (Hammond, 1981). Mixed infections are now more easily detected by the extensive use of the enzyme-linked immunosorbent assay (ELISA) in virus surveys conducted in various crops (i.e., Davis and Mizuki, 1987; Lecoq *et al.,* 1991).

A specific case of a mixed infection is the presence of two strains of the same virus in the host. This is probably the most common form of mixed infections, and may arise either from separate infections from different sources or from virus evolution within the host. It is generally accepted that virus strains exist as quasispecies as a result of the lack of proofreading during viral replication. A "quasispecies" may be defined as a population of molecules that can be represented by a consensus (or master) sequence. Within the population there will be every possible single and double nucleotide variant, and varying proportions of triple and higher-order variants from the consensus sequence (Domingo *et al.,* 1985). A mixture of strains of the same virus may be regarded as a mixture of more than one quasispecies—i.e., more than one consensus sequence is required to represent the population adequately. Particular sequence variants may have a selective advantage in different host species or genotypes or under different environmental conditions. However, competing quasispecies (two or more distinct consensus sequences) can coexist for many replication cycles before competitive exclusion of one or the other occurs (Domingo *et al.,* 1996). For example, Raccah *et al.* (1980) showed that both poorly aphid-transmitted and highly aphid-transmissible isolates of citrus tristeza virus (CTV) were maintained within a single tree. Genetic bottlenecks, in which small populations are isolated, may result in significant changes in the consensus sequence composition; these changes may or may not result in phenotypic effects. Serial bottleneck

events may cause accumulation of deleterious mutations and a decrease in viral fitness, a concept described in population genetics as "Muller's ratchet" (Muller, 1964; Domingo *et al.,* 1996). Genetic bottlenecks are probably frequent during the infection cycle of many viruses, particularly as a consequence of vectored transmission and establishment of new infections from relatively small numbers of virus particles (Domingo *et al.,* 1996). Thus Muller's ratchet is engaged, and there is a tendency for sequential incorporation of debilitating mutations. The effect of such mutations may be essentially irreversible unless compensatory mechanisms such as recombination can restore mutation-free classes of genomes (Muller, 1964; Domingo *et al.,* 1996). Recombination between related virus isolates in mixed infections is thus probably a frequent event allowing maintenance of productive virus infections.

Within a single infection (a quasispecies represented by a single consensus sequence), many mutations are silent (not resulting in a change in amino acids or resulting in a change in amino acids in regions that are not conserved and therefore have minor or no effect on the biological functions of the virus). Mutations that do occur in sites of biological importance must usually have a selective advantage in order to increase within the virus population. This advantage could be faster replication, transmissibility by vectors, better movement in the host, better passage through the seeds, and so on (e.g., Aldaoud *et al.,* 1989; Rodríguez-Cerezo *et al.,* 1989); however, a phenotypic change may not become apparent without transmission to a new host or without an environmental change affecting the host in which the mutant sequence arose.

1. Ways to Distinguish Mixed Infections and Identify the Components

One way to determine the presence of more than one virus in the host is by using local lesion hosts and starting a new virus population from a single lesion or single-vector transmissions. Failure to reproduce the original symptoms on the original host will often indicate absence of one component of a mixture. Alternatively, inoculation of differential or filter hosts (susceptible to one virus but not another) can be used to separate viruses or strains (e.g., Smith, 1931); this is less likely to separate strains of one virus than two distinct viruses. Serological methods will usually readily differentiate viruses of different groups (e.g., Matthews, 1991), and especially with monoclonal antibodies, it may be possible to differentiate strains of a single virus within an individual plant (e.g., Candresse *et al.,* 1998). Analysis of viral double-stranded RNAs can be used to detect mixed infections and will often

allow at least tentative identification of components of a mixed infection (Dodds *et al.,* 1984; Dodds, 1993). Hybridization with nucleic acid probes or the polymerase chain reaction (PCR) may also be used to detect and differentiate between distinct viruses and, in some cases, between strains of the same virus (e.g., Candresse *et al.,* 1998). Electron microscopy of leaf extracts may reveal multiple infections (e.g., Hitchborn *et al.,* 1966; Hammond, 1981), and examination of thin sections of embedded material may reveal virions and cytopathological effects characteristic of different viruses (Matthews, 1991) or, in some cases, of coinfections of different isolates of a single virus (Hull and Plaskitt, 1970). Mixed infections of distinct strains have been revealed by PCR amplification of specific sequences combined with restriction fragment length polymorphisms (e.g., Hammond *et al.,* 1998) or cloning of multiple different sequence variants obtained from a single plant (e.g., Magome *et al.,* 1997).

B. Types of Interactions Between Viruses in Mixed Infections

When two or more viruses are present in the same host, a variety of interactions may occur. Possible interactions between related or nonrelated viruses present in the same host include:

1. Lack of interaction, with each of the viruses replicating and being transmitted from the host independently.
2. Antagonism, or cross-protection, in which one virus adversely affects the replication, movement, or presence of another virus.
3. Enhancement of replication (synergism), in which one virus facilitates replication of another virus to a higher level than it would reach in a single infection. Enhancement of movement within a doubly infected plant may have similar effects.
4. Enhancement of transmission (dependent transmission) of another related or nonrelated virus that otherwise will not be transmitted or will be transmitted inefficiently.
5. Creation of hybrid viruses composed of segments of genomes of two viruses that coinfect the same host. The term used for this phenomenon is "recombination" (or "pseudorecombination" in the case of genome segment exchange in multipartite viruses), and this relationship is believed to be responsible for the evolution of new viruses.

1. Lack of Interaction

Apparently, lack of interaction reflects a situation in which either there is no exchange of genome sections or heteroencapsidation (see

below) or, if interactions occur, they are not reflected in any phenotypic change in the participating viruses. For example, Hull and Plaskitt (1970) used electron microscopy to demonstrate the accumulation, within the same cell, of aggregates of two distinct strains of alfalfa mosaic virus (AMV). In some cells the cytoplasm appeared to be divided into two areas, one containing strain Caldy aggregates and the other containing aggregates of strain 15/64. In other cases, 15/64 and Caldy aggregates were found in adjacent cells or groups of cells. As the aggregate type is characteristic of the virus isolate, and possibly of the coat protein (CP) (Hull *et al.,* 1970), it is apparent that nucleic acids of both isolates were able to replicate in the same cell, in this instance without apparent interaction. However, Hull and Plaskitt (1970) noted that it is possible to create pseudorecombinants of AMV by mixing appropriate nucleoprotein components (or RNAs) and that a vector could form new strains in nature as a result of feeding on a plant with such a mixed infection.

A second illustration is the presence of multiple strains of plum pox potyvirus (PPV) in leaves from the same branch of a single apricot or plum tree (Hammond *et al.,* 1998). Here the presence of multiple isolates with distinct restriction fragment length polymorphism patterns was not shown to occur at the level of single cells, but there was clear evidence of the replication of several distinct strains within a single branch. The differences in restriction patterns were such that it is unlikely that they represent sequence microheterogeneity within a single isolate (i.e., a single quasispecies). A more probable explanation is the introduction of distinct isolates to the tree by multiple aphids, possibly initially in different branches, with subsequent mixing as the isolates spread into other parts of the tree. Similar cases have been observed in fruit trees infected with multiple sequence variants of apple stem grooving virus (ASGV) (Magome *et al.,* 1997)

Absolute lack of interaction between viruses in mixed infections is not well documented because usually it is when interactions are observed that there is a report in the literature. For example, Demski and Jellum (1975) found no synergism between different pairings of four viruses affecting soybean; effects on stunting or yield reduction were additive, or less than additive, compared to single infections. Similarly, in a study on a possible increase in multiplication of cucurbit aphid-borne yellows virus (CABYV) in mixed infection with other cucurbit viruses, it was observed that there was no interaction between CABYV and cucumber mosaic virus (CMV) or squash mosaic virus (SqMV), in contrast to what was observed with potyviruses (Bourdin and Lecoq, 1994).

2. Antagonism and Cross-Protection

In a number of situations, infection by one virus or virus strain can prevent or limit multiplication of another virus or virus strain. Ponz and Bruening (1986) define two general classes of antagonism between viruses: indirect and direct. Indirect antagonism occurs when the agent induces the host to resist the challenging virus at a distance ("induced resistance"). Direct antagonism between two viruses or two strains of the same virus must take place in the same tissue or host cell ("cross-protection").

a. Indirect Antagonism. Indirect antagonism between viruses has been described in plants reacting with local lesions to the protecting and challenging virus inoculations, such as tobacco mosaic virus (TMV) in tobacco plants carrying the *N* resistance gene. In his classical work, Ross (1961) observed that TMV inoculation on lower leaves of Samsun-*NN* tobacco rendered noninoculated parts of the plant less receptive to a further inoculation by the same virus or by other unrelated viruses forming local lesions [such as tobacco necrosis virus (TNV) or turnip mosaic virus (TuMV)]. The resistance was estimated by lower numbers and/or reduced sizes of local lesions in comparison to untreated controls. This situation, in which the protecting virus is not systemic in the plant, was referred to as "systemic acquired resistance (SAR)." SAR is essentially nonspecific: it can be induced by other pathogens as well as by chemicals such as salicylic acid, and it is effective against various challenging viruses or pathogens (Fraser, 1985; Sticher *et al.,* 1997). In cucumber, for example, a primary inoculation with the fungus *Colletotrichum lagenarium* induces SAR against several diseases caused by fungi, bacteria, and viruses; a similar situation is observed when the primary inoculum is TNV (Sticher *et al.,* 1997). Molecular mechanisms involved in SAR are not well established. A number of pathogenesis-related (PR) proteins, including hydrolases (chitinases and glutanases), have been correlated with SAR, although their role in induced resistance is still controversial (Fritig *et al.,* 1990; Fraser, 1982). Another protein, known as "inhibitor of virus replication" (IVR), has been detected in the intercellular fluid of induced-resistant noninoculated tissues of Samsun-*NN* tobacco (Spiegel *et al.,* 1989). IVR was shown to be neither virus nor host specific (Loebenstein and Gera, 1990). Although extensively studied, SAR has not yet found a direct practical application for controlling virus diseases in the field. However, isolation and cloning of the gene coding for IVR (or eventually for PR proteins if they are involved in SAR) could lead to a new generation of virus-resistant transgenic plants.

b. Direct Antagonism. (*i*) *Antagonism between distinct viruses.* Direct antagonism between viruses inducing systemic infections has been described in a number of virus/host combinations. Although reports of direct antagonism between strains of the same virus are abundant, they are scarce for distinct viruses. Antagonism between distinct viruses is most often not reciprocal and may be expressed in different ways, including prevention of virus infection. When mechanically inoculated together with papaya ringspot virus (PRSV) or watermelon mosaic virus 2 (WMV2), zucchini yellow mosaic virus (ZYMV) generally predominates and is transmitted to a higher percentage of inoculated plants than are the other viruses, suggesting a competitive advantage in the early stages of infection (Davis and Mizuki, 1987; Bourdin and Lecoq, 1991). An extreme situation was described between two unrelated nepoviruses: tomato ringspot virus (TmRSV) and cherry leaf roll virus (CLRV). Infection of tobacco by TmRSV prevents infection by CLRV, while infection by a number of other nepoviruses has no effect on CLRV infection (Fulton, 1975). This interaction is strictly unilateral because CLRV-inoculated plants can be easily infected by TmRSV. Reduced multiplication of one virus in a mixed infection may also be observed, but this depends upon the second virus involved in the double infection and possibly also on the host plant. When mixed infections of WMV2 and ZYMV are established in zucchini squash, the WMV2 multiplication rate, as estimated by quantitative double antibody sandwich-ELISA (DAS-ELISA), is reduced in comparison to singly infected plants, while ZYMV multiplication remains unchanged (H. Lecoq, unpublished). In contrast, in CMV-ZYMV mixed infections in cucumber, ZYMV multiplication is reduced,while that of CMV is enhanced (Poolpol and Inouye, 1986). The molecular mechanisms involved in these different types of antagonism have not yet been elucidated. Antagonism expressed as a reduced level of virus replication has also been reported in certain associations between viruses and their satellite RNAs. In the case of CMV, for example, some satellites strongly attenuate symptom expression and significantly decrease virion production (Jacquemond and Tepfer, 1998).

(*ii*) *Antagonism between strains of the same virus.* McKinney (1929) observed that a tobacco plant systemically infected by a green strain of TMV was protected from infection by another TMV strain inducing a yellow mosaic. This phenomenon, called "cross-protection," was subsequently generalized not only to many other plant virus groups (except for a limited number of viruses including geminiviruses) but also to viroids and satellite RNAs (Lecoq, 1998; Singh *et al.,* 1990; Jacquemond and Tepfer, 1998).

Protecting and challenging strains can be inoculated mechanically, by grafting, or by using their natural vectors. Cross-protection is most often reciprocal, but it is not immediately effective after the inoculation of the protective strain. Hull and Plaskitt (1970) observed differences between isolates of AMV in the ability to exclude a second isolate and also a dependence on the time between inoculations. Generally, 1 or 2 weeks are required for the protecting strain to become systemic in the plant and for the cross-protection to be fully effective (Walkey *et al.,* 1992). If the challenging strain is inoculated during this period, or if the challenging and protecting strains are inoculated at the same time, both strains will multiply in the same host (Desbiez *et al.,* 1997). Experiments using quasi-isogenic mutants of ZYMV showed that cross-protection could occur within 24 to 48 hours after inoculation of the protecting strain (Desbiez *et al.,* 1997). As a general rule, observation of ZYMV reveals that the closer the molecular homologies between the protecting and challenging strains, the shorter the incubation period before cross-protection is fully effective (C. Desbiez and H. Lecoq, unpublished).

c. Practical Application of Cross-Protection. Cross-protection has been used in many host/virus combinations as an experimental means to establish virus–strain relationships (Matthews, 1991). Cross-protection has also been applied in the fields to control tobamo-, poty-, and closteroviruses in a number of annual and perennial crops, mainly vegetables and fruit trees, because virus infections drastically affect the quality of these products (Lecoq, 1998). For this purpose, the protecting strain used should cause attenuated symptoms and should reduce marketable yields only slightly, if at all, compared to those of healthy controls.

Mild strains have been generally selected by empirical approaches. Some were isolated as naturally occurring variants directly from plants with mild symptoms in the fields (Costa and Muller, 1980). Others were obtained in the laboratory, either after single local lesion isolations from samples with severe symptoms or from plants inoculated by severe isolates that spontaneously developed axillary branches with mild symptoms (Lecoq *et al.,* 1991). Mild isolates have also been obtained after heat or cold treatment (Oshima, 1975; Kosaka and Fukunishi, 1993); after nitrous acid mutagenesis treatment alone (Rast, 1972; Yeh and Gonsalves, 1984); or followed by ultraviolet (UV) light treatment of purified virus preparations (Tan *et al.,* 1997) or by site-directed mutagenesis (A. Gal-On and B. Raccah, unpublished).

To improve the efficiency of cross-protection and to minimize the risks involved in its use, an "ideal" mild isolate should have several characteristics (Lecoq, 1998):

1. It should induce mild symptoms that will not reduce the market value of the crop (or reduce it only slightly, to an economically acceptable level).
2. It should not inflict disease on other crops that are not targets for the cross-protection.
3. It should be completely systemic because the effectiveness of cross-protection depends on the presence of the mild strain in all tissues to be protected from severe strain inoculation.
4. It should be genetically stable, not reverting to the severe form.
5. It should not be easily disseminated by vectors in order to limit unintentional spread to other crops or fields.
6. It should provide protection against the widest possible range of severe isolates.
7. The protective inoculum should be easy to produce, to check for purity, and to store.
8. Finally, a simple inoculation procedure should be applicable so that it does not require expensive equipment or specific training to be applied in nurseries or in the fields.

Inoculum quality control is a particularly important issue in implementing mild strain protection. It should include careful testing of the inoculum to ensure that it does not contain contaminants. This can be quite easily done for distinct viruses by standard biological (differential host analysis), serological [DAS-ELISA, ISEM (immunospecific electron microscopy)], or molecular (PCR) techniques. More difficult to control is the absence of contamination by a severe strain of the same virus. This is generally done empirically by inoculating test plants for symptom expression, but it requires a long incubation time. When the genetic determinants for mildness are identified, it will be possible to develop specific molecular or serological tools to ensure that only sequences containing the "mildness" mutation(s) are present in the inoculum. Finally, specific tests should be developed to control the infectivity level of the mild strain inoculum (by inoculating serial dilutions to systemic or local lesion hosts) and its cross-protective potential (by a challenge inoculation with a range of severe strains).

Although not all of these requirements were always met, cross-protection has been successfully applied to control a number of virus diseases throughout the world. Mild strain protection of tomato against tomato mosaic virus (ToMV) is probably one of the most successful applications of this method. It was widely used in Europe, Canada, New Zealand, and Japan in the 1970s and early 1980s (Rast, 1972; Oshima, 1975; Fulton, 1986). In France alone, more than 13 million

tomato plants were cross-protected every year with the use of inoculum consisting of purified virus preparations that were produced and checked for specific infectivity and purity by a qualified Technical Institute. Cross-protection proved to be a very efficient and popular method to control ToMV in tomato, and only the release of resistant cultivars of good agronomic quality has progressively reduced its use (Lecoq, 1998).

Cross-protection has also been applied successfully to control potyviruses. In papaya, PRSV is causing severe epidemics in tropical regions. A mild PRSV variant was obtained from a severe strain from Hawaii through nitrous acid mutagenesis (Yeh and Gonsalves, 1984). It was widely used in Hawaii to protect papaya plantations very efficiently. However, in Taiwan the same mild variant was only partially protective, and in Thailand it did not provide a useful level of disease control. These failures were attributed to genetic diversity among PRSV isolates (Fuchs *et al.,* 1997). In cucurbits, ZYMV causes a very destructive disease worldwide. A mild ZYMV variant was used successfully to control severe strains in different parts of the world (France, Taiwan, Great Britain, Hawaii, Israel, and elsewhere), with yield increases reaching up to 40 times those of unprotected plants (Lecoq, 1998). However, as with PRSV, the mild ZYMV strain failed to protect against a few isolates of ZYMV from Reunion and Mauritius Islands.

The largest application of cross-protection was probably achieved in Brazil to control CTV, which causes a very destructive disease in citrus crops. Mild isolates were obtained from trees observed to show no or very localized symptoms in orchards in which all other trees were severely infected. The protective strain was inoculated by grafting scions originating from mild strain-infected mother plants, which limited the problems of inoculum production and storage. However, an additional constraint in this case was the need to select a mild variant for each scion/rootstock combination (Muller and Costa, 1987). Cross-protection has been used with great success in Brazil; more than 8 million Pera orange trees were cross-protected in 1980 (Muller, 1980), increasing to more than 50 million in 1987 (Urban *et al.,* 1990).

An original form of cross-protection has been developed for controlling CMV. Mild CMV strains were "constructed" by addition of a nonnecrogenic satellite RNA that attenuates symptoms. This type of mild inoculum has a double protective effect: protection against severe CMV strains (through "classical" cross-protection between virus strains) and protection against necrogenic satellite RNA (through cross-protection between satellites) (Jacquemond and Tepfer, 1998). This strategy was applied successfully on a large scale in China to control CMV (Tien

and Wu, 1991), and preliminary field tests to control necrogenic satellite RNAs were very promising (Jacquemond and Tepfer, 1998).

More than any other control method, cross-protection has been associated with potential hazards (Hamilton, 1985; Fulton, 1986; Lecoq, 1998). Some limitations or possible risks have been associated with the mild strain itself: (a) possible mutation of the protecting virus toward a more severe form that would cause a destructive disease and (b) possible spread of the protecting strain to other hosts in which it may be more severe. Other risks are associated with potential interactions of the mild strain with other viruses that are not targets of the cross-protection: (a) amplified disease symptoms caused by synergism with other viruses, (b) heteroencapsidation or heteroassistance in a mixed infection that may modify virus transmission specificity or efficiency, or (c) genetic recombinations between the protecting strain and another virus in a mixed infection. None of these risks are different from those that may arise from natural mixed infections.

Despite these anticipated difficulties, only a few problems have been associated with the use of cross-protection (Lecoq, 1998). In some cases (ToMV, ZYMV), more pronounced symptoms were observed in cross-protected plants, generally associated with stress situations for the plants or with aging. It is not known whether they were signs of cross-protection "breakdowns" or whether they were only more severe responses of the plants to mild strain under certain environmental conditions or to senescence. The mild ToMV strain belongs to pathotype 1, which induces necrotic reactions on plants possessing the $Tm2^2$ gene in the heterozygous form, and some necrosis was noticed on resistant plants when they were grown next to susceptible cross-protected crops. Synergism was also noticed between CMV and the mild ToMV strain; therefore ToMV cross-protection has been strictly limited to tomato crops grown in greenhouses, where CMV occurs very rarely.

Cross-protection is still used against a number of virus diseases in different crops, and more mild strains are being evaluated for future use [i.e., vanilla necrosis potyvirus (VNPV) (Liefting *et al.,* 1992), soybean mosaic virus (SMV) (Kosaka and Fukunishi, 1993), and cucumber green mottle mosaic virus (CGMMV) (Tan *et al.,* 1997)]. A multi-cross-protection strategy using a mixture of three mild strains has been tested to control CMV, ZYMV, and WMV2 in cucumber (Kosaka and Fukunishi, 1997). Nevertheless, due to the constraints linked to its implementation in the fields, cross-protection should be contemplated only for viruses that present a real threat to a crop and for which no alternative control method is available. Under these conditions, the use of cross-protection should not introduce additional specific risks in

comparison to those that may arise by letting a severe strain spread naturally in the fields without appropriate control measures.

d. Possible Mechanisms of Cross-Protection. Although it has been successfully applied in the fields for decades, little is known about the mode(s) of action of cross-protection. Several mechanisms have been proposed for the interrelation between competing strains of the same virus (Palukaitis and Zaitlin, 1984; Ponz and Bruening, 1986; Sherwood, 1987; Urban *et al.,* 1990). The common denominator is that a double infection occurs in the host cell.

1. *Depletion of host-derived precursors or structure.* The inducing virus may exhaust one or more of the essential compounds needed for the replication of the challenging strain. This hypothesis is consistent with a number of proven situations (e.g., Barker and Harrison, 1978). There is no direct evidence of a specific substance or factor that is limiting to a degree that will allow this hypothesis to be tested.
2. *Specific virus-encoded inhibitors.* This presumes that the inducing virus produces substances that inhibit the replication of challenging related viruses but not unrelated viruses.
3. *Template substitution.* If the replicase of the second virus is not selective enough, it may synthesize more strands of the inducing virus, whose template is more common in the host cells (Ross, 1974). Variations on this approach were proposed by Gibbs (1969).
4. *Coat protein sequestration.* The CP of the inducing virus encapsidates the nucleic acid of the related challenging virus, and the virions thus produced are not in an environment within the cell that enables their nucleic acid to be released and initiate replication (de Zoeten and Fulton, 1975). Some of the experiments conducted on the uncoating of TMV during RNA translation (e.g., Wilson, 1985) may serve to explain this hypothesis. Proof of this approach is not available from the cross-protection situation. However, expression of viral CP in plants has provided several lines of proof for the disassembly approach (Baulcombe, 1996). An elegant proof of the role of CP in cross-protection was given in an experiment by Culver (1996) in which a potato virus X (PVX) vector expressing the TMV CP in *Nicotiana benthamiana* conferred resistance to challenge infection with TMV.
5. *Sequestering the challenging genome.* This assumes that the (+) strands of the challenging RNA are reduced because the nascent (−) strand RNAs are hybridized by the more abundant (+) strands of the inducing strain (Palukaitis and Zaitlin, 1984).
6. *Inhibition of systemic spread.* In this approach, it is assumed that the inducing strain develops specific movement mechanisms that prevent the movement of the challenging strain (Dodds *et al.,* 1985).

7. *Gene silencing.* The progress made in understanding the mechanisms of CP-mediated virus resistance in transgenic plants may shed new light on classical cross-protection. In some instances, the direct role of the coat protein has been established; in others, gene silencing, a phenomenon involving sequence-specific RNA degradation in the cytoplasm, has been suspected. In these plants, the transgenic mRNAs (derived from the virus) are selectively and rapidly degraded in the cells. Homologous RNAs of invading viruses then undergo a similar degradation (de Haan, 1998). In cross-protection, the protecting virus inoculation could stimulate gene silencing, which would prevent, in a way still unknown, challenge virus infection (Ratcliff *et al.,* 1997).
8. Finally, in some cases, symptom expression of the challenge isolate is suppressed, but replication of the challenge isolate is not markedly inhibited (e.g., Cassells and Herrick, 1977).

Apparently, it is quite possible that more than one mechanism is involved in different viruses or in different stages of double infection in the same host cell.

C. Synergism: One Virus Enhancing the Symptoms and/or Titer of Another

Synergistic viral disease in plant hosts is the result of the presence, and more than additive interaction, of two independent viruses in the same host. Cases of enhancement of symptoms (or synergism) have been documented in the literature since the early 1920s. A famous experiment was reported by Smith (1931); a mixture of PVX and potato virus Y (PVY) caused severe veinal necrosis instead of the mild mottling or vein banding seen with either virus alone. In potato the mixture of PVX and PVY causes rugose mosaic, which is more severe than that caused by either virus alone (e.g., Matthews, 1991). More recent examination of the PVX–PVY interaction demonstrated that the titer of PVX was markedly increased in the mixed infection, while the titer of PVY remained similar to that in a single infection (Goodman and Ross, 1974a; Vance, 1991; see below). More reports of viral synergism are available in the literature, with many examples including a potyvirus as one of the components of the synergistic combination (e.g., Pio-Ribeiro *et al.,* 1978; Davis and Mizuki, 1987; Scheets, 1998). However, combinations of other viruses can also be synergistic, such as the mixture of TMV and PVX in tomato causing leaf-drop streak and often killing the plants (Matthews, 1991). Synergism has also been reported

in mixed infections of beet western yellows luteovirus (BWYV) with the ST9 satellite-like virus (Sanger *et al.,* 1994) and in mixed infections of panicum mosaic virus (PMV) with satellite panicum mosaic virus (SPMV) (Scholthof, 1999). Increased symptom severity has been reported when pepper plants are infected with satellite tobacco mosaic virus (STMV) as well as with TMV helper virus (Rodríguez-Alvarado *et al.,* 1994).

Other examples are the ability of TMV to move and replicate systemically in barley only in the presence of brome mosaic virus (BMV) (Hamilton and Nichols, 1977) or to replicate to very high levels in barley in the presence of barley stripe mosaic hordeivirus (BSMV) (Dodds and Hamilton, 1972), while TMV alone replicates hardly at all. Synergism often results in an impressive aggravation of disease symptoms and in the increased accumulation of at least one of the coinfecting viruses (Palukaitis and Kaplan, 1997; Walkey and Paine, 1990; Poolpol and Inouye, 1986). Not all mixed infections result in synergism.

The synergism that frequently occurs between a potyvirus and a virus of a different genus may be to some extent host dependent. Typically, the level of replication of the potyvirus component is similar in single and mixed infections, whereas the nonpotyvirus replicates to a higher level in the mixed infection (see Scheets, 1998, and references therein). In some instances, the concentration of the potyvirus is reported to decrease, while the concentration of the other virus increases (Poolpol and Inouye, 1986). The cause of the synergism has been elegantly dissected for the classic PVX/potyvirus synergism, in which a significant increase in the amount of PVX (−) strand RNA has been shown (Vance, 1991). Further work with the use of transgenic plants expressing 5′ proximal portions of the potyvirus genome showed that the potyvirus HC-Pro protein causes the increase in PVX pathogenicity, while both HC-Pro and P1 are required for the increased accumulation of PVX (−) RNA (Vance *et al.,* 1995; Pruss *et al.,* 1997). Mutations in HC-Pro but not in P1 eliminated the synergism (Shi *et al.,* 1997). The P1 and HC-Pro sequences were also found to enhance the pathogenicity of CMV and TMV (Vance *et al.,* 1995). Scheets (1998) has demonstrated that wheat streak mosaic tritimovirus (WSMV) RNA replication increases in mixed infections with maize chlorotic mottle machlomovirus (MCMV) under certain environmental conditions, whereas MCMV replication is increased in the mixed infection under a broader range of conditions. In contrast, Goldberg and Brakke (1987) reported that in combination with sugarcane mosaic potyvirus (SCMV)-MD-B, only MCMV replication increased.

PVX/PVY synergism is maximized when both viruses enter the tissue at the same time (Goodman and Ross, 1974b), and the same is probably true for WSMV/MCMV (Scheets, 1998) and other pairings. This is presumably because replication of the two viruses is then synchronized. The synergistic effect may be displayed differently in different parts of the plant as a consequence of the timing of the two infections. In the case of PVX/PVY synergism, the greatest synergistic effect is observed in rapidly developing leaves where both viruses are invading the tissue simultaneously (Rochow and Ross, 1955; Goodman and Ross, 1974b). In contrast, in young corn plants doubly infected with MCMV and any of three potyviruses, once necrosis starts to develop, it spreads throughout the plant until the plant dies (Niblett and Claflin, 1978; Uyemoto *et al.,* 1980).

D. Vector Specificity

As discussed previously (Section II,A), phenomena of multiple infection are well documented in many hosts. In order to evaluate the epidemiological significance of interviral interactions, it is important to understand virus–vector relationships and their role in vector specificity. Only then can the extent and limitations of potential epidemiological effects of such interactions be evaluated in a meaningful way.

1. Specificity of Plant Viruses for Their Vectors

The dependence of plant viruses on vectors for survival is explained by two factors: (a) The virus cannot enter intact cells of a plant to establish infection, due in the first instance to the impermeable cuticle. Most vectors are equipped with piercing-sucking mouthparts well adapted for introducing virus into the cytoplasm. (b) Unlike animals, plants are devoid of independent mobility, being rooted in the soil. Thus, transport of virus to adjacent or more distant hosts depends on the flight or movement capacity of their vectors.

a. Specificity and Modes of Transmission. Specificity seems to be more restricted when the association between the virus and the vector is more intimate. In the past, classification of the modes of transmission was based on the duration of retention of the virus in the vector (Watson and Roberts, 1939). Different virus groups demonstrate different forms of association with the vectors. A detailed discussion of the various forms of association between plant viruses and vectors is beyond the scope of this article. However, a simplified description of virus–vector relations can be divided into two types: (a) circulative or internal, in

which the virus crosses body barriers and enters the circulatory system of the vector, and (b) noncirculative or external, in which the virus remains attached to the cuticle of the vector and does not cross body barriers (Hull, 1994a; Pirone and Blanc, 1996).

The specificity of plant virus groups for particular insect vector groups is remarkable. Various aphids transmit potyviruses, cucumoviruses, luteoviruses, caulimoviruses, closteroviruses, and carlaviruses with different degrees of specificity. Individual viruses within a virus group may be transmitted by different types of insect vector, but a particular virus is transmitted by only one type of vector. For example, tomato yellow leaf curl geminivirus (TYLCV) is transmitted by the sweet potato whitefly, and maize streak geminivirus (MSV) is transmitted by a leafhopper.

Whitefly-transmitted viruses include some geminiviruses, the sweet potato mild mottle ipomovirus (SPMMV), and the criniviruses. Transmission of geminiviruses is internally borne and primarily determined by CP (Briddon *et al.,* 1990), whereas by analogy with aphid-transmitted potyviruses and closteroviruses, SPMMV and criniviruses are probably externally (stylet-) borne and dependent on a virus-encoded helper component (HC) as well as the CP. Only *Bemisia* spp. are reported to transmit geminiviruses and SPMMV, whereas some criniviruses are transmitted by both *Bemisia tabaci* and *Trialeurodes vaporariorum* (Wisler *et al.,* 1998).

Leafhopper-transmitted geminiviruses and several other virus groups transmitted by leafhoppers, planthoppers, and treehoppers are mainly internally borne, and in some cases (e.g., hopper-transmitted rhabdoviruses) the virus replicates in the vector as well as in the plant host (Hull, 1994a; Nault and Ammar, 1989). Machloviruses are externally borne and semipersistent (ability to transmit being retained for some time after acquisition) (Nault and Ammar, 1989).

Several genera of thrips transmit tospoviruses, which also replicate within the vector (Wijkamp *et al.,* 1993). There is both species and biotype specificity in the vector, determined by whether the virus can replicate in the vector (Peters *et al.,* 1996). The glycoproteins of tospoviruses that interact with cell surface receptors in the vector may confer specificity (Hull, 1994a). Eriophyid mites transmit members of the *Potyviridae* genera *Rymovirus* and *Tritimovirus.* Although aphid-transmitted potyviruses are externally (stylet-) borne, mites remain infective for long periods, suggesting a circulative relationship; however, this remains to be proven. Rymoviruses are transmitted only by *Abacarus* spp. and Tritimoviruses only by *Aceria* spp. (Salm *et al.,* 1996; Stenger *et al.,* 1998).

Chrysomelid beetles transmit four groups of viruses: comoviruses, sobemoviruses, tymoviruses, and some bromoviruses; all of these viruses reach high concentrations in their plant hosts and have highly stable virions (Matthews, 1991). The particles of some other viruses are readily acquired by beetles but are not transmitted, apparently as a result of RNase activity in beetle regurgitant (Gergerich *et al.,* 1986). Transmission may be a combination of purely mechanical activity during feeding and circulative passage through the hemolymph, as beetles can become infective very rapidly but vector efficiency and retention of infectivity increase with prolonged feeding. Further evidence of circulative passage comes from gain of transmission in beetles injected with virus into the hemocoel (Fulton *et al.,* 1987). However, some efficiently beetle-transmitted viruses do not enter the hemolymph, whereas some viruses not transmitted by beetles do enter the hemolymph and are deposited on the leaf surface in regurgitant (Gergerich and Scott, 1996). Thus, both vector-specific interactions and RNase resistance play roles in beetle transmissibility.

Some badnaviruses and the closterovirus-like pineapple mealybug wilt-associated virus (PMWaV), grapevine leafroll-associated viruses, and some trichoviruses are transmitted by mealybugs (Sether *et al.,* 1998); transmission characteristics of the badnaviruses suggest an externally borne relationship with the vector (Hull, 1994a). Although the mechanism of transmission of PMWaV is not clear, it appears to be transmitted in an external, semipersistent fashion similar to that of grapevine leafroll-associated virus 3 (GLRaV-3) (Sether *et al.,* 1998).

Known nematode vectors are all found within two subgroups of the *Dorylaimidae,* with nepoviruses being transmitted by species of *Xiphinema* and *Longidorus,* and tobraviruses by species of *Trichodorus* and *Paratrichodorus.* Both nepoviruses and tobraviruses can be retained by their nematode vectors for weeks or months, although retention of virus in weed hosts and their seed may frequently be more important in long-term survival in field situations (Harrison, 1977).

Some virus groups have fungal vectors among the *Plasmodiophorales* (mainly *Polymyxa* spp.) and others among the *Chytridiales* (primarily *Olpidium* spp). Association of bymoviruses and furoviruses with *Polymyxa* spp. is internal, and infectivity can be maintained within the resting spores for months. A CP readthrough domain is essential for transmission of beet necrotic yellow vein furovirus (BNYVV) (Tamada and Kusume, 1991), while the bymoviruses appear to have an HC analogous to that of the aphid-transmitted potyviruses (Dessens *et al.,* 1995). Necroviruses are absorbed to the external surface of zoospores of *Olpidium* by a specific interaction (Temmink *et al.,* 1970), whereas

tobacco stunt virus (TStV) is found internal to spores of *O. brassicae* (Hiruki, 1987).

The different types of relationships apparent in "internal" versus "external" specificity, and with different types of vector, demonstrate that there are many different types of interaction. These are largely dependent on interactions with the CP and, in some cases, with additional viral gene products. This presumably explains the decreasing probability of interactions between more distantly related viruses noted by Rochow (1970). Lack of compatibility between different types of CP, and between different CPs and nucleic acid motifs involved in initiation of encapsidation, is likely to be a major impediment to interactions affecting vector specificity between unrelated viruses. Different virus groups are stabilized by different types of interaction (protein–protein, protein–RNA, and RNA–RNA interactions in different combinations) that determine virion morphology and the amount of RNA that can be encapsidated, as well as the sequence specificity of some RNA–protein interactions (Matthews, 1991). It is thus extremely unlikely, for example, that a nepovirus RNA would be encapsidated by a potyvirus CP to permit aphid transmission or that a potyvirus RNA would become encapsidated in a nepovirus CP to facilitate nematode transmission. The CP–RNA interactions of different virus groups are quite distinct, and there are also constraints on the size of RNA that can be packaged in isometric particles. However, in *in vitro* reconstitution experiments, TMV CP was able to encapsidate the RNA of the spherical turnip yellow mosaic virus (TYMV) to form elongated particles (Dodds and Hamilton, 1976). Similarly, TMV RNA was encapsidated by CMV CP to form isometric particles that were acquired *in vitro* and transmitted by aphids (Chen and Francki, 1990). However, when heterologous encapsidation has been observed *in vivo* within the potyviruses, there appear to be adverse effects on the stability of virions (Hammond and Dienelt, 1997).

b. Viral Structural Proteins and Their Role in Specificity for Vectors. Vectors can transmit intact virions but not naked nucleic acids. However, in many cases, viral RNA and DNA are infectious when inoculated mechanically into plant tissues. This suggests that the CP is essential for transmission by vectors. The role of CP in transmission of "external" viruses was demonstrated for potyviruses when mutations were made in the conserved Asp-Ala-Gly (DAG) motif within the otherwise highly variable, surface-exposed amino-terminal region (C. D. Atreya *et al.,* 1990; P. L. Atreya *et al.,* 1991; Gal-On *et al.,* 1992). It was demonstrated that the DAG domain is involved in binding to the potyviral HC-Pro (Blanc *et al.,* 1997; Peng *et al.,* 1998) rather than interacting directly

with the vector. The role of CP has also been shown for cucumoviruses (Gera *et al.,* 1979; Perry *et al.,* 1998). Loss of aphid transmissibility as a result of mutation (e.g., Atreya *et al.,* 1990) or deletion (e.g., Maiss *et al.,* 1989) in the CP can be complemented by heterologous encapsidation in a mixed infection with an isolate having CP competent for aphid transmission (e.g., Bourdin and Lecoq, 1991).

An "internal" virus, the barley yellow dwarf luteovirus (BYDV), was the first for which the role of heteroencapsidation in vector specificity was demonstrated (Rochow, 1970). It is now known for both luteoviruses and for the pea enation mosaic enamovirus (PEMV) that there are two viral proteins that encapsidate the virion: the predominant one is the capsid protein (CP; ~22–24 kDa), and the minor component is the readthrough domain (RTD) protein (~55–58 kDa). The RTD is the outcome of a larger protein translated via a weak stop codon in the CP gene (Brault *et al.,* 1995; Demler *et al.,* 1997). Virions lacking the RTD protein are nontransmissible by aphids, but ingested particles containing only CP are found in the hemolymph. This suggests that virions encapsidated with the CP may cross the hindgut barrier of aphids, but the RTD is needed to recognize the accessory salivary glands (ASG).

Transmission of luteoviruses and enamoviruses (Van den Heuvel *et al.,* 1997), and possibly also whitefly transmission of geminiviruses (H. Csoznek, personal communication), requires a protein that is produced by endosymbiotic bacteria of the genus *Buchnera,* namely, the symbionin or GroEL chaperonin. Symbionin is produced by the bacteria in specialized cells located in the abdominal mycetome of aphids. The viral RTD protein was found to interact with GroEL, and mutation of the BWYV RTD disrupted binding to GroEL. In addition, *in vivo* studies showed that BWYV virions encapsidated with CP but with no RTD were significantly less persistent in aphid hemolymph than virions containing RTD. It is proposed that the interaction between *Buchnera* GroEL and the RTD protects the virus from rapid degradation in the aphid (van den Heuvel *et al.,* 1997).

The glycoproteins of tospoviruses may be the viral determinant of specificity for the thrips vectors, as they are postulated to interact with cell surface receptors (Hull, 1994a). Similar specificity of other viruses for their nematode and fungal vectors may also be related to the appropriate CP or RTD proteins.

c. Nonstructural Proteins Required for Vector Specificity. A nonstructural viral protein, termed "helper component" (HC) in potyviruses or "aphid transmission factor" (ATF) in caulimoviruses, is also essential for vector transmission (reviewed by Pirone and Blanc, 1996). Other

virus groups may also require a helper function for vectored transmission. Indirect evidence suggests that helper is involved in several other systems. The semipersistent parsnip yellow fleck virus (PYFV) is not transmissible by aphids unless acquired with the Anthriscus yellows virus (AYV) (Elnagar and Murant, 1976), and a dense material with virus-like particles was seen in aphids mouthparts after acquisition of the virus (Murant *et al.,* 1976). Another semipersistent virus that apparently acts to provide helper assistance to a distinct virus is the rice tungro spherical virus (RTSV), which is transmissible by several *Nephotettix* leafhopper species. RTSV assists the transmission of rice tungro bacilliform virus (RTBV) (Hibino *et al.,* 1979). However, sequence analysis of RTSV does not shed light on the gene that may encode the helper activity (Shen *et al.,* 1993). Another semipersistent leafhopper-borne virus, maize chlorotic dwarf virus (MCDV), is also considered to have a helper component (Hunt *et al.,* 1988). There is speculation on the need of helper for carlaviruses and closteroviruses as well because of the lack of aphid transmissibility of infectious purified virions, but experimental evidence is still lacking (Murant *et al.,* 1988). The nematode-transmitted tobraviruses may also employ a helper component, as nematode-nontransmissible isolates differ from transmissible isolates in RNA 2. In the case of pea early-browning virus (PEBV), the putative HC is a 30-kDa open reading frame (ORF) on RNA 2 that differs by two residues from the product of a transmissible isolate (MacFarlane and Brown, 1995).

In the HC of aphid-transmitted potyviruses there are two domains that are required for transmission. These are the motifs Lys-Ise-Thr-Cys (KITC; Atreya *et al.,* 1992) and Pro-Thr-Lys (PTK), which was found to be associated with HC activity in ZYMV (Huet *et al.,* 1994). Following comparisons of aphid-transmissible tobacco etch virus (TEV) or tobacco vein mottling virus (TVMV) virions with either functional PVY or TVMV HC (motif KITC) or nonfunctional HC (motif EITC), it was proposed that the KITC domain binds to the aphid stylet (Wang *et al.,* 1996). The PTK domain apparently binds to the DAG domain of the CP (Peng *et al.,* 1998).

The identification of the aphid transmission factor in cauliflower mosaic virus (CaMV) was achieved by an analysis of deletion mutants and the formation of recombinants between aphid-transmissible and -nontransmissible isolates (Howarth *et al.,* 1981; Armour *et al.,* 1983; Woolston *et al.,* 1983). Direct evidence for the role of P18 was obtained by the use of a gene II product expressed in insect cells for complementation of aphid transmissibility (Blanc *et al.,* 1993). Evidence for the association of CaMV P18 with virus particles comes from the fraction-

ation studies of Espinoza *et al.* (1992). Mutated ATF of caulimoviruses failed to assist aphid transmission and also failed to bind to cauliflower mosaic virions (Schmidt *et al.,* 1994).

E. Enhancement of Transmission: Dependent Transmission from Mixed Infections

1. Heterologous Encapsidation

Vectors may transmit more than one virus from a host coinfected with two viruses (e.g., Falk and Duffus, 1981). In the majority of cases, both viruses are transmissible by vectors, and therefore the phenomenon of transmission of one virus with the aid of viral gene products of the other virus was often overlooked. This was due in part to the difficulty of distinguishing between pseudorecombinant, heteroencapsidated, and normal viruses. Dependent transmission is a situation in which one virus is able to replicate in the absence of a second virus and may be mechanically transmissible, but is transmissible by a specific vector only in the presence of a helper virus. Some forms of dependent transmission are based on an exchange of capsid protein. These exchanges are documented in the literature under several terms: "transcapsidation," "heteroencapsidation," and "phenotypic mixing." All these terms refer to the situation in which some or all of the capsid protein subunits around the nucleic acid of one virus are replaced by subunits of another virus or isolate. Proof of heteroencapsidation is available from transmission experiments. The first proof came from the work of Rochow (1970). He showed that the BYDV strain that is normally transmitted by the aphid *Macrosiphum avenae* can also be transmitted by *Rhopalosiphum padi,* but only from a mixed infection with the BYDV strain normally transmitted by *R. padi.* He explained this phenomenon by the fact that capsomers of one strain encapsidate the second strain. Luteoviruses can assist transmission not only of other luteoviruses, but also of viruses from other groups. Watson *et al.* (1964) reported that carrot red leaf luteovirus (CtRLV) acts as the helper virus for carrot mottle virus (CMoV), and Falk *et al.* (1979) showed that BWYV served as helper virus for transmission of lettuce speckles mottle virus (LSMV).

In more detailed study of the heterologous encapsidation of luteoviruses in mixed infections, Wen and Lister (1991) examined the interactions among four isolates of BYDV with differential vector specificities that are also differentiated serologically. Using both immunohybridization techniques with isolate-specific cDNA probes and antibody sand-

wich ELISA with different isolate-specific antibodies, they were able to detect and differentiate two types of interaction. The interaction between more distantly related isolates was primarily transcapsidation of the RNA of one isolate completely in the CP of the unrelated isolate; in contrast, the interaction between closely related isolates was primarily phenotypic mixing, with RNA encapsidated in a mixture of the CP subunits of both isolates. Heterologous encapsidation in certain combinations occurred only in one direction—that is, RNA of one isolate was encapsidated in CP of the second isolate, but not vice versa, as had been previously suggested by Rochow (1982) on the basis of transmission studies. The degree of relatedness of the isolates correlated with both the capacity for different types of heterologous encapsidation and the ability of the different isolates to cross-protect against each other (Wen and Lister, 1991) and may reflect compatibility of the subunits of the different isolates to assemble together. Compatibility of a particular CP with the RNA of another isolate presumably dictates the direction of the one-way interactions observed by Wen and Lister (1991).

In another example, an aphid-nontransmissible ZYMV isolate became transmissible when coinoculated with an aphid-transmissible PRSV isolate (Bourdin and Lecoq, 1991). Although potyviruses are typically aphid-transmissible, aphid-nontransmissible isolates can be readily obtained by serial mechanical transmission (e.g., Swenson *et al.,* 1964). Lack of aphid transmissibility may arise because of mutations in the CP or the HC (Atreya *et al.,* 1990, 1992). Transmission from mixed infections may be due either to heterologous encapsidation with the transmission-competent CP of the coinfecting isolate or to interaction with the effective HC protein of the other isolate (Kassanis and Govier, 1971; Bourdin and Lecoq, 1991). Isolates defective for aphid transmission can persist in nature by virtue of complementation in mixed infections (e.g., Kassanis and Govier, 1971; Bourdin and Lecoq, 1991) or in agriculture by vegetative propagation of infected crop plants. Loss of aphid transmissibility following mechanical transmission has also been reported for viruses other than potyviruses, including PEMV (Tsai and Bath, 1974) and CMV (Mossop and Francki, 1977), and natural populations of PEMV may contain mixtures of aphid-transmissible and aphid-nontransmissible isolates (Demler *et al.,* 1997). Nematode-transmitted tobraviruses can also readily lose vectored transmissibility as a result of mechanical transmission, and this has been linked to deletions in a nonstructural gene in RNA 2 (e.g., Hernandez *et al.,* 1996; MacFarlane and Brown, 1995). Fungus-transmitted furoviruses and bymoviruses can also lose vector transmissibility as a result of

mutations or deletions (Tamada and Kusume, 1991; Dessens *et al.,* 1995).

Heterologous encapsidation has also been reported in other virus groups (Gera *et al.,* 1979; Huth *et al.,* 1984; Sarkar, 1969). Heteroencapsidation may be demonstrated by electron microscopy with the use of specific antibody to decorate the capsid protein subunits of the coinoculated virus (Bourdin and Lecoq, 1991). Production of hybrid progeny virions was described for plant, animal, and bacterial viruses (see reviews by Rochow, 1977; Dodds and Hamilton, 1976; Falk *et al.,* 1995). Distinction between components of a hybrid virus is now possible due to the progress in developing epitope-specific monoclonal antibodies (e.g., Hammond and Dienelt, 1997) and the use of immunocapture reverse transcription polymerase chain reaction (RT-PCR) with antibodies specific to one virus and primers specific to the other (e.g., Candelier-Harvey and Hull, 1993).

In certain cases, heterologous encapsidation is responsible for expanding the vector range (a typical case is BYDV). There are also instances in which related viruses that differ in vector specificity do not appear to interact. As noted in Section II,D,1,a, different genera of the *Potyviridae* and the *Geminiviridae* have different types of vector, but there is no evidence of interactions between viruses in different genera affecting vector specificity. There are also criniviruses that differ in vector specificity, but in preliminary experiments no alteration of whitefly vector specificity was observed in mixed infections of tomato infectious chlorosis virus (TICV) and tomato chlorosis virus (ToCV) as a result of transcapsidation (G. Wisler, personal communication). However, in examples of dependent transmission, heteroencapsidation is the mechanism by which the helper virus (BWYV and LSMV, CtRLV and CMoV, RTSV and RTBV) transmits vector-nontransmissible viruses (see Falk and Duffus, 1981).

Persistent viruses are known to have high specificity for their vectors. In these cases, heterologous encapsidation may determine if a virus is spread or not (e.g., Rochow, 1972). However, in one study (Wang *et al.,* 1998), it has been shown that a lesser but still substantial degree of specificity for aphid vectors exists even within the nonpersistent potyviruses. Thus, encapsidation with a foreign capsid may result in a change in vector species affinity for the virus. This vector may have a different preference for host plants. In this way, the virus may ultimately be introduced to host plants that can support virus replication but would not have been infected in the absence of a mixed infection. However, many viruses are transmitted by polyphagous insects, which would facilitate introduction of the virus to many potential hosts.

2. Transmission Dependent on Nonstructural Gene Products

Another form of dependent transmission occurs when one virus uses a nonstructural protein encoded by a different virus or isolate for movement within the plant (for systemic spread) or for movement among hosts (for helper-assisted vector transmission). In certain cases, the invading virus is devoid of the ability for systemic spread or mechanical transmissibility to a particular host and depends on another virus for its movement. For example, LSMV is mechanically transmissible to some hosts but will infect others only when aphid-transmitted in the presence of the helper virus BWYV (Falk and Duffus, 1981). Other examples include rice tungro bacilliform and spherical viruses (Hibino *et al.,* 1979), groundnut rosette virus (GRV), and PEMV (see below).

The original example of dependence on a virus-coded nonstructural protein for vectored transmission from a mixed infection was discovered by Kassanis (1961) and was later characterized by Kassanis and Govier (1971). In this case, assistance for transmission of potato virus C (PVC) was achieved by feeding the vector on plants infected with the transmissible PVY and then on plants infected with the normally nontransmissible PVC. Therefore, it was not strictly necessary for the two viruses to be present in the same host in order to be dependent on each other. This phenomenon of "helper-assisted transmission" was described first for the potyviruses and for potyvirus-assisted transmission of potato aucuba mosaic potexvirus (PAMV) (Kassanis, 1961; Kassanis and Govier, 1971) but was later also found among caulimoviruses (Lung and Pirone, 1973). In some cases, the helper protein of one virus may assist the transmission of a virus of a different group (Elnagar and Murant, 1976; Kassanis, 1961; Kassanis and Govier, 1971). That this depends on an interaction between HC and the viral CP was further demonstrated by Baulcombe *et al.* (1993), who showed that the PVY HC could effect aphid transmission of PVX potexvirus engineered with the CP N-terminal DAG motif from PAMV. The importance of this phenomenon was also observed in the fields with the HC-deficient, aphid-nontransmissible ZYMV mild strain used for cross-protection; it was observed that in a mixed infection with WMV2, the mild ZYMV strain could be transmitted by aphids through the mediation *in trans* of the WMV2 HC (Lecoq *et al.,* 1991).

The presence of helper-assisted transmission has been reported or implied for other groups of viruses. Elnagar and Murant (1976) reported the dependence of the semipersistent PYFV on coinfection of AYV for aphid transmission by *Cavariella aegopodii.* As with the potyviruses and caulimoviruses, PYFV transmission could also occur from plants

infected with PYFV alone if the aphids previously acquired AYV from separate plants. Hibino *et al.* (1979) have reported the dependence of RTBV on mixed infection with RTSV for transmission by the leafhopper *Nephotettix virescens.* Lack of aphid or whitefly transmissibility of purified virions of closteroviruses or lettuce infectious yellows virus (LIYV) also suggests the requirement of a helper factor (Pirone and Blanc, 1996).

There are interactions between some viruses of different groups in mixed infections that may over time lead to further evolution. One example is the interaction between the umbraviruses and luteoviruses—and the satellite RNAs that are associated with these complexes. Umbraviruses have been found in nature only in mixed infections with luteoviruses, on which they depend for encapsidation and aphid transmission despite being experimentally transmissible mechanically (e.g., Gibbs *et al.,* 1996). Lacking an apparent CP of their own (Gibbs *et al.,* 1996), the umbraviruses are highly dependent on their luteovirus partner for survival in nature. PEMV (the sole member of the Enamovirus group) appears to have evolved as a further stage in the close association of umbraviruses with luteoviruses; in this case, RNA 1 of PEMV appears to be a defective luteovirus, lacking systemic movement that is effected by the movement protein from the umbravirus-like RNA 2 (Demler *et al.,* 1996). The satellite RNA associated with the groundnut rosette disease complex is essential for the luteovirus-assisted aphid transmissibility of the umbravirus component of the complex (Murant, 1990), demonstrating a further level of complexity in the interaction. It has been shown that the satellite RNAs of PEMV and GRV can be replicated by the heterologous virus but that the PEMV satellite RNA cannot substitute for the GRV satellite RNA to enable aphid transmission of GRV (Demler *et al.,* 1996). This demonstrates that there are multiple levels of interaction in luteovirus–umbravirus complexes that are presumably still evolving. Synergism (see Section II,C) is another example of interviral interactions.

3. Potential Epidemiological Consequences of Dependent Transmission

It is likely that many more interactions resulting in dependent transmission occur than have been reported. The epidemiological consequences are rather limited in most cases, the principal reason being that nucleic acid encapsidated in the protein of another virus and thus transmitted will produce only its own protein in the new host. In the absence of the helper virus the effect is a single case transfer, and no further vectored transmission can occur. In the presence of the helper

virus, the original mixed infection is reproduced. It is clear that dependent transmission does occur, as illustrated by isolation of aphid-nontransmissible isolates of potyviruses (e.g., Bourdin and Lecoq, 1991) and PEMV (Demler *et al.,* 1997) from natural populations and the survival of umbraviruses solely in association with luteoviruses (e.g., Gibbs *et al.,* 1996).

The specificity of transmission of nonpersistent viruses by aphid vectors is low (Pirone and Harris, 1977) compared to that of persistently transmitted viruses. Encapsidation in a "new" CP that is recognized by one aphid species but not another (as in the BYDV example) is somewhat of an exception. Encapsidation in a different CP may allow the vector to carry the virus to a new host range, potentially broadening the virus range and exposure to new potential vectors. Many viruses are transmitted by multiple different aphid species, and many of the vector species are polyphagous and will probe or feed on a wide variety of plant species. However, different aphid species have been found to transmit viruses with different efficiency (Halbert *et al.,* 1981; Markham *et al.,* 1987; Raccah *et al.,* 1985; Wang *et al.,* 1998). The difference in efficiency may reflect the intrinsic properties of the virus (the capsid protein or an accessory transmission protein), the feeding behavior or other characteristics of the aphid species, the attraction of the target host to the aphid, and susceptibility of the host to the transmitted virus. A scenario may be envisaged in which a virus is acquired from a mixed infection by a different aphid species that has stronger affinity for the heterologous CP. If the aphid is then attracted to a different set of hosts that are able to support replication of the virus, the infection may appear to be a new virus. Presence of the virus in a new host may expose the virus to additional vectors and allow transfer to further hosts.

It is difficult to prove that such events occur in nature, but the periodic appearance of "new" viruses often occurs with the expansion of cropping areas into new environments, where new interactions between potential vectors, viruses in the endemic vegetation, and crops previously unexposed to the viruses may occur. Any such transfers may result in population bottleneck events for the virus followed by subsequent rapid sequence evolution as the virus adapts to the new environment in a different host (Domingo *et al.,* 1996). It is significant that new viruses appear to emerge in crops but are then typically discovered to have preexisting host ranges in endemic plants. In other words, the virus is discovered by virtue of damage to a crop, whereas a seed-borne virus introduced to an area in a crop, or a virus introduced in vegetative

planting material, is unlikely to be recognized as a new virus if transmitted by vectors to endemic plants.

F. Recombination

One form of interaction is the structural interchange of components between two distinct strains or viruses by exchange of nucleic acid sequences. While heterologous encapsidation or HC-assisted transmission results in unigenerational effects, recombination results in alteration of the phenotype of all subsequent progeny virions. The capsid protein is an important component of the virion surface, and is believed to be the principal factor in recognizing host cell and vector binding sites and to be involved in movement between host cells (Falk *et al.,* 1995). Exchange of capsid protein in a recombinant virus could thus have profound effects, as could exchange of sequences affecting host range, symptoms, and pathogenicity.

1. Natural Recombination

Most known plant viruses have small genomes composed of ssRNA, usually 10 kb or less. RNA–RNA recombination is a rare event in plant virus replication, but it contributes to evolution of the viral genome. Indeed, under strong selective pressure for the recombinant RNA, intermolecular RNA–RNA recombination has been demonstrated for several groups of RNA plant viruses, including bromoviruses (Bujarski and Kaesberg, 1986; Allison *et al.,* 1989), cucumoviruses (Fernández-Cuartero *et al.,* 1994), carmoviruses (Cascone *et al.,* 1990), hordeiviruses (Edwards *et al.,* 1992), nepoviruses (Le Gall *et al.,* 1995b), and tombusviruses (White and Morris, 1994a,b). Recombination may be more frequent among virus groups that have multiple genomic RNAs rather than unipartite genomes, as multipartite genomes have common terminal sequences recognized by the replicase complex. However, different families of viruses appear to vary in their ability to recombine (King *et al.,* 1987), and this is not necessarily related to their genome organization. Indeed, the frequency of recombination events may be a function of the viral polymerase rather than of the host cell, suggesting that ability of the polymerase to reinitiate on a new template after dissociation from the original is required (King *et al.,* 1987). Alternatively, polymerases of viruses in which recombination is common may be more prone to stall at specific sequences or secondary structures, thus leading to dissociation more frequently. Sequence analysis has led to the suggestion that recombination is a cause of some of the

diversity within the potyviruses (Cervera *et al.,* 1993; Revers *et al.,* 1996), luteoviruses (Rathjen *et al.,* 1994; Gibbs and Cooper, 1995), nepoviruses (Rott *et al.,* 1991; Le Gall *et al.,* 1995a), and tobraviruses (Robinson *et al.,* 1987; Goulden *et al.,* 1991), although no experimental evidence of recombination has been obtained in the luteoviruses. Recombination, at least to the extent of loss of introduced sequences (presumably intramolecular recombination), has been documented in engineered infectious clones of several virus groups, including potyviruses (Dolja *et al.,* 1992, 1993), potexviruses (Chapman *et al.,* 1992), tombusviruses (Scholthof *et al.,* 1993), and tobamoviruses (Kearney *et al.,* 1993). Recombination between RNA 1 and RNA 2 of a tobacco rattle tobravirus (TRV) isolate derived from infectious cDNAs has also been reported (Hernandez *et al.,* 1996). Recombination has also been observed between two defective ZYMV transcripts coinoculated into plants (Gal-On *et al.,* 1998), providing the first experimental evidence of intermolecular recombination in the potyviruses.

There is also evidence of recombination in the closteroviruses and related viruses (Dolja *et al.,* 1994a). The duplication of the CP gene followed by divergence of the two copies present in all true closteroviruses, and the reversal of the order of the major capsid protein and its diverged homolog in the bipartite closteroviruses (genus *Crinivirus*), is one indication of recombination; another is the presence of a homolog of the cellular heat-shock protein, HSP70, and a possible divergent copy. The HSP70 gene was presumably captured from the cellular genome and may be necessary to allow expression of the very large closterovirus genome (Dolja *et al.,* 1994a). The variability in the size of different *Closteroviridae* genomes, with different numbers of genes and significantly different sizes of the ORF 1a, plus the occurrence of both monopartite and bipartite genomes, all support the occurrence of recombination. Dolja *et al.* (1994a) have reviewed the evolution of closteroviruses and similar viruses and have proposed a sequence of steps through which these extremely large RNA genomes may have been derived from simpler genomes and other viral groups. Further evidence of recombination comes from the discovery of multiple species of defective RNAs replicated by CTV that appear to reflect recombination of subgenomic RNAs with distant 5′ parts of the genome (Bar-Joseph *et al.,* 1997), suggesting another mechanism for generating diversity within closterovirus genomes.

2. Types of Recombination

Recombination occurs not only between closely related RNA molecules but also between dissimilar RNAs—possibly at sites of similar

RNA structure (Lai, 1992; Nagy and Bujarski, 1993). Three types of recombination have been distinguished (Lai, 1992). These are classified as Type 1 (homologous recombination), Type II (aberrant homologous recombination), and Type III (nonhomologous or illegitimate recombination). Type I occurs between two similar or closely related molecules with extended sequence homology and is due to crossing over at an exact position from one molecule to the next. The switch may, however, be at a position where there is no nucleotide homology; thus the progeny recombinant molecule retains the original organization of the parental molecules but with a chimeric sequence. Most RNA recombinants involving full-length viral genomes, such as those between poliovirus serotypes, are Type I recombinants (Lai, 1992). Type II recombination also occurs between molecules that have significant stretches of homologous sequence, but it does not result in exact crossovers from one parental strand to the other; the polymerase may slip or stutter. The result may be either deletion and/or duplication of nucleotides from one or both parental strands or insertion of nontemplated nucleotides. Nonhomologous recombination is especially common in recombination involving defective RNAs (Lai, 1992), as exemplified by recombination between defective interfering RNAs and satellite RNAs associated with turnip crinkle virus (TCV) (Cascone *et al.,* 1990). Type III recombination occurs between RNA molecules of unrelated sequence, and the mechanism is therefore not clear; possibly the parental strands share a similar secondary structure. Type III recombination is less common within RNA than are Types I and II, but it may explain gene rearrangements, insertions, and deletions (Lai, 1992). Nagy and Simon (1997) have suggested new terminology reflecting insights into the different mechanisms of recombination. The loss of inserted genes from viruses used as vectors is presumably due to nonhomologous recombination (e.g., Dolja *et al.,* 1992, 1993; Chapman *et al.,* 1992). Nonhomologous recombination may also be a major force in the modular evolution of viruses (see below).

3. Recombination in Viral Evolution

Recombination is thought to be one major cause of virus evolution (Holland *et al.,* 1982; King *et al.,* 1987; Strauss and Strauss, 1988; Koonin, 1991; Lai, 1992; Simon and Bujarski, 1994). Another is the gradual accumulation of changes that result from lack of a proofreading function in RNA-dependent RNA polymerases (e.g., Rao and Hall, 1993). Viral RNAs may on occasion recombine with nonviral RNAs, for which there is evidence in some apparently aberrant luteo-

virus, potyvirus, and tobravirus genomes (Mayo and Jolly, 1991; Sano *et al.,* 1992; Simon and Bujarski, 1994).

There is considerable homology between specific genes and gene products of different families of viruses, and also conservation of genome organization, even across plant and animal kingdom boundaries and between viruses with genomes of positive and negative polarities (Haseloff *et al.,* 1984; Goldbach, 1986, 1987; Koonin, 1991). This has led to the concept of modular evolution of viruses, with two basic types of genome organization and replication strategies that are typified by Sindbis virus and the picornaviruses among the single-stranded, positive-sense RNA viruses (Gibbs, 1987; Goldbach, 1987; Goldbach and Wellink, 1988; Zimmern, 1988). Cases that strongly support such modular evolution are the existence of both monopartite and bipartite genomes among the *Potyviridae, Closteroviridae,* and *Geminiviridae* and both 10- and 12-segmented reovirus genomes; further evidence comes from the fact that some furovirus and reovirus genome segments may be lost in the absence of vectored transmission, and that pseudorecombinants can be created within several virus groups having multipartite genomes (see Mayo, 1992). Modular recombination presumably occurs through Type III (nonhomologous) recombination at unrelated sequences, which is thought to be quite rare; the probability of obtaining a viable recombinant, and especially one that will have a competitive advantage for survival, must be even lower by several orders of magnitude. It has been noted, however, that recombination and rearrangement of RNA genomes are necessary for genome repair as well as to increase genetic variability, and that recombinant viruses do have a selective advantage in certain circumstances (Simon and Bujarski, 1994; Nagy and Bujarski, 1992). The frequency of replicase errors results in a significant proportion of nonviable progeny molecules, some of which may be repaired by recombination (e.g., Bar-Joseph *et al.,* 1997; Dolja *et al.,* 1994a; Simon and Bujarski, 1994). Genome division between multiple RNA segments also facilitates exchange and reassortment without physical recombination (Zaccomer *et al.,* 1995; Graves *et al.,* 1996).

In most instances, recombination between unrelated viruses will result in nonviable molecules that lack recognition signals for transcription by the appropriate replicase. It is possible that chimeric molecules with 5′ sequences derived from one parental strand and 3′ sequences derived from the other parental virus could be maintained in mixed infections. There are also some virus groups that have relatively relaxed requirements for replicase recognition. For example, Ishikawa *et al.* (1991) created chimeric BMV RNA 3 molecules with the 3′ noncoding

region from TMV. These molecules were propagated in tobacco protoplasts coinoculated with wild-type BMV RNAs 1 and 2, and variable populations of molecules were recovered from different experiments. In most cases, the newly rearranged or recombined molecules hybridized to both BMV RNA 3 and TMV 3′ probes, indicating that the introduced TMV 3′ sequence was functional in the heterologous system. In contrast, Skuzeski *et al.* (1996) showed that the tymovirus 3′ sequence is critical for replicase recognition, as molecules with heterologous tRNA-like structures were nonviable even though they could be correctly aminoacylated; other 3′ noncoding sequences that are known to confer RNA stability were similarly unrecognized by the viral replicase.

There thus appear to be differences in the tolerance of different viral groups for variation in the replicase recognition sequences. In cases where the replicase is intolerant of change, it seems likely that only double internal recombination could result in a viable molecule for further replication. This would result from a switch from the parental template to a heterologous template and then back to either the original or a second template molecule of the parental virus. Alternatively, the recombinant molecule would have to include the most variation-tolerant replicase gene of the two parental types. However, it is significant that more evidence of natural recombination between viral groups has been observed in those viral groups (carmoviruses, tombusviruses, and luteoviruses) whose replicases lack the helicase and NTP-binding domains found in the bromovirus replicase (Koonin and Dolja, 1993). This probably leads to a difference in enzymatic properties between these groups that affects their tolerance of heterologous recombination (Simon and Bujarski, 1994).

An intramolecular recombinatory repair has been reported in a partially defective chimeric tobamovirus to yield a fully functional recombinant (Deom *et al.,* 1994). In this instance, a functional movement protein (MP) gene from sunn-hemp mosaic virus (SHMV; a tobamovirus able to move from cell to cell but not long distance in *N. tabacum* cv. Xanthi) was inserted into a clone of TMV from which the 5′ portion of the TMV MP gene was deleted. Transcripts from the chimera were infectious and spread systemically, but progeny virions were found to have a recombinant MP gene with the 5′ portion from SHMV fused to the 3′ portion of the TMV gene. Total replacement of the TMV MP gene with the SHMV MP gene resulted in a chimeric virus defective for long distance movement in cv. Xanthi, whereas SHMV MP-transgenic plants were able to complement fully an MP-defective isolate of TMV. This suggests that viral determinants other than MP are required

for full systemic movement (Deom *et al.,* 1994) and that many other recombinants will not be fully functional.

Carlaviruses and potexviruses have similar genome organizations, differing in part by the presence of an additional 3′ gene and an N-terminal extension of the CP in the carlaviruses compared to the potexviruses. This suggests that the aphid transmissibility of the carlaviruses compared to the potexviruses may result from presence of one of these regions (Memelink *et al.,* 1990). This distinction may have resulted from the gain of one or more genes between the common ancestor of carlaviruses and potexviruses and an unknown source, or the loss of aphid transmissibility by the carlavirus progenitor followed by deletion of defective aphid transmission functions. As noted above, both furovirus and reovirus genome segments may be lost in the absence of vectored transmission, with no apparent effect on mechanical transmissibility or replication functions.

The apparent gain of a gene from within the same virus group has been reported, where a strain of TRV had apparently "captured" a gene from PEBV (Angenent *et al.,* 1986). In this instance, the TRV RNAs 1 and 2 retained the 3′ homology typical of other tobravirus isolates (and common to most other multicomponent viruses). However, a pseudorecombinant between TRV strains with distinct 3′ terminal sequences was shown to retain the nonhomologous sequences through 25 passages in greenhouse experiments, although field isolates apparently exchange genes by recombination rather than reassortment into pseudorecombinants and thus retain terminal homology (Angenent *et al.,* 1989). Recombination between genome segments of multicomponent plant viruses restoring such intercomponent terminal homology was first demonstrated by Bujarski and Kaesberg (1986). More recently, Mueller *et al.* (1997) demonstrated that at least for tobraviruses, the specificity of template recognition is determined by 5′ noncoding sequences, not by 3′ noncoding sequences. The CP gene subgenomic promoter was also recognized by the replicase of the heterologous virus. Amplification of subgenomic RNAs by the replicase of a heterologous virus might prove to be an initial step to template switching to the homologous genomic RNA during transcription of the (−) strand.

Gene exchange between different viruses presumably can occur only in mixed infections in plants, or possibly in insect vectors in the case of those virus groups that are also capable of replication within insects. Indeed, Goldbach (1986) has discussed the possibility that the common ancestor of the animal picornaviruses and the plant picorna-like viruses (comoviruses and potyviruses) was an insect virus. For recombination to occur, it is presumably also necessary for replication of the two virus

types to occur in the same cellular compartment and at the same time. Although replication of some virus groups is associated with certain ultrastructural changes in the host cell, such as the chloroplast invaginations observed in tymovirus infection (e.g., Matthews, 1973), it is not clear whether replication is wholly contained within such structures or that other RNAs are excluded from such sites. However, recombination is unlikely to occur between a virus replicating strictly in the nucleus and one replicating in the cytoplasm.

4. Recombination as a Normal Part of the Viral Life Cycle

Recombination between viral RNAs and either cellular mRNAs or heterologous viral RNAs may prove to be a normal occurrence in some virus groups. Single-stranded viral RNAs are vulnerable to RNase activity and would benefit from a repair mechanism. Two types of repair mechanisms have been reported. Nagy *et al.* (1997) have detected a mechanism that repaired deletions of up to 6 nt from the 3′ end of (+) transcripts from satellite RNA C of TCV; the origin of the repaired sequence was apparently abortive synthesis from the 3′ end of the viral genomic RNA. Neither base pairing nor ligation reactions are thought to be necessary for these abortive products to then be incorporated into the repaired (−) strand (Nagy *et al.,* 1997). A short region of base pairing between the donor and acceptor molecules and a hairpin in the acceptor RNA region contribute to determination of recombination sites (Nagy *et al.,* 1998). Estabrook *et al.* (1998) have shown that cap snatching by a tenuivirus can occur from another plant viral RNA as well as from cellular RNA. A stretch of 8 nt at the 5′ and 3′ ends of the tenuiviral genomic RNA is self-complementary, but viral mRNAs have additional, nontemplated nucleotides between the 5′ cap structure and the conserved 5′ octanucleotide sequence. Maize stripe tenuivirus (MSpV) was shown to recruit the 5′ cap and adjacent nucleotides from all three BSMV genomic RNAs in coinfected seedlings, with sufficient frequency to allow reproducible detection of the chimeric RNAs (Estabrook *et al.,* 1998). Replication of MSpV and BSMV is thus obviously not exclusively compartmentalized in the infected cells.

5. Experimentally Documented Recombination

Within plant virus groups—and even a single virus—all three types of RNA recombination have been reported. Rao and Hall (1993) reported that homologous recombination within BMV occurred more frequently than nonhomologous recombination: all observed events were precise recombinations (Type I), leading to the suggestion that the viral polymerase switches templates during (−) strand synthesis, i.e., on a (+)

strand template. The evidence further suggested that the polymerase switched from the less abundant mutant RNA 2 template to the more abundant RNA 3 template, as expected for the copy-choice mechanism (Rao and Hall, 1993). These experiments were carried out with a mutant RNA 2 that accumulated to <10% of wild-type RNA 2, with mutations in a region important for replicase recognition, and thus imposed strong selection for recombinants that restored function (Rao and Hall, 1993). Nagy and Bujarski (1996) also examined recombination in BMV and showed that aberrant homologous (Type II) recombination occurs more frequently near AU-rich sequences. AU-rich sequences also occur around viral translational frameshift sequences recognized by ribosomes (e.g., Miller *et al.,* 1995) and thus may generally cause slippage in enzyme–RNA interactions. Nagy and Bujarski (1993) also reported that nonhomologous (Type III) recombination can be experimentally targeted to sequences upstream of heteroduplexes in BMV. Short direct repeat sequences have been reported to occur at both donor and acceptor sites in poliovirus recombination (Pilipenko *et al.,* 1995).

Carpenter and Simon (1996) examined recombination between TCV genomic RNA and a TCV-associated satellite RNA in both whole plants and protoplasts over time. They determined that the majority of recombinants occur within a 24-nucleotide repeat that forms part of a stable hairpin (rather than adjacent to heteroduplex sequences, as suggested for BMV). The results were somewhat surprising in that populations of recombinants in plants became progressively shorter, but populations in protoplasts became progressively longer over time (Carpenter and Simon, 1996). Additionally, the most commonly recovered recombinants were determined to be defective for replication and appeared to result from multiple individual recombination events, thus demonstrating the high frequency of recombination to yield nonviable molecules (Carpenter and Simon, 1996). The change in size of the recombinants may reflect changes in the ratio of polymerase to template overall, or to templates viable and nonviable for replication; it has not been established whether the TCV genomic RNA is the donor or the acceptor template (Carpenter and Simon, 1996).

The replication competence of the recombinant sequence is of critical importance in the detection of recombination in many systems and in the estimation of recombination frequency. In many studies, only functional, amplifiable molecules would have been detected, and thus bias the estimate of the extent of recombination that may occur (Lai, 1992); however, it is only viable recombinants that are of any potential epidemiological significance. Mutations in the polymerase can have a significant effect on both recombination frequency and the fidelity and

location of crossovers (Nagy *et al.,* 1995). Indeed, Dinant *et al.* (1993a) showed that compatibility between bromovirus polymerase and helicase gene products was critical for efficient replication; combinations of BMV and cowpea chlorotic mottle virus (CCMV) 1a (helicase) and 2a (replicase) proteins transcribed from *in vitro* transcripts were quite different in activity. Both homologous 1a + 2a combinations supported high levels of RNA synthesis in protoplasts; however, no RNA synthesis was detected from the CCMV 1a/BMV 2a combination. BMV 1a/CCMV 2a yielded significantly reduced amounts of (+) strand and subgenomic RNA, but similar amounts of (−) strand RNA to the homologous combinations, indicating that interactions between 1a and 2a proteins differentially affect (+) and (−) strand synthesis (Dinant *et al.,* 1993a). This suggests that polymerase/template compatibility is a major factor in the creation of viable recombinants even within a virus group. Other reports further substantiate this possibility: (a) Tobravirus field isolates that appear to have gained sequences from other isolates have identical termini on the two genomic RNAs (Angenent *et al.,* 1989), and (b) Fernández-Cuartero *et al.* (1994) created a pseudorecombinant between two cucumoviruses, with RNAs 1 and 2 derived from CMV, and RNA 3 from tomato aspermy virus (TAV). A recombinant RNA 3 was identified after several years of repeated plant passage in tobacco. The recombinant had apparently gained the 3′ terminal sequence of CMV RNA 2 (thus presumably increasing polymerase compatibility) and also had an increased fitness relative to either CMV or TAV RNA 3 (Fernández-Cuartero *et al.,* 1994).

Another report documents the creation of a hybrid virus as a consequence of mixing TAV RNAs 1 and 2 with CMV RNAs 2 and 3 (Masuta *et al.,* 1998). The hybrid virus recovered from these plants was a quadripartite virus consisting of TAV RNA 1, CMV RNAs 2 and 3, and a hybrid RNA resulting from recombination between CMV RNA 2 and the 3′ 320 nt of TAV RNA 2 (probably by aberrant homologous recombination). Thus the hybrid virus contains the TAV helicase and the CMV polymerase subunits, creating an interspecific replicase complex. The formation of the chimeric CMV–TAV RNA 2 is presumed to have been an early event necessary to establish the quadripartite system, and the chimeric RNA to be well adapted to the hybrid replicase complex (Masuta *et al.,* 1998).

Osman *et al.* (1998) have shown that encapsidation competence is a critical factor in systemic infectivity of chimeric viruses, demonstrating additional limitations on recombinant viruses even when they are competent for replication. The failure may have been due to lack of compatibility between viral MP and CP (Osman *et al.,* 1998). Other interactions

required for long-term viability may include compatible CP-HC combinations for vectored transmission. Rubio *et al.* (1999a) suggest that most recombinant viruses that may occur in nature should be at a competitive disadvantage because of lack of compatibility between heterogeneous gene combinations, and state they they are unlikely to survive.

Recombination has also been reported between isolates of viruses with DNA genomes. Perhaps the best-documented case is that of the Ugandan cassava mosaic virus isolate, UgV, which has been shown to be a recombinant between African cassava mosaic (ACMV) and East African cassava mosaic (EACMV) geminiviruses (Zhou *et al.,* 1997). ACMV and EACMV have readily distinguished DNA genomes and largely nonoverlapping geographical distributions (see Harrison *et al.,* 1997). UgV alone produced more severe symptoms than either parental isolate alone, but that severity was further enhanced (symptomatic synergism) when UgV occurred in mixed infections with ACMV. Similar severity was observed in mixed infection of the two parental isolates, ACMV and EACMV (Harrison *et al.,* 1997). The total virus concentration in mixed UgV/ACMV infections was not greater than in infections of either isolate alone, indicating that the enhanced severity of the recombinant isolate UgV was not due to increased replication. The detection of ACMV and EACMV in a mixed infection without detectable UgV suggests that the two parental viruses can coexist in infected plants without recombining (Harrison *et al.,* 1997). There are several crops in which multiple geminiviruses with overlapping geographical distributions are already known to occur (e.g., Brown and Bird, 1992), in which recombination may provide a mechanism for genesis of new variants (Harrison *et al.,* 1997; Zhou *et al.,* 1997).

Evolution of variant geminiviruses may also occur from the subgenomic DNAs observed in cultures of several viruses. These include the leafhopper-transmitted beet curly top virus (BCTV) (Frischmuth and Stanley, 1992; Stenger *et al.,* 1992) and both monopartite and bipartite whitefly-transmitted geminiviruses (Liu *et al.,* 1998, and references therein). In some cases, the subgenomic DNAs are simple deletions from the genomic DNAs (e.g., Frischmuth and Stanley, 1992), but in others, multiple deletions and rearrangements have occurred, including incorporation of sequences unrelated to the geminiviral genomic DNA (Stanley *et al.,* 1997; Liu *et al.,* 1998). Many of the recombination events involved in creating the subgenomic DNAs appear to be illegitimate, with few shared nucleotides between the parental sequences at the recombination junctions (Liu *et al.,* 1998). With some viruses, deleted and/or rearranged molecules are detected early in infection

(Frischmuth and Stanley, 1992; Stenger *et al.,* 1992); in the case of cotton leaf curl virus (CLCuV) the smaller DNAs were not observed 3 months after infection but were present after 6 months (Liu *et al.,* 1998).

The effects of the defective DNAs also differ among viruses, causing symptom amelioration in some instances (Stanley *et al.,* 1990, 1997), but with no apparent phenotypic effects in others (Liu *et al.,* 1998). The variation among isolates of CLCuV (Zhou *et al.,* 1998) suggests that the defective DNAs may play a role in emergence of novel geminivirus variants, possibly including incorporation of gene sequences from the host genome (Liu *et al.,* 1998). Two further observations lend credence to this possibility: Bejarano *et al.* (1996) found geminiviral-related sequences within the genome of *Nicotiana* species, which might be available for recombination; and Etessami *et al.* (1989) showed that CP gene deletion mutants of ACMV revert to close to the wild-type size by gaining sequences from elsewhere in the viral genome. Thus recombination of defective, subgenomic geminiviral sequences either with host genomic DNA or with other geminiviral sequences may result in the creation of viable new geminiviruses.

From the above information, we can conclude that (a) recombination occurs naturally in mixed infections of plant virus species and isolates; (b) some virus groups are much more prone to recombination than others, probably as a function of the degree of specificity of the polymerase complex for homologous template; (c) the vast majority of recombinants that are produced are nonviable molecules that are not replicated; and (d) even where viable recombinants are formed, it is rare for the recombinant to have a selective advantage over the parental viruses.

G. Virus Variability and Emergence of New Viruses

The emergence of new viruses may be related to passage of a viral population into a new host species or into a new ecosystem. Despite the lack of proofreading in viral RNA replication, plant virus populations appear to be remarkably stable. Replicase misincorporation has been shown to result in slow accumulation of nucleotide changes in plants infected with infectious transcripts from cloned cDNA of TMV (Kearney *et al.,* 1993); the mutation rate was calculated at $\leq 10^{-4}$ mutations per base per plant passage. TMV has been shown to evolve slowly in both natural populations and greenhouse-maintained cultures (Rodríguez-Cerezo *et al.,* 1991; Rodríguez-Cerezo and García-Arenal, 1989). However, Domingo *et al.* (1985) have calculated that all possible single and double nucleotide mutations, plus a proportion of triple and higher-

order mutations, are present in a typical RNA virus infection. Viral populations thus exist as "quasispecies" in which most individual molecules differ from the consensus population sequence at one to many nucleotide positions (Domingo *et al.,* 1985). Mutations at one or more sites may alter the phenotype, but unless this mutant becomes predominant in the population, it may well be masked. The phenotype can be altered by passage in a different host, which may affect the balance of different variants within the initial population (MacNiell and Boxall, 1974; Yarwood, 1979). Passage through the original host may restore the original phenotype, presumably by reverse selection of the dominant sequence (Koenig, 1976). Different hosts have been shown to exert opposite selection pressures on the initial population of TMV (reviewed by Dawson, 1992).

Villegas *et al.* (1997) showed that considerable microvariation occurred within a greenhouse-maintained isolate of RTBV, which has a dsDNA genome but replicates via an RNA intermediate. Variation was also found within and between field isolates from the same region. RTBV is insect-transmitted only in the presence of RTSV, and transmission may result in the transfer of relatively few virions to initiate infection in the new host. The necessity for compatibility with RTSV for vectored transmission may impose strong selection pressure on quasispecies variation, leading to maintenance of greater stability in the population than might be expected (Villegas *et al.,* 1997; Pirone and Blanc, 1996).

The effect of introducing an existing virus to a new host species may be compounded by rapid selection of mutants from within the population that are better adapted to the new host. Adaptation to one species may lead to the ability to infect additional species, leading to further variation and ultimately to a stable population of a new virus. It has been observed in several instances that new plant viral diseases are identified after crops are grown in new areas. Expansion of the growing area may bring the crop into proximity with additional wild plant species that harbor viruses to which the crop has not previously been exposed, or potential new vector species. Such changes in crop ecology may lead to the emergence of new diseases. The introduction of beets in proximity to desert areas of California may have led to the emergence of BCTV as a major disease of multiple crops in this part of the United States (Bennett, 1971). The emergence of the whitefly-transmitted closteroviruses in the southwestern desert regions of the United States was presumed to be due to changes in temperature and increased insecticide use; dramatically increased whitefly activity resulted in transmission of these viruses from native plants in which

the virus was presumably well established (Duffus *et al.,* 1986). Cocoa swollen shoot virus (CSSV) had already caused widespread devastation over some 200 square miles before it was recognized that the disease was of viral origin (Posnette, 1941). Because of the severity of the disease, cacao production was abandoned in many areas (Posnette and Todd, 1951). The cacao crop in West Africa was derived from a very narrow germplasm base from Brazil, and it appears that viruses spread to cacao from a few widespread forest tree species. Virus transmission from the indigenous reservoirs to cacao seems to be rare, as virus tends to be of exceedingly low availability to vectors in the indigenous virus hosts (Posnette and Todd, 1951). All of the indigenous species identified as hosts were, like cacao, in the order Tiliales (Tinsley and Wharton, 1958). Once transferred to cacao, the availability to vectors increases markedly, and rapid spread occurs within cacao (Posnette and Todd, 1951). Thus the introduction of a crop susceptible to the virus changed the whole ecology of the virus.

Emergence of a new virus, possibly as the result of adaptation from an endemic plant host to a crop plant, may be followed by further adaptation to new hosts and increased variability. Such may have been the case with ZYMV. Although two other potyviruses were widely distributed in cucurbit crops throughout the world, in the early 1970s ZYMV was isolated from zucchini in Italy (Lisa *et al.,* 1981). Within a few years this "new" virus was detected in many other countries, and strains differing in host range, symptoms, and aphid transmissibility were described from different locations (Lecoq and Pitrat, 1984; Lecoq and Purcifull, 1992). No symptoms typical of ZYMV were detected on the island of Martinique prior to 1992, although other cucurbit viruses were widespread (Desbiez *et al.,* 1996). A survey of the variability of ZYMV isolates collected in Martinique in 1992 and 1993 detected significant biological and antigenic variability among 14 isolates from various hosts and locations. Some of these isolates were able to overcome the *Zym* resistance gene of muskmelon, suggesting that durable control of ZYMV would not be possible in Martinique by the use of this resistance gene, and that other measures are necessary (Desbiez *et al.,* 1996). At least three pathotypes could be distinguished among the 14 Martinique isolates, with further possible differentiation based on antigenic or symptom differences, despite very similar CP sequences. This suggests either multiple introductions of the virus to Martinique over a very short period or (more likely) rapid evolution from a single or limited number of introductions of the virus through a "foundation" effect (Desbiez *et al.,* 1996). Despite the low molecular variability observed in the N-terminal domain of the CP, variants with new biological

properties appeared very easily within the viral populations (Desbiez *et al.,* 1996).

One question that remains is whether the range of possible variation has been reached within viruses that have coevolved with crops over longer periods such as PVY, compared to recently recognized viruses such as ZYMV. New pathotypes have been described for PVY as well as for ZYMV, and probably more will occur with the release of resistant cultivars (Blanco-Urgoiti *et al.,* 1998). In addition, previously recognized pathotypes may periodically reemerge as a locally predominant type as a consequence of changes in crop genotypes or growing areas.

III. Transgenic Plants

A. Selection of Viral Genes to Be Expressed in Plants

Currently, many different viral sequences have been expressed in transgenic plants. The rationales behind the original choices have been somewhat varied but are summarized in the following eight sections.

1. Coat Protein

There are multiple reasons why CP genes have been tested for so many viruses. Perhaps the simplest is that what was thought to be CP-mediated resistance was the first type of transgenic, virus-derived resistance reported (Powell-Abel *et al.,* 1986). The initial assumption was that CP mediated traditional cross-protection (Sherwood and Fulton, 1982) and that CP-transgenic plants would be protected to equivalent levels (Powell-Abel *et al.,* 1986). Transgenic resistance would therefore have the benefits of traditional cross-protection without the yield loss due to infection by the protective isolate or the necessity for inoculation of large numbers of plants (Powell-Abel *et al.,* 1986).

It is also generally true that CP genes are relatively easy to clone. In many cases (e.g., potyviruses), the CP gene is at the 3′ end of the viral genome on a polyadenylated RNA, making first-strand cDNA very easy to prepare. However, potyvirus CPs are normally processed from the genomic polyprotein and require introduction of an initiation codon in a suitable context (e.g., Beachy *et al.,* 1990). CP clones are also often easily identified from plasmid or phage expression libraries, by screening colonies or plaques with virion-specific antisera (e.g., Hammond and Hammond, 1989). The availability of specific antibodies also allows simple screening for both qualitative and quantitative evaluation of CP transgene expression once transgenic plants are generated.

The relative resistance of the first transgenic plants expressing CP of economically important viruses was a huge motivating factor—both to join the field and to evaluate the mechanisms involved and to obtain resistance to viruses in crops in which no resistance genes had been identified. There were also cases where introgression of available resistance genes into agronomically acceptable varieties was an extremely laborious and lengthy process.

CP expression has also been shown to be less isolate-specific than several other transgenic approaches to resistance (e.g., Lomonossoff, 1995; Spillane *et al.,* 1997). This may be of great importance in some crops where significant virus diversity has been demonstrated.

2. Replicase

Initial reports of replicase-mediated resistance suggested that there was great potential for the use of a dominant negative mutant, resulting in extreme resistance to infection (e.g., Longstaff *et al.,* 1993). Other reports revealed that even native, presumed fully functional replicases could confer resistance (Braun and Hemenway, 1992; Rubino and Russo, 1995). Further testing in different systems has continued to demonstrate extreme resistance where resistance is observed, but it has also been shown that replicase-mediated resistance is typically more isolate-specific than CP-mediated resistance (e.g., Golemboski *et al.,* 1990; Audy *et al.,* 1994; Lomonossoff, 1995; Spillane *et al.,* 1997). This is true even despite the greater intraspecies conservation of the replicase gene than any other potyvirus gene, for example (Shukla *et al.,* 1994). Jones *et al.* (1998) found that pea plants expressing the replicase gene of pea seed-borne mosaic virus (PSbMV) isolate DPD1 were highly resistant to the homologous (pathotype 1) isolate. However, results from challenge with the pathotype 4 isolate PSbMV-NY were highly variable, indicating that the sequence identity of the NIb genes of these two isolates is at the borderline for induction of resistance. Challenge of virus-free upper leaves of plants that had recovered from infection suggested that different sequences are involved in induction of gene silencing than in consequent RNA degradation (Jones *et al.,* 1998).

3. Movement Protein

Interference with virus movement is an obvious approach to limiting the effects of virus infection, as the hypersensitive response has been one of the most effective and lasting forms of resistance to many pathogens from natural resistance genes. The hypersensitive response is not necessarily induced by the movement protein, but the response effectively limits viral movement. Indeed, resistance conferred by the

N′ gene in tobacco is induced by the TMV CP (Culver and Dawson, 1989), and the *Tm-1* resistance response in tomato is elicited by the TMV replicase (Meshi *et al.,* 1988). *Tm-2* resistance to TMV induced by the movement protein can be overcome by mutations in the virus (Meshi *et al.,* 1989). However, the relationship between both structure and function of the MPs from different viruses (Atabekov and Taliansky, 1990; Melcher, 1990) indicated that a broader resistance might result from expression of an MP able to confer resistance to the homologous virus (Cooper *et al.,* 1995). The triple gene block of potexviruses has been shown capable of conferring resistance to other viruses having similar genome organization, including carlaviruses (Beck *et al.,* 1994; Seppänen *et al.,* 1997). Whereas resistance to TMV from a functional BMV MP was reported in transgenic tobacco, a nonsystemic host of BMV (Malyshenko *et al.,* 1993), there is also the danger of complementation or enhancement if a functional MP is used (e.g., Atabekov and Taliansky, 1990; Ziegler-Graaf *et al.,* 1991; Cooper *et al.,* 1995). The use of defective MP mutants is now strongly preferred (see Section IV,B,2 and Lapidot *et al.,* 1993; Cooper *et al.,* 1995).

4. Antisense RNA

Antisense RNA had been shown to have a significant regulatory effect on expression of nuclear genes, but it was not clear that it would have the same effect on a replicating viral RNA (reviewed by Tabler *et al.,* 1998). In many of the initial experiments, plants expressing antisense versions of the CP-expressing constructs were generated as controls for the CP expressors as much as for any effect the antisense RNA was expected to have on virus resistance (Tabler *et al.,* 1998). In many cases it was observed that resistance from antisense RNA was less effective than from CP expression, and fewer subsequent studies paid much attention to antisense constructs. A few studies have, however, reported significant resistance from expression of antisense RNA (e.g., Hammond and Kamo, 1995a; Tabler *et al.,* 1998). The use of ribozymes to confer resistance may be regarded as an extension of the antisense RNA strategy (e.g., De Feyter *et al.,* 1996; Tabler *et al.,* 1998).

5. Untranslatable RNA

Untranslatable RNA was initially examined as a means of distinguishing CP-mediated protection from the possibility of RNA-mediated resistance from the CP transcript. Lindbo and Dougherty (1992a) observed a higher level of resistance from untranslatable RNA than from the translated TEV CP gene, and a new area began to emerge. In theory almost any sequence can function in this fashion, and antisense RNA

may be regarded as a special case of untranslatable RNA (Tabler *et al.,* 1998). RNA-mediated resistance is likely to be effective largely through homology-dependent gene silencing mechanisms, which has been reviewed by Baulcombe (1996).

6. Satellite RNA

The expression of viral satellite RNAs (satRNAs) as a transgenic resistance strategy was first demonstrated with CMV satRNA (Baulcombe *et al.,* 1986; Harrison *et al.,* 1987) and tobacco ringspot virus (TRSV) satRNA (Gerlach *et al.,* 1987). SatRNAs typically ameliorate the symptoms of virus infection, an effect that is not dependent on the level of viral inoculum applied; however, in some cases, satRNAs cause an intensification of disease (e.g., Collmer and Howell, 1992). In at least one case, a transgenically expressed satRNA had no protective effect against the virus but suppressed defective interfering RNA accumulation (Rubino *et al.,* 1992).

7. Defective Interfering RNAs (or DNAs)

Defective interfering (DI) RNAs are replicating deletion mutants of genomic RNAs that also typically act to reduce replication of the parental genomic RNA (Roux *et al.,* 1991), although at least one DI RNA intensifies symptom expression (Li *et al.,* 1989). DI RNA-mediated resistance is also not overcome by high levels of viral inoculum (Burgyàn *et al.,* 1991). DI DNAs have similar competitive effects on DNA viruses (Stanley *et al.,* 1990). Transgenic resistance has been shown for both DI RNAs (Kollàr *et al.,* 1993) and DI DNAs (Frischmuth and Stanley, 1991).

8. Other Genes

The potyvirus proteinase, NIa, has been shown to confer resistance to the homologous virus (Maiti *et al.,* 1993; Vardi *et al.,* 1993), as has the PVY helper component (unpublished data, cited in Vardi *et al.,* 1993). Both potyvirus P1 and P3 genes have been shown to confer resistance to the homologous virus, probably by RNA-mediated mechanisms (Pehu *et al.,* 1995; Moreno *et al.,* 1998). Resistance conferred by the P1 gene appeared to be more isolate-specific than that conferred by the P3 gene (Moreno *et al.,* 1998). Blanc *et al.* (1993) showed that prior feeding on a nonactive form of bacterially expressed CaMV P18 aphid transmission factor inhibited subsequent transmission of CaMV from infected plants. Defective helper components expressed in transgenic plants might therefore block virus transmission (Hull, 1994b). Other reports include the 3′ noncoding region of TYMV (Zaccomer *et*

al., 1993). This region was subsequently shown to be necessary for specific virus replication functions, as it could not be replaced by generic tRNA-like elements or by heterologous 3′ untranslated regions (Skuzeski *et al.,* 1996). It is probable that almost any portion of a viral genome may confer some degree of resistance (Lomonossoff, 1995).

Future criteria for gene construct selection may be driven more by risk avoidance or reduction than was the case with the first generation of virus-resistant transgenic plants. However, there is little evidence to suggest that there is sufficient risk from the first generation of transgenic plants that these lines should not be deployed, other than those expressing functional MPs or genes involved in pathogenicity enhancement (see Sections III,C and III,D). As we learn more about the mechanisms of resistance mediated by portions of the viral genome, we will rapidly increase the sophistication of both approaches and selection of virus-resistant plant lines. Some of the potential risks are described in Sections III,C to III,E, and means of minimizing these perceived risks are addressed in Section IV,B.

B. The Enigma of Coat Protein-Mediated Resistance

The mechanism of CP-mediated resistance was initially assumed to be similar to the phenomenon of cross-protection, with CP accumulation interfering with the uncoating of virions and inhibiting both the establishment of infections and the spread of virus from cell to cell (Wisniewski *et al.,* 1990; Beachy *et al.,* 1990). The necessity for expression of the CP itself was first demonstrated for TMV. Plants expressing a TMV CP construct lacking an initiation codon showed none of the resistance conferred by the equivalent CP-expressing construct (Powell-Abel *et al.,* 1986; Powell *et al.,* 1990). Strong correlations between the level of CP expression and resistance were also noted with AMV and PVX (Loesch-Fries *et al.,* 1987; Hemenway *et al.,* 1988). In these cases, plants not expressing readily serologically detectable levels of CP were not resistant to infection, and there was a positive correlation between CP expression and degree of resistance. It was further noted that decreased levels of TMV and CMV CP accumulation in plants grown at elevated temperatures were associated with decreased resistance to infection and spread (Nejidat and Beachy, 1989; Okuno *et al.,* 1993).

It was not long, however, before contrasting results were obtained with potyvirus and luteovirus CP-expressing plants. In the case of PVY or potato leafroll virus (PLRV) CP expressed in potato, there was no correlation between CP detection and resistance. Some lines expressing

the lowest amounts of PVY CP (Lawson *et al.,* 1990), and some PLRV CP transgenic lines in which no CP was serologically detectable (Kawchuk *et al.,* 1990), were found to be highly resistant. Indeed, Lindbo and Dougherty (1992a,b) found that an untranslatable CP RNA conferred higher levels of resistance than either full-length or truncated translatable CP constructs of TEV. Remarkably, an N-terminally truncated CP construct conferred no resistance to TEV infection, but infected plants showed a recovery phenotype in which upper leaves were symptomless and free of virus infection (Lindbo and Dougherty, 1992a). Taken together, these results suggested that the protection observed was RNA-mediated rather than protein-mediated in some instances. Induction of the virus-resistant state in recovered tissue required initial systemic infection, suggesting that stimulation or infection of cells at the apical meristem is necessary; the transgene transcript and the replicating viral genome may act additively to trigger a natural cellular response (Lindbo *et al.,* 1993). In highly resistant plants the virus-resistant state may be fully induced by the transgene transcript (Dougherty *et al.,* 1994).

Additional evidence was provided by de Haan *et al.* (1992), who showed that high levels of resistance to tomato spotted wilt virus (TSWV) were obtained whether the TSWV N-gene construct was translated or not; the resistance was restricted to isolates and strains of TSWV and not to other tospoviruses with considerable nucleotide homology in the N-gene sequence. This contrasted with the results of Pang *et al.* (1992), who had demonstrated some differential resistance to TSWV isolates from a translated CP construct.

1. *Occurrence of Multiple Resistance Mechanisms from the Same Construct*

Further results suggest that a single type of construct may confer resistance via both protein- and RNA-mediated mechanisms. Pang *et al.* (1993) showed that a tospovirus N-gene sequence conferred resistance to heterologous tospoviruses only in plants with the highest levels of expressed protein, but that the most effective resistance to the homologous virus occurred in those plants with the lowest steady-state levels of RNA and little protein; the RNA-mediated resistance appeared to be due to a direct inhibition of replication.

Multiple types of resistance have also been observed in transgenic lines expressing various potyvirus CP constructs. In some bean yellow mosaic virus (BYMV) CP lines, only resistance to initial BYMV infection was observed, and plants that did become infected supported wild-type infections; other lines showed no resistance to infection but displayed the recovery phenotype in symptomless, virus-free upper leaves. Recov-

ery was complete in some lines and incomplete (resulting in significantly reduced symptoms and virus titer) in others. Both initial resistance to BYMV infection and recovery phenotypes were combined in a third class of lines (Hammond and Kamo, 1993, 1994, 1995b; Hammond, 1996). Some resistance to infection was observed against several heterologous potyviruses in lines expressing BYMV or chimeric potyvirus CPs, but recovery was observed only in BYMV-infected plants or in PVY-infected plants expressing a chimeric BYMV/PVY CP gene (Hammond, 1996). Thus, it was apparent that not only was more than one resistance mechanism activated by a single construct, but also that the mechanisms could be differentially activated in different plant lines.

Resistance to heterologous viruses has typically been associated with the expression of the CP rather than RNA. Resistance to other potyviruses has also been shown for plants expressing the CP of SMV (Stark and Beachy, 1989), ZYMV or WMV-2 (Namba *et al.,* 1992), and lettuce mosaic virus (LMV) (Dinant *et al.,* 1993b), among others. Maiti *et al.* (1993) reported that plants transformed with the CP gene of TVMV were also resistant to other potyviruses, whereas lines expressing the NIa proteinase were resistant only to the homologous virus, and lines expressing CI protein were fully susceptible. Resistance to heterologous viruses has been reported only for plants expressing CP (and not CP RNA alone) or MP (see Section III,A,3 and Lomonossoff, 1995). Virus-specific (e.g., Lindbo *et al.,* 1993; Smith *et al.,* 1994) and isolate-specific resistance of some lines (Farinelli *et al.,* 1992) is primarily associated with CP RNA-mediated rather than protein-mediated mechanisms.

Hence the enigma of CP-mediated resistance: is it the expressed CP or the nucleic acid that confers resistance? The answers are not always clear, but in several cases the mechanism has been shown to be due to protein, while in other cases the RNA is clearly the responsible factor. Both types of resistance may operate in some instances. What, then, are the actual mechanisms? A detailed examination is beyond the scope of this article, but reviews have been published by Lomonossoff (1995) and Baulcombe (1996). We will present here only brief summaries of what has been determined and the questions that remain. We also note that the question of whether RNA or protein confers resistance is not limited to CP constructs but also applies to replicase-mediated resistance (reviewed by Palukaitis and Zaitlin, 1997) and possibly resistance mediated by other gene constructs.

2. Types of Resistance Mechanism

Difficulties in elucidating mechanisms occur for a number of reasons. One is the variability in phenotypes among different lines transformed

with the same construct. This may be related to both copy number and chromosomal position effects, thus affecting transcription efficiency or temporal control of expression; these effects are discussed by Lomonossoff (1995). Attribution of resistance to specific transgene loci has been addressed by Goodwin *et al.* (1996) and Mueller *et al.* (1995). The second reason is that it is possible for mutations to be introduced in the transgene prior to or during plant transformation. For example, Donson *et al.* (1993) showed that a line expressing resistance to a broad spectrum of tobamoviruses had an insertion of a bacterial transposable element in the middle of the TMV-183K transgene, while lines expressing wild-type 126K or 183K transgenes were not resistant. Similarly, Duan *et al.* (1997a,b) found that unexpected mutations in tomato mottle geminivirus (ToMoV) BC-1 protein also resulted in resistance. In at least one case, however, the resistant transgene has been demonstrated to be functional by recloning the Cymbidium ringspot virus (CymRSV) replicase gene from transgenic plants and substituting it into an infectious viral clone (Rubino and Russo, 1995).

a. RNA-Mediated Mechanisms. To the extent that no promoterless transgenes have been shown to confer resistance (e.g., Pang *et al.,* 1993), it is assumed that transgene transcription is required to induce resistance (Lomonossoff, 1995). However, steady-state transcript levels are not a good predictor of resistance in any system. Where transcription levels have been carefully examined (in cases where RNA-mediated mechanisms were suspected), three general classes of resistance phenotype have been observed: (a) plants that are fully susceptible; (b) those that become infected and recover; and (c) those that are highly resistant to infection. These classes appear to correlate with plants that have (a) low to moderate transgene transcription and steady-state RNA; (b) moderate to high transgene transcription and steady-state RNA in uninfected plants but low-level steady-state RNA in recovered tissues; and (c) high levels of transgene transcription with low steady-state RNA levels (Smith *et al.,* 1994). The effect of resistance appears to be mediated by destruction of both the transgene RNA transcript and the closely related genomic sequences of the challenge virus. The mechanism has similarities to RNA sense suppression (cosuppression) and gene silencing mechanisms, which have been reviewed with special reference to virus resistance by Lomonossoff (1995), Baulcombe (1996), and Palukaitis and Zaitlin (1997).

Other possible means of RNA-mediated resistance include the action of a (+) sense transgene transcript acting as an antisense RNA interacting with the viral (−) strand; as viral (−) RNA is present in much lower concentrations than the genomic (+) strand RNA once infection

is established, competition with the small amount of (−) RNA present during initial replication could interfere with infection (Tabler *et al.,* 1998). Lindbo and Dougherty (1992a,b) speculated that untranslatable or truncated sense TEV RNA conferred superior protection to fully functional, translatable CP constructs because their transcripts would not be associated with ribosomes and therefore would be more accessible for intermolecular RNA interactions with the viral genomic (−) strand. A CP transgene transcript, especially one with a full-length viral 3′ noncoding region (NCR) containing replicase recognition sequences, could potentially also titrate out viral or host factors and competitively inhibit replication (e.g., Zaccomer *et al.,* 1993).

b. Protein-Mediated Mechanisms. CP-mediated resistance might function through several possible mechanisms related to cross-protection. One of these is inhibition of virion disassembly in the initially infected cells, as suggested by Register and Beachy (1988). This is consistent with evidence that free CP subunits inhibit cotranslational disassembly of TMV *in vitro* (Wilson and Watkins, 1986). Osbourn *et al.* (1989) provided evidence that both virion disassembly and a later step in replication were inhibited by examining infection of protoplasts from TMV CP plants. Further evidence of a requirement for CP in the initial stages of TMV infection was provided when it was shown that tissue-specific expression significantly affected resistance (Reimann-Philipp and Beachy, 1993). Whether plant-expressed CP directly inhibits uncoating or blocks a receptor site within the cell at which disassembly is initiated is not clear (Lomonossoff, 1995), but TMV CP plants were more resistant to inoculation with virions than to inoculation with RNA (Powell-Abel *et al.,* 1986). However, Clark *et al.* (1995a,b) showed that TMV CP-mediated resistance involves interactions distinct from potential virion surface amino acid residues, and that the specificity of resistance is determined by the CP of the challenge virus rather than by the genome *per se.* In TMV CP-mediated resistance there is also an inhibitory effect on long-distance transport (Wisniewski *et al.,* 1990), and intergrafted CP-transgenic stem segments were able to inhibit virus transmission between an infected nontransgenic rootstock and a nontransgenic scion. Strangely, this was true only if the intergrafted stem segment carried a leaf, suggesting the possible necessity of a source-sink flow from the transgenic stem piece into the nontransgenic rootstock. This evidence for potential mechanisms applies specifically to TMV, but some aspects may also apply to other viruses.

There is evidence that CP interference with virion disassembly also contributes to AMV resistance. In lines with low-level expression of AMV CP, resistance was effective only against virions, and not against

RNA inoculum; high-level expressors were resistant to both virion and RNA inoculation (Taschner *et al.,* 1994), again suggesting two distinct mechanisms of resistance. PVX CP-mediated resistance is effective against both virion and RNA challenge (Hemenway *et al.,* 1988). PVX CP has been shown to be necessary for cell-to-cell movement of the virus (Chapman *et al.,* 1992), so transgenic expression of PVX CP may interfere with transport as well as possible effects on disassembly (Baulcombe, 1996). In any case, CP-mediated resistance to PVX is active at a later stage of infection than virus disassembly; replication is suppressed in initially infected cells, and both cell-to-cell and systemic movement are inhibited (Spillane *et al.,* 1997). The situation with other viruses may also differ significantly.

In many cases, viral gene products are multifunctional and interact with other gene products in support of those functions; for example, potyvirus CP is required not only for encapsidation of the genomic RNA, and for aphid transmission (by interaction with HC-Pro), but also has functions in both cell-to-cell and long-distance movement of the virus (Dolja *et al.,* 1994b, 1995). Disruption of any one of these functions might occur by expression at inappropriate times or amounts. Differences in the functions of the CP of different virus groups other than RNA encapsidation may help explain differences in the effectiveness of CP-mediated resistance, despite the remarkable similarity of effects of cross-protection across different virus groups.

Inhibition of disassembly may not be an important part of potyvirus CP-mediated resistance. Hammond and Dienelt (1997) noted a lack of correlation between the ability of various CP transgene constructs to confer resistance and the extent to which the transgene CP was able to incorporate into virions of the challenge virus. Further evidence comes from the fact that modified PPV CP constructs, which are unable to assemble into virions, were still able to confer high-level resistance to PPV (Jacquet *et al.,* 1998a,b).

Tospovirus resistance conferred by N-gene expression may be due to interference with replication, as the amount of free N protein is thought to determine whether the polymerase acts to transcribe mRNA from the (−) sense genomic RNAs or to replicate the genomic RNAs; transcription is favored at low concentration of the N protein and replication at high N protein levels. Transgene-expressed N protein could thus prevent transcription and interfere with the viral life cycle (Goldbach and de Haan, 1993). Free protein levels may also act as a feedback mechanism, controlling the balance between translation and replication in viruses with (+) sense genomes.

c. Resistance versus Complementation. It is also interesting that CP-mediated resistance is situation dependent. It has been demonstrated with TMV that plants that did not support replication of wild-type virus isolates were able to fully complement CP-defective isolates (Osbourn *et al.,* 1990) and that TEV CP plants partially complemented an isolate of TEV unable to spread on nontransgenic plants (Dolja *et al.,* 1994b). Spillane *et al.* (1997) have shown that PVX-resistant CP-transgenic plants can complement PVX with defects in the CP gene, suggesting that both resistance and complementation result from the roles of the CP in the normal PVX infection cycle. This may occur through a single interaction between the CP and the viral origin of assembly (Spillane *et al.,* 1997), apparently distinct from prevention of disassembly.

3. Dominant Negative Mutations

A nonfunctional CP (or other viral gene) might also function in a way quite distinct from cross-protection. It has been suggested that defective proteins (including MP and replicase) might have the properties of a dominant negative mutation (Herskovitz, 1987; Lomonossoff, 1995; Palukaitis and Zaitlin, 1997).

There are thus many ways in which expression of viral CP genes could interfere either with establishment of viral infection, with cell-to-cell or long-distance movement, or directly with viral replication. It is also likely that any given CP gene construct will induce multilayered effects on infection, and that different mechanisms induced by the same gene may predominate in any particular plant as a result of chromosomal position effects and differences between hosts in the particular virus–host interactions.

C. Recombination between Viral cDNA (Transgene Transcripts) and the Genome of an Invading Virus

There are multiple possible interactions between a transgenic plant expressing a viral sequence and a plant virus introduced to that plant. The intended consequence is, in most cases, resistance to a specific virus or group of related viruses. The actual end result will depend on the viral sequence expressed by the plant, on the degree of relatedness of the incoming virus to the expressed sequence, and on the type of mechanism by which resistance is conferred. Other possible outcomes include (a) complementation of virus species or isolates poorly infectious or noninfectious to nontransgenic plants of the same species; (b) synergism (increased replication of a virus infectious to nontransgenic plants)

as a consequence of expression of the transgene; (c) heterologous encapsidation affecting vectored transmission (see Section IV,E,1,a); and (d) recombination between the virus and either the transgene DNA (for DNA viruses) or its transcript, leading to production of a hybrid virus.

Hull and Davies (1992) suggested that levels of viral protection conferred by transgenes should be standardized for easy comparison of results from different laboratories and, perhaps more importantly in the future, so that farmers can make rational choices between different genotypes. The proposed levels of protection are as follows (Hull and Davies, 1992):

1. Full immunity to a range of viruses.
2. Full immunity to a range of strains of a virus (e.g., TEV untranslatable RNA; Lindbo and Dougherty, 1992b).
3. Full immunity to a few closely related strains of a virus (e.g., TMV 54K protein; Golemboski *et al.,* 1990; PVY replicase; Audy *et al.,* 1994).
4. Subliminal infection, with inability of the virus to spread from initially infected cells.
5. Delay in systemic infection with a description of duration (e.g., Powell-Abel *et al.,* 1986).
6. Reduction in severity of systemic symptoms associated with a reduction of virus titer (e.g., Hammond and Kamo, 1995a,b).
7. Reduction of systemic symptoms with no reduction of virus titer.

These proposed levels have some utility, but they must be modified to take into account some other protection phenotypes that have subsequently been observed or may be anticipated. Locally limited symptomatic infections, inducing a novel hypersensitive response that would prevent systemic movement, would fall between levels 4 and 5 of the scale proposed by Hull and Davies (1992). Initial infection followed by recovery (production of leaves with progressively lower titer and then virus-free tissue if recovery is complete; e.g., Lindbo *et al.,* 1993; Hammond and Kamo, 1995a,b) would fall between levels 4 and 5 or between levels 5 and 6, depending on the degree of recovery observed. Limited resistance to a range of viruses, such as that conferred by a defective movement protein (Cooper *et al.,* 1995), would fall somewhere between levels 1 and 4. Lack of resistance or lack of other interactions will result in infection typical of the virus in nontransgenic plants. In most cases, a nontransformed plant and a virus-resistant transgenic plant of the same species would be equally susceptible to a second, unrelated virus. In other cases, there may be synergy between an invading virus and a gene expressed in a transgenic plant, increasing disease severity.

Although synergy has been reported in studies that were not designed to examine resistance (see below), this is a possibility that should be assessed in the evaluation of transgenic plants intended to be virus-resistant. It is unlikely that synergy would be observed in transgenic plants except with virus combinations where synergy is observed in mixed infections (see Section II,C).

1. Complementation in Transgenic Plants

Complementation has been used to assay gene function and to dissect the interdependence between different genes or gene products of a virus. Mutant virus isolates, debilitated in a particular gene function such as systemic movement, are inoculated into a transgenic plant expressing a functional copy of the gene in question. For example, Cronin *et al.* (1995) created a series of amino acid substitution mutants in the HC-Pro gene of TEV, each of which was still able to replicate in protoplasts; however, one mutant was confined to the inoculated leaves of whole plants. Systemic infection was observed when this mutant was inoculated into transgenic plants expressing functional HC-Pro, indicating that the protein supplied *in trans* by the transgenic plant complemented the debilitated mutant. This provided the first evidence of a long-distance potyvirus movement function of HC-Pro, in addition to its activities as an aphid transmission HC and as a proteinase required for self-processing (Cronin *et al.,* 1995). Complementation of CP-defective strains of TMV, but no recombination to yield wild-type TMV, has been observed in TMV CP-transgenic tobacco (Osbourn *et al.,* 1990; Holt and Beachy, 1991); similar complementation of MP-defective TMV by MP-transgenic plants has also been reported (Holt and Beachy, 1991). Defective genomes have also been complemented in plants expressing functional replicase proteins (Taschner *et al.,* 1991; Mori *et al.,* 1992; Li and Carrington, 1995). Complementation has also been observed within the caulimoviruses (Schoelz *et al.,* 1991), where the gene VI transgene from an isolate of CaMV able to infect solanaceous hosts systemically was shown to complement systemic infection of a CaMV isolate unable to establish an infection in nontransgenic *Nicotiana* species.

2. Synergism in Transgenic Plants

The synergism between PVX and potyviruses has been studied with the use of transgenic plants, resulting in the demonstration that the increased level of replication of PVX is dependent on the HC-Pro and P1 proteins of TEV (Vance *et al.,* 1995; Pruss *et al.,* 1997). Expression of HC-Pro alone was sufficient to increase PVX symptom severity (Pruss

et al., 1997). Shi *et al.* (1997) have reported that mutations in the central domain of the HC-Pro transgene, but not within the P1 coding region, eliminated the synergism. No significant virus resistance has been reported from potyvirus HC-Pro expression, and it is unlikely that such plants would be deployed in the field. The potential for a general enhancement of severity of other viral diseases (Pruss *et al.,* 1997) would render any such crops more susceptible; it is extremely unlikely that any plant breeder or seed company would release such a line. However, resistance to one component of a synergistic disease complex may have highly beneficial effects. For example, Murry *et al.* (1993) reported that CP-mediated resistance to maize dwarf mosaic virus (MDMV) was highly effective in inhibiting replication of MCMV, the nonpotyvirus component of the synergistic mixed infection causing corn lethal necrosis. A mutant HC-Pro might be a candidate for resistance, provided that both HC function and synergism were disabled.

3. Recombination of RNA Viruses in Transgenic Plants

Recombination between a viral transgene and a virus able to replicate in transgenic plants has been demonstrated in a limited number of cases, mainly under high selective pressure for the presence of recombination. As noted above, there are differences between viral groups with respect to frequency of intraviral and interviral recombination, and this is expected to hold true for recombination between virus genomes and transgenes as well. Thus, even when recombination would yield a readily observable wild-type TMV in complementation experiments, none was observed (Osbourn *et al.,* 1990). Lommel and Xiong (1991) provided the first report of recombination between a plant virus and a related transgene; in this case, a deletion mutant of red clover necrotic mosaic virus (RCNMV) was restored by recombination with an RCNMV transgene. Selection pressure (detection of replicating, systemically invasive virus) was high in this example, as the deletion mutant was unable to spread in nontransgenic plants. A similar observation was made by Greene and Allison (1994), who created a partial CP deletion mutant from an infectious clone of CCMV and inoculated transcripts into transgenic plants expressing a wild-type CP gene with the full viral 3′ noncoding region. Viable recombinants were identified from 4 of 125 transgenic plants inoculated, and recombinants were further identified as such by the presence of nucleotide substitutions unique to the transgene transcript; each recombinant was distinct from the parental sequence (Greene and Allison, 1994). Despite design of the experiment to favor homologous recombination (and despite documentation of homologous recombination in bromoviruses; Rao and Hall,

1993), each recombinant resulted from distinct aberrant recombination events (Greene and Allison, 1994). In further experiments, additional transgenic lines were prepared in which portions of the transgene's viral 3′ NCR were deleted; no recombination was detected in any of these lines (Greene and Allison, 1996) even though the selection pressure was as high as in the initial experiments. It is postulated that the 3′ noncoding region may recruit host factors necessary for replication, which may in turn target the transcript to the replication complex. Therefore, omission or mutation of the 3′ noncoding region may significantly reduce the potential for formation of viable recombinants, requiring a double crossover to generate a viable recombinant (Greene and Allison, 1996).

Borja *et al.* (1999) have reported restoration of a wild-type tomato bushy stunt virus (TBSV) following challenge of TBSV CP transgenic plants with transcripts of TBSV or chimeric tombusvirus constructs with a defective TBSV CP gene. As in the experiments reported by others, the transgenic plants conferred no resistance to wild-type virus. In each case, a double recombination event was necessary to regenerate a wild-type virus, which occurred in up to 20% of transgenic plants inoculated with defective transcripts. The CP mutants were chosen because they can establish systemic infections, albeit with mild symptoms, whereas wild-type virus induces a severe infection resulting in plant death; this therefore provided a highly selective bioassay for recombination (Borja *et al.,* 1999). These results also showed that the recombinant virus, with the more aggressive phenotype, readily predominated in infected plants. However, the level of recombination was significantly reduced in experiments with infectious transcripts carrying a partially deleted CP gene fused to a reporter gene; this shows that reducing the extent of shared sequence between the challenge virus and the transgene can reduce recombination (Borja *et al.,* 1999). The authors note several factors that may have influenced the high level of recombination observed. These include the general lack of fidelity in tombusvirus transcription, which leads to frequent generation of DI RNAs, and the ability of different tombusviruses to recombine in protoplasts; these may result from the lack of a helicase domain in the tombusvirus replicase (Borja *et al.,* 1999). Another potential contributing factor is the ability of the challenge defective viruses to spread systemically in the absence of recombination, thus increasing the number of infected cells in which recombination could potentially occur. As was demonstrated by experiments with a construct with a marker gene fused to a partially deleted CP gene, the extent of homology between

the transgene and the challenge virus also had a profound effect on the rate of recombination (Borja *et al.,* 1999).

4. Recombination in Viruses with DNA Genomes

Recombination has also been reported in caulimoviruses and geminiviruses in transgenic plants. Gal *et al.* (1992) tested CaMV in transgenic plants because recombination had already been observed when two mutant CaMV isolates were coinoculated into nontransgenic plants (Howell *et al.,* 1981). In this instance, one CaMV gene was integrated in transgenic plants, and a second construct containing the remainder of the circular genome, plus overlaps at both termini, was supplied by agroinfection; alternatively, both constructs were introduced by agroinfection (Gal *et al.,* 1992). Thus two partial, defective genomes were supplied, neither of which contains sufficient information for replication. In addition, agroinfection provided an ongoing source of the defective construct. Both RNA and DNA recombination events were possible because of the replication of CaMV through an RNA intermediate (see Gal *et al.,* 1992). Indeed, the recombinants recovered included one type most easily explained by RNA-only mechanisms and a second type more likely to have been derived from DNA–DNA homologous recombination. Both types were recovered from a single plant (Gal *et al.,* 1992).

Schoelz and Wintermantel (1993) set out to use complementation in transgenic plants to determine which CaMV genes were host-range determinants for solanaceous plants. They observed complementation with some isolates, but other isolates gained infectivity by recombining with the transgene rather than through complementation. Nucleotide sequence analysis indicated that the transgene sequence became incorporated into the genome during reverse transcription by a template switch (Schoelz and Wintermantel, 1993). The recombinant isolates caused milder symptoms than the parental isolate in turnip, a host for all CaMV isolates, while having a broader host range (Schoelz and Wintermantel, 1993). It was observed that no recombinants were recovered from another CaMV isolate tested; this isolate was able to infect both the transgenic plants and their nontransgenic counterparts. Although recombinants might be expected to form from this isolate at the same rate as with the other, putative recombinants may be unable to compete with the host-competent parental isolate inoculated. In the absence of selection pressure, a recombinant virus may not become established, as one CaMV tends to predominate to the exclusion of other variants (Schoelz and Wintermantel, 1993; Zhang and Melcher, 1989).

In further experiments, Wintermantel and Schoelz (1996) inoculated the transgenic plants with different isolates of CaMV that were able

to infect nontransgenic *N. bigelovii* plants; there was therefore moderate or weak selection pressure for recombination in the transgenic plants. A recombinant virus was recovered from 3 of 23 transgenic plants inoculated with one isolate and from 3 of 32 plants inoculated with a second isolate (Wintermantel and Schoelz, 1996). In contrast, under strong selective pressure inoculation with an isolate unable to infect nontransgenic *N. bigelovii,* recombinants were recovered from all of 24 systemically infected plants (Wintermantel and Schoelz, 1996).

Wintermantel and Schoelz (1996) also evaluated the relative competitiveness of the recombinant and parental isolates, and determined that the recombinant was able to induce systemic symptoms in *N. bigelovii* faster than the parental type. However, symptom development was similar to that observed with the isolate from which the transgenes was derived, and no selective advantage of the recombinant was observed in turnip plants (Wintermantel and Schoelz, 1996).

Frischmuth and Stanley (1998) reported recombination between an ACMV CP transgene and an agroinoculated ACMV genomic DNA with a deletion in the CP gene. In this case several transgene constructs were examined, and recombination was observed only when geminiviral noncoding sequence was present at both sides of the CP transgene. No recombination was observed when there were homologous sequences only at the 3′ side of the genomic deletion (Frischmuth and Stanley, 1998). The ACMV deletion mutant examined was previously reported to gain sequences by recombination in systemic infections of nontransgenic plants, so that the deleted genome was restored almost to the wild-type size (Etessami *et al.,* 1989).

5. *Effect of Resistance Status and Selection Pressure on Recombination*

It should perhaps be pointed out that in none of the experiments discussed above (with RCNMV, CCMV, TBSV, CaMV, or ACMV) was there any resistance to the wild-type virus. Complementation of CP-defective TMV isolates (but no recombination) has been observed in TMV CP-transgenic plants that have partial resistance to wild-type TMV isolates (Osbourn *et al.,* 1990). The occurrence of CaMV and ACMV recombination in nontransgenic plants has been reported (e.g., Howell *et al.,* 1981; Etessami *et al.,* 1989). There are multiple reports of RNA virus recombination from both laboratory experiments and naturally occurring field isolates (see Section II,F). The significance of the transgene recombinants discussed above must therefore be considered in this broader context and any potential or perceived risks evaluated accordingly. It must also be remembered that most transgenic

plants engineered to express viral genes and intended for field release are expected to be viral resistant (unlike the examples presented above). They will also typically express transgene transcripts at levels orders of magnitude lower than would be expected in a wild-type viral infection.

The selection pressure utilized in all of the experiments described above must be compared to the selection pressures found in natural infections. It was necessary to apply high selection pressures in these experimental situations in order to be able to detect the occurrence of recombination within a manageable number of plants. In most of the studies described, no viral replication, and hence no symptom expression, would have occurred in the absence of recombination. This is obviously not the case in the typical natural situation, in which a wild-type virus isolate would be able to replicate in a transgenic plant with incomplete resistance or resistance to an unrelated virus. For a recombinant to survive and be transmitted to other plants, it must not only be viable, but also have some selective advantage in at least one of the hosts to which it is transmitted.

It is most likely that viable recombination will occur between a virus isolate and a transgene with a closely related sequence, so we will address the factors potentially affecting this situation. It is generally accepted that plant viruses exist as quasispecies, with mixtures of sequences, of which one type predominates in a specific host (see Section II,G). In some situations a different sequence variant may have a selective advantage—in a particular host, or by having a better affinity for transmission by a certain vector species or biotype. Among plant viruses with RNA genomes, an established infection of a host with one isolate typically protects against superinfection with a related strain; this is the basis of cross-protection (see Section II,B). If a recombinant is generated in an infected plant, the recombinant is likely to be similarly repressed and unlikely to become a major component of the sequence mixture unless it has a selective advantage in the transgenic plant.

Indeed, a recombinant arising in a transgenic plant with even partial resistance will probably be inhibited, or even eliminated, by the resistance mechanisms expressed in the transgenic plant. This is especially likely in the case of resistance conferred by posttranscriptional gene silencing, as activation of host surveillance mechanisms degrading transgene-related sequences should select against novel recombinant viruses (Rubio *et al.,* 1999a). It is unlikely that a recombinant would become phenotypically apparent (expressing distinctive symptoms) or dominate the infection unless it is transferred to a host of a genotype in which it has a selective advantage. The most likely scenario for this event is transmission by an arthropod vector. Transmission via insect

vectors may result in the establishment of a new infection from relatively few virus particles (e.g., Pirone and Blanc, 1996), which could lead to the emergence of the recombinant sequence as the dominant one of the mixture. In this case, the next question that must be addressed is whether the recombinant will spread further. Insect transmission can occur over long distances, but unless there are environmental cues to stimulate long-range dispersal of the insects, most transmissions will be to nearby plants; transgenic plants grown in a field would be surrounded by other plants of the same genotype. As resistance is typically greatest against isolates that are most closely related to the transgene, there is no *a priori* reason to expect that a recombinant would replicate to a higher level than the parental isolate, even when introduced to other transgenic plants as a major component of the sequence mix. Indeed, there might be greater protection against the recombinant than against the parental isolate.

In the case of introduction to a nontransgenic plant, in which the recombinant is able to replicate freely, are there any indications that the recombinant may be more severe than the parental isolate? This would depend in part on the viral gene present in the transgenic plant and the characteristics of the isolate from which the transgene was derived. If the transgene was derived from a severe isolate, or one able to replicate with greater efficiency, or with distinctive vector relationships, then it is possible that these characteristics would be transferred to the recombinant. However, if the transgene was derived from a typical endemic isolate (as recommended in Section IV,C), there is little reason to suggest that the recombinant would be of any more consequence than any other isolate of the virus in question. Indeed, detailed molecular characterization would probably be required even to identify the new isolate as a recombinant.

Is there any evidence to suggest that recombination is more likely to occur between a transgene (or transgene transcript) and an invading virus than between two viruses in a mixed infection? As noted above (Section II,F,2), different virus groups typically replicate in different subcellular locations, and in many instances replication is thought to be compartmentalized. Hull and Covey (1996) have discussed replicative compartmentalization as an adaptive feature of different types of retroelements, including retroviruses and the plant pararetroviruses, allowing separation of their replication from the restraints of cellular nucleic acid synthesis, yet maintaining the necessary integrity of the host cell. To some extent this restricts "promiscuous" transcription of nonviral nucleic acids; additional controls may be present in plant viruses with RNA genomes. Although not eliminating template switch-

ing, replication compartmentalization would minimize initiation of transcription on cellular RNAs, including related transgene transcripts. The identification of cellular RNA sequences in isolates of PLRV (Mayo and Jolly, 1991) and TuMV (Sano *et al.,* 1992) indicates that compartmentalization of viral replication is not complete, but the rarity of such reports is notable.

There is presumably less chance of recombination between genomic RNAs of viruses with different subcellular localization of replication than between two viruses utilizing the same site within the cell, and therefore viruses in different taxonomic groupings are less likely to recombine than related viruses. However, two viruses replicating in the same subcellular location will have high levels of both genomic RNAs present and presumably available for interaction. A transgene transcript driven by a constitutive promoter would be present in every cell of the transgenic plant (Allison *et al.,* 1996), and presumably at most stages of plant development, and therefore for longer periods than a virus might be actively replicating. A transgene transcript would typically be present at much lower levels in the cell than would the replicating viral genomic RNA, and would also likely be excluded from the replication complex. Thus there is more likely to be recombination between viruses in a mixed infection than between a viral genomic RNA and a transgene transcript. Experiments described by Allison *et al.* (1996) bear this out; a greater percentage of viable recombinant viruses were recovered when mixtures of two types of CP-defective viral transcripts were directly inoculated into nontransgenic cowpea plants than when a single type of defective transcript was inoculated into transgenic plants expressing a wild-type CP transcript. The increased quantity of transcript available to the replication complex in plants inoculated with mixtures of defective transcripts, and the increased length and possibly increased stability of the longer transcripts inoculated into nontransgenic plants, may explain this difference (Allison *et al.,* 1996).

There is one report of a large-scale effort to detect possible effects of recombination or adverse effects on vector specificity in field trials of transgenic plants expressing either the CP or replicase gene of potato leafroll luteovirus. Over a 6-year period, Thomas *et al.* (1998) examined over 25,000 plants from 442 lines expressing various PLRV CP constructs and nearly 40,000 plants representing 512 lines expressing one of seven PLRV replicase constructs; viruses other than PLRV were identified and examined for alterations in transmission characteristics, serological affinity (to determine potential incorporation of PLRV CP), host range, and symptoms. Viruses that were propagated in selected

transgenic lines in a greenhouse setting were also examined (Thomas *et al.,* 1998). No evidence was found for any alteration in transmission characteristics or for heterologous encapsidation; nor was there any synergism or infection of any of the transgenic lines by any virus not normally infecting potato. Specific attempts were also made to infect transgenic potatoes with the heterologous luteoviruses BWYV and pea leafroll virus (PeLRV) with the use of appropriate virus-specific aphid vectors; no infection was detected, and thus it appears that expression of PLRV transgenes was not able to complement infection of viruses that do not normally infect potato (Thomas *et al.,* 1998). No evidence was obtained of any recombination, synergism, or altered transmission, or of any infection by any virus unable to infect nontransgenic potato (Thomas *et al.,* 1998). Thus, in the largest survey carried out to date, there is no evidence of any adverse effects or "new" viruses resulting from large-scale deployment of transgenic plants.

There is, of course, evidence that severe isolates of various viruses arose in the past—before the advent of transgenic plants. Whether such isolates arose from recombination between isolates or distinct viruses, or from sequence selection and adaptation to one host that coincidentally increased replication or symptom severity in a second host, cannot be determined in most instances. There is, however, good evidence that a severe Ugandan isolate of cassava mosaic virus is a recent recombinant between ACMV and EACMV from a natural mixed infection (Zhou *et al.,* 1997; Harrison *et al.,* 1997). This certainly does not free us from the obligation to try to understand the mechanisms of recombination and to design transgene constructs that should minimize the possibility of recombination. However, knowledge and study of natural recombination will aid analysis of isolates identified in the future. The derivation of most transgenes from well-characterized virus isolates of known sequence will certainly be useful in cases where recombination between a virus isolate and a transgene is suspected as being the origin of "new" viruses identified in the future.

D. Nonstructural Proteins That May Affect Systemic Infection in Mixed Infections or in Transgenic Plants

1. Potential Risks Deriving from Heterologous Use of Movement Proteins

In order to achieve systemic spread in hosts, plant viruses encode for proteins that facilitate movement. These proteins are termed "movement proteins" (MP) and are found in many groups of plant viruses.

It is possible that a host infected with one virus may assist the systemic spread of another virus coinfecting the same host. Plant tissue restriction is a phenomenon where by a certain virus will not invade a particular tissue in the host. This type of restriction can sometimes be overcome by mixed infection with heterologous viruses. Thus, the restriction on movement of the luteovirus PLRV into mesophyll cells from phloem cells is overcome by coinfection with potyvirus PVY (Barker, 1987). The ability of viruses from different groups to exchange and complement the movement functions of a distinct unrelated virus group suggests that different viruses can be transported by common intercellular movement routes. In some cases, structural proteins (such as the capsid protein) or genome replication proteins may also function in cell-to-cell or long-distance transport (e.g., Dolja *et al.,* 1994b, 1995; Carrington *et al.,* 1996).

A transgenically expressed MP might therefore facilitate systemic spread of an incoming virus in the transgenic plant, although the virus would not spread systemically in a nontransgenic plant. A number of reports indicate that MPs encoded by one virus group may assist the movement of certain other viruses (e.g., De Jong and Ahlquist, 1992; Giesman-Cookmeyer *et al.,* 1995). Additionally, movement defects of one virus can often be complemented by coinfection with an unrelated virus that is movement competent (Atabekov and Taliansky, 1990). For example, the bromovirus BMV gains the ability to move through tomato if it is coinoculated with TMV (Taliansky *et al.,* 1982).

Complementation of movement-defective tobamoviruses in TMV MP-transgenic plants was reported by Holt and Beachy (1991), as was cell-to-cell but not systemic spread of an MP-defective CMV (Cooper *et al.,* 1996). In contrast, CMV-MP-transgenic plants supported only subliminal infection of a movement-defective TMV isolate (Cooper *et al.,* 1996). Deom *et al.* (1994) showed that SHMV MP-transgenic plants supported efficient long-distance spread of an MP-defective TMV isolate, but a recombinant TMV expressing SHMV MP rather than the homologous MP spread only locally and not systemically in nontransgenic plants. Thus, the effects on heterologous viruses seem limited, even within a single virus group, and the results of Deom *et al.* (1994) suggest that an efficient systemic infection requires interaction between multiple viral gene products. Experiments by Lauber *et al.* (1998) substantiate this observation: substitution of single components of the BNYVV triple gene block with the equivalent genes from the distantly related peanut clump virus (PCV) did not result in complementation, but substitution of the whole PCV triple gene block did. Lauber *et al.* (1998) propose that highly specific interactions among

the cognate triple gene block proteins are important for function and/or stability.

The level of complementation observed in other instances of experimental substitution of MP genes is variable. The RNA 3 (encoding MP and CP) of prunus necrotic ringspot virus (PNRSV) was able to complement encapsidation of AMV RNA 1 and 2 but supported only limited cell-to-cell movement (Sanchez-Navarro *et al.,* 1997). Osman *et al.* (1997) showed that MPs of either BMV or CCMV were unable to complement systemic spread of the heterologous RNA 1 and 2, whereas substitution of CP genes alone permitted essentially wild-type infections. Both TMV and RCNMV can act as helper viruses or their transgenically expressed MPs can complement the heterologous movement-defective virus; this shows that their MPs, with similar secondary structures but minimal sequence homology, are functionally equivalent for cell-to-cell movement (Giesman-Cookmeyer *et al.,* 1995). Indeed, a chimeric TMV expressing RCNMV MP in place of the TMV MP was functional despite the differences in virion and genome structure between TMV and RCNMV (Giesman-Cookmeyer *et al.,* 1995). As noted above, TMV MP is also able to complement cell-to-cell movement of CMV but not vice versa (Cooper *et al.,* 1996). Thus, although it is possible that a functional transgenically expressed MP of one virus might somewhat enhance the ability of a tissue-restricted virus to spread outside that tissue, or enhance the ability of a movement-defective isolate to spread slowly throughout a transgenic plant, radical changes in invasiveness are unlikely to occur. Virus movement appears to require highly specific interactions between multiple gene products, and complementation seems unlikely to drastically affect the ability of a virus to infect a transgenic host. Even a recombination event adding a complete heterologous MP gene to a wild-type viral genome would presumably lack the specific interactions with other viral gene products necessary for effective systemic movement that might allow alteration of the viral host range.

We should point out at this juncture that although functional complementation does occur in nature (e.g., transmissibility of aphid-nontransmissible potyviruses from mixed infections; see Section II,E,1), this occurrence is quite rare. That functional complementation of movement functions is very rare can be illustrated by the common practice of separating the different components of a mixed viral infection by inoculating differential hosts. If functional complementation were common, such host-based separation would be impossible.

2. Potential Risks Deriving from Other Nonstructural Gene Products

The use of any gene products potentially involved in synergism or increased pathogenicity has been discussed in Section II,C. These gene

products include the potyviral P1 and HC-Pro proteins, which together are responsible for the synergy observed in multiple infections involving a potyvirus and members of various other groups (e.g., Vance *et al.,* 1995; Pruss *et al.,* 1997). The potyviral HC-Pro is sufficient to cause increased pathogenicity of a PVX infection (Pruss *et al.,* 1997), and the use of functional copies should be avoided for this reason, as well as because of its ability to assist aphid transmission of some other isolates or viruses that are not normally transmitted by aphids. P1 protein alone can confer resistance (Pehu *et al.,* 1995; Moreno *et al.,* 1998), but potential synergism must be evaluated in such plants. Other sequences identified as pathogenicity factors or enhancers, such as necrogenic or severity-increasing satellite RNAs (Collmer and Howell, 1992), are also best avoided. However, symptom-ameliorating satellite or DI RNAs have been demonstrated to be of significant value in reducing losses to virus infection in both cross-protection and transgenic applications (see Sections II,B and IV,B,6), and the potential advantages and disadvantages for each new situation must be carefully weighed in each case. For example, Shi *et al.* (1997) reported that mutations in HC-Pro eliminated the synergism with PVX, which could potentially allow use of HC-Pro if the mutated gene confers any resistance. Similarly, mutations could be applied in the two conserved domains of the HC-Pro involved in aphid transmission (Huet *et al.,* 1994) in order to abolish the capacity of the HC-Pro to mediate aphid transmission.

The expression of gene products directly associated with replication has so far shown little likelihood of increasing replication of any replication-competent viruses. Indeed, replicase-mediated resistance has proven highly successful, and no synergy has been reported in plants expressing any replicase protein (Palukaitis and Zaitlin, 1997). Transgenic plants expressing the replicase proteins of multipartite viruses such as AMV and BMV are able to complement the remaining genomic RNAs of these viruses (Taschner *et al.,* 1991; Mori *et al.,* 1992), and the transgenically expressed polymerase of TEV (Li and Carrington, 1995) is able to complement defective transcripts of the homologous virus. Plants expressing the polymerase alone are generally resistant to infection with wild-type virus (Palukaitis and Zaitlin, 1997).

E. Vector Specificity and Potential Epidemiological Consequences of Virus-Infected Transgenic Plants

1. Epidemiological Consequences

The discovery of phenotypic mixing led Rochow (1977) to discuss the epidemiological consequences of the phenomenon, suggesting that

heterologously encapsidated virions could cause a "new" disease. Thus, the nucleic acid of virus A (Rochow, 1977) packaged in capsomers of virus B could now be transmitted by an aphid species other than the usual vector. If this different aphid species has a plant host range distinct from that of the usual vector, the nucleic acid of virus A could thus be introduced to a "new" host. Although the virus produced in the new host will be only A, as a consequence of being on a new host it may now be transmitted by aphid species that favor the new host. Rochow (1977) concludes, "Because of the change of both plant host and aphid vector, the identity of virus A before and after these events might not be easily recognized."

Falk *et al.* (1995) discussed two potential effects of interactions in transgenic plants expressing viral capsid proteins similar to those detailed by Rochow (1977) in mixed infections; these interactions can be taken to include CP RTD proteins

a. Heterologous Encapsidation in Transgenic Plants Could Alter or Facilitate Vector Transmissibility of the Progeny Virions. As discussed in Section II,D, in most cases only one type of vector (e.g., aphids but not nematodes or whiteflies) will transmit a particular virus, indicating coevolution of the virus and vector involving development of specific interactions that control the ability of the vector to transmit the virus (Hull, 1994a). This specificity between virus and vector is not absolute. For example, melon necrotic spot virus (MNSV) has been reported to be transmitted by both a chytrid fungus (Hibi and Furuki, 1985) and a chrysomelid beetle (Coudriet *et al.,* 1979). On the other hand, specific viruses may be transmitted by multiple species of aphids, and a particular aphid species may transmit multiple viruses (Kennedy *et al.,* 1962), but different biotypes of aphids (or other vector species) may differ in their ability to transmit the same virus isolate (Hull, 1994a). Similarly, the ability of a specific vector to transmit virus A and virus B often does not correlate with the ability of a second species, which may transmit A but not B or B but not A.

Heterologous encapsidation in transgenic plants has been reported in a number of cases, but to date, these all deal with experimental situations (see below). No potential problems of the kind envisioned by de Zoeten (1991) have been observed. Indeed, the only large-scale study of field-grown plants examining the possibility revealed no evidence of any interactions other than resistance between transgenes and viruses able to infect the transgenic plants (Thomas *et al.,* 1998). On the contrary, Thomas *et al.* (1997) observed significantly decreased transmission of PLRV in fields of transgenic plants, presumably due to the reduced replication in resistant transgenic plants.

Osbourn *et al.* (1990) and Holt and Beachy (1991) reported that TMV mutants with defective CP genes were complemented by infection of transgenic plants expressing wild-type TMV CP and that wild-type virions were formed. As TMV has no specific vector, and as CP-defective strains are not prevalent in nature, this is of no apparent ecological consequence. Farinelli *et al.* (1992) demonstrated partial heterologous encapsidation between transgenic PVY^N CP and PVY^O infecting the transgenic plants. In uninfected transgenic plants the PVY^N CP was not detectable, and it was suggested that incorporation into PVY^O particles stabilized the transgene CP. Farinelli and Malnoë (1993) further showed that transgene PVY^N CP was also incorporated into particles of potato virus A (PVA) and potato virus V (PVV) potyviruses. These viruses are commonly found in mixed infections with PVY, are transmitted by some of the same aphid species, and have a more limited host range than PVY, so there is not likely to be any detectable adverse consequence of field-scale plantings in this case either.

A further, more detailed laboratory study was carried out by Lecoq *et al.* (1993). They demonstrated that an aphid-nontransmissible isolate of ZYMV could not only be partially encapsidated in PPV CP from transgenic plants, but also that the phenotypically mixed particles were aphid transmissible as a result. ZYMV-NAT transmission from the PPV CP transgenic plants was considerably less efficient than for an aphid-transmissible isolate from nontransgenic control plants, but these experiments demonstrate that vectored transmission can occur as a result of heterologous encapsidation in transgenic plants. In a further examination of this system using mutated or deleted PPV CP transgenes, Jacquet *et al.* (1998a) demonstrated that an N-terminally deleted PPV CP was still competent to heteroencapsidate ZYMV-NAT but not to effect aphid transmission. They also showed that mutating PPV transgene CP residues involved in RNA binding eliminated detectable heterologous encapsidation and therefore aphid transmissibility of ZYMV-NAT from the transgenic plants. As the mutated and deleted PPV CP constructs still conferred high levels of resistance to PPV (Jacquet *et al.,* 1998b), these types of constructs represent a proactive means of risk reduction in a situation in which little risk was present in the first place. The combination examined (ZYMV and PPV) is one that is extremely unlikely to occur in an agricultural situation, as there are no crops that are host to both viruses. Aphid-nontransmissible isolates of potyviruses (and of some other normally aphid-transmitted viruses) do exist naturally, and are presumably maintained by transmission from mixed infections as a consequence of complementation with effective HC (e.g., Kassanis, 1961) or heterologous encapsidation

(Bourdin and Lecoq, 1991). The only crops in which PPV is found naturally are various *Prunus* Spp. (e.g., Kegler and Hartmann, 1998), in which no other potyviruses are found, and thus possible transgene–virus interactions would be limited to different isolates of PPV. As individual trees appear to be commonly infected with multiple distinct PPV sequences (Hammond *et al.,* 1998), there are many opportunities for different isolates to interact naturally, and no novel interactions are likely from infection of transgenic plants.

Hammond and Dienelt (1997) used a monoclonal antibody specific for an epitope unique to the transgene CPs to show that wild-type and chimeric potyvirus transgene CPs were incorporated into particles of several different potyviruses. There was no apparent correlation between the extent of heterologous encapsidation and the degree of resistance to infection in the different transgenic lines. Chimeric CPs were incorporated into particles but adversely affected the stability of the phenotypically mixed virions, presumably as a consequence of reduced compatibility with the CP of the infecting virus (Hammond and Dienelt, 1997). Reduced stability of heterologously encapsidated virions might be expected to lessen vector transmissibility. As predicted, inoculation of heterologously encapsidated virus into nontransgenic hosts restored the wild-type serological properties of virions; no reaction with the transgene CP-specific monoclonal antibody was detected from such plants (Hammond and Dienelt, 1997).

Candelier-Harvey and Hull (1993) showed that AMV CP from transgenic plants was able to encapsidate CMV RNA, by trapping virions from extracts of CMV-inoculated plants with AMV-specific antiserum prior to CMV-specific RT-PCR, and thus demonstrated that heterologous encapsidation could occur in transgenic plants between different viral groups; in further work, CMV RNA was detected in up to 92% of the plants analyzed (R. Hull, personal communication). In related experiments with AMV CP transgenic plants, no heterologous encapsidation of either TMV or TRV was detected; nor was any heterologous encapsidation detected in plants expressing either TMV or grapevine chrome mosaic nepovirus (GCMV) CPs that were challenged with CMV or TRV (R. Hull, personal communication). It is therefore apparent that some relationship between the CP donor and the superinfecting virus was necessary for heteroencapsidation to take place. No relationship was observed between virus concentration and heteroencapsidation levels in different leaves. These results suggest that heterologous encapsidation across virus groups is likely to be limited to related viruses with virion types stabilized primarily by protein–RNA interac-

tions, not protein–protein interactions (R. Hull, personal communication).

Maiss *et al.* (1994) challenged plants expressing PPV CP with various potyviruses and also with TMV, PVX, and BNYVV. PPV CP was readily detectable in newly formed potyvirus particles but not in any virions of the nonpotyviruses. They also confirmed that heterologous encapsidation occurs in mixed potyvirus infections. Maiss *et al.* (1994) also challenged BNYVV-CP transgenic sugarbeet with beet yellows closterovirus (BYV), beet mosaic potyvirus (BtMV), beet mild yellowing luteovirus (BMYV), TNV, and TMV; no heterologous encapsidation was detected in any of these combinations. In field release experiments, some BNYVV-CP transgenic beet became naturally infected with beet soil-borne furovirus (BSBV) and BtMV, but, again, no heterologous encapsidation was detected (Maiss *et al.,* 1994). This further supports the suggestion that vector interactions across virus groups, and hence alteration of viral ecology, are rather unlikely. As mixed infections are common in many crops, nature has already had the opportunity to provide such potential interactions many times over (see Section II,D).

b. Dependent Transmission Could Be Due to Genes Other Than Coat Protein. Together with effects of capsid proteins, we can consider accessory transmission factors, such as assistance of the transmission of one virus by the HC of another, as the potential for alteration of vector specificity is similar. There are no reports of dependent transmission from transgenic plants other than those due to CP expression. The first report of interviral assistance in nontransgenic plants was potyvirus-assisted transmission of PAMV from mixed infections (Kassanis, 1961). Kassanis and Govier (1971) showed that other potyviruses could also assist transmission of PAMV and that aphid transmission was dependent on a proteinaceous factor designated "helper component" (HC) that could be purified from infected plants separately from virions (Govier and Kassanis, 1974). However, a more common phenomenon is the situation in which HC of one potyvirus assists the transmission of virions of another potyvirus (Kassanis and Govier, 1971; Sako and Ogata, 1981; Pirone, 1981; Sako *et al.,* 1984; Lecoq and Pitrat, 1985; Lopez-Moya *et al.,* 1995). Heterologous assistance between HC of one potyvirus and virions of another is not always bidirectional (Lecoq and Pitrat, 1985). For example, WMV2 HC and PVY HC assist transmission of TuMV, but TuMV HC does not effect transmission of PVY virions (Sako and Ogata, 1981). However, from the above, it is expected that a highly expressed HC (through mixed viral infection or from a transgene) may assist the transmission of another potyvirus. The effect will be limited to only one passage from a transgenic plant, and the phenotype

of the resultant virus will be that conferred by homologous HC alone unless both viruses are transmitted from a mixed infection. Affinity for HC may also play a role in specific aphid species that may transmit better using the HC of one potyvirus rather than another (Wang *et al.,* 1998).

CP readthrough domains (RTDs) act as integral HCs (Hull, 1994a), with effects on vector specificity analogous to those of separate HCs. Transgenic expression of the readthrough protein could therefore result in altered vector specificity. Natural mixed infection with different BYDV isolates resulted in transmission by aphid species that normally do not serve as vectors. In an elegant study, it was proven that this nonspecific transmission is the result of heterologous encapsidation (Rochow, 1970). Since the time of these early findings, additional knowledge has been obtained from studies on PEMV and luteoviruses (Demler *et al.,* 1997; Gildow and Gray, 1993; Gildow and Rochow, 1980; Peiffer *et al.,* 1997). In these studies, it was established that in the process of entering and leaving the insect body, the viruses have to cross barriers in the hindgut. These barriers are located in the accessory salivary gland, basal lamina, and plasmalemma. It is evident that different virus isolates have preferential affinity for some or all barriers in the vector body (Gildow and Gray, 1993; Peiffer *et al.,* 1997).

In PEMV and in luteoviruses, two viral proteins were found to interact with the barriers in the insect to facilitate aphid transmission, the major capsid protein and the minor RTD protein. The RTD protein is an outcome of translational readthrough (beyond a weak stop codon) into an inframe open reading frame (ORF) downstream of the CP ORF (Brault *et al.,* 1995; Demler *et al.,* 1997). Readthrough occurs at low efficiency, and the RTD protein is therefore present at very low concentration. Thus, the virion may include only a few subunits of the RTD protein. However, the RTD protein plays a determining role in virion entry to the salivary accessory glands. Therefore, it has a key role in the release of the virus and the infectivity of the vector. The presence of a compatible RTD protein in virions from transgenic plants could therefore lead to transmissibility by vectors other than the species able to transmit the wild-type virus. However, as mixed infections of various luteoviruses are commonly detected, it is most likely that transgene RTD protein incorporation will not create a situation that does not exist quite commonly already, unless RTD proteins from viruses exotic to the location or the crop are used. As genes from virus isolates closely related to the target isolate generally confer the best resistance, use of transgenes from exotic viruses is unlikely.

Falk *et al.* (1995) analyzed the epidemiological consequences of virions of a nonhomologous virus encapsidated with transgene-expressed CP subunits, which might lead to transmission by a "new" vector, as discussed earlier by Rochow (1977) for mixed infections. The effects of an HC-mediated interaction could be very similar. If this virus is transmitted to susceptible crops that are not transgenic, the virus will be established there. Transmission by the "new" vector can occur only to establish a primary infection because in nontransgenic plants the virions will be encapsidated solely with their own CP subunits. Secondary spread would be dependent on the properties of the virus in the absence of the transgene CP or HC interaction. In view of the fact that most of the spread in annual crops is secondary, this risk is of limited economic importance, except eventually for the transgenic crop itself.

c. Heterologous Encapsidation or CP Interactions Could Facilitate Systemic Movement of Progeny Virion Within Transgenic Plants for Viruses That Normally Do Not Move in That Host. This takes into consideration the role that CP may have in cell-to-cell movement and long-distance movement (e.g., Dawson *et al.,* 1988; Hull, 1989; Maule, 1991; Dolja *et al.,* 1994b, 1995). Thus viruses to which the crop is immune, by limiting systemic spread (e.g., local lesion reaction to potyviruses in the Chenopodiaceae), might be able to spread systemically if movement is facilitated by CP expressed in the transgenic host. This possibility is predicated on a functional interaction between the transgene CP and virions or nucleic acid of the infecting virus, which is most likely with closely related viruses. No such movement complemented by CP of distinct viruses has been reported.

2. Differences between Crops and Noncultivated Plants

Viruses affecting agricultural crops and noncultivated plants include a broad spectrum of taxonomic groupings, which have many different transmission characteristics. In natural stands of mixed species, the effects of virus infection are often not readily apparent for a number of reasons. First, plants of a susceptible species will typically be of different genotypes with varying responses to infection. Second, in a mixed plant community, competition between species will often obscure differences in the phenotype of individuals within a particular species. Third, in a mixed stand, a vector may move between plants of different species and lose infectivity by probing a plant species that is not host to the virus acquired. It may be that the effects of virus infection become apparent only when a virus is introduced into fields of cultivated plants where there are many plants of similar susceptibility.

The planting in one place of many plants of the same species facilitates virus transmission by mechanical means, by seed transmission, and through specific viral vectors. Close proximity of infected and susceptible plants facilitates both virus acquisition and virus inoculation and, where required by the vector, ready access to a food source (although not all viral vectors colonize or parasitize virus-susceptible hosts).

IV. Analysis of Risk and Proactive Risk Reduction

A. Gene Bank Data as a Tool for Analysis of Foreign Domains in Viruses

The occurrence of recombinant viruses may not be immediately apparent, as there may not be a significant phenotypic difference from the parental isolate to allow recognition of the occurrence of a new genotype. Differences in symptom type or severity, host range, transmissibility, or vector specificity may become apparent only after separation of the new genotype from the parental types. Symptom severity may be the most readily detectable phenotypic difference. Lack of differentiation from the parental types may hinder recognition of recombinants whether the new isolate is a result of intraviral or interviral reassortment or recombination, or even if it is a recombinant with either a viral transgene or a cellular nucleic acid. Indeed, most reports of recombinants do not note any obvious symptom differences from parental isolates, and insertions of additional genes into viral vectors often result in milder symptoms and reduced replication (e.g., Chapman *et al.,* 1992; Hammond-Kosack *et al.,* 1995). Where pseudorecombinants have been constructed by mixing purified genomic RNAs from different isolates, phenotypic differences of the recombinant from the parental isolates are much more apparent. The pseudorecombinant is separated from the parental types *de facto* (e.g., Shang and Bujarski, 1993), as the purpose of creating a pseudorecombinant is usually to map particular phenotypes to a specific genome segment. The increased severity of a recombinant CaMV genome created *in vitro* by Anderson *et al.* (1992) is one exception. Intermolecular recombination within a bipartite geminivirus pseudorecombinant (Hou and Gilbertson, 1996) is another. Perhaps the only well-documented natural plant virus recombinant with increased severity is UgV (Harrison *et al.,* 1997; Zhou *et al.,* 1997). Even in this case, the severity of the recombinant was similar to that of a mixed infection of the two parental isolates in which no recombination was detected (Harrison *et al.,* 1997). Many other naturally occur-

ring severe isolates may have arisen by mutation rather than recombination.

Most naturally occurring putative recombinant isolates have been identified as such—rather than as products of sequence drift—as a result of routine sequence analysis of virus isolates not initially suspected to be unusual. The parental isolates of many presumed recombinants cannot be identified with any certainty (e.g., Revers *et al.,* 1996; Rathjen *et al.,* 1994). Cervera *et al.* (1993) identified PPV-õ6 as a presumed recombinant between a D-serotype and an M-serotype isolate of PPV, but the specific parental isolates were not identified and there is no means to determine the recency of the recombination. Further adaptation is likely to occur following recombination, which may involve sequences on either side of the crossover point as well as in distant portions of the genome. It is therefore likely that parental isolates will be accurately identified only in cases where the recombination event was very recent and one or both of the parental isolates are present nearby.

Pairwise comparison of any newly sequenced isolate with all available related sequences of the same or closely related viruses would be the first step in identifying any unusual features of the sequence. Related sequences in the databases are easily identified by searching GenBank with either the virus name or group name or by carrying out a BLAST search. Any discontinuity of significant sequence homology within a pairwise comparison, at a position where other pairwise comparisons of related sequences have continued homology, may indicate recombination. Higher homology to one sequence upstream of a particular point, and to a different sequence downstream of that point, is a strong indicator of recombination. Other indications may be insertions or deletions compared to related sequences. However, the increased length of some potyvirus genes (and especially the CP N terminus) compared to other isolates of the same virus, together with repeated amino acid motifs, may indicate replicase slippage rather than intermolecular recombination (Hammond, 1992). Once an apparent insertion is identified, the inserted sequence can be separately compared with the databases by BLAST search, with a greater probability of obtaining significant matches.

A PLRV isolate from Scotland that has apparently captured part of a chloroplast RNA transcript was identified as unusual only following the sequencing of two additional PLRV isolates. Then the Scottish isolate and two additional isolates from Scotland were examined, and the chloroplast-related sequence was detected from each of the three isolates; further tests revealed that the additional sequence was present

in only a small proportion of the RNA population in two of the isolates (Mayo and Jolly, 1991). It is not clear whether recombination had occurred in multiple instances or whether the isolates represented mixed infections, but Mayo and Jolly (1991) presented a mechanism by which the recombination could have occurred independently in different locations. The probable origin of the additional sequence from a chloroplast transcript exon was determined initially by comparison of the putative translation product with the EMBL database, with subsequent nucleotide-level matches with chloroplast genes from additional species (Mayo and Jolly, 1991).

Sano *et al.* (1992) sequenced part of the genome of a Japanese isolate of TuMV to compare it with previously sequenced isolates from China and Canada because these isolates had been reported to have 3′ NCRs of very different length. Sano *et al.* (1992) found that the 3′ NCR of TuMV-Jap was exactly the same length (212 nt) as that of TuMV-Chi and had 82.8–99.5% homology to the first 201 nt of both TuMV-Chi and TuMV-Can 3′ NCRs. Following this region of homology, TuMV-Can had an apparent insertion of 467 nt. A database search revealed an 89.9% homology of the insertion to a soybean chloroplast ribosomal protein gene transcript and similar levels of homology to equivalent transcripts of additional species (Sano *et al.,* 1992).

It is particularly interesting that both documented cases suggest recombination with chloroplast transcripts, as it is rare for virus particles to be observed in chloroplasts (Schoelz and Zaitlin, 1989), although TMV particles are known to associate with chloroplasts (Zaitlin and Boardman, 1958). Few virus groups cause obvious chloroplast abnormalities like the invaginations of the chloroplast membrane associated with replication of tymoviruses, although many viral infections lead to increased starch accumulation and chloroplast degradation (Matthews, 1991). Kitajima and Costa (1973) have reported chloroplast clumping in TuMV-infected plants, and both proteins and RNA of PVY and of TMV have been reported in chloroplasts (Gunasinghe and Berger, 1991; Reinero and Beachy, 1989; Schoelz and Zaitlin, 1989; Banerjee and Zaitlin, 1992). The finding that TMV CP can encapsidate multiple specific chloroplast transcripts in pseudovirions (Rochon and Siegel, 1984) suggests that this might be a means of delivery of chloroplast transcripts to the viral replicase complex. Banerjee and Zaitlin (1992) have shown that TMV CP is imported into chloroplasts rather than synthesized there, supporting the hypothesis that replication occurs in the cytoplasm. It may be that the specificity of the replicase becomes somewhat relaxed late in the infection cycle, which is presumably when chloroplast breakdown occurs and other cellular abnormalities are

more pronounced; thus, pseudovirions containing chloroplast transcripts might be released and gain access to the replicase complex. Carpenter and Simon (1996) noted that the location of recombination sites changed over the course of infection in the TCV/satellite RNA system, suggesting that cellular conditions do influence recombination. It may also be significant that typically (−) strand RNA synthesis ceases relatively early in the infection cycle, whereas (+) strand synthesis continues (e.g. Ishikawa *et al.,* 1991). This would support suggestions that many recombinations, including internal deletions, occur during synthesis of the (+) strand (e.g., Kaplan and Palukaitis, 1998).

Mayo and Jolly (1991) noted that the chloroplast transcript incorporated into the PLRV genome encodes a 5-kDa polypeptide that might possibly have some function during virus multiplication. If this is so, it would represent capture of an additional function by the viral genome with potential value in increased viral fitness. In contrast, the chloroplast ribosomal protein gene transcript detected in TuMV-Can is only a partial fragment, including the 3′-terminal 65 nt of the first exon and a major portion of the adjacent intron (Sano *et al.,* 1992). As such, it is unlikely that the insertion in TuMV-Can has any functional relevance.

Sequence alignments and comparisons have been used to determine the probable origins of apparent recombinants between distinct tobraviruses (Angenent *et al.,* 1989; Goulden *et al.,* 1991) and between potyvirus isolates (Cervera *et al.,* 1993). The evidence for multiple origins of luteoviruses by recombination with other virus groups (Martin *et al.,* 1990), or between distinct luteoviruses (Rathjen *et al.,* 1994), is also based on sequence comparisons. Recombination between and within the tobraviruses, and also in the luteoviruses, appears to be largely related to similarities in the noncoding and intergenic regions or subgenomic promoters (Goulden *et al.,* 1991; Miller *et al.,* 1995, 1997). In contrast, potyvirus recombination commonly occurs within the coding region (Cervera *et al.,* 1993; Revers *et al.,* 1996; Gal-On *et al.,* 1998), as well as the 3′ NCR (Sano *et al.,* 1992; Revers *et al.,* 1996).

There is evidence for the acquisition of cellular genes by the closteroviruses, which have apparently gained the heat-shock protein analog from a cellular source (Dolja *et al.,* 1994a). Additional genes have apparently been acquired by some closteroviruses, as there are several differences in the gene complement of CTV and BYV; however, database searches revealed no apparent homologs of at least one of these proteins, the 33K ORF 2 of CTV (see Dolja *et al.,* 1994a). Whether this and other genes present in CTV but not BYV originated from cellular or other viral sources is not clear.

Systematic approaches using sequence information to find evidence of recombination were first applied to plant viruses by Chenault and Melcher (1994), who examined multiple isolates of CaMV. Initial evidence of the likelihood of recombination was obtained when isolates appeared in different clusters in phylogenetic trees based on different short regions of the viral genome; related nonrecombinant isolates are expected to cluster similarly whichever portion of the genome is examined. When an isolate appears in a different cluster based on comparison of different regions of the genome, additional statistical tests are employed to confirm that recombination has probably occurred, either within one of the sampled regions or in the sequence between the regions analyzed.

Revers *et al.* (1996) applied similar tests to multiple isolates of eight different potyviruses, using three regions of the genome known to exhibit variability between both isolates and distinct viruses (e.g., Rybicki and Shukla, 1992; Shukla *et al.,* 1994) and three separate statistical methods. With this approach, several potyvirus isolates were predicted to have arisen by recombination, and one isolate of PVY was found to have undergone two separate probable recombination events. Most of the recombination breakpoints were tentatively localized with the use of the maximum chi-squared approach of Maynard-Smith (1992), including a calculation of percentage identity between the sequence domains derived from each putative parental sequence. This allows determination of both the most likely breakpoint and the direction of the transition (Revers *et al.,* 1996). One notable observation from this study was that all of the necrotic-tuber necrosis (NTN) isolates of PVY, a significant biological phenotype, appeared to be recombinants between subgroups I and II (as revealed by the analysis) with breakpoints over a very short (14-nt) region. Other nucleotide variations unique to NTN isolates indicate that these isolates may be derived from a common recombinant precursor (Revers *et al.,* 1996). This emphasizes the potential value of such information in epidemiological studies.

Revers *et al.* (1996) point out several weaknesses of this type of analysis. First, only viable recombinants are typically isolated and analyzed. Second, the database for a particular virus must be large enough to establish clear clustering of isolates and to increase the chance that the database contains a recombinant. Third, no recombination will be detected between closely related isolates, as no visible shift between clusters would result. Fourth, double recombinants may cluster similarly, depending upon the location of the breakpoints relative to the regions analyzed. Fifth, recombination upstream (or downstream) of the regions analyzed would not be detected. However, the

methods are obviously powerful and could be extended by inclusion of additional regions of the genome as more complete sequences become available.

As a practical matter, some of the limitations identified are of little relevance to risk analysis. Nonviable recombinants cannot cause disease, let alone more severe disease than the viable parental isolate. Recombinants between closely related isolates are unlikely to be significantly different from the parental types and would also be very hard to differentiate from sequence variation within the quasispecies. There is high sequence microheterogeneity in any viral population, and diverse RNA viruses have been estimated to display spontaneous mutation rates of about 1 nt per genome per replication (Drake, 1993). Thus, many closely related sequences will exist after a few replication cycles even if infection is established by a minimal number of virions.

The difficulty of detecting double recombinants is of greater relevance to risk assessment in transgenic plants. However, as the structure of the transgene will be known, appropriate genomic regions can be chosen for analysis. Only those regions included in the transgene transcript, and those immediately upstream or downstream in the viral genome, would be relevant, as the transgene transcript is the only part of the genome available for either single or double recombination. The only possible exception to this would be the situation in which two distinct viral genomes could recombine with each other upstream of the transgene region *and* with the transgene transcript, resulting in a recombinant with domains from three parental molecules. A triparental recombination event is presumably at least an order of magnitude rarer than any bimolecular interaction, and would require all three types of molecule to be present within the same intracellular location or two distinct recombination events to take place in separate cycles of replication. In many cases, cross-protection would reduce the probability of this occurrence (see Section II,B). Pairwise comparisons of a number of sequences including a double recombinant would likely reveal a region with differential homology to the parental isolates that would only be detected by the methods of Revers *et al.* (1996) if this region were one of those analyzed.

There are some features that have been associated with recombination between viral RNAs and that may also be relevant to recombination between viral and nonviral RNAs. These include intragenic regions and subgenomic RNA promoters (e.g., Goulden *et al.,* 1991; Miller *et al.,* 1995, 1997; Bar-Joseph *et al.,* 1997), AU-rich regions with or without upstream GC-rich regions (Nagy and Bujarski, 1996; Bujarski and Nagy, 1996; Simon and Nagy, 1996), and regions with homology to

terminal sequences (e.g., Simon and Nagy, 1996). Simon and Nagy (1996) have also noted that recombination in the TCV/satellite RNA system is significantly influenced by the presence of imperfect tandem repeats that can form part of a stable hairpin structure; this may cause replicase pausing and thus facilitate template switching. The repeat sequences also have homology to the 5′ ends of subgenomic RNAs (Simon and Nagy, 1996). Longer heteroduplexed regions support recombination at higher frequency, while regions of less than 30 nt homology do not induce recombination at a detectable level in the BMV system (Bujarski and Nagy, 1996). Different factors affect homologous and nonhomologous recombination (e.g., Bujarski and Nagy, 1996). Means of reducing the possibility of recombination by deliberate transgene design are discussed in Section IV,B.

B. Tailoring of Genes to Avoid Risk

The possible adverse effects of use of transgenic plants have been discussed in a number of communications, including de Zoeten (1991), Hull (1990), Palukaitis (1991), Tepfer (1993), Greene and Allison (1994), Falk and Bruening (1994), Rissler and Mellon (1993), Hoyle (1994), and Miller *et al.* (1997).

The perceived risks include interactions between viral CPs expressed in plants and the genomic RNA of a virus able to infect the transgenic plants (de Zoeten, 1991; Hull, 1990; Rissler and Mellon, 1993; Tepfer, 1993; Miller *et al.,* 1997). The possibility of such interactions is clear and has been demonstrated in several cases (Lecoq *et al.,* 1993; Farinelli *et al.,* 1992; Maiss *et al.,* 1994; Candelier-Harvey and Hull, 1993; Hammond and Dienelt, 1997). It is also clear that the likely epidemiological consequences are limited, as the virus would have only its own genomically encoded CP available after the initial transfer to a nontransgenic plant (Hull, 1990; Lecoq *et al.,* 1993; Tepfer, 1993; Hammond and Dienelt, 1997). Even the possible case of umbraviruses being encapsidated in transgenically expressed luteovirus CP (Tepfer, 1993) could only result in a single vectored passage to another plant. As umbraviruses require coinfection with a luteovirus for transmission in the first instance (e.g., Gibbs *et al.,* 1996), it is unlikely that an umbravirus would have infected a transgenic plant expressing CP in the absence of the helper luteovirus, unless the helper virus was not able to infect the transgenic plant (due either to transgene-derived resistance or to virus–host incompatibility). This risk is further minimized in plants that express only the luteovirus CP domain, as there is strong evidence

that the CP RTD is required for aphid transmissibility and possibly for cell-to-cell movement of luteoviruses (Brault *et al.,* 1995; Chay *et al.,* 1996). In any event, an umbravirus thus transmitted would be unable to be further aphid-transmitted, as is the case if an aphid transmits an umbravirus from a mixed infection to plants resistant to the helper virus (Falk and Duffus, 1981).

A second potential adverse consequence is recombination between the transgene transcript and a virus capable of replicating in the transgenic plant (de Zoeten, 1991; Rissler and Mellon, 1993; Tepfer, 1993; Hull, 1994b; Miller *et al.,* 1997). Recombination has also been demonstrated in transgenic plants (e.g., Greene and Allison, 1994; Schoelz and Wintermantel, 1993; Frischmuth and Stanley, 1998). There is thus likely to be recombination in transgenic plants; however, in the vast majority of instances, it is likely that recombinants will be either nonviable or very similar in characteristics to the parental isolate (Falk and Bruening, 1994). Indeed, novel combinations would probably be selected against, as they would most likely be unable to compete with a wild-type parental isolate that has been selected over thousands of replication cycles (Falk and Bruening, 1994; Miller *et al.,* 1997). This is not to deny that recombination may lead to new virus isolates with characters distinct from those of the parental types. Indeed, as discussed above (Section II,F), modular virus evolution by recombination between distinct virus groups appears to have been a significant cause of emergence of new viruses over an evolutionary time scale (Gibbs, 1987; Goldbach and Wellink, 1988). The question is whether recombination between a transgene and a viral genome is any more likely to yield a new virus than recombination between two distinct viruses in a mixed infection. Allison *et al.* (1996) suggest that the concern most unique to virus-resistant transgenic plants is that recombination could occur between the transgene and a virus that is capable of replication in the transgenic plant but not of movement. The nonpathogenic virus could then be converted to a systemically invasive pathogen by gaining movement functions from the transgene. It is not clear how this differs from the potential for recombination in a mixed infection unless the transgene is derived from a virus that does not normally infect the plant species concerned. In such a case, there would be no necessity for the transgene to obtain virus resistance, and such plants are unlikely to be deployed; it is therefore difficult to understand any credible concern. There are many instances in which subliminal infections have been reported, and they must often occur in plants previously infected systemically with another virus, thus providing the potential for recombination with high levels of replicating genomic RNA. We agree with

Falk and Bruening (1994) that there is no reason to suppose that the use of transgenic plants will result in greater frequency of viral recombinants, or of any more severe isolates, than occur as a result of natural mixed infections. Indeed, as noted by Rubio *et al.* (1999a), pathogen-derived resistance may play a prominent role in elimination of possible recombinants, and would also significantly reduce the proportion of mixed and codependent infections within the crop. This would both reduce reservoirs of virus for transmission to other crops and reduce the probability of the emergence of viable recombinants.

However, the use of transgenic plants may provide new opportunities for two viral genomes to be present in the same plant, although they would have little chance to occur in mixed infections. This may happen when genes derived from viruses not infecting a specific crop are used to confer heterologous resistance; for example, LMV CP provided resistance to PVY in tobacco, a nonhost for LMV (Dinant *et al.,* 1993b). In this case, it is conceivable that LMV CP gene transcripts might coexist with the genome of a virus infecting tobacco that would have no common host with LMV and consequently would have no chance to occur in a mixed infection with LMV. The second situation is when a plant has transgenic multiresistances, such as cucurbits resistant to CMV, WMV2, and ZYMV (Fuchs *et al.,* 1997). Such plants probably will be deployed in all regions where ZYMV is prevalent, including tropical areas where WMV2 is never or only rarely detected. In this case, WMV2 sequences might recombine with a local potyvirus strain that again would have had no chance to occur in mixed infections with WMV2.

There are other potential interactions that are of more practical concern. One of these is transgene interactions with other viruses that might result in synergy (Hull, 1990; Vance *et al.,* 1995; Pruss *et al.,* 1997). Another concern is alteration of invasiveness of a virus by transgenic expression of a functional MP (Ziegler-Graaf *et al.,* 1991; Cooper *et al.,* 1995) that either permits escape from locally limited infection or increases the number of infected cells. In either of these cases, disease severity would be increased in the transgenic plants, but there would be little effect on nontransgenic crops beyond the possibility of an increased virus reservoir in the transgenic plants. Any such interactions would probably be identified in the course of a thorough evaluation of a transgenic line before release. It is unlikely that any seed company would release a line found to be more susceptible to a particular virus than the parental line because of the potential adverse effects on its own business and reputation. If such a line were released to farmers, increased susceptibility to particular diseases would become apparent over subsequent growing seasons and lead to abandonment of the line

by growers. Farmers routinely abandon disease-susceptible, conventionally bred varieties in favor of more productive ones, and the same would be expected with transgenic varieties. It is thus doubtful that extensive losses would result, or that there would be significant, lasting epidemiological or ecological impacts. HC or analogs that can alter vector-specific interactions would have a more limited effect analogous to heteroencapsidation, affecting only a single passage to a new host plant.

Hull (1990) also raised the possibility that expression of viral gene products might cause, or increase the degree of, seed transmission. There is variation between virus isolates in the efficiency of seed transmission, which suggests that efficiency of seed transmission is controlled by one or more as yet unidentified viral gene products (Hull, 1990; Johansen *et al.,* 1996; Maule and Wang, 1996). Increasing the rate of seed transmission would be highly deleterious. Selection of less efficiently seed-transmitted virus isolates as a source of transgenes would be a wise precaution until the viral genes responsible for this trait are identified and can be avoided, at least for crops in which seed transmission is known to occur.

There is little evidence that any transgene product–virus interactions or transgene–virus recombination events would result in evolution of any "new" viruses that could not occur naturally equally easily in mixed infections of distinct viruses (Falk and Bruening, 1994; Miller *et al.,* 1997). As has been discussed above (Sections II,A to II,F), mixed infections and interactions between virus isolates are common both in noncultivated plants and in agricultural situations. New viruses continue to be discovered, frequently as the result of introduction of new crops or new germplasm to areas in which they have not previously been grown. In many cases, the "new" viruses can then be traced to noncultivated plants in the same area and may not be new at all, but only of recent transition to any effect on agriculture. Adaptation of a virus to a new host plant may also be facilitated by introduction or migration of a new vector species into an area, providing a link between the natural reservoir and the crop plant.

However small we may perceive the risks posed by deployment of transgenic plants to be, there is always the possibility of minimizing the potential risks by altering the design of the transgene itself. It should be noted that in many instances the mechanism of resistance is not well understood and that some approaches to risk minimization might adversely affect virus resistance. In these cases, the options of using alternative viral (or nonviral) genes to obtain resistance should be examined; if no alternatives are found to confer effective resistance,

then the economic and ecological benefits gained from resistance must be weighed against the potential risks of using transgenic resistance. The combination of multiple resistance genes with different mechanisms of action is likely to result in longer-lasting resistance than any one form of resistance alone (see below). Thus, combinations of viral transgenes, or of viral transgenes and other types of resistance, are likely to find usage in many crops. Both Falk and Bruening (1994) and Miller *et al.* (1997) have concluded that the benefits of increased yields and reduced pesticide applications outweigh the identified potential risks from deployment of viral transgenes. We concur with their conclusions but support the rationale that mitigating risk by design ("sanitizing" the transgene; see Hull, 1994b; Allison *et al.,* 1996) is preferable to managing risk *post facto.* Therefore some possibilities for risk reduction are outlined below.

1. Use of Defective Genes

Wherever possible, defective copies of genes can be used—with deletions, insertions, or mutations of active sites. One means of ensuring that altered genes are not functional is to substitute them in an infectious clone and to inoculate different host species. The functionality of genes required for replication, systemic movement, vectored transmission, or ability to infect a specific host can all be tested in this manner. Where this is not possible because infectious clones are not available, known active motifs can be mutated or deleted. Even where gene function analysis is incomplete (as is the case for many multifunctional plant viral genes), sequence comparison has been used to identify structural motifs that have similarity to genes with identified functions from other organisms. Thus, nucleotide binding site motifs are known to be conserved across a wide variety of organisms (Gorbalenya *et al.,* 1988). Deletion or disruption of sequences known or predicted to be replication complex initiation sites is recommended (Allison *et al.,* 1996) unless they are required for resistance.

Examples include deletion or mutation of the CP genes of aphid-transmitted viruses to reduce or eliminate the possibility of altering vector specificity as a consequence of heteroencapsidation. Lindbo and Dougherty (1992a) have shown that TEV CP lacking N- and C-terminal domains is capable of conferring some virus resistance. C. D. Atreya *et al.* (1990) and P. L. Atreya *et al.* (1991) have shown that deletion or mutation of the DAG motif in the N terminus of the CP is sufficient to abolish aphid transmissibility of an infectious TVMV clone. Jacquet *et al.* (1998a) have shown that various deletions and mutations in the PPV CP are effective in minimizing heteroencapsidation and conse-

quent aphid transmission of a non-aphid-transmissible isolate of ZYMV. Deletion of the DAG motif allowed heteroencapsidation but no aphid transmission, whereas deletion of amino acid residues implicated in RNA binding (Jagadish *et al.,* 1991, 1993) abolished detectable heteroencapsidation as well as aphid transmission (Jacquet *et al.,* 1998a). Resistance to PPV was maintained with both types of mutated CP (Jacquet *et al.,* 1998b).

Similarly, deletion or mutation of the CP RTDs of luteoviruses, PEMV, and furoviruses would minimize or eliminate the potential for transgenically expressed proteins to alter the vector specificity of these virus types, as the RTD is essential for vectored transmission (e.g., Brault *et al.,* 1995; Demler *et al.,* 1997; Tamada and Kusume, 1991). Indeed, Demler *et al.* (1997) demonstrated that specific mutations in the RTD of PEMV were sufficient to abolish aphid transmission. Bruyère *et al.* (1997) showed that a deletion in the RTD of BWYV reduced virus accumulation, abolished symptoms, and/or abolished aphid transmissibility. If such constructs confer virus resistance, they would pose a minimal risk of adversely affecting any viruses able to infect transgenic plants.

Some deleted (Braun and Hemenway, 1992) or mutated forms of the PVX replicase gene with substitutions in the active site (Longstaff *et al.,* 1993) were shown to confer resistance to the virus to about the same extent as full-length wild-type constructs (Braun and Hemenway, 1992). The presence of mutated or deleted sequences should reduce the probability of generating a viable recombinant virus from any isolate capable of replicating in such plants.

A mutated form of the MP of TMV conferred partial resistance to TMV and unrelated viruses (Lapidot *et al.,* 1993; Cooper *et al.,* 1995), whereas the wild-type MP enabled systemic infection by a debilitated isolate (Deom *et al.,* 1987). Expression of a mutant, nonfunctional MP is greatly preferred for resistance over a functional copy because of the potential increased severity of disease in some combinations (see below).

2. Avoidance of Functional Copies of Genes Known to Interact with Other Viruses

The use of functional copies of genes that have known potential to cause interaction with other viruses should be avoided as a means of conferring resistance. As discussed above, a wild-type TMV MP was shown to complement a movement-defective isolate of TMV (Deom *et al.,* 1987) and also enabled somewhat increased accumulation of several unrelated viruses (Cooper *et al.,* 1995). Malyshenko *et al.* (1993) showed

that the bromovirus BMV MP conferred partial resistance to TMV despite the fact that TMV MP was earlier shown to substitute for the BMV MP in a hybrid virus constructed by De Jong and Ahlquist (1992). Thus, the MP of one virus is known to permit the spread of an unrelated virus. There are also reports that luteoviruses, which are normally regarded as phloem-limited, can "escape" into the mesophyll in some mixed infections, especially with potyviruses (Barker, 1987). This may be at least in part the result of increased replication efficiency in the presence of a potyvirus, as the potyvirus P1 and HC-Pro proteins have been suggested to be a general transactivator of virus replication (Pruss *et al.,* 1997). The HCs of several virus groups are able to affect the vector specificity of related viruses (e.g., Pirone and Thornbury, 1983; Lecoq and Pitrat, 1985), and the potyvirus HC is also known to contribute to synergistic interactions with other viruses (Vance *et al.,* 1995; Pruss *et al.,* 1997). Such transmission accessory proteins thus would be best not used, even if expression conferred resistance to the virus from which they were derived; however, no resistance has yet been reported in plants expressing HC. Shi *et al.* (1997) have demonstrated that mutations in the HC-Pro can eliminate the synergistic effect, and such defective genes could be valuable if they confer effective resistance. Any other genes known to act as pathogenicity factors would also be better avoided unless mutated to disable such functions.

Although the potential damage from increased replication resulting from synergism would primarily affect the transgenic crop, the resulting increase in potential virus inoculum could also pose a threat to nontransgenic crops. There have been examples of major disease losses caused by widespread deployment of naturally occurring genes, of rather different causes but significant effects. In 1970 there was a devastating epiphytotic outbreak of southern corn leaf blight in maize caused by *Cochliobolus heterostrophus*; the cause was the widespread use of germplasm containing the *t-urf*13 gene responsible for cytoplasmic male sterility, which was used to simplify propagation of hybrid seed varieties (Pring and Lonsdale, 1989). The gene conferring cytoplasmic male sterility also confers high susceptibility to *C. heterostrophus,* a factor not apparent when the germplasm was released; favorable weather conditions for development of the disease together with the large proportion of the crop having the susceptible phenotype led to unparalleled crop losses (Pring and Lonsdale, 1989). Deployment of single genes for resistance to TMV in tomato led to rapid emergence of a new strain of TMV that became widespread very quickly (Pelham *et al.,* 1970). Thus, it is clear that significant or even devastating losses can result from overreliance on any single gene that affects disease

resistance either positively (Pelham *et al.,* 1970) or negatively (as with southern corn leaf blight). The lessons learned from these two cases with naturally occurring genes should be remembered as transgenes are brought to market. In order to permit long-term use of any resistance gene, thought should be given to prevention of too high a selective pressure for the pathogen to develop a resistance-breaking strain. This can be achieved through the use of artificial multiline varieties in which different plants carry different resistance genes, or by incorporation of multiple resistance genes (either transgenes, natural genes, or combinations of the two) effective through different mechanisms into a single genotype (pyramiding of resistance genes). The strategy chosen will depend on the availability of resistance genes and the difficulty of breeding in the particular crop, as well as on the known variability of the pathogen of concern.

3. Use of Genes from Mild Endemic Isolates

Wherever feasible, it would be generally be wiser to use genes derived from endemic isolates of the virus and mild strains if these can be identified. There are two major reasons for this recommendation. The first is that resistance mediated by RNA-based mechanisms has been shown to be rather isolate-specific; thus, a transgene derived from an endemic isolate is likely to be most effective. Although resistance derived from expression of CP might be expected to be more broadly effective, it has been shown that there is considerable variability in protection against different isolates in transgenic papaya expressing the PRSV CP (Tennant *et al.,* 1994). Genes cloned from endemic PRSV isolates are currently being used for deployment in different countries (Lius *et al.,* 1997; Gonsalves *et al.,* 1997). The second reason is to reduce the possibility that any recombinant between the transgene and an invading virus will have properties significantly different from those of existing endemic strains; a recombinant incorporating part of the genome of an exotic strain would be more likely to have altered pathogenicity or host range.

There are exceptions to the general recommendation. One exception may be in citrus crops, where severe seedling yellows isolates of CTV are the most significant problem (Garnsey *et al.,* 1998). Although conventional cross-protection by preinfection with a mild CTV isolate confers significant protection against severe isolates (Garnsey *et al.,* 1998), it remains to be shown whether the CP gene from mild isolates is sufficient to confer broad resistance in transgenic trees. If the CP gene from mild isolates is not effective as a transgene, then it may be necessary to use the CP gene (or another gene) from a seedling yellows

isolate to obtain effective resistance. It would still be advisable to utilize genes from an endemic, rather than an exotic, seedling yellows isolate.

A second example is that of PPV, a virus not yet established in North America. Considering the life span of stonefruit trees, and the extended times necessary to propagate trees and establish orchards of fruit-bearing size, it is desirable to have resistant trees planted in case the virus is introduced despite the rigorous quarantine measures designed to exclude PPV (Kegler and Hartmann, 1998). In this instance, it is obviously not possible to deploy a gene derived from an endemic isolate, but it would be advantageous to use the CP gene from a non-aphid-transmissible isolate (Laimer da Câmara Machado *et al.,* 1992) or to use mutated genes defective for encapsidation and/or aphid transmission (Jacquet *et al.,* 1998a,b).

Selection of an endemic isolate also requires adequate determination of the identity of local virus isolates, which is not always possible. The spectrum of isolates prevalent in an area will also change with time, as a consequence both of nonselective viral evolution and of the response to selection pressures in the form of resistance genes (of both transgenic or host origin) deployed in crops. International trade in seed and vegetative propagating material of many crops will also complicate the selection of "endemic" isolates to utilize as the source of transgene constructs. However, international trade can also result in introduction of other virus isolates in planting material and thus affect what may be considered endemic isolates. The severity of potential losses from virus infection, and the availability and effectiveness of other resistance genes or disease control measures, must be balanced against the perceived risks of utilizing genes from severe or exotic virus isolates—i.e,. the threat of *actual* risks of disease losses must be compared to *possible* risks from the use of transgenic resistance.

4. Avoidance of Replicase Recognition Sequences

Subgenomic promoters are best avoided, as these contain replicase recognition sequences and are potential sites of recombination both within and between viral genera (Duggal *et al.,* 1994; Miller *et al.,* 1995,1997; Timmerman-Vaughan, 1998). Genomic 5′ and 3′ viral noncoding regions also contain replicase recognition sequences. These can result in transcription of complementary transcripts from transgene templates related to natural DI RNAs (Kollàr *et al.,* 1993), from satellite RNAs (Harrison *et al.,* 1987; Tousch *et al.,* 1994), and (by inference) from other transgenes (e.g., Greene and Allison, 1994, 1996). Such sequences should be excluded from constructs unless they are necessary for resistance (Allison *et al.,* 1996). Indeed, it has been postulated that

viral satellite RNAs evolved from host plant RNA transcripts that were nonspecific templates for the viral replicase (Francki, 1985). It has also been shown that an increase in DI RNAs occurred in transgenic plants expressing a functional tombusvirus replicase (Rubino and Russo, 1995). Lack of replicase recognition sites in transgene transcripts reduces the potential for creation of functional recombinant genomes or creation of new satellite RNAs that might develop pathogenicity. Many naturally occurring recombinants resulted from recombination in noncoding sequences (Lai, 1992; Mayo and Jolly, 1991; Sano *et al.,* 1992), which also tend to have more AU-rich sequences that are prone to imprecise recombination (Nagy and Bujarski, 1996).

However, terminal sequences may make a significant contribution to resistance in some cases (e.g., Zaccomer *et al.,* 1993), and elimination of the terminal sequences may reduce the effectiveness of resistance (see Section IV,B,5). In other cases, substitution of other 3′ untranslated regions known to enhance mRNA expression or stability (e.g., Skuzeski *et al.,* 1996) may reduce the probability of generating a functional recombinant while maintaining the transgene characters required for resistance. In cases where noncoding sequences are necessary for resistance, minimal mutation may serve to disable any potential recombinant. Turner and Buck (1999) have shown that mutation of three nucleotides in the loop of a terminal stem–loop structure in the 3′ untranslated region of RCNMV RNA 2 was sufficient to abolish replication. They also showed that sequences across the whole 5′ untranslated region were necessary for production of the (+) but not the (−) strand. Potential resistance must be balanced against reduced potential risks, as discussed above.

As noted in Section II,F,5, homologous RNA recombination has been particularly associated with AU-rich regions (Nagy and Bujarski, 1996; Bujarski and Nagy, 1996; Simon and Nagy, 1996). Nagy and Bujarski (1998) showed that GC-rich regions downstream of recombination hot spots markedly reduced the incidence of recombination, even though the length of sequence identity was increased; the effect was similar whether or not the GC-rich region had significant secondary structure. Insertion of GC-rich regions upstream increased the recombination frequency in the downstream AU-rich recombination hot spot (Nagy and Bujarski, 1998). How applicable this knowledge is to prevention of recombination in transgenic plants is questionable; any sequences known or thought to be recombination hot spots should be excluded from transgene constructs. However, if AU-rich sequences were identified as important elements in inducing RNA-mediated resistance, downstream placement of GC-rich sequences would minimize recombination. Avoid-

ance of potential hairpin structures in the transgene that might function as acceptor structures for the replicase complex (Nagy *et al.,* 1998) would also be advisable. Mutations that disrupted the motif 1 hairpin of TCV sat-RNA C eliminated detectable recombination (Nagy *et al.,* 1998). Insertion of stop codons downstream of any AU-rich regions or probable hairpins would further minimize the viability of any recombinants formed.

5. Use of Untranslatable or Antisense Sequences

The use of untranslatable or antisense sequences minimizes risks—especially if the expressed transcripts lack terminal sequences. No functional protein product can be produced from either untranslatable or antisense transgenes, and there is therefore no potential for transgene protein–viral RNA interactions. Sequences mutated to be untranslatable further reduce the potential for generation of a viable recombinant molecule by reducing the length of the transcript within which recombination can generate a functional genome. The smallest viral fragment that confers effective resistance should be used, as longer fragments provide larger recombination targets and are more likely to encode functional modules that could become incorporated into another virus as a single unit (Allison *et al.,* 1996). Multiple mutations to distribute nonsense codons along the transcript would be preferable to a single termination codon close to the initiation codon. The presence of terminal NCRs may contribute to resistance conferred by antisense constructs through recruitment of replicase complexes to a nonviable template (Hammond and Kamo, 1995a). Powell *et al.* (1989) showed that the effect of a TMV antisense RNA was reduced when the viral 3′ NCR was deleted from the construct. However, reduction of TMV replication by the construct including this region was minimal, and the influence of 3′ NCRs in antisense constructs remains to be demonstrated clearly.

6. Use of Satellite and DI Sequences

Effective resistance has been demonstrated from expression of both satellite RNAs (Harrison *et al.,* 1987; Gerlach *et al.,* 1987) and DI RNAs (Burgyàn *et al.,* 1991; Kollàr *et al.,* 1993). Rubio *et al.* (1999b) have shown that TBSV DI RNA constructs flanked by ribozymes were amplified more effectively than constructs with unprocessed nonviral termini, and that these constructs also conferred protection against multiple tombusviruses. SatRNA and DI RNA resistance results from the replication of the transgene transcript and its consequent ameliorative effect on virus replication and symptomology through competition with the genomic RNA (Collmer and Howell, 1992; Rubino *et al.,* 1990). There

are few sequence differences between satRNAs causing more severe symptoms and ameliorating satRNAs, and only a few mutations would be required to cause a change from one to the other (Sleat and Palukaitis, 1990; Tepfer, 1993). However, preinoculation of vegetable crops in China, with the use of a protective satRNA and a mild strain of CMV, has not caused any reported problems despite usage on a massive scale (Tien and Wu, 1991). Subsequently, transgenic crops were also field-tested in the United States and China (Tien and Wu, 1991; Stommel *et al.,* 1998). As protective, symptom-ameliorating satRNA would be continuously produced in the transgenic plants by transcription of the transgene, any mutation derived by viral replicase error would have to yield a highly competitive symptom-intensifying satRNA to be of much consequence. Eigen and Beibricher (1988) have shown that even an RNA molecule with enhanced replication efficiency does not necessarily come to dominate an RNA population of which it is initially a minor constituent. A threshold amount of necrogenic CMV satRNA is necessary before it can outcompete a nonnecrogenic (protective) satRNA, although transmission to a new plant host may lead to reversal of minority and majority sequence types (see Tepfer, 1993). It is not yet clear whether satRNAs can be identified that are less likely to mutate to symptom-intensifying types, or indeed, whether satRNAs with internal deletions can be functional. There would always be the possibility that, as a replicating RNA (in the presence of the helper virus), a transgene satRNA could also act as a template for recombination. However, the natural equivalent of this has been reported only in TCV, where satRNAs have recombined with DI RNAs (Cascone *et al.,* 1990; Zhang *et al.,* 1991).

A specific problem raised by satRNA-mediated resistance to CMV is that the satRNA can escape the transgenic crop through aphid transmission of its helper virus. Then the satRNA might be able, with its helper virus, to multiply, spread, and eventually evolve in nontransgenic crops. To limit this possibility, it would be advisable to identify regions on the satRNA genome involved in encapsidation in order to delete or mutate these sites (Jacquemond and Tepfer, 1998). There are, however, a large number of satRNA variants that naturally infect various crops together with CMV, and the presence of satRNA often results in decreased aphid transmission of CMV (e.g., Jacquemond and Tepfer, 1998). The effects of satRNA escape from transgenic crops might thus be difficult to differentiate from natural epidemics.

DI RNAs occur naturally in a limited number of virus groups, but artificial DI RNAs have been produced from BMV (Marsh *et al.,* 1991) and TMV (Raffo and Dawson, 1991). DI RNAs occur as a consequence

of natural recombination events and are often a mosaic of viral (and sometime nonviral) sequences. As DI RNAs typically modulate symptoms, it is unlikely that recombination between a functional virus and a dysfunctional DI RNA would give rise to a new functional virus. To overcome DI RNA-mediated resistance would probably require fundamental replicase alterations affecting recognition specificity for *cis* sequence elements, coupled with coordinated 5′ and 3′ terminal mutations to accommodate the altered replicase properties; it is therefore extremely unlikely that even widespread release of transgenic varieties resistant via a DI RNA-mediated mechanism would lead to selection of resistance-breaking mutants (Rubio *et al.,* 1999b).

DI DNAs have also been reported to confer virus resistance (Frischmuth and Stanley, 1991). A later report of recombination between a defective geminiviral DNA and a wild-type CP transgene (Frischmuth and Stanley, 1998) suggests that recombination can be significantly reduced by lack of homologous sequences on one side of the transgene coding region. If recombination does occur primarily in NCRs, as has been suggested (Lai, 1992), then DI RNAs or DI DNAs that have nonfunctional sequence mosaics between the required replicase recognition sequences are unlikely to contribute to viable recombinant viruses. As DI RNAs and DI DNAs are by definition dysfunctional, DI molecules are probably preferable to satRNAs, which have greater potential for mutating to symptom-intensifying variants.

7. Minimizing Effective Transgene Expression Levels

Selection of transgenic lines that provide maximum resistance with minimal transgene expression is a further means of reducing recombination potential; the fewer the transcripts, the less likely they are to be involved in recombination (Allison *et al.,* 1996). Experiments in which more recombinants were recovered after inoculation of mixtures of defective CCMV transcripts to nontransgenic plants than when a defective transcript was inoculated into transgenic plants with a functional CP gene suggest that recombinant recovery is indeed proportional to the quantity of transcript available (Allison *et al.,* 1996). Application of this strategy for risk reduction depends on the mechanisms involved, and the strategy is more readily applied in the case of RNA-mediated resistance when posttranscriptional gene silencing mechanisms active prior to virus infection are involved (e.g., Lindbo *et al.,* 1993; Mueller *et al.,* 1995). Examples of effective resistance from small transgene transcripts have been reported by Sijen *et al.* (1996) and Pang *et al.* (1997). Where resistance is positively correlated with levels of CP expression (e.g., Powell-Abel *et al.,* 1986; Spillane *et al.,* 1997),

selection of lines with reduced transcript levels would compromise resistance.

8. Combining Transgenes or Pyramiding Transgenes with Natural Resistance

Combinations of several of the strategies outlined above may be regarded as a distinct strategy for resistance and risk reduction. The use of partial gene sequences derived from functional genes may itself be a viable strategy. Cassidy and Nelson (1995) suggested that the presence of an initiation codon is necessary for induction of resistance by an untranslatable version of the peanut stripe virus (PStV) CP gene, possibly by causing recognition of the transcript as dysfunctional. Duplication of partial cowpea mosaic virus (CPMV) sequences was found to induce highly effective resistance (Sijen *et al.,* 1996), possibly by causing similar cellular recognition of the transgene transcript as dysfunctional. Lindbo and Dougherty (1992b) proposed that greater resistance was a function of the degree of dysfunctionality of the construct, and that translation of some constructs interfered with their ability to confer resistance. Deliberate out-of-frame duplications or in-frame ligation of fragments producing a peptide that cannot fold correctly may cause recognition of other sequences as dysfunctional and target both aberrant transgene RNA and related sequences for degradation, as proposed by Lindbo *et al.* (1993) and English *et al.* (1996). Ligation of multiple fragments from different viruses, especially with reading frame disjunctions, may be a means of obtaining multiple virus resistance with a minimal risk of creating a viable recombinant virus.

RNA-mediated resistance effected through degradation of both transgene transcript and invading virus, as described by Lindbo *et al.* (1993) and Mueller *et al.* (1995), offers one of the avenues for specific virus resistance with minimal risk of adverse effects. Unfortunately, examples to date suggest that RNA-mediated resistance is one of the most isolate-specific types of transgenic virus resistance, depending on very high degrees of homology between the transgene and the challenge virus to be effective (Mueller *et al.,* 1995; Baulcombe, 1996). It is possible that pyramiding genes (Hull and Davies, 1992; Hull, 1994b) or gene fragments from different isolates may allow broader resistance to multiple isolates of a particular virus. However, this approach will require considerable examination of different sequences and more complex constructs.

Hull and Davies (1992) suggested that it is better to use a variety of approaches to attain multilevel resistance, so that if the virus is able to overcome one form of resistance, it will be faced by other levels of

protection. They stated that at least one of the forms of protection utilized should be prevention of viral replication. This is critical because viral variation occurs only as a result of replication, and hence inhibition of replication will necessarily reduce the frequency of mutations with the potential to overcome the resistance (Hull and Davies, 1992). Pyramiding of a gene conferring highly effective RNA-mediated resistance to the most prevalent severe isolates with CP-mediated resistance that may be somewhat less effective against a broader spectrum of isolates may be a more practical approach in the shorter term. As we begin to understand enough about RNA-mediated resistance to predict which sequences will be effective, more complex constructs may become a more logical way to proceed.

Multiple forms of resistance, especially those based on different mechanisms of action, reduce the possibility that any pathogen mutation will be able to overcome all forms of resistance (e.g., Fraser, 1986; Hull and Davies, 1992). Combinations of host genes and transgenes (Xu *et al.,* 1997) or different transgenes effective against the same virus (Yie *et al.,* 1992; Spillane *et al.,* 1997) result in more effective, and probably more durable, resistance than either alone. The total "gene load" (number and complexity of introduced sequences) can be reduced by using broad-spectrum rather than virus- or isolate-specific resistance and by designing resistance constructs to be expressed where the virus enters the plant. Thus, resistance to viruses transmitted by soil-inhabiting fungi would be most effectively attained with the use of root-specific rather than leaf-specific promoters (Hull and Davies, 1992).

Eventual failure of transgenic forms of resistance is to be expected, as virus variability and evolution have overcome many natural resistance genes. The way in which transgenic resistance is deployed may have a significant effect on the durability of the resistance. Uniform application of a single resistance strategy would create the greatest pressure on the virus in question to mutate to escape the limitation imposed and would probably result in rapid spread of a resistance-breaking strain. This has been observed in the case of resistance to ToMV in tomato (Pelham *et al.,* 1970), for example, although this situation was remedied by incorporation of multiple resistance genes (Fraser, 1986).

Transgenic resistance derived from viral genes may be more durable than natural resistance genes precisely because of its origin. Natural resistance genes have evolved over long associations between the pathogen and its host, or at least between the host and closely related pathogens. The sequence similarities between some resistance genes specific for viral, fungal, and bacterial pathogens (Lawrence *et al.,* 1995;

Staskawicz *et al.,* 1995) suggest that plants have tailored long-established pathways to meet new challenges, but they may also allow pathogens to derive new variations on virulence determinants to overcome these resistances. At no previous point in their evolution have viruses encountered selection pressure based on antagonism from inappropriate expression of their own genomes, except in the case of viruses supporting DI RNAs (or DI DNAs, as appropriate). In all cases reported to date, DI RNAs act as molecular parasites and typically result in decreased viral accumulation and symptom expression. Despite the fact that some forms of transgenic resistance are highly strain-specific, and that in other cases the phenotype is better described as tolerance rather than resistance, there are presumably no preexisting viral variants that are capable of overcoming resistance based on their own genome. The closest situation in nature appears to be the recovery from symptoms in certain virus–host combinations such as nepoviruses in various *Nicotiana* species (Ratcliff *et al.,* 1997). In this example, nontransgenic plants of *N. clevelandii* inoculated with tomato black ring virus (TBRV) initially show symptoms, but leaves developing later are symptomless and have reduced levels of viral RNA compared to symptomatic leaves. Inoculation of upper, asymptomatic leaves with related isolates of TBRV resulted in lower viral accumulation than in equivalent leaves of control plants not previously inoculated. No such reduction in accumulation was observed when recovered leaves were inoculated with unrelated viruses, suggesting that there is a similarity between viral defense and gene silencing in such plants, presumably by the same RNA-mediated mechanism (Ratcliff *et al.,* 1997). This being the case, the reason for the absence of virus recovery in most virus–host combinations is not clear, especially as many other viruses reach higher concentrations in hosts that exhibit recovery from nepovirus symptoms.

It is also probable that single point mutations will not suffice to overcome viral gene-derived resistance mechanisms. It is certain that multiple resistance genes deployed in a single host will be more durable than any one alone, as multiple simultaneous mutations would be required. A comparison to the evolution of human immunodeficiency virus type 1 (HIV-1) may serve as an illustration. HIV-1 is able to mutate quite rapidly to overcome individual drug treatments, and may be able to mutate again if a different drug is substituted (e.g., Tisdale *et al.,* 1995). However, it has been shown in several instances that a mutation that confers resistance to one drug is incompatible with resistance to a second drug (e.g., Tachedjian *et al.,* 1996). This explains

the success of multidrug combinations, as the virus is not able to evolve resistance to all treatments simultaneously.

It must also be remembered that in many cases even transgenic plants susceptible to infection support lower levels of virus replication and/or spread than their nontransgenic counterparts, which will reduce the frequency of mutants arising on a per-plant (or per-infection) basis. A reduction of virus replication levels in transgenic plants, and levels of transgene transcripts typically orders of magnitude below the levels of a replicating virus genomic RNA, reduce opportunities for RNA recombination in comparison to two viruses in a mixed infection of the same species. A further point is that even when a viable viral recombinant is produced as a consequence of interaction between a replicating virus and a transgene transcript, the recombinant has significant homology to the transgene—the situation in which resistance is typically more complete.

V. Concluding Remarks

Several types of interaction may occur between a virus infecting a transgenic plant and either the transgene transcript or its translation product. These include heterologous encapsidation or interaction with a transmission accessory factor, which have the potential to alter vector specificity; complementation from a protein involved in cell-to-cell or long-distance movement, with possible effects on tissue tropism or the ability to establish a systemic infection; synergism resulting in increased replication and/or increased symptom severity; and recombination resulting in gain of function or alteration of pathogenicity (e.g., Hull, 1990; de Zoeten, 1991; Tepfer, 1993; Miller *et al.,* 1997).

All of these interactions are also possible, and have been documented, in mixed infections of either related or distinct viruses. These risks are not unique to transgenic plants, and there is little reason to believe that the consequences of interactions in transgenic plants will be more severe than those resulting from natural mixed infections of different viruses or virus isolates. It is possible that the frequency of such interactions will increase when large areas of transgenic crops are infected with heterologous viruses or virus isolates; however, it must be remembered that mixed infections are extremely common in some natural plant populations and in crops that are vegetatively propagated. Also, effective transgenic virus resistance would be expected to substantially reduce the proportion of multiple and codependent infections within cultivated crops, and thus impose a significant constraint on the possible occurrence of viable recombinants (Rubio *et al.,* 1999a).

The consequences of alteration of vector specificity through heterologous encapsidation are likely to be limited in effect. As has been noted previously, after transmission to a nontransgenic plant, any virus able to replicate will have only its own CP (and any viral accessory proteins). Further transmission will then be dependent solely on the wild-type viral phenotype. It has been suggested that alteration of the vector specificity of heterologously encapsidated particles could cause transmission to a susceptible host to which wild-type virus would otherwise never be transmitted, resulting in expansion of the plant host range and accessibility to new potential vectors (de Zoeten, 1991). But is this a significant risk? Many potential vectors, especially aphid vectors, are polyphagous and feed on a wide variety of plants; even aphids with narrow host ranges will alight and probe on nonhosts to determine plant palatability. Indeed, Kennedy *et al.* (1962) suggest that a vector relationship between an aphid and a virus sharing the same host plant is least likely when the aphid is closely adapted to that plant as its host, although data from Wang *et al.* (1998) show that *Lipaphis erysimi* can transmit TEV only in the presence of TuMV-HC; TuMV is adapted to crucifers, as is the vector *L. erysimi.* Mixed infections of both similar and dissimilar viruses are common, and thus opportunities for heterologous transcapsidation are also common. It is therefore unlikely that a virus-resistant transgenic plant will create a heterologous combination that has not previously occurred naturally, with a few exceptions, as outlined in Section IV,B. Such situations can be largely avoided by selecting more appropriate transgenes or through reduction of risk by sanitizing transgenes to minimize potential interactions.

Recombination that might add or substitute a transmission-associated gene conferring vector specificity would be of greater potential concern. An example would be exchange of the CP gene between a leafhopper-transmitted and a whitefly-transmitted geminivirus, effecting a complete change of vector specificity, as shown experimentally by Briddon *et al.* (1990). However, if virus genes are only employed for resistance in crops susceptible to that virus, such recombination could occur only between viruses that already have the opportunity to recombine in mixed infections.

Potential complementation in a transgenic plant would also be limited in epidemiological effect, as the complementing transgene would not be present in nontransgenic plants. One potential difference between complementation in a transgenic plant and in mixed infections would be the expression of a transgene in essentially all tissues, and at all growth stages, from a constitutive promoter. Thus, it is possible that a complemented infection of the transgenic plant might be more

severe or that a larger reservoir of virus might be available in the crop for transmission to other plants. Most such potential interactions would probably be observed in laboratory or field trials prior to variety release and would result in a decision not to use that particular gene. Even if a breeder or seed company released any transgenic line with such characteristics, farmers would be likely to shun such a line after experiencing disease losses; any increase in virus reservoirs would thus be temporary.

Recombination to create a "new" virus (de Zoeten, 1991) has perhaps the greatest probability of causing a significant epidemiological effect. It is generally accepted that viral recombination in natural infections is a major force in evolution of new viruses and that currently recognized viral taxa have arisen by rearrangement of several basic modules (e.g., Goldbach, 1986; Dolja *et al.,* 1994a). Further, although a viral transgene driven by a constitutive promoter will result in the presence of the transcript in most tissues and most developmental stages of the plant, transgene transcript levels are typically far lower than levels of viral genomic and subgenomic RNAs in a natural infection. The transgene transcript will also typically be significantly shorter than viral genomic RNAs, reducing the potential regions for recombination. Allison *et al.* (1996) noted both of these factors as probable reasons for a lower frequency of recombinant recovery in transgenic plants than in experiments where two sets of defective genomic RNAs were coinoculated into nontransgenic plants.

Recombination can, and almost certainly will, occur in transgenic plants, mainly between a virus and a closely related transgene. What, then, are the potential risks? How will we be able to attribute occurrence of putative recombinants to interactions between a virus and a transgene, rather than to interactions between two viruses in a mixed infection or to selection of a minor sequence in a quasispecies population following a genetic bottleneck? A bottleneck event could occur as the result of transmission from a minimal viral population in a resistant transgenic plant, but it could equally well occur as a result of reduced levels of replication in a "naturally resistant" nontransgenic plant. Bottleneck events may also occur as a consequence of establishment of infection from small numbers of particles following vectored transmission and may form a normal part of the infection cycle of many viruses (Domingo *et al.,* 1996). Sequence analysis of putative recombinants could identify likely crossover points and the level of homology to transgenes deployed in the field. Actual transgene sequences will usually be available for comparison, whereas there are few reports of the level of sequence variability of any particular virus in the field at

any particular location. Thus, some putative recombinants (selected by virtue of symptom or serological differences from previously prevalent isolates) might be shown to have no identifiable sequences homologous to transgenes and distinct from prevalent isolates. Absence of identifiable homology to the transgene but difference from previously prevalent field isolates would suggest introduction of a new virus isolate from elsewhere rather than recombination. In most cases, however, recombination will occur between very similar transgene and viral sequences, and will result in recombinants that cannot be distinguished from the parental quasispecies distribution. Indeed, it is becoming accepted that recombination is a normal part of the replication of many viruses to restore function to genomes damaged as a consequence of the lack of fidelity in viral replication (e.g., Simon and Bujarski, 1994; Domingo *et al.,* 1996; Gal-On *et al.,* 1998). There is no *a priori* reason to suppose that the product of recombination between a virus and a related transgene would be any more severe than that between two isolates of the same virus in a mixed infection. Nor is there any reason to suspect that recombination between a viral transgene and an unrelated virus would be of greater consequence than that between two unrelated viruses in a natural infection.

Laboratory experiments with pseudorecombinant or chimeric viruses have demonstrated that substitution of genes from heterologous viruses can yield viruses competent for replication (e.g., De Jong and Ahlquist, 1992; Mise *et al.,* 1993; Giesman-Cookmeyer *et al.,* 1995; Taliansky and GarcÌa-Arenal, 1995; Solovyev *et al.,* 1996, 1997; Salánki *et al.,* 1997). In most instances, however, the recombinant viruses are seriously debilitated for cell-to-cell or long-distance movement. Hybrid viruses expressing heterologous MP genes were shown to infect only hosts common to both parental viruses (Solovyev *et al.,* 1997), and there was little or no cell-to-cell movement in species that were hosts to only one of the parental viruses (e.g., De Jong and Ahlquist, 1992; Mise *et al.,* 1993; Solovyev *et al.,* 1996, 1997). Most recombinant viruses may be compromised in virulence because the heterologous gene combinations are not fully compatible (Rubio *et al.,* 1999a); even insertion of a foreign gene into a plant viral vector frequently lessens replication and symptom severity (e.g., Chapman *et al.,* 1992; Hammond-Kosack *et al.,* 1995). Compatibility of MPs and other viral sequences is required for efficient cell-to-cell movement (Solovyev *et al.,* 1997). Compatibility may also be required between the CP and other viral sequences or the host for long-distance movement (e.g., Taliansky and García-Arenal, 1995). Compatibility between viral replicase components (e.g., Traynor *et al.,* 1991; Dinant *et al.,* 1993a; Weiland and Edwards, 1994) may

determine the viability of an interspecific viral recombinant. As a consequence of lack of compatibility between genes derived from different viruses, the vast majority of hybrid viruses that might appear through either interviral recombination or recombination between a virus and a viral transgene should be debilitated with respect to the parental virus(es) and unlikely to survive (Rubio *et al.,* 1999a). Factors influencing the ability of a recombinant to compete with the parental genotype include the recombinational ability of the parental virus(es), the type of recombination event and the functionality of recombinant gene products, ability to move systemically, and selection pressure on the recombinant in comparison to the parental virus(es) for both replication and transmission to new hosts (Rubio *et al.,* 1999a). Despite the plasticity of viral genomes anticipated as a consequence of RNA polymerase infidelity and the quasispecies nature of viruses, the literature suggests that viral genomes and phenotypes have remarkable stability, and few probable recombinants with distinctive phenotypes are detected.

Resistance obtained by genetic engineering is not the last word in plant protection. Viruses will continue to evolve, and will surely at some point overcome resistance derived from transgenic expression of viral sequences, just as resistance-breaking isolates have arisen in the face of genetic resistance bred into the crop by traditional means. Transformation with the appropriate gene from the resistance-breaking isolate would likely confer new resistance; however, development of additional resistance genes from various sources will continue to be necessary. Resistance conferred by expression of viral sequences may be somewhat more durable, as such resistance is not known to have formed any previous selection pressure in virus evolution. Evolution to escape resistance based on a homologous sequence may require multiple mutations and many cycles of replication; as replication would be much reduced in resistant plants, the mutable population available to yield a resistance-breaking sequence would be smaller. The durability of any individual form of pathogen-derived resistance will be extended by deployment in combination with other resistance genes functioning through different mechanisms, as it is far more difficult for a pathogen to mutate to overcome multiple separate genes than to overcome a single gene. Pyramiding of virus-derived transgenes with host resistance genes, with other types of virus-derived transgenes, or with other types of nonconventional resistance such as plant-expressed antibodies or dsRNA-specific nucleases (reviewed by Hull, 1994b; Hammond, 1996, 1997) will increase both the effectiveness and the durability of virus resistance in crops.

In this article we have discussed all those potential risks of which we are aware and have offered suggestions for minimization of each. The significance of each type of potential risk will depend on the virus concerned and, to a lesser extent, on the crop. For example, heterologous encapsidation could potentially affect transmission of nepoviruses or tobraviruses by different species or biotypes of nematodes, but only through a single vectored passage. Both types of nematode-transmitted virus are more prone to geographical variation in genotype and serotype than most other virus groups as a consequence of the vector's restricted mobility, and nematodes may spread disease within a field by less than 50 cm per year (Harrison, 1977). In contrast, given the soil-linked nature of nepovirus transmission, a few varieties of transgenic nepovirus-resistant rootstocks could potentially suffice to protect grape varieties worldwide against nepovirus infection (Brault *et al.,* 1993) if combined with virus-indexed scion material. Seed transmission of nepoviruses in weed species may be of far more concern in local persistence and spread than the nematode vectors in many situations (Harrison, 1977). Heterologous encapsidation of viruses with fungal vectors may similarly be of little concern, as there are few vector species of limited motility. Heterologous encapsidation is of more concern for viruses with aerial vectors such as aphids and whiteflies because of the potential distances involved and the possibility of movement between different ecosystems. Even so, as outlined above, potential risks from heterologous encapsidation in transgenic plants are of no greater apparent significance than those from commonly occurring mixed infections.

We agree with Falk and Bruening (1994), Miller *et al.* (1997), and Rubio *et al.* (1999a) that the potential risks attributed to deployment of transgenic plants currently appear minimal and that the benefits to be obtained outweigh the perceived risks. Recombination between viral groups presents perhaps the greatest risk of "new viruses" or alteration in viral ecology, but it could occur with equal probability in mixed infections. Efforts to detect and document any potential adverse epidemiological effects of transgenic potato expressing PLRV CP or replicase found no evidence of recombination, altered transmission, or altered virion properties (Thomas *et al.,* 1998) in the most extensive survey reported to date. The benefit of reduced field spread of PLRV in CP transgenic potato had previously been demonstrated, with significant restriction of transmission between plants in the field (Thomas *et al.,* 1997). We encourage further such studies to examine both the benefits and risks of virus-resistant plants in field conditions. It is prudent to minimize the potential risks that have been identified by taking any known factors into account in transgene design, and we recommend

the proactive approach to risk reduction taken by Jacquet *et al.* (1998a,b). This is a sound approach for second- and third-generation transgene design, but it should not preclude deployment of first-generation transgenic plants that do not present specific risks or prevent the use of such first-generation plants in breeding programs to combine transgenic resistance with host resistance.

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RESPIRATORY VIRUSES

Guest Editor: Joseph L. Waner

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ADVANCES IN VIRUS RESEARCH, VOL. 54

INTRODUCTION

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Viruses cause the greatest number of acute respiratory infections. In addition to morbidity and mortality, the economic burden of these infections on the health care system due to lost workdays, hospitalization, and therapeutics is in excess of $1 billion a year. The vaccine that is available for influenza A and B viruses has an efficacy of approximately 70%, and the available antiviral therapy is effective only against influenza A. No treatments or vaccines are available for the other respiratory viruses. The following articles deal with the virus groups that are responsible for the most important and most prevalent respiratory virus infections: the orthomyxoviruses, paramyxoviruses, and rhinoviruses. Each of these groups and individual members within the groups have evolved strategies of replication that include ways to elude the protective immune responses of their hosts to the benefit of the virus family.

Influenza virus infections cause serious morbidity in all age groups and are associated with significantly increased mortality in the elderly. Epidemics of influenza have been recognized historically since the Middle Ages and possibly back to ancient times. The natural history of influenza is characterized by the ability of the virus to change its antigenic mosaic in response to the acquisition of immunity to the strains circulating in the host population. This is reflected in subtle but effective antigenic drifting or in the total exchange of one of the two dominant antigenic proteins through antigenic shifting. The influenza virus was first isolated in 1933, and the currently used formalin-inactivated vaccine represents technology of the 1950s. The three articles that deal with influenza review the current knowledge of virus replication on the molecular level and show how that knowledge has resulted in new concepts for vaccine development and for therapy that is efficacious against influenza types A and B.

The family *Paramyxoviridae* includes respiratory syncytial virus (RSV) and the four human parainfluenza viruses (PIV1–PIV4). In contrast to the influenza viruses, these agents are dealt with as stable antigenic types. Reinfections are common but are associated with di-

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minished pathogenesis, indicating that immunity follows primary infection but is imperfect. RSV is the most important cause of lower respiratory tract disease in infants and young children. It is also an important cause of lower respiratory tract disease in the elderly, possibly approaching the importance of influenza in that high-risk group. Collectively, the parainfluenza viruses are the most important causes of lower respiratory tract infections in infants and children after RSV. PIV1–PIV3, particularly PIV1, are the major causes of laryngotracheobronchitis, or croup; PIV3 is also an important cause of pneumonia and bronchiolitis in infants and children. PIV3 does not have a seasonal appearance, as do the other parainfluenza viruses, RSV, and the influenza viruses. There are no vaccines available for these viruses. An attempt in the 1960s to duplicate the inactivated vaccine strategy for RSV resulted in the induction of an atypical immune response. Enhanced disease resulted in some of the vaccine recipients when they were infected with naturally occurring strains of RSV. This experience showed the need for fundamental knowledge about the virus before realistic efforts could be made to develop a vaccine.

Rhinovirus infections are responsible for up to 50% of common colds. In fact, the orthomyxoviruses and paramyxoviruses identified above also contribute to occurrences of the syndrome. Rhinoviruses are members of the picornavirus family but do not traverse the gastrointestinal tract due to their sensitivity to low pH. There are at least 100 different strains; each serotype conveys specific protective immunity but does not induce cross-immunity to other serotypes. The large number of virus strains, therefore, seems to preclude meaningful vaccine development. Although efforts have been made to develop therapeutic treatments, none are currently available. Investigations in pursuit of these goals, however, have revealed an interesting picture of viral pathogenesis that is less viral than it is host in origin. In contrast to the orthomyxoviruses and paramyxoviruses, therefore, treatment concepts may reasonably address modulation of the host response.

The articles in this section are intended to present up-to-date reviews of the molecular biology of replication of these groups and thereby provide a background for the rationale behind the concepts for new vaccines and for novel approaches to antiviral therapy. In total, the articles demonstrate that clinical approaches to disease begin with a foundation in basic research.

ADVANCES IN VIRUS RESEARCH, VOL. 54

REPLICATION OF ORTHOMYXOVIRUSES

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I. Introduction

The *Orthomyxoviridae* family comprises the influenza A, B, and C viruses, as well as the Thogoto/Dhori group of tick-borne viruses. These are enveloped viruses whose genome consists of several segments of single-stranded, negative-polarity RNA. They all share a similar genetic organization, nuclear phase of replication, and coding strategies (for general reviews, see 1–3). The influenza A viruses are the best-known members of the *Orthomyxoviridae* family. This review focuses on this group of viruses, with reference to others whenever appropriate.

Influenza A viruses bind to their host cells by interaction of the hemagglutinin (HA) with cell surface molecules containing sialic acid, and virions enter cells by receptor-mediated endocytosis. In the secondary endosomes, the low pH causes a conformational change in HA that allows fusion of the viral envelope with the membrane of the endosome (4–6). This fusion event delivers the viral genome into the cytoplasm,

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and thereafter it rapidly enters the nucleus (7), where transcription and replication occur. The functional units for these processes are viral ribonucleoprotein (vRNP) complexes. They contain the viral genomic RNA associated with four virus-encoded proteins, the nucleoprotein (NP) that interacts with the RNA throughout its length, and a heterotrimeric complex composed of the three subunits of the viral polymerase (PB1, PB2, and PA). Within the nucleus, vRNPs are transcribed into viral mRNAs and replicate by means of complementary ribonucleoprotein complexes (cRNPs) (8). The progeny vRNPs exit from the nucleus to the cytoplasm in association with newly synthesized matrix (M1) and NS2 proteins. Finally, they move to specific sites on the plasma membrane, modified by the insertion of the viral integral membrane proteins HA, neuraminidase (NA), and M2 protein, where new virus particles are formed by budding.

In this review we shall discuss the results of recent studies on orthomyxovirus replication without attempting to be comprehensive. Only certain topics on which new data have accumulated will be considered. Other topics have been covered by reviews (7,9–14).

II. Ion Channel Protein

The M2 protein is a 97-amino acid (aa) protein encoded by a spliced mRNA derived from genomic RNA segment 7. It is expressed at the plasma membrane of virus-infected cells and has a 24-aa-long N-terminal extracellular domain, a 19-aa transmembrane (TM) domain, and a 54-residue cytoplasmic tail (15). Initial clues to the role of the M2 protein in viral replication came from studies that analyzed the inhibitory effect of amantadine and rimantadine on influenza virus infection. Amantadine and rimantadine are antiinfluenza drugs that specifically inhibit influenza A virus replication. Amantadine-resistant influenza virus mutants have been isolated (16), and genetic studies indicate that drug resistance is linked to genome RNA segment 7, which encodes the M1 and M2 proteins. Nucleotide sequence analysis of RNA segment 7 indicated that the target of antiviral action mapped to four residues in the M2 protein (17). In a natural infection the incoming RNPs dissociate from M1 and enter the cell nucleus (see below). However, in the presence of the drug, the incoming RNPs and M1 protein are not detected in the cell nucleus but colocalize in places throughout the cytoplasm (18,19). M1 protein is selectively removed from the RNP structure at acidic pH, and it was suggested that this change in the RNP–M1 protein interaction occurs when the virion is

in the endosomal compartment (20). However, because the M1 protein resides inside the viral lipid bilayer, a mechanism must exist to make the interior of the virion accessible to a pH change, and various types of evidence support the notion that acidic pH serves as a switch allowing dissociation of M1 protein from the vRNPs.

Besides the early effect of amantadine on influenza virus replication described above, for some subtypes of avian influenza virus, which contain an HA that is cleaved intracellularly and has a high optimum pH of fusion (e.g., fowl plague virus Rostock), the drug has a second effect late in replication. Addition of amantadine to cells late in infection causes a premature conformational change in HA that occurs in the trans-Golgi network (TGN) during the transport of HA to the cell surface (21,22). This form of HA is indistinguishable from the low-pH-induced form of HA that results following exposure of the native protein to low pH. Thus, the M2 activity susceptible to amantadine is thought to function in the TGN, and the associated transport vesicles are believed to regulate intracompartmental pH and keep the pH above the threshold at which the HA conformational change occurs. The consequence of this irreversible conformational change, which causes a wrong extrusion of the fusion peptide, is that the HA trimers aggregate and viral budding is greatly restricted (23). Alteration of the same aa positions in the M2 TM domain abolishes susceptibility to both early and late effects of amantadine (17).

The first direct evidence that M2 forms an ion channel came from studies of Lamb and co-workers showing that injection of wild-type (wt) M2 mRNA into *Xenopus laevis* oocytes produced a pH-regulated inward current that could be blocked by addition of amantadine (24). Oocytes expressing M2 proteins containing mutations that confer resistance to the drug were not blocked by addition of amantadine (24). Other direct evidence for the function of the M2 channel was obtained from electrophysiological studies, including heterologous expression in mammalian cells (25,26) and in yeast (27). It was also shown that M2 protein expressed in *Escherichia coli* increased membrane permeability (28), and lipid vesicles reconstituted with purified M2 protein expressed by baculovirus worked as an ion channel (29). Therefore, it has been proposed that the M2 ion channel activity allows the flow of protons into the viral particle and that this acidification induces a change in M1–vRNP interactions that causes the release of nucleocapsids.

A. M2 Protein Structure

M2 is a posttranslational modified protein. It is phosphorylated and palmitylated at the cytoplasmic tail, and cysteine residues 17 and 19 in

the ectodomain form intermolecular disulfide bonds (21,30). However, disulfide bonds and acylation are not required for virus viability or ion channel activity (31,32). Other posttranslational M2 modifications, such as phosphorylation of residues on the ectodomain, are also unnecessary for ion channel activity (33).

The TM domain of M2 protein was predicted to have an α-helical secondary structure (34). In this model, TM residues whose specific changes lead to an ion channel that is resistant to amantadine were located on the same face of the putative α helix (35). Structural analysis with the use of M2-TM mutants expressed in *X. laevis* indicated that changing any residue on this face of the putative α helix altered the properties of the channel (24). Location of the amantadine in an area between Val-27 and Ser-31 of the TM domain was reported, consistent with the formation of a steric block within the ion channel by the drug (36,37).

Further characterization of the M2 protein provided evidence that the active form of the channel consists of a tetrameric array of TM domain peptides forming a parallel bundle of α helices. Using Cys scanning mutation to generate a series of variants with successive substitution in the TM segment of the protein (38) or a simulation study (39), two independent groups proposed a similar ion channel model. In that model the helix bundle forms a left-handed supercoil surrounding a central pore, with His-37 involved in the activation of the pore (Fig. 1). The orientation of the imidazole ring lies roughly parallel to the superhelical axis, and its orientation within the channel may provide the strong proton selectivity of the channel. In a deprotonated state, the pore of the channel should be occluded due to the orientation toward the lumen of the side chains of His-37, whereas a fully protonated His-37 should allow its low-pH activation (Fig. 1). Alkali metal ions or chloride ions will have difficulty passing beyond the His-37 residue, but protons may be transmitted by a proton relay mechanism. The model also explains the effects of amantadine-resistant mutations on the TM region because each of the resistant mutant projects toward the lumen of the channel, a picture consistent with the presence of larger and more hydrophilic aa found in amantadine-resistant strains of the virus.

B. Influenza B and C Analogs to Channel Protein

Proteins analogous to M2 protein have been described in other influenza viruses. These analogs include the NB protein from influenza B

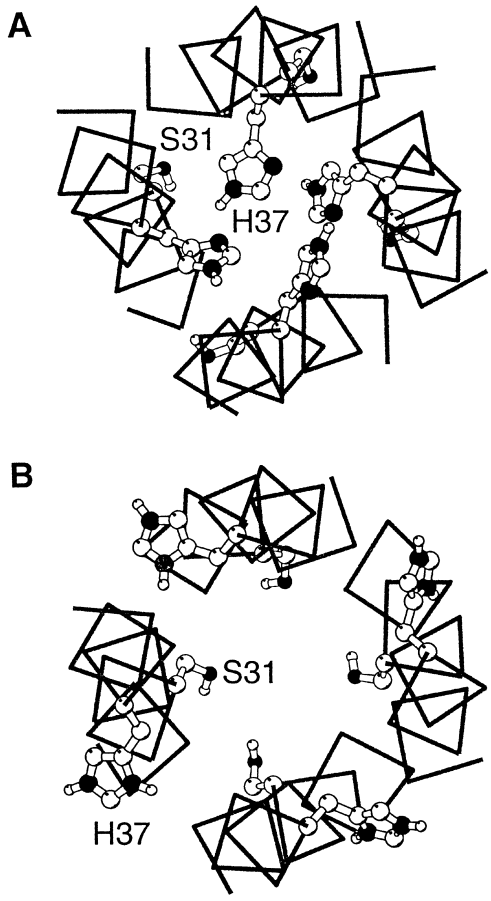

FIG 1. Model for the ion chanel structure of the influenza A M2 protein. Reproduced from Ref. 39. See text for further details.

(40) and the CM2 protein from influenza C (41). The three members share structural characteristics: they are small integral membrane proteins having an ectodomain that is glycosylated in NB and CM2 but not in M2, a TM domain, and a cytoplasmic tail (42–45). The NB glycoprotein is synthesized in virus-infected cells in amounts similar to those of the other viral membrane proteins but is incorporated into the virus particles in low amounts, resembling M2 protein (42). Although no role for NB in virus entry has been reported, the above-mentioned data, together with the finding that NB incorporated into

lipid bilayers forms a cation-permeable channel at physiological pH (42), support the notion that NB is the functional counterpart of the influenza A virus M2 protein. Some of the structural properties of the CM2 protein resemble those of the NB and M2 proteins. Although it is tempting to speculate that CM2 may represent the ion channel protein of the influenza C virus, there are no data providing experimental support for this assumption. The requirement for ion channel activity in influenza C virus uncoating is not compelling due to the reported dissociation of the nucleocapsids at neutral to mildly alkali pH *in vitro,* in contrast to the required acidic pH for influenza A or B disassembly. However, a functional ion channel in influenza C virus could be required for other functions, such as the trafficking of the HEF viral glycoprotein across the lumen and the TGN.

III. Return Ticket for RNP: Nucleocytoplasmic Transport of Influenza Genome

During a lytic infection, the RNP complexes and the RNP-associated proteins undergo three nuclear traffic events: the bidirectional transport of RNPs into and out of the nucleus and the nuclear import of the isolated RNP protein components. The NP and the three polymerase subunits each have sequences responsible for their individual nuclear transport; therefore, any of these proteins could be responsible for targeting the RNPs into and out of the nucleus. However, it appears that the RNP transport events are mediated by interactions involving the NP, which is the major component of RNPs.

Macromolecular communication between the nucleus and cytoplasm occurs through the nuclear pore complex (NPC). Protein import and export are active, energy-dependent, signal-mediated events. The best-characterized nuclear import pathway is the one affecting target proteins that contain an aa sequence termed the "nuclear localization signal" (NLS). The canonical NLS is rich in basic residues, but there are proteins with no canonical NLS that also use the same NLS-dependent protein import pathway. This pathway involves interactions of the target protein with the NLS receptor, which is constituted from two proteins, importin α and importin β (also known as "karyopherin α" and "karyopherin β"). Other proteins then cooperate to translocate the protein–NLS receptor complex through the NPC. One of the best-characterized nuclear export signals (NES) is a short leucine-rich sequence present in the human immunodeficiency virus (HIV) Rev protein. It should be mentioned that other nuclear import and export

pathways that do not involve the NLS and NES signals are just starting to be characterized (all these topics have been reviewed in 46,47).

A. Nuclear Import of Nucleoprotein

The influenza A virus NP is a basic protein of 498 aa that does not contain a canonical NLS. However, the protein contains sequences that mediate its import into the cell nucleus when synthesized from recombinant DNA (48). Early experiments dealing with the intracellular localization of recombinant deleted NPs (derived from the A/NT/60/68 gene) in *X. laevis* oocytes led to the conclusion that the NP region required for nuclear accumulation was located between residues 327 and 345 (49). More recently, the group of P. Palese has shown that the N-terminal 13 residues of NP (of the A/PR/8/34 strain) are involved in binding to members of the importin α protein family and that the NP can enter the nucleus through the NLS-dependent pathway (50–52). The same authors also showed that the NP contains a sequence within the 20 N-terminal residues of NP that allows accumulation of fusion proteins containing this region in the nucleus of HeLa cells (52). Unexpectedly, they observed that an NP protein lacking both this N-terminal karyophilic signal and the one determined earlier (49) localized mostly to the cell nucleus. All these data indicate that the NP contains several karyophilic sequences, one N-terminal and other(s) not yet characterized. These conclusions were confirmed by analysis of the intracellular localization of deleted NPs derived from the A/WSN/33 strain in COS-1 cells (53). In this study, it was also observed that the wt protein and some deleted NPs were found exclusively in the cell nucleus at early times postransfection (9 hours) but that the wt protein was found only in the cytoplasm at later times (24 hours). Similar results have been reported for the A/PR/8/34 and B/Ann Arbor/1/66 NPs (54). These results appear to contradict data showing that the NP expressed alone behaves as a shuttling protein (55). It should be mentioned that the NP does not act as a shuttling protein in infected cells because the newly synthesized protein remains in the nucleus until the RNPs undergo M1-mediated export (see below) (55). The discrepancies between the results of the experiments described above may be due to the use of different cell lines and expression vectors, which would lead to different levels of accumulation of NP. Neumann and colleagues (53) have proposed that overexpression of the NP triggers the formation of NP multimers, which may be a prerequisite for nuclear export. These authors also reported data suggesting that phosphorylation/dephosphorylation (of

a cellular protein or the NP itself) may be another regulator of nuclear export (53). In fact, it was shown that incubation with a protein kinase inhibitor prevented nuclear export of the wt NP and other NP-deleted proteins. In this regard, it is worth mentioning that the A/Victoria/3/75 NP is phosphorylated at the serine in position 3 (56). This residue, which is conserved in the A/PR/8/34 but not in the WSN strain, is located within the region involved in binding to importin α. It is conceivable that phosphorylation/dephosphorylation of this aa could modulate some of the NP nuclear traffic events.

B. Nuclear Import of RNPs

Following fusion of viral and endosome membranes, the incoming RNPs separate from M1 (see the preceding section) and enter the nucleus through the NPC by an active process (20,57). The incoming M1 protein remains entirely cytoplasmic or distributes between the nucleus and the cytoplasm, depending on the cell type used for infection. Entry of M1 into the nucleus occurs independently of the presence of vRNPs, apparently by a passive diffusion process (20).

In the virions, the M1 protein appears to form a shell that interacts with the viral envelope and the vRNPs (3). It has been proposed that the M2 ion channel activity plays a role in allowing dissociation of the M1–RNP complexes during virus entry (see the preceding section) (7,19). Consistent with this concept, M1-free RNPs, prepared at either neutral or acidic pH, when microinjected into the cells can enter the nucleus and are functional for expression of the viral genome (57). Therefore, these experiments indicate that exposure to acidic pH affects the binding properties of M1 and that the vRNPs themselves do not require prior exposure to low pH to be competent for nuclear import. It has also been shown that in the presence of amantadine, vRNPs microinjected into the cytoplasm are efficiently imported into the cell nucleus, indicating that this drug does not affect uptake itself but does appear to mediate its effect by inhibiting the M2 protein (57). The nuclear import of vRNPs has also been studied in cells expressing a recombinant M1 protein. When these cells were infected with influenza virus or were microinjected with purified vRNPs, the incoming vRNPs localized in the cytoplasm. However, the RNPs were able to reach the nucleus if the cytosol of the M1-expressing cell was acidified following internalization of vRNPs (58). To explain the mechanism of M1-mediated inhibition of vRNP nuclear import, it has been proposed that M1 masks the vRNP nuclear import signals. Alternatively, M1 binding

could result in the association of RNPs into complexes too large to be imported through the NPC or in the formation of complexes that would be bound to cytosolic membranes via the lipid-binding domain of M1.

C. Nuclear Export of RNPs

After late viral gene expression takes place, viral transcription is inhibited by interaction of M1 protein with the RNPs (59). In addition to this role of M1 protein, several lines of evidence indicate that expression of nuclear M1 protein promotes RNP export from the nucleus (19,60). In fact, it has been shown that when the synthesis of the late influenza virus proteins (HA, M1, M2, and NA) is blocked, the NP is confined to the nucleus. Moreover, microinjection of anti-M1 antibodies into infected cells (which prevents newly synthesized M1 from entering the nucleus) results in the retention of the RNPs in the cell nucleus (19).

The characterization of the M1 mutant WSN *ts*51 raised questions about the role of M1 protein in RNP export (61). This mutant contains a single aa substitution in the M1 gene that causes hyperphosphorylation of the protein in infected cells (62). In contrast to the wt M1 protein, which is distributed into the cytosol and the nucleus, the mutant protein accumulates only in the nucleus. Despite the nuclear accumulation of M1 at restrictive temperature, the export of newly synthesized vRNPs into the cytoplasm is not affected (61,62). Therefore, from the results obtained with the *ts*51 mutant, it appears that only a few molecules of M1 need to be bound to the vRNPs to promote their export from the nucleus or that M1 mediates RNP export without forming a stable complex with RNPs.

Experiments involving heterokaryons have suggested a new role for the M1 protein related to the nuclear traffic of RNPs (55). When L929 cells infected with the WSN strain were fused with uninfected HeLa cells, the vRNPs that exited the L929 nucleus did not reenter the HeLa cell nucleus (55). A similar experiment was performed at a restricted temperature with the mutant *ts*51 (at this temperature the M1 is nuclear), and the RNPs reentered the HeLa cell nucleus. This effect was reversed if the HeLa cells expressed a recombinant M1 protein. Therefore, these experiments suggest that M1 protein prevents nuclear reimport of vRNPs. It should be mentioned that this M1-mediated effect is specific for the NP in RNPs complexes because fusion of cells expressing recombinant NP with HeLa cells expressing M1 does not interfere with the nuclear import of NP.

More recently, it has been shown that NS2, an influenza virus nuclear protein, is also involved in the nuclear export of RNPs (63). It is

well established that the HIV Rev protein contains an NES and that the function of the NES correlates with its ability to bind to nucleoporins (most of which are components of the NPC). With the use of the yeast two-hybrid system, it was observed that NS2, but neither NP nor M1 nor the polymerase subunits, was able to interact with the same nucleoporins as Rev protein does. Moreover, it was demonstrated that the N-terminal 30 residues of NS2 contain an NES signal. Importantly, it was shown that NS2 cooperates in the export of RNPs because microinjection of anti-NS2 antibodies into infected cells, which presumably sequestered the newly synthesized NS2 in the cytosol, blocked influenza virus RNP export. On the basis of evidence indicating that M1 is involved in RNP export and that NS2 interacts with M1 (64,65), it was suggested that the role of NS2 in RNP export may be mediated by interaction with M1 protein (63). Given the new role of the NS2 protein, O'Neill *et al.* (63) propose to rename the NS2 protein "influenza virus nuclear export protein (NEP)."

IV. Transcription and Replication: Structure and Regulation of RNP Complex

The influenza virus RNA polymerase complex is responsible for three separate activities during the virus infection cycle. Immediately after the parental vRNPs enter the cell nucleus, they are transcribed to produce the primary mRNAs (primary transcription). This step does not require newly synthesized viral protein. Instead, it provides viral factors, in particular the NP, needed to proceed to viral RNA replication. The switch to RNA replication includes three mechanistic changes compared to transcription. There is *de novo* initiation instead of priming with a capped primer, the RNA product is encapsidated by NP molecules, and there is no premature termination, leading to a complete positive-sense RNP (cRNP). These cRNPs are copied back in a similar fashion to yield large amounts of progeny vRNPs. Thus, RNA replication involves two phases, cRNP synthesis and vRNP amplification. Once RNA replication has taken place, the progeny vRNPs are used to transcribe large quantities of viral mRNAs (secondary transcription) before they migrate to the cytoplasm to participate in virion morphogenesis.

Therefore, a number of questions arise in regard to the regulation of these processes: What determines the switch from cap-primed initiation during transcription to *de novo* initiation during cRNP synthesis? How is this switch linked to RNP encapsidation and to antitermination? Is

the change from cRNP synthesis to vRNP synthesis regulated? If it is, what are the regulatory mechanisms? Does the change from transcriptase to replicase (and back to transcriptase) involve modifications of the polymerase complex (e.g., posttranslational modifications)? In the last few years, new information on some of these questions has been gathered and is described below.

A. Structural Studies of RNP

The overall conformation of the virus RNP has been analyzed by chemical and enzymatic probing of virion RNA before and after its binding to NP. The RNA–NP interaction lacks sequence specificity and leads to the destruction of any RNA secondary structure (66). The RNA binding domain of the NP has been mapped to the N-proximal sequences (positions 1–180) (67,68), and its interaction with the RNA backbone leave the bases exposed to the solvent, allowing the virion RNA present in the RNP complex to perform as a template in transcription and replication (66). As documented earlier, the RNP has a supercoiled circular conformation (69). Dissociation of the polymerase complex by detergent treatment determines a change to a linear form (70). In addition, removal of the polymerase correlates with the loss of the observed protection of the vRNA terminal sequences to chemical and enzymatic attack, suggesting that the polymerase complex is the factor responsible for the maintenance of the panhandle structure of the RNP (70).

The transcriptase present in the virion is a heterotrimer, and all three subunits seem to be required for replication because reconstitution of cRNP or vRNP synthesis from cloned genes demonstrated that omission of any of the polymerase subunits abolished the activity (71). With the use of a different experimental setting, some RNA synthesis activity was obtained with the isolated PB1 subunit (72,73) or by coexpression of PB1 and PA proteins (74), but the relevance of such activities in the context of the virus infection remains to be ascertained.

The structure of the polymerase complex has been studied by a variety of methods. The earlier observation that the PB1 subunit is the core of the complex (75) was confirmed independently (76,77), and the regions of each subunit involved in complex formation have been determined (76–80). The N-terminal sequences of the PB1 protein interact with the C-terminal region of the PA subunit, and sequences next to the C terminus of the PB1 protein interact with the N-terminal

region of the PB2 subunit (Fig. 2). This overall structure of the complex fits with other accumulated data because there is no apparent overlap of these interaction domains with other functional domains (see below). Although no experimental evidence has been reported for an interaction of isolated PA and PB2 proteins, its existence in the ternary complex cannot be excluded. Furthermore, the possible role of vRNA or cRNA in the structure of the complex(es) has not been considered, nor has the possible modifications of this overall structure in the processes of transcription and/or replication.

The interaction of the polymerase complex with vRNA in virion RNPs was studied by ultraviolet (UV) cross-linking. Direct contact of PB1 and PB2 subunits with the 3′-terminal sequences was observed, as well as interaction of all subunits with the 5′-terminal sequences (81,82). The polymerase complex prepared by expression of the three subunits with vaccinia recombinant viruses was used to study the interaction of the complex with model vRNA probes (83). Binding was observed with a 5′-terminal probe, but it was most efficient with a probe containing both termini of vRNA. Modification interference analyses identified specific residues at the 5′-terminal sequence that may be contact sites for the polymerase (83). These sequences correspond to the loop predicted in the panhandle model of the promoter (see below). These results support the implication that both ends of vRNA are

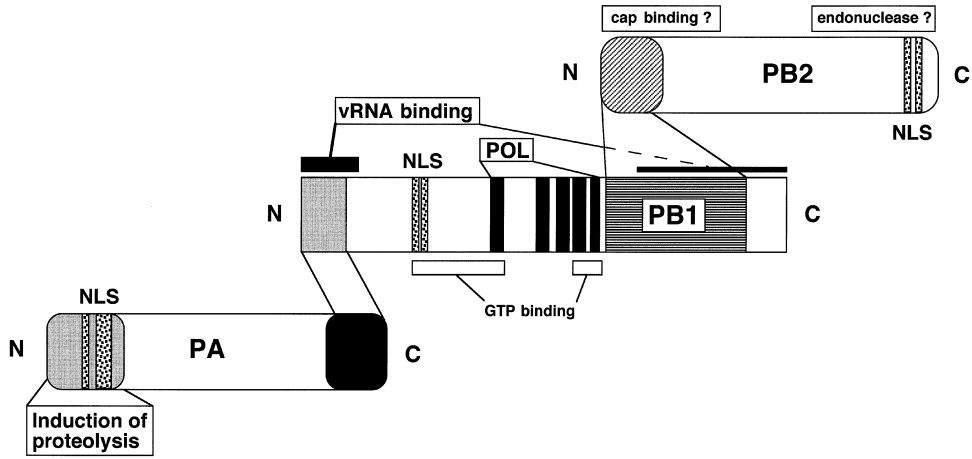

FIG 2. Overall organization of the influenza A polymerase complex. The regions of interaction among the polymerase subunits are shown, together with identified functional domains. See text for further details.

essential elements of the promoter, but they do not provide information on the role of each polymerase subunit in the recognition of the template. The *in vitro* interaction of isolated PB1 protein with vRNA probes has been studied (84). Specific binding was demonstrated, and an apparent dissociation constant around 10^{-8} *M* was determined. Binding to the 5′-terminal sequences was prominent, and binding to the 3′-terminal region was also detectable. Interestingly, the interaction with the 5′-terminal sequences allowed efficient binding to the 3′-terminal sequences. Two regions of the protein, located at the N and C termini of the PB1 subunit, were identified as responsible for binding (84) (Fig. 2). Much remains to be learned about the interactions of the polymerase complex with the template. In particular, it is important to identify the RNA binding domains in other subunits to better understand the structure of the polymerase–template complex(es) and their modification during transcription and replication (see below).

A number of functional domains of the polymerase subunits have been mapped. The sequence motifs predicted to be relevant to polymerase activity in the PB1 protein (85) were experimentally tested by site-directed mutagenesis and were found to be essential for the activity of an *in vivo* reconstituted polymerase with the use of the vaccinia-T7 expression system (86) (Fig. 2). These results are in agreement with the polymerase activity observed with short model templates when PB1 protein is expressed independently of the rest of the subunits (72). Earlier studies had shown that PB2 protein is the cap-binding subunit, and now this activity has been verified by expression of the isolated subunit (87). This activity and the phenotype of temperature-sensitive mutants in the PB2 gene indicate that this gene is involved in the initiation of transcription. Inhibition of this step *in vitro* by monoclonal antibodies directed to the N terminus of the protein reinforced this assumption (88). It is not known whether cap binding or cap snatching was inhibited by the anti-PB2 monoclonals, but the phenotype of a PB2 mutant at position 299 (79) and the limited homology of the PB2 subunit with the eIF-4E protein suggest that the cap-binding activity of PB2 may be located at its N-proximal region (Fig. 2). Localization of the cap-dependent endonuclease activity within the polymerase complex has been difficult because it depends on the presence of the complete heterotrimer and the vRNA template (89). Inhibition of the endonuclease activity with serum anti-PB2, but not with sera anti-PB1 or anti-PA, suggested that PB2 codes for such activity (90). Furthermore, an antipeptide serum specific for the C terminus of the protein inhibited the endonuclease activity but not the cap-binding activity *in vitro* (91) (Fig. 2).

The phenotype of temperature-sensitive mutants in the PA gene indicated that this subunit of the polymerase is involved in viral RNA replication, yet the only biochemical function identified so far for the PA protein has been its capacity to induce proteolysis. Thus, the PA subunit is a phosphorylated protein (92) whose coexpression with a number of other viral or nonviral proteins leads to their proteolytic degradation (93). The capacity of the protein to induce proteolysis was mapped to its N terminus (94), partially overlapping the sequences responsible for nuclear transport (95) (Fig. 2). The relevance of this biological activity of PA protein for viral RNA replication is still under investigation.

B. Structure of Promoter

In the last few years, considerable advances have taken place in the analysis of the promoter structure of viral RNA. In addition to the polymerase–vRNA template binding studies referred to above, *in vitro* RNA synthesis has been used to study the phenotype of mutant templates. In addition, the *in vivo* CAT activity obtained after reconstitution of the viral transcription–replication machinery with mutant model vCAT RNAs has been used to measure the activity of the promoter. Interpretation of these results is difficult because most of the *in vitro* studies were limited to primer-dependent initiations (transcription), and the *in vivo* studies involve transcription and replication processes that are difficult to distinguish.

In spite of earlier results indicating that the 3′ terminus of vRNA can promote transcription, it was later established that both vRNA terminal sequences are essential elements of the promoter (82,89,96). These results, together with those of the polymerase-vRNA binding studies described above, have clarified the role of different vRNA sequences in transcription initiation. The conformation of the 5′- and 3′-terminal sequences in the promoter has been a subject of debate in recent years. Since the time of the original proposal of the panhandle structure (69), a number of studies have shed light on its actual conformation and its relevance for transcription activity. The existence and structure of the panhandle in naked RNA were analyzed by chemical and enzymatic probing (66). The sequence arrangement obtained is essentially compatible with that determined by nuclear magnetic resonance (NMR) with a short model vRNA (97) and contains a distal stem including both RNA termini, an internal loop more extended in the 5′

arm, and an internal stem (Fig. 3). The functional relevance of such structural elements for the promoter activity has been tested by mutational analysis of model vRNAs, including single and multiple nucleotide exchanges, as well as double mutations that alter the sequence but preserve the structure of the stems (82,98–100). These results support the notion that the formation of the internal stem is essential for promoter activity and that this stem structure is more important than its sequence, although some sequence restrictions are apparent. By contrast, changes in the distal sequences (positions 1–7 from each end) did not substantially affect the activity of the promoter. These results led to the proposal of the fork model for the vRNA promoter (82,98,100) (Fig. 3). In an extensive mutation analysis with the use of *in vivo* CAT activity as a reporter, evidence has been presented supporting the presence of intrastrand base pairing in the terminal nine residues at each end of the vRNA (hook/corkscrew model) (101) (Fig. 3). Although a promoter-up mutant was used for these analyses, mutations intended to compensate for a theoretical distal stem did not rescue activity, in agreement with the fork model, but other single mutations were compensated for by secondary mutations that restored the presumptive intrastrand base pairs (101). Similar results were obtained for the 5′-terminal sequences of Thogoto virus vRNA (102), as predicted by an RNA folding algorithm (103).

A more precise definition of the promoter structure must await a detailed analysis with the use of *in vitro* and/or *in vivo* assays in which both transcription and replication can be measured independently. In this context, it should be mentioned that the nonconserved sequences present in the untranslated regions (UTRs) seem to be important in vRNA amplification (104).

C. Mechanistic Studies of Polymerase Complex

Some enzymatic properties of the transcriptase complex have been studied. The K_m values for the different triphosphates have been determined in complete transcription reactions with the use of RNPs from virions (105). The value found for ATP was 10-fold higher than the values obtained for the other NTPs. The most likely explanation for this difference is that the polymerase needs to undergo a conformational change to copy the template from position 4 to position 5. On the other hand, the endonuclease activity of the complex is unique in generating a 3′-OH terminated primer instead of the 2′-3′ cyclic form (106). In addition, the rate-limiting step of the enzyme is the release of the

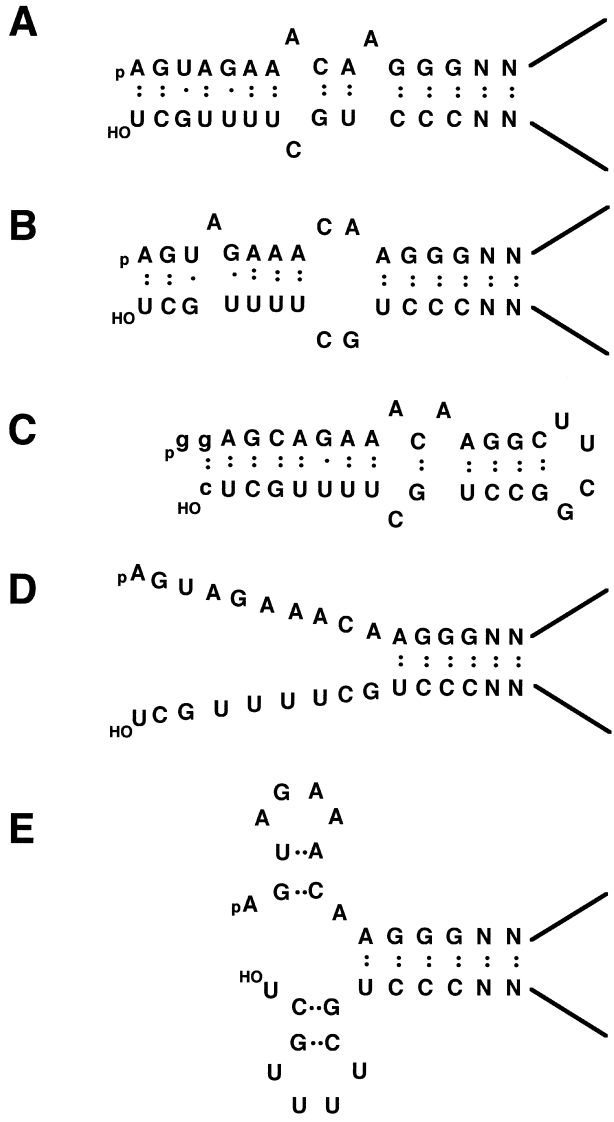

FIG 3. Alternative models for the secondary structure of the influenza virus vRNA promoter. Several models for the secondary structure of the interacting 5′- and 3′-terminal vRNA sequences are shown. (A) Original panhandle model (69). (B) Panhandle model as determined by RNA probing (66). (C) Panhandle model as determined by NMR (97). (D) Fork model (82). (E) Hook/corkscrew model (101,103).

capped oligonucleotide in agreement with its function as a primer (106). In the Thogoto orthomyxovirus, the viral mRNAs contain a cap structure at their 5′ terminus, but no extra sequences of cellular origin can be detected (107,108). *In vitro* transcription experiments indicate that the polymerase is able to steal the cap structure from exogenous mRNAs, generating a primer with just one nucleotide (preferentially A) downstream of the cap (109).

In spite of the reported heterogeneity of the sequences of the cellular primers used by the transcriptase, some preferences have been described. The influence of the mRNA sequence on its cleavability and the capacity of the capped oligonucleotide to serve as a primer have been studied *in vitro.* The complementarity of the sequence around the cleavage site to the 3′-terminal sequence of the vRNA is not important for cleavage, but it is essential for elongation of the capped oligonucleotide by the polymerase (110). In agreement with these results, the endonuclease activity of the polymerase required interaction with the 5′- and 3′-terminal sequences of the vRNA template, whereas the binding ability of the capped RNA required binding only to the 5′ end of vRNA (96). In fact, the interaction of the polymerase with the 5′-terminal sequence of vRNA appears to be essential in defining a transcriptase mode. Thus, mutations that affect the formation of the 5′ hook structure (see above) abolish the transcriptase activity of Thogoto vRNA *in vitro,* and the introduction of mutations leading to a hook structure in cRNA allows its use as template for transcription (111). In addition, alterations of the 5′-terminal sequence known to affect interaction with the polymerase affect the polyadenylation of mRNA *in vitro* (112), and mutations at the internal stem lead to diminished mRNA levels *in vivo* (113). It is not known whether the interaction of the PB1 subunit with the 5′-terminal sequence of vRNA (see above) is responsible for these effects or whether interactions with other subunits of the polymerase are also involved.

The switch from the transcriptase to the replicase mode implies a change to *de novo* initiation, coupling of elongation to encapsidation, and inhibition of the polyadenylation step. Such a switch may involve alterations in the polymerase complex, the participation of other viral proteins in RNA synthesis, and the participation of specific cellular factors. The phosphorylation of the PA subunit of the polymerase (92) might correlate with its role in viral RNA replication, but definite evidence is still lacking. The interaction of NP with the PB1 and PB2 subunits of polymerase in infected cells (114) is in line with the established requirement of newly synthesized NP for antitermination (8). In addition, a role for NS1 protein in the transcription–replication

switch is also possible in view of its association with RNPs in infected cells (115). In regard to the implication of cellular factors, a number of protein fractions have been isolated biochemically by complementation of the replication activity of RNPs *in vitro* (116,117). The characterization of these protein fractions will provide a better understanding of the transcription–replication switch.

V. Virus Assembly

Three major components are thought to be involved in the formation of influenza virus particles at the plasma membrane: (i) the cell surface-expressed integral membrane proteins HA, NA, and M2; (ii) the M1 protein; and (iii) the viral RNP/M1/NS2 complexes. During the maturation process, influenza virus HA and NA proteins are concentrated at the cell surface in areas active in virus assembly from which cellular proteins are efficiently excluded. The M1 protein associates with membranes in a manner characteristic of integral or peripheral membrane proteins even in the absence of other viral proteins (118–120) and is therefore thought to be the connecting element between the transmembrane-cytoplasmic tail regions of the surface glycoproteins and the viral RNPs. Direct interaction of M1 with HA and NA could not be demonstrated, but it was shown that the membrane association of M1 was stimulated by HA and NA expression (119); however, similar studies with the use of other cell lines did not confirm this result for unknown reasons (120,121). The high level of sequence conservation of the short cytoplasmic tail of HA (10–11 aa) and NA (6 aa) among all the influenza virus subtypes and the presence of three palmitylated cysteine residues in all HA tails suggest an important role for these domains in virus replication (122,123), but several groups reported the rescue of mutant viruses lacking either one or both tail domains (124–127). The HA tail deletion mutant (HA tail$^-$) showed normal HA incorporation and was only slightly less infectious than wt virus (124,127). The NA tail deletion mutant (NA tail$^-$) generated by two groups, as well as the mutant lacking both tails (HA/NA tail$^-$), produced about 10-fold fewer particles in tissue culture with a fairly normal protein content (125,127). The wt virus and the HA tail$^-$ virus formed mostly normal spherical particles, whereas the NA tail$^-$ mutant showed a tendency to form elongated, irregularly shaped particles (127) that aggregated in filaments at the cell surface (126). Furthermore, in the case of the HA/NA tail$^-$ mutant, the particle morphology was drastically altered and elongated; extended particles of irregular shape were frequently seen (127). Although the deletion of the HA cytoplasmic tail

did not affect viral growth in tissue culture, it would be risky to claim that this domain is not important. A revertant virus in which a point mutation restored the cytoplasmic tail could be selected (124), and in two cases it was reported that mutants with changes in the conserved cysteine residues reverted to cysteine (128,129), suggesting that the possession of a palmitylated cytoplasmic HA tail is preferred. In conclusion, these results suggest that normal budding requires the presence of the cytoplasmic tails as adapters for the inner viral protein (M1 protein). Both the NA tail and the HA tail can interact with M1 independently, and having one of the two tails seems to be sufficient for normal budding, although the NA tail is more important than the HA tail. In support of this finding, Yang *et al.* (130) described mutant viruses that were selected in the presence of NA-specific antibodies and exogenous neuraminidase. Several independent mutants with large internal deletions in the NA gene were isolated, but in all cases the sequence coding for the TM domain and the cytoplasmic tail was maintained. Thus, it might be concluded that this amino-terminal domain of NA is absolutely required for efficient virus assembly. In addition to the cytoplasmic tail domain of NA and HA, it was found that the filamentous morphology of influenza virus particles may also depend on the M1 and/or the M2 protein, and that the morphology of the influenza virus particle is determined not only by viral structural proteins but also by host cell factors (131 and references therein). Whereas spherical particles are usually found in laboratory-adapted strains, the virions from original human isolates and early egg-passaged virions are usually filamentous (131 and references therein). What mechanisms are responsible for the exclusion of cellular proteins from the virions? *In vivo* complementation experiments have shown that chimeric HAs with heterologous TM domains and/or cytoplasmic tails were basically excluded from the viral surface (132), suggesting that nonspecific sequences in this region are not compatible for interaction with M1. Interestingly, only a few copies of M2 are found in virions even though significant amounts of M2 are expressed on the cell surface. The mechanisms for this partial exclusion of M2 are not well understood. However, *in vivo* complementation experiments showed that the ectodomain of M2 was required for viral incorporation but that neither the TM region nor the cytoplasmic tail was needed (32). Thus, a weak interaction of the M2 extracellular domain with HA and/or NA may be responsible for the limited incorporation of M2.

A. Random or Selective Packaging of Viral RNPs

One possible model for efficient packaging is that membrane-associated M1–surface protein complexes attract cytoplasmic M1/RNP/

NS2 (NEP) complexes by M1–M1 or M1–NS2 (NEP) interaction. As previously mentioned, M1 and NS2 (NEP) are involved in the nuclear export of viral RNPs (60,63). However, such protein–RNP complexes might be packaged randomly or in a segment-specific manner. Both mechanisms are in agreement with the observation that the RNA segments in a stock of purified virus are represented in equimolar amounts. In selective packaging, all virions would contain the full set of eight RNA segments as the result of a gene-specific recognition and counting mechanism. Alternatively, random packaging of more than eight RNPs, in which only a few of the particles contain the eight different genes, is possible. It was calculated that the random packaging of 10–11 RNPs per virion would cause about 5–10% of the particles to be infectious (133), which is in agreement with published ratios of infectious to noninfectious particles (134,135). Most of the results in favor of the random packaging model have been obtained by reverse genetics. It was shown that a heterologous gene flanked by the influenza 5′ to 3′ virus-specific nontranslated sequences of NS-RNA was amplified and packaged into virions as an additional segment when infected with a helper virus (136) or as the only segment if all the structural proteins were provided *in trans* (137). These results suggest that the noncoding regions are sufficient for packaging. Interestingly, mutant viruses that contained the NA gene flanked by the noncoding regions of the PB1-RNA, the NS-RNA (104), or the NS-RNA of the influenza B virus were rescued (138). These mutant viruses showed a reduction in the level of NA-specific viral RNA in the cytoplasm that correlated with a reduced level in the virions, which would be expected in random packaging. The fact that these mutant viruses contain two segments with the same noncoding regions (e.g., PB1 and NA-PB1) indicates that these regions cannot contain the signal for segment specific-packaging or suggests that a putative recognition sequence extends into the coding regions. Using another approach, Enami and colleagues (133) generated a mutant virus that expressed functional NS1 and NS2 mRNAs from two separated segments and not from one segment, as in the wt virus. Thus, this virus must contain nine different segment rather than eight to be infectious. The ratio of infectious to noninfectious particles in this NS mutant virus was lower than that of the wt virus in agreement with random packaging of 10–11 RNPs per virion.

Support for selective packaging comes from reports showing that the levels of individual RNA segments in infected cells are not equimolar and thus differ from those in the virions (139). It was proposed that the longest segments 1, 2, and 3 were underrepresented in the viral particles because of the presence of defective RNAs that may compete

for packaging in a segment-specific manner. Duhaut and McCauley reported the generation of avian influenza virus clones that contained, in addition to the eight standard segments, a single extra defective RNA of segment 1 or 2 (135). In two cases, it was shown that the presence of this defective RNA did not inhibit the accumulation of the parental RNA in the infected cell but instead competed efficiently for its packaging in a segment-specific manner. Similar observation were made previously by others (140; for review, see 141). These results suggest that the packaging of influenza virus segments is not a purely random process and also suggest the existence of a segment-specific packaging signal. In summary, with the cumulative data presented up to now, it is not possible to reach any firm conclusion concerning the existence of regulation in the packaging of the various vRNPs into the influenza virion.

VI. Future Prospects

The disease caused by influenza virus is still a major worldwide human health threat. Prevention through vaccination, although effective, is not optimal, and there are no effective drugs to control the disease. Therefore, there are not only basic biological questions but also practical problems that justify intense influenza virus research.

The three-dimensional structures of an NS1 protein domain, the M1 protein, and the low pH conformation of HA have been determined (142–144). These structures have been added to those of the viral proteins HA and NA that were determined previously. We predict that a major effort will be made to determine the three-dimensional structures of M2, NS2, NP, and the three polymerase subunits (or fragments thereof). This information, together with that gained from the numerous biochemical studies already carried out on these proteins (particularly the M2 protein and the polymerase subunits), will allow the identification of the proteins' functional domains. Moreover, knowledge of these structures will help us to design specific compounds that can interfere with virus replication, as has been done with drugs targeted to the NA, that appear to have pharmaceutical potential. We are still far from having a detailed picture of the mechanisms by which M1 and NS2 help to modulate RNPs transport. Moreover, many questions remain to be answered regarding the role of the different NP nuclear accumulation signals during the viral replication cycle. Further studies are needed, and the information obtained could also shed light on the mechanism and the proteins that modulate nucleocytoplasmic trans-

port of proteins and RNAs in eukaryotic cells. A picture of the organization of the polymerase complex is starting to emerge, but we lack structural data. The interaction domains, as well as a number of functional domains, have been grossly defined. This information will be especially valuable when added to the three-dimensional structure, even a low-resolution one. Some aspects of the regulation of transcription and replication of the influenza virus genome are also being elucidated, but as discussed above, many questions remain. A number of reports have focused on the process of virus assembly. It is anticipated that studies (involving both genetically altered viruses and biochemical assays) aimed at identifying the protein–protein and protein–RNA interactions that determine virus assembly will continue.

Influenza virus was the first example of a negative-sense, single-stranded RNA whose genome was altered with the use of DNA recombinant technology (136). Since then, it has been possible to substitute RNAs derived from genes cloned in plasmids for several of the viral segments. However, the efficiency of the rescue process is still low and depends on the availability of a selection strategy (145). The rescue of several negative-sense, single-stranded RNA viruses with the use of viral genes cloned in plasmids has been described, but such a method is not yet established for influenza virus. This is an active field of research because the availability of a method to recover an infectious influenza virus entirely from cDNA would speed up the preparation of genetically altered viruses. This would allow the controlled preparation of attenuated vaccines and should permit proper evaluation of the usefulness of influenza virus as a vector for expression of foreign genes.

Acknowledgments

The work of the authors' group was supported by Programa Sectorial de Promoción General del Conocimiento (Grants PB94-1542 and PM-0015), Comunidad Autónoma de Madrid (Grant A0063), and Fondo de Investigaciones Sanitarias (Grant 98/0315). T. Z. was supported by a fellowship from the Swiss National Foundation (823A050353).

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ADVANCES IN VIRUS RESEARCH, VOL. 54

INFLUENZA VACCINES: PRESENT AND FUTURE

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I. Introduction

Influenza viruses cause sporadic, epidemic, and pandemic outbreaks of disease with significant attendant morbidity and mortality. Although the very young and the very old are at greatest risk for serious morbidity and mortality, people of all ages can be infected, and school-age children have been proposed to be the main vectors for the spread of influenza in the community (1). The two principal options for controlling influenza are vaccines and antiviral drugs. The following discussion will focus on vaccines.

Three types of influenza viruses circulate and cause disease in humans: A, B, and C. Influenza C appears to cause minor illness, but annual outbreaks of influenza A and B disease result in a significant disease burden. Since 1977, influenza A H1N1 and H3N2 viruses have been circulating together with influenza B viruses. The viral antigens that are the targets of the protective immune response are hemagglutinin (HA) and neuraminidase (NA). There are two major mechanisms by

which influenza viruses undergo antigenic change to evade immunity: antigenic shift and antigenic drift. Antigenic shift, a rare phenomenon seen only with influenza A viruses, occurs when a virus containing a novel HA, with or without a novel NA, is introduced into the human population. This occurs either as a result of genetic reassortment between a human influenza A virus and a nonhuman influenza A virus (2) or by the direct introduction of a nonhuman influenza virus into the human population (3–5). Antigenic drift is much more common and is an almost continuous process that results when point mutations occur in genes encoding the HA or NA molecules of influenza A or B viruses, resulting in mutations in two or more antibody-binding sites that allow the virus to evade neutralization by preexisting antibodies.

Some considerations unique to the field of influenza vaccines are the need for a multivalent vaccine to protect against both influenza A and influenza B strains and the need to modify the composition of the vaccine to keep pace with antigenic drift (6). Despite the use of standardized amounts of HA, the inclusion of different antigens in vaccines can create difficulties in comparing the efficacy of different preparations of influenza vaccines. The closeness of the antigenic match between the vaccine strain and the epidemic strain is an important determinant of the efficacy of influenza vaccines.

The World Health Organization (WHO) coordinates a global influenza surveillance network of national collaborating laboratories and four collaborating centers at which influenza viruses from around the world are analyzed antigenically and genetically. The goals of the network are to identify viruses with novel HA or NA types (antigenic shift) and to monitor the appearance and spread of antigenic variants of the same subtype (antigenic drift). The WHO, the Food and Drug Administration (FDA) in the United States, and responsible authorities in other countries recommend the influenza viruses (A and B) to be included in the annual vaccine formulation (7,8). The committees base their recommendations about vaccine composition primarily on (1) antigenic analyses, (2) genetic analyses, (3) epidemiologic data, and (4) serologic data from volunteers who were vaccinated with the previous year's vaccine. An additional requirement is that the viruses used in the vaccine formulation be isolated and passed in eggs rather than in a continuous cell line, a condition imposed by regulatory considerations.

Antibodies directed against the HA have neutralizing activity (9). Anti-NA antibodies may also play a role in modulating the severity of disease (10). In the mouse model of influenza, antibodies protect against infection, and both antibodies and cytotoxic T cells (CTLs) play roles in recovery (11,12). The most commonly measured correlate of protec-

tion in humans is the serum hemagglutination inhibition (HAI) antibody titer; susceptibility to infection is inversely related to HAI antibody titers (6,13–15). Postvaccination titers of 1 : 30 to 1 : 40 represent the 50% protective level of antibody (13,16). Nearly all influenza vaccines in use or in development aim to elicit an anti-HA immune response. Experimental vaccines that elicit a mucosal antibody response and those that elicit a cellular immune response against CTL epitopes present on internal proteins may provide cross-protection against antigenically drifted strains.

II. Trivalent Inactivated Vaccine

The only influenza virus vaccine that is currently licensed in the United States is a trivalent formalin-inactivated (TIV) preparation that includes influenza A (H3N2), influenza A (H1N1), and influenza B virus components. Because this vaccine has been discussed at length in several articles and textbooks (17–19), comments in this review are limited to a few salient points.

A. Overview

1. Principle

A protective, HA-specific antibody response is elicited by intramuscularly administered killed influenza virus vaccines.

2. Historical Development and TIV Formulations

Historically, the vaccine was standardized in the United States by chicken red blood cell agglutinating units (CCA), but since 1980–1981, vaccines have been standardized to micrograms of HA, and the recommended dose is 15 μg of each HA per 0.5 ml. The vaccine is available as both a whole virus and a split product (or subvirion) preparation; the split product vaccine results in fewer local and systemic side effects and is recommended for use in children. "High-growth reassortant" viruses are generated bearing the HA and NA gene segments of the wild-type (wt) influenza A viruses and at least the M gene segment of the influenza A/Puerto Rico/8/34 (H1N1) virus (PR8) because (1) protective humoral immunity in influenza is directed against the HA and NA but (2) growth to high titer in eggs, a desirable characteristic in candidate vaccines, is conferred primarily by the M gene segment of PR8. Wt viruses are used for the influenza B virus component of the TIV.

B. Clinical Evaluations

1. In Children

In 1976, influenza A/NJ/76 (H1N1) vaccines were tested in 2300 healthy children (20) and 1200 children with high-risk conditions such as cardiac, pulmonary, and hematologic diseases and cancers, that increased the risk of severe illness or complications from influenza (21–26). These and similar studies (27–32) established the safety and immunogenicity of the vaccine but noted high rates of side effects of the vaccines in younger children and resulted in the recommendation to use split product vaccines in children <13 years of age (20). Immunogenicity studies with the A/NJ/76 and subsequently with A/USSR/77 vaccines in healthy children also established the need for two doses of vaccine in unprimed persons (20,27). The immunogenicity of TIV in infants with bronchopulmonary dysplasia and congestive heart disease is variable and is dependent on the age of the child and the vaccine antigen (33,34). Efficacy of TIV in children was inferred from the immunogenicity data collected from vaccine trials in children and by extrapolation from efficacy trials in adults.

2. In Adults

Extensive experience has been gathered after decades of use of TIV in adults (reviewed in several virology and vaccine textbooks, 17–19). In general, these vaccines induce immunity in 60–90% of recipients, and efficacy rates of 70–90% are reported (35). Persons vaccinated for the first time had higher antibody titer increases than those who had been vaccinated previously (36–38), but mean postvaccination titers achieved in both groups were similar (36), and influenza infection rates in revaccinated adults were lower than in first-time vaccinees (37). Immunogenicity is often reduced in the elderly; a higher dose of TIV (39,40) or of a highly purified HA in the elderly and the very young (41,42) resulted in improved immunogenicity.

3. Effectiveness

Vaccine effectiveness endpoints differ in different age groups. In children, influenza vaccination decreased the incidence of acute otitis media during the influenza season in two studies (43,44); among elderly persons, immunization was associated with reductions in the rates of hospitalization (45–48) and death from influenza (45–51) and related complications and was cost effective (52). In healthy working adults 18–64 years of age, influenza vaccination resulted in fewer episodes of

upper respiratory illness (53), fewer days of sick leave from work, and fewer visits to physicians' offices for upper respiratory illnesses (54).

4. Indications and Contraindications

Tables I and II list the patient groups in whom TIV is recommended to prevent influenza. In the United States, TIV is recommended for use in people with a variety of high-risk conditions (Table II). Although human immunodeficiency virus (HIV)-infected persons develop rises in antibody titers to influenza following TIV (55), literature reports about the effect of influenza immunization on the viral load of HIV-1 are contradictory (56–58). Because the vaccine is grown in eggs, it is

TABLE I

RECOMMENDATIONS FOR USE OF INFLUENZA VACCINE[a]

Advisory Committee on Immunization Practices (ACIP)	Red Book Committee (Committee on Infectious Diseases) of the American Academy of Pediatrics
Strongly recommended for: Any person ≥6 months of age who is at increased risk for complications of influenza (see Table II)	*Recommended for:* Children ≥6 months of age with one or more specific risk factors (see Table II) Health-care personnel in contact with pediatric patients. Household contacts of high-risk children Children who are members of households with high-risk adults
Should be used in: Health care workers and others (household members) in close contact with persons in high-risk groups	*Should be considered in:* Persons traveling to foreign areas in which influenza outbreaks are or may be occurring Groups of persons whose close contact facilitates rapid transmission and spread, such as college or school students, particularly those who reside in dormitories; members of athletic teams; and persons living in residential institutions
May be administered to: Any person who wishes to reduce the chance of becoming infected with influenza	*May be administered to:* Any healthy child or adolescent

[a] From Refs. 59 and 60.

TABLE II

GROUPS AT INCREASED RISK FOR COMPLICATIONS OF INFLUENZA[a]

Advisory Committee on Immunization Practices (ACIP)	The Red Book Committee (Committee on Infectious Disease) of the American Academy of Pediatrics
Persons aged ≥65 years	Asthma and other chronic pulmonary disorders
Residents of nursing homes and chronic-care facilities who have chronic medical conditions	Hemodynamically significant heart disease
Persons with chronic pulmonary and cardiovascular disorders, including asthma	Immunosuppressive disorders and therapy
Persons with chronic metabolic diseases, including diabetes mellitus, renal dysfunction, hemoglobinopathies, and immunosuppression, including that induced by medication	HIV infection
Children (6 months to 18 years old) on long-term aspirin therapy (due to the risk of Reye's syndrome)	Sickle cell anemia and other hemoglobinopathies
Women who will be in the second or third trimester of pregnancy during the influenza season	Diseases requiring long-term aspirin therapy (due to the risk of Reye's syndrome)
	Children who should be considered potentially at risk for complicated influenza illness:
	Diabetes mellitus
	Chronic renal disease
	Chronic metabolic disease
	Any underlying condition that may compromise children, including young age (<1 year)
	Pregnancy (see ACIP recommendation)

[a] From Refs. 59 and 60.

contraindicated in persons with serious egg allergies. An association with an increased incidence of Guillain-Barré syndrome was described with the 1976 swine influenza vaccine, but a causal association with subsequent vaccine strains is less clear (59,60).

5. Disadvantages

The disadvantages of TIV are mild local reactions at the injection site; low mucosal immunoglobulin A (IgA) antibody responses; a poor

cell-mediated immune response; varying efficacy, which ranges from 70% to 90% in most studies; and reduced immunogenicity and efficacy in the elderly (13). A practical disadvantage is the dependence on the supply of embryonated eggs. Furthermore, growth of influenza viruses in eggs selects for mutations in the HA gene; egg-grown variants can also be distinguished antigenically from the epidemic strain (61,62).

There is controversy regarding the benefits of repeated annual influenza immunization; one study showed a decrease in vaccine effectiveness in children who had been vaccinated previously compared with those who were vaccinated for the first time (63). When rises in antibody titer were measured as a predictor of vaccine efficacy, some investigators found that repeated annual vaccination resulted in lower antibody titers (38,64), whereas others did not (36,65).

Despite the safety and efficacy profile and recommendations for its use, TIV is underused in the target population. A discussion of the reasons for and strategies to address the underuse of the vaccine are important areas beyond the scope of this review (66).

III. Recombinant and Subunit Vaccines

Although the licensed influenza vaccine is safe, immunogenic, and effective, advances in the fields of vaccine research and biotechnology that can improve options for preventing influenza are being actively pursued. Several experimental approaches with the use of recombinant influenza protein vaccines have been reported, some have been evaluated in animal models, and others have been tested in clinical trials in human volunteers. For example, purified nucleoprotein (NP) (67), chimeric proteins expressing HA or NP epitopes in the flagellin gene of *Salmonella* (68), and a recombinant modified vaccinia virus vector (MVA) expressing HA and NP (69) were evaluated in mice. Baculovirus expressed H1 and H3 HA (70,71), and a recombinant protein consisting of the carboxyl terminal of the HA2 subunit fused to the amino-terminal residues of the NS1 nonstructural protein (72) was evaluated in small-scale trials in human volunteers.

Alternative methods of vaccine delivery to improve immunogenicity, such as encapsulation of formalin-inactivated vaccines in biodegradable polymeric microspheres (73) or interleukin-2 (IL-2) liposomes (74) and iscoms (75) showed promise in mice and cynomolgus macaque monkeys. An iscom vaccine is licensed for use in Europe.

Efforts to develop vaccine alternatives to the TIV, including live attenuated vaccines, have been underway for several decades. The cold-

adapted influenza vaccine has been under development for nearly 30 years and, in its current form as a trivalent formulation, is a promising live attenuated influenza vaccine candidate.

IV. Live Attenuated Influenza Vaccines

A. Overview

1. Principle

The principle underlying the use of intranasally administered attenuated viruses as vaccines is that the vaccine virus is sufficiently attenuated to be restricted in replication to the upper respiratory tract, yet is sufficiently immunogenic to elicit a specific protective immune response. The most promising live attenuated influenza vaccine (LAIV) candidate is the cold-adapted (ca) influenza vaccine. The current formulation of the ca vaccine is a trivalent preparation comprising reassortant ca viruses bearing the HA and NA genes of the recommended influenza A (H1N1 and H3N2) and influenza B viruses.

2. Historical Development and Generation of ca LAIV Formulations

The ca reassortant viruses in the trivalent vaccine, derived by genetic reassortment between appropriate wt influenza viruses and the ca "master donor" strains, bear the HA and NA genes of the wt viruses and attenuating internal gene segments of the master donor strains. The two master donor strains that have been evaluated in the United States are an influenza A virus, A/Ann Arbor/6/60 (H2N2), and an influenza B virus, B/Ann Arbor/1/66, which were derived by serial passage of wt viruses at successively lower temperatures by Dr. H. F. Maassab (76). Both viruses replicate efficiently at 32°C and display three important phenotypes: the virus is *temperature sensitive* (ts), which describes a >100-fold reduction in virus titer at 39°C; *cold-adapted* (ca), which describes efficient replication of the virus at 25°C; and *attenuated* (att), which describes decreased replication of the virus in the respiratory tracts of mice, ferrets, and humans. These three characteristics of the ca viruses can be reliably and reproducibly transferred to reassortant viruses by transfer of the six internal gene segments (77). An additional property of the ca LAIV is "transdominance," the ability of the ca virus to interfere with the replication of wt influenza A viruses in mixed infections (78). Transdominance of the ca LAIV was demonstrated *in vitro* and in ferrets but not to a significant degree in humans (79,80). Russian investigators independently developed influenza A/Leningrad/134/47/57 (H2N2) and B/USSR/60/69 ca master do-

nor strains, from which reassortant trivalent vaccine strains were generated and evaluated; these vaccines have been in widespread use in Russia for several years (81).

3. Genetic Basis for Phenotypic Properties

The ts phenotype of the influenza A/Ann Arbor/6/60 ca master donor strain was specified independently by the PB2 and PB1 gene segments, the ca phenotype was specified by the PA gene segment, and the att phenotype was specified independently by the PB2, PB1, and PA gene segments (82,83). The nucleotide sequences of the A/Ann Arbor/6/60 ca virus and the wt virus from which it was derived by serial passage were compared, and coding mutations were identified in each gene segment (84,85). When the sequence analysis results are combined with those from the analysis of the single gene reassortant viruses, the amino acid residues that are probably responsible for the ts, ca, and att phenotypes of the A/Ann Arbor/6/60 ca master donor strain can be deduced.

Although sequence analysis identified 105 differences in five of six internal protein genes of the influenza B/Ann Arbor/1/66 ca master donor strain compared with its wt parent (86), it is not known which mutations encode the ca and att phenotypes of the ca influenza B virus.

B. Clinical Evaluations

1. Clinical Trials

LAIV were evaluated in clinical trials, with specific attention to safety and reactogenicity, infectivity, immunogenicity, genetic stability, transmissibility, efficacy, and interference with routine childhood vaccination. Clinical trials with experimental influenza virus vaccines were first carried out in healthy adults and proceeded in a stepwise fashion to include successively younger children (children >36 months, 6–36 months, and <6 months of age). Because children shed influenza viruses for 7–10 days, clinical trials in children provide the most sensitive data on the attenuation and genetic stability of LAIV. However, assessment of vaccines in children is complicated by high rates of intercurrent respiratory illness, and the data from pediatric trials may not be uniformly applicable to adults. The infectivity of ca LAIV is 100-fold lower in children than in adults (87–90). Although the data presented in this review are limited to a summary of the published vaccine trials in children, the ca LAIV has been evaluated extensively in adults; more than 65 clinical trials have been carried out, over more than 20 years,

in volunteers of different ages (77). Clinical studies of ca LAIV in the elderly indicate that the immune response induced was short-lived (91) and that the combined use of TIV and ca LAIV was better than the use of either one alone (92).

Early clinical trials that established the 50% human infectious dose (HID_{50}) safety, tolerability, immunogenicity, and reproducibility of the principle of generating attenuated ca vaccine strains with HA and NA gene segments of different wt viruses were carried out with monovalent LAIV preparations (A/H1N1, A/H3N2, or B). In the next phases of development, bivalent influenza A vaccines containing appropriate titers of H1N1 and H3N2 viruses and, subsequently, trivalent combinations of A/H1N1, A/H3N2, and B viruses were evaluated in clinical trials.

2. Safety

In more than 25 studies involving 3360 infants and children and including more than 108 infants less than 6 months of age (87–89, 93–116), the ca LAIV vaccine was safe and well tolerated. Mild rhinorrhea and low-grade fever were observed in some patients, but high fever and symptoms of lower respiratory tract involvement were not reported.

3. Infectivity and Immunogenicity

The infectivity and immunogenicity of the A/H1, A/H3, and B components of monovalent, bivalent influenza A, and trivalent ca LAIV trials in children immunized with doses $\geq 10^{6.0}$ $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$) are summarized in Table III. Some of the important determinants of immunogenicity of LAIV in seronegative subjects are the vaccine antigen used, age of the subject, titer of LAIV vaccine doses administered, antigens used to measure the antibody response, treatment of antigens used to measure the serologic response, vaccine manufacturer, and mode of administration (nose drops vs. spray) (117). In general, rates of seroconversion ($\geq$fourfold titer rise) tend to be lower in previously seropositive vaccinees than in previously seronegative vaccinees, but the vaccine appears to be efficacious even in previously seropositive vaccinees who fail to demonstrate a fourfold titer rise.

4. Efficacy

In various clinical trials, the efficacy of LAIV has been defined as the ability of the vaccine to protect against illness, against culture-proven influenza, or against serologic evidence of an influenza infection. In clinical trials in children, the efficacy of influenza vaccines was measured against "natural challenge," on exposure to epidemic influ-

TABLE III

INFECTIVITY[a] AND IMMUNOGENICITY[b] OF COLD-ADAPTED LIVE ATTENUATED INFLUENZA VACCINE COMPONENTS IN CHILDREN[c]

Influenza type and subtype	Infectivity			Immunogenicity		
	Monovalent	Bivalent	Trivalent	Monovalent	Bivalent	Trivalent
A/H1N1	56% ± 8%	68% ± 24%	80% ± 15%	71% ± 3%	55% ± 9%	48% ± 20%
A/H3N2	76% ± 11%	89% ± 8%	100% ± 0%	94% ± 3%	70% ± 24%	96% ± 3%
B	81% ± 10%		50% ± 32%	47% ± 25%		54% ± 22%

[a] Infectivity of the vaccine is the percentage of vaccinated subjects who shed vaccine virus in the 10 days following vaccination.

[b] Immunogenicity of the vaccine is the percentage of vaccinees in whom a fourfold rise in serum antibody titer was detected.

[c] Immunized with $\geq 10^{6.0}$ $\log_{10}$ $TCID_{50}$ of vaccine virus in monovalent,[d] Bivalent[e] influenza A (H1N1 and H3N2) and trivalent[f] formulations.

[d] Refs. 87–89, 93–95, 97, 101, 103, 104, 107, 113, 114.

[e] Refs. 99, 102, 105, 108, 114, 117.

[f] Refs. 107, 109, 115, 116.

enza in the community, or against "experimental challenge" with the attenuated ca vaccine virus. The efficacy of each component of a multivalent LAIV depends on the actual antigen used, and as with TIV, the efficacy is influenced by the closeness of the antigenic match between the vaccine strain and epidemic strains. Two large-scale field studies of the efficacy of the ca LAIV have been reported; one evaluated a bivalent influenza A vaccine (110), and the other evaluated a trivalent preparation (116). The first was a comparative trial of TIV and a bivalent ca influenza A vaccine carried out over 5 years in 5210 normal persons ranging in age from 1 to 65 years (110). The measures of efficacy used in this study were culture-positive influenza illness and infection defined by a fourfold rise in antibody titer during the influenza season. The efficacies of TIV and bivalent ca LAIV were equivalent against culture-positive H3N2 illness and against H1N1 influenza infections, regardless of the definition of the illness. The ca LAIV was less efficacious than TIV in preventing serologically defined H3N2 infections (110). Much higher efficacy rates were reported for a trivalent ca LAIV in a multicenter trial in which 1070 young children received the vaccine and 532 received a placebo (116). Vaccine efficacy in children who received two doses of vaccine, measured as protection against culture-positive influenza, was 96% (95% confidence interval 90–99%) against influenza A (H3N2) and 91% (95% confidence interval 78–96%) against influenza B (116).

5. Use in High-Risk Persons

Evaluation of the ca LAIV in high-risk patient groups in the United States has been limited to studies of asthmatic adults (118); these patients tolerated the vaccine well. Studies carried out in Japan using the U.S. ca LAIV in asthmatic children and children with severe psychomotor retardation indicated that the vaccine was safe in these patient groups and was effective against nosocomial outbreaks of influenza (119,120). The ca LAIV was also evaluated in elderly persons who had chronic cardiac, pulmonary, endocrine, or hematologic conditions (121).

6. Advantages

Intranasally administered ca LAIV can elicit a mucosal immune response in addition to the systemic immune response that involves both the humoral and cellular arms of the immune system. In practical terms, the ease of administration and the acceptability of an intranasally administered vaccine are advantages over parenterally administered TIV. Theoretically, a live virus vaccine will elicit a longer-lasting or broader immune response than TIV, which could translate into the

ability to provide protection for more than one season or from influenza viruses that have undergone greater antigenic drift. Also, by analogy with oral polio vaccines, the use of LAIV could provide herd immunity and help to limit the spread of an epidemic. Clinical evidence must be gathered to support these hypotheses.

7. Disadvantages

The primary concern about the widespread use of LAIV is the genetic stability of the att phenotype of the vaccine virus, with the attendant risk of reversion to the growth characteristics and virulence of a wt influenza virus. The genetic stability of the ts and ca phenotypes of the ca LAIV was monitored extensively in clinical trials, as surrogate markers for the att phenotype and the ca LAIV have been genetically stable. Another concern about the ca LAIV is the risk of transmissibility of the vaccine virus to unvaccinated contacts. The vaccine was not transmitted from vaccinees to placebo recipients in several clinical trials (89,103,104,107,112,113) involving infants and young children, in which vaccinees and placebo recipients spent several hours together in a playroom following administration of the vaccine or placebo. There are no published data on the use of the ca LAIV in immunocompromised hosts, including persons with acquired immunodeficiency syndrome (AIDS). There is a potential risk of inadvertent use of the vaccine in immunocompromised hosts if the vaccine is widely used in the general population.

8. Target Populations

As stated earlier, TIV is the only licensed influenza in the United States. The recommendations of the Advisory Committee on Immunization Practices (ACIP) of the Centers for Disease Control and Prevention (CDC) and the Committee on Infectious Diseases (Red Book Committee) of the American Academy of Pediatrics on the use of influenza vaccine are summarized in Tables I and II. More widespread use of TIV, i.e., universal immunization of the general population, has not been recommended because factors such as availability, acceptability, side effects, efficacy, effectiveness, and cost effectiveness of TIV influence the balance between the benefits of prevention and the risks of usually uncomplicated influenza in healthy persons. Some of these factors are likely to be different for LAIV and, therefore, if a LAIV is licensed in the future, vaccine recommendations may be revised. In specific populations, TIV or LAIV may offer distinct advantages over one another; in some populations they may simply be acceptable alternatives to one another; and in populations such as the elderly, a combination of TIV

and LAIV may offer better protection against influenza than does TIV alone (92).

Unprimed persons who are immunologically naive to influenza require two doses of TIV (122–124). Usually, children who have not previously received the influenza vaccine fall within this category. However, in a pandemic, in which infection with an influenza virus with a novel HA and/or NA is the target of the vaccine, people of all ages would most likely require two doses of vaccine for optimal protection. In previously primed persons, who have had prior exposure to influenza virus or vaccine, one dose of TIV is sufficient.

V. Future Developments in Vaccines

A. Adjuvanted Vaccines

Several new adjuvants are under evaluation for their ability to enhance the immunogenicity of TIV. These include an oil-and-water emulsion with the use of squalene and detergents (MF59) (125), a saponin derivative (Qs21), and monophosphoryl lipid A (19). A derivative of muramyl dipeptide induced major reactions in a small group of human volunteers (126).

B. Cell Culture-Derived Vaccines

The use of cultured cells such as Madin Darby canine kidney (MDCK) cells to generate an inactivated influenza vaccine would circumvent two problems related to egg-derived vaccines: the mutations in the HA that result from adaptation of the virus to growth in eggs (61,62) and the dependence on a reliable supply of embryonated chicken eggs for vaccine manufacture. An MDCK-derived bivalent subunit vaccine was safe, well tolerated, and immunogenic in clinical trials in Europe (127). Further development of cell culture-derived influenza vaccines is underway.

C. Vaccines Against Potential Pandemic Strains

Three influenza pandemics have occurred in the twentieth century, with the appearance of H1N1 viruses in 1918, H2N2 viruses in 1957, and H3N2 viruses in 1968. Although neither the timing nor the virus that will cause the next pandemic can be predicted, another pandemic is inevitable. It has been suggested that seed viruses bearing different

HA and NA subtypes should be prepared and stockpiled for use in vaccines, should the need arise. One possible approach would be to generate a priority list representing subtypes including, for example, H5, H7, and H2 viruses that are thought to be the most likely pandemic strains. The limitation of such an approach is that avian influenza viruses belonging to several lineages within the subtype may be circulating simultaneously in birds, and the choice of the "parent" avian virus will be somewhat arbitrary. In addition, although it can be argued that a vaccine that is an imperfect match will still elicit an antibody response that could modify the risk of disease, decades of experience with inactivated vaccines against H1 and H3 viruses suggest that a close antigenic match between the vaccine strain and the epidemic strain is essential for a vaccine to be efficacious in humans.

D. DNA Vaccines

The influenza virus NP protein gene has been explored singly (128) and in combination with the HA and matrix (M) protein genes (129) as an intramuscularly administered DNA vaccine in mice. An influenza virus HA gene DNA vaccine has also been immobilized on gold particles and administered intradermally by a "gene gun" as a vaccine to mice and chickens (130,131). In the mouse model, the HA constructs administered by gene gun elicited strong antibody responses and protected mice from subsequent homologous virus challenge (130). The NP construct administered intramuscularly elicited antibody as well as CTL responses and provided protection from antigenically drifted challenge virus strains and from challenge with influenza A viruses of different subtypes (128). Murine and primate studies suggest that the immune protection is long-lasting (132); the plasmids remain episomal and are not integrated into the host cell genome. Results of influenza DNA vaccine studies in humans are awaited; it will be of interest to compare the immunogenicity of DNA vaccines with that of the licensed TIV.

E. Genetically Engineered LAIV

The advent of reverse genetics techniques (133) made it technically feasible to generate a transfectant influenza virus with several site-specific attenuating mutations engineered into a single gene segment (134). Combinations of ts and attenuating mutations were introduced into the PB2 gene of an influenza A virus, with the intent of producing a vaccine strain that could reliably attenuate different wt viruses by

transfer of its PB2 gene. Although the vaccine strain exhibited the desirable degree of temperature sensitivity and attenuation, when the mutant PB2 gene was transferred into a different wt virus, the level of temperature sensitivity and attenuation was reduced (135). Clearly, a constellation of genes in the vaccine virus contributed to the level of temperature sensitivity and attenuation, and this contribution was lost when the PB2 gene alone was transferred to a new genetic background. It is possible that an additional mutation(s) that attenuates the PB2 gene by a mechanism other than temperature sensitivity or the introduction of one or more mutations in a companion gene segment would overcome this particular obstacle. This example illustrates some of the difficulties encountered in vaccine development.

We are entering an exciting time in the field of influenza vaccines. The prospect of the ca vaccine as a licensed alternative to the inactivated vaccine is a long-awaited development that will have a significant impact on immunization practices. Preclinical studies with DNA vaccines offer the hope of inducing broader cross-strain protection, and the application of advances in reverse genetics will facilitate the rapid generation of recombinant vaccine strains.

VI. Summary

Immunization is the most feasible method for preventing influenza. Vaccination against influenza is recommended for everyone 65 years of age and older and for persons less than 65 years of age who are at risk for developing complications of influenza. Immune correlates of protection have been established, and a global network is in place to monitor the appearance and circulation of antigenic variants of influenza viruses, as well as the appearance of novel subtypes of influenza A. Antigenic and genetic analyses of circulating viruses and testing of serum from vaccine recipients guide vaccine composition updates. The efficacy of influenza vaccines depends in part on the closeness of the antigenic match between the vaccine strain and the epidemic strain. Currently licensed influenza vaccines are trivalent, formalin-inactivated, egg-derived vaccines; their efficacy ranges from 70 to 90% in young, healthy populations when there is a close antigenic match between vaccine strains and epidemic strains. Development of intranasally administered alternative vaccines and improvement of the existing vaccine are areas of active research. A trivalent, ca live vaccine is the most promising LAIV candidate. In a field trial, efficacy rates of LAIV in young children were 96% against influenza A (H3N2) and 91%

against influenza B. However, few data are available to compare this formulation of the trivalent ca live vaccine with the trivalent, inactivated vaccine. Influenza vaccine recommendations will most likely be revised on licensure of LAIV; each vaccine may offer distinct advantages in specific populations.

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ADVANCES IN VIRUS RESEARCH, VOL. 54

INFLUENZA NEURAMINIDASE AS TARGET FOR ANTIVIRALS

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I. Introduction

The outbreak of a "new" H5N1 influenza virus in Hong Kong during 1997 served to remind us how fragile are our defenses against this disease (1). It takes several months to make a vaccine against a newly emergent strain, and an additional problem is that the H5N1 virus is lethal to chickens and cannot be grown in embryonated chicken eggs, the standard host for vaccine production. The approaches that may be used in the future to make a vaccine more rapidly against a new strain are described elsewhere in this volume (see the article by Subbarao). Fortunately, in the case of the Hong Kong H5N1, the virus did not adapt to human transmission, and it was contained by mass slaughter of chickens and ducks in Hong Kong. Six people died from this influenza virus.

The currently circulating human influenza viruses are variants of type A subtypes H1N1 and H3N2 and type B. In most winters one of the three is dominant, but there is no definitive way to predict which one. Vaccines currently in use contain antigens of a representative of each of these three types and subtypes, and each year at least one of the components is updated due to antigenic drift. It is clearly complex, expensive, and not very effective to make new vaccines every year. An alternative approach is to develop antiviral drugs. Influenza replication involves many steps that are specific to the virus and not involved in

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cellular functions. The virus-coded proteins that mediate these steps must conserve their function amid the ongoing antigenic variation, and thus they are potential targets for development of new antiviral agents that will be effective against all strains of influenza virus.

Two drugs are currently approved in the United States for the specific prophylaxis and therapy of influenza virus infections: amantadine and its close relative, rimantadine. Amantadine was discovered by random screening. It interferes with virus uncoating by blocking the virus-coded ion channel protein, M2. However, mutants resistant to amantadine are readily isolated after a single passage (2), thus limiting its usefulness. A more serious deficiency of amantadine is that it is totally ineffective against influenza B infections because the type B virus uses a different ion channel. With the constant threat of a new pandemic influenza strain emerging, there has been renewed interest in developing antiviral drugs targeted to the viral neuraminidase (NA) and the availability of crystal structures of NA from several different viruses has enabled the structure-based design of inhibitors, some of which are showing considerable promise in clinical trials.

This article describes the function of NA, its structure, how inhibitors are designed, and the current state of development of these new agents against influenza.

II. Antiviral Agents

A. Need for Antiviral Drugs

The most important protective response against invading pathogens is immunological. For many viruses, a single infection of a young child confers lifelong immunity. Vaccination has been successful in eliminating smallpox from the world, and now poliovirus is also approaching extinction. However, many viruses have proved more difficult to control by vaccination. Some vaccines are expensive, and some must be refrigerated. Thus diseases such as measles are well controlled in wealthier parts of the world but remain highly prevalent in the less developed regions. More significantly, many viruses have developed ways to escape from the immune system. Mechanisms to evade immune surveillance include replication in sites inaccessible to the immune system, replication in and destruction of cells of the immune system, viral coding of factors that interfere with the immune response, and escape by changing the structures of antigens. Antigenic variation of influenza viruses was recognized in the 1940s. The immune system is fully functional in influenza infection, but by the time it provides good protection,

the virus has changed its antigenic structure and so the disease persists in the human population.

There is obviously a limit to how much variation can be tolerated by a virus. The integrity of the outer surface that is the target of neutralizing antibodies must be maintained, and mutation to escape antibodies must preserve the functions of the surface proteins. Thus an ideal vaccine would be directed against essential regions where mutation would result in loss of viability. Unfortunately, crystal structures show that these essential regions are usually in depressions on the surface of the virus or viral protein, and these "canyons" are generally inaccessible to antibodies (3). They are, however, accessible to small molecules, and this is the reason much effort is being directed toward the development of antiviral drugs that bind to conserved sites and inhibit viral functions.

B. Potential Targets for Anti-influenza Drugs: Influenza Virus Replication Cycle

Influenza is an enveloped virus containing a segmented, negative-sense RNA genome. The eight RNA segments code for 10 proteins, most of which are packaged in the virion (Fig. 1). Two proteins, hemagglutinin (HA) and NA, protrude out of the lipid envelope, and a small ion channel (M2) is also exposed to the exterior.

The influenza virus replication cycle is shown schematically in Fig. 2. Infection begins with recognition of receptors on the surface of the host cell by the viral HA. For many years it has been assumed that the receptors are sialic acids, and this conclusion has been substantiated by crystal structures of sialyl compounds bound to HA (4,5). The first method that could be used for antiviral design is to block attachment by using a ligand that binds to the receptor binding site on the HA. However, the interaction between HA and sialic acid is weak (millimolar) and involves few contacts; hence the binding site is neither extensive nor well conserved. The interaction is stabilized by multivalent attachment, and polyvalent inhibitors have a better effect than monomers (6,7), but these are larger than optimal for good pharmacological properties or cost-effective production. More seriously, it is becoming clear that many viruses use more than one type of molecule to enter the cell, and the concept of a single receptor species that allows entry of the virus and initiation of its replication is an oversimplification of the events involved (8). Our results show that the presence of sialic acid increases the efficiency of influenza virus infection of target cells,

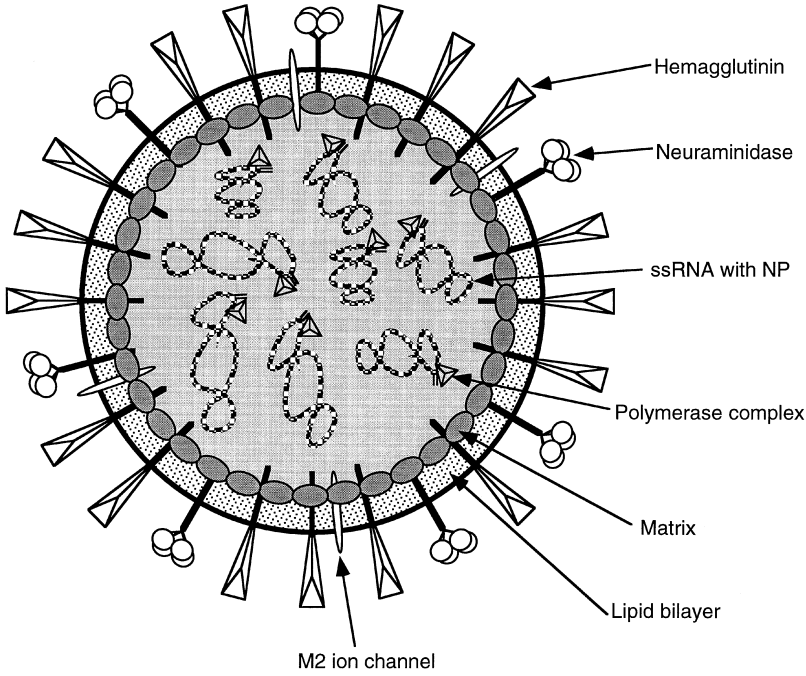

FIG 1. Components of the influenza virion. The virus has a lipid envelope derived from the host cell. The two surface antigens, hemagglutinin and neuraminidase, are anchored in this membrane, together with smaller amounts of the ion channel protein, M2. The major structural protein (matrix) is thought to be associated with the inside surface of the membrane, and within this shell are the nucleocapsid complexes, consisting of the eight segments of the RNA genome complexed with nucleoprotein (NP) and the three polymerase proteins (PA, PB1, PB2).

but sialic acid seems not to be essential for virus entry (8a). Removal of accessible sialic acid reduces virus binding only up to 70%, although virus entry and replication are reduced 10- to 100-fold, depending on the strain. We showed that broad-specificity sialidases do not cleave all sialic acid from MDCK cells, and periodate reduced ganglioside sialic acid by only 50%. This raised the question of whether virus is binding to gangliosides, but we also demonstrated that the gangliosides in MDCK cells are inherently sensitive to sialidase, consistent with observations that sialidase accessibility of neuronal cell gangliosides is regulated by the physiological state of the cells (9). Thus, although MDCK cells treated with sialidase still have significant amounts of sialic acid on glycoconjugates, the sialic acid is not accessible to small sialidases and hence must be inaccessible to the much larger virus. We

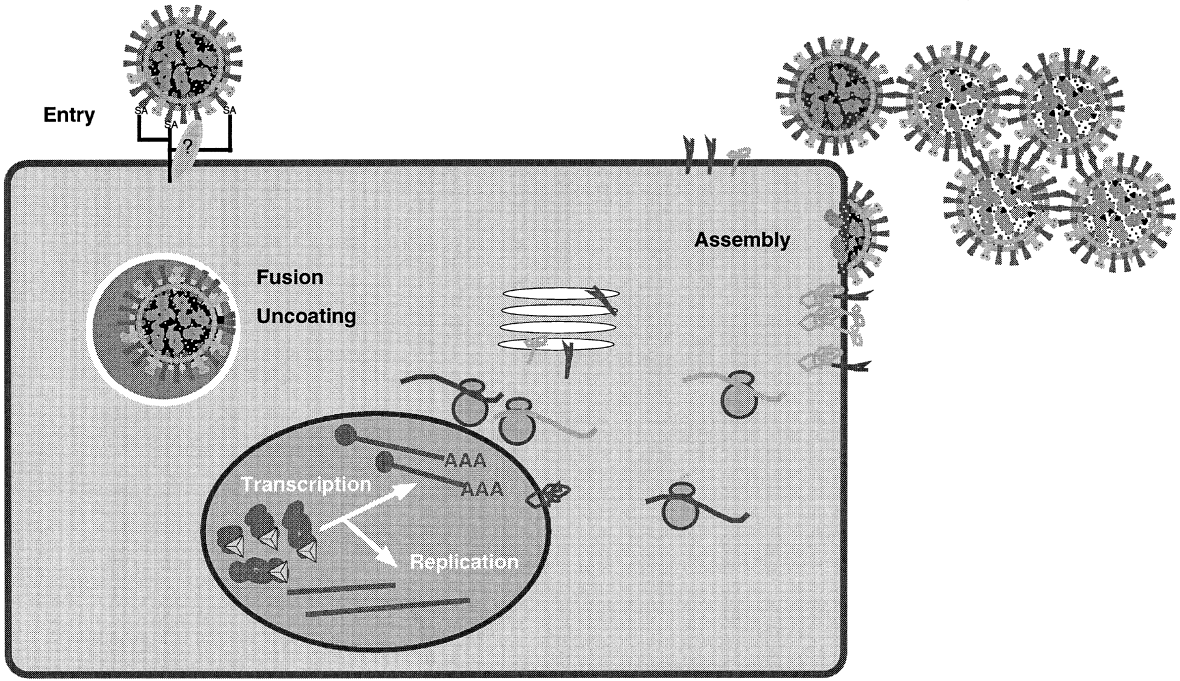

FIG 2. The replication of influenza virus. Entry into the host cell is assisted by binding of the HA to sialic acid on the cell surface. The virus is taken up into endosomes. As these acidify, the HA undergoes a conformational change to expose a short hydrophobic domain that mediates fusion of viral and endosome membranes to release nucleocapsids into the cytoplasm. These nucleocapsids travel to the nucleus and begin transcription of viral mRNA and replication of new negative-sense vRNA. The viral mRNAs are translated by the normal cell machinery. The virion components assemble at the plasma membrane, and virions bud out. In the absence of viral NA activity, HA on newly formed virions binds to the sialic acid on viral glycoproteins, causing aggregation of viral particles that then cannot spread to new cells.

propose a multistep model for influenza virus infection in which initial binding to an abundant low-affinity receptor (sialic acid) may be circumvented at higher virus concentrations. Influenza virus can enter cells without the assistance of sialic acid (8a) and also in the absence of a functional sialic acid binding site (10), so blocking the sialic acid binding site on the HA may not be the best strategy for designing antiviral agents against influenza.

Influenza viruses are believed to enter the host cell via the endosomal pathway, and fusion of viral membrane with host cell membrane is mediated by a massive conformational change in the HA triggered by the low-pH environment in the endosome (11,12). Inhibitors that act against the fusion event have been sought (13), but there is little conservation of amino acid sequence except for the fusion peptide itself, which is inaccessible until after the conformational change. The nucleocapsids must also be exposed to low pH to be freed from the structural matrix protein before being released into the cytoplasm, and this is mediated by the virus-coded ion channel protein (M2) that is present in the virion at low levels. The only currently licensed anti-influenza drugs (amantadine and rimantadine) act to block this ion channel. They are effective inhibitors, but only against type A viruses, and resistant strains readily develop from those viruses.

Once the eight viral nucleocapsids are released into the cell, they move to the nucleus and begin the replication cycle using the attached viral polymerase complex. Viral polymerases are attractive targets for inhibitor design, and most of the currently marketed antivirals are nucleoside analogs that have been modified empirically to have a greater degree of specificity for the viral enzyme than for the equivalent protein in the host replication system, or they are activated by viral enzymes so that they inhibit polymerases only in virus-infected cells. However, there are no published studies of drug candidates that inhibit the influenza polymerase.

Assembly of new virus particles is an ordered process that must be well regulated to coordinate the packing of genome, required viral enzymes, and structural proteins within a sealed protein and lipid shell. The HA of influenza viruses must be cleaved to activate the fusion peptide before the next round of infection, but this cleavage is mediated by cellular proteases, so for influenza there is no viral target for inhibiting this step.

The egress of newly formed virus particles from the cell is even less well understood than the entry. Enveloped viruses gather their external glycoproteins in the cell membrane and bud out nucleocapsids with a matrix shell through that membrane. Unlike bacteriophage, animal

viruses are not known to code for lysis factors. There are probably potential targets for antivirals, proteins or domains that are required for virus exit or budding, but none have been characterized. There is, however, a further stage that has shown surprising promise. Influenza virus requires its second surface glycoprotein, NA, to cleave sialic acid receptors from the glycoproteins of the virus and on the cell surface so that the virus can spread. In the absence of NA, HA binds to sialic acids of other HA molecules, forming large aggregates of virus and terminating infection (14,15). Inhibitors of NA described in this article have been developed on the basis of the structure of NA complexed with substrate or transition-state analog.

C. Neuraminidase

The NA accounts for about 5–10% of influenza virus protein, forming a mushroom-shaped spike on the surface of the virion. It is a tetramer with a box-shaped head measuring 100 × 100 × 60 Å, made of four identical coplanar subunits and a centrally attached stalk containing a hydrophobic region by which the NA is embedded in the viral membrane (Fig. 3). For biochemical studies, a soluble form of the NA can be released from the virus particles by treatment with proteases. These proteases cleave the stalk and release the enzymatically and antigenically active head of the NA, which in some cases can be crystallized. Some influenza viruses have "stubby" NA molecules in which the stalk is shortened by deletions of up to 18 amino acids (16). The enzyme activity of the stubby viruses is the same as that of the wild type with small substrate molecules, but is somewhat reduced when the glycoprotein fetuin is used as substrate, presumably due to accessibility problems. It is intriguing that the H5N2 and H5N1 viruses that are highly pathogenic have stubby NAs (1,17–19).

Several roles have been suggested for the NA. The enzyme catalyzes cleavage of the α-ketosidic linkage between terminal sialic acid and an adjacent sugar residue. In mucin, removal of sialic acid lowers the viscosity and may permit access of the virus to the epithelial cells. This possible role was suggested many years ago, but so far there is little experimental evidence. NA may destroy the sialic acid receptors on the host cell, allowing elution of progeny virus particles from infected cells. However, the most important role of NA may be removal of sialic acid from the carbohydrate moiety of newly synthesized HA and NA to prevent self-aggregation of the virus (14,15). In general, then, the role of NA may be to facilitate mobility of the virus both to and from the

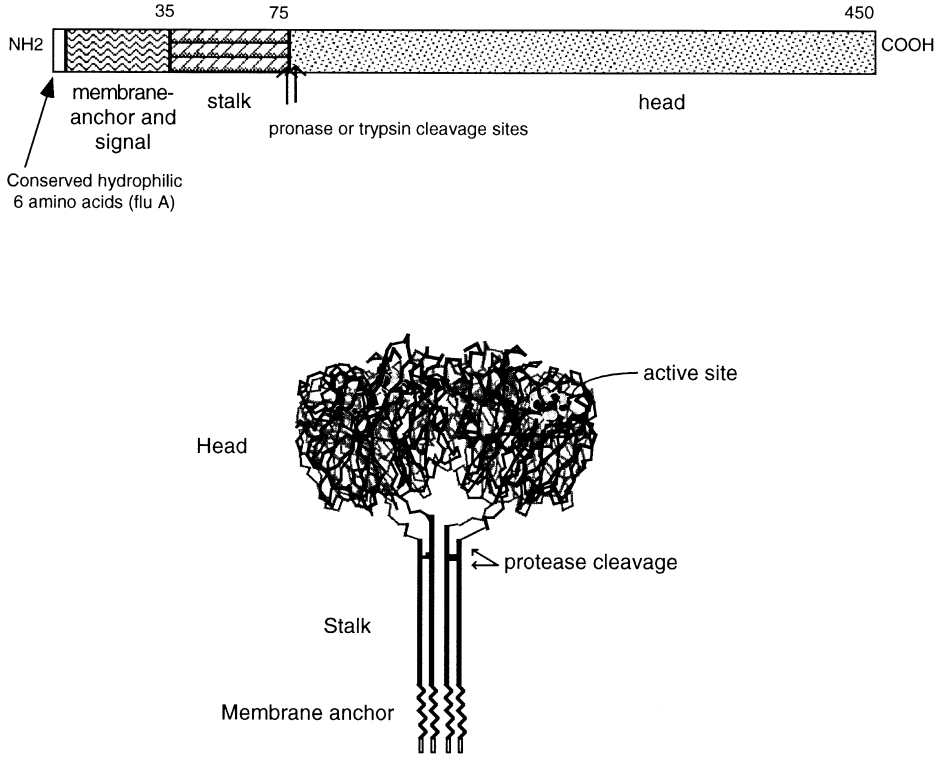

FIG 3. Overall structure of the influenza NA. *Top:* The linear amino acid sequence begins with six amino acids that are conserved in all influenza A strains. The sequence in influenza B is different but also conserved. Then follows a hydrophobic sequence that acts as a signal for insertion into the membrane and anchors the protein in the viral membrane. The following stalk sequence is highly variable. Then follows the head domain, which contains the enzyme active site. *Bottom:* Topology of the NA. The intact NA is a tetramer of four identical polypeptide chains anchored in the membrane at the N terminus. The structure of the stalk is unknown, but it is seen to be long and thin under the electron microscope. The head domain is globular and can be released from the stalk by proteases for crystallization. (For color reproduction, see color section.)

site of infection. This sounds a little vague, but the NA induces antibodies that protect against lethal influenza viruses (20) and thus provides an essential function for the virus.

It is generally believed that inhibition of NA markedly slows viral replication rather than prevents infection. Therefore an important reason for targeting antiviral agents to the NA is that it is possible that enough virus will replicate to give an immune response but not enough

to cause disease. Thus the anti-NA agent will modify an infection so that it acts as an attenuated live vaccine (21).

1. Crystal Structures of Influenza Neuraminidase

The three-dimensional structures of several subtypes of influenza A NA and of three influenza B NAs are now known. There is considerable sequence variation, but the overall architecture is highly conserved. Each monomer is composed of six topologically identical β sheets arranged in a propeller formation (22). The tetrameric enzyme has circular fourfold symmetry stabilized in part by metal ions bound on the symmetry axis. Insertions and deletions are accommodated in the loops connecting the β sheets or in the sheets themselves. The catalytic sites are located in pockets that occur on the upper surface of the box-shaped tetramer (Fig. 4) (24,25). Comparison of the main chain trace of the various NA structures shows some variation at the perimeter of the subunit but close superposition around the active site (24,25). The NA active site is a rather large cavity lined by 11 conserved amino acids, many of which are charged. The interaction of a transition state analog with NA is shown in Fig. 5. The binding of sialic acid in the active site is dominated by the charge–charge attraction between the carboxylate anion and a pocket of three positively charged arginine residues (Arg-118, Arg-292, and Arg-371). Opposite the arginine pocket, the methyl group of the acetylamido moiety fits into a small hydrophobic pocket formed by residues lle-222 and Trp-178, as well as the side chain of Arg-224. The acetylamido carbonyl oxygen forms a hydrogen bond with the guanidinium group of Arg-152. The hydroxyl group at C-4 forms hydrogen bonds with Asp-151 and Glu-119, whereas the last two hydroxyl groups of the glycerol moiety form hydrogen bonds with Glu-276. The Tyr-406 hydroxyl is directly underneath the C-2 and O-6 sugar ring atoms (24–26).

There is close to 80% amino acid sequence difference between type B and type A NA, but the amino acids that bind the glycosidically linked sialic acid or its transition state are totally conserved in spatial arrangement as well as in chemistry. The similarity extends even further, to bacterial sialidases in which no homology in amino acid sequence was detected when compared to influenza NAs. The X-ray crystal structures of bacterial sialidases (*Vibrio cholerae* and *Salmonella typhimurium*) and influenza NAs show a remarkable similarity in the overall fold and in the catalytic site (27,28). In all the active sites there are three arginine residues to stabilize the carboxylate of the sialic acid, a glutamic acid stabilizes the position of one of the triad arginines,

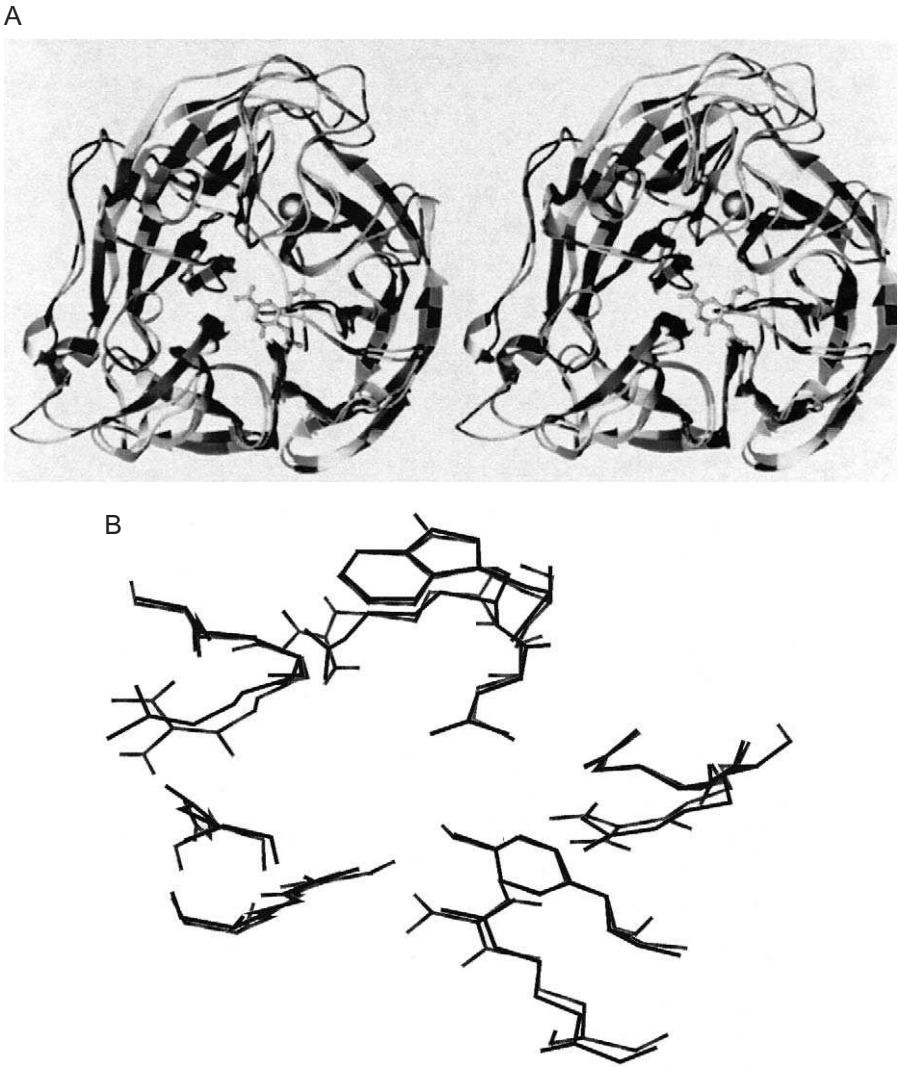

FIG 4. (A) The folding of influenza virus NAs of types A and B. The stereo view shows superimposed Cα RIBBON drawings (23) of an influenza type A NA (A/tern/Australia/G70c/75, N9) with the inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu5Ac2en) bound in the active site and a type B NA (B/Beijing/1/87). The monomer is viewed from the top, looking toward the viral membrane. The chains are color-coded according to the types of amino acid side chains: green, hydrophobic; blue, polar; orange, charged. Note that the chain paths diverge at the surfaces but come together in the area immediately surrounding the bound Neu5Ac2en. The coordinates used are 1NNB (24) and 1NSB (25) from the Brookhaven Protein Database. (B) A more detailed view of the active site showing the positions of side chains in influenza type A and type B that bind to the substrate. (For color reproductions, see color section.)

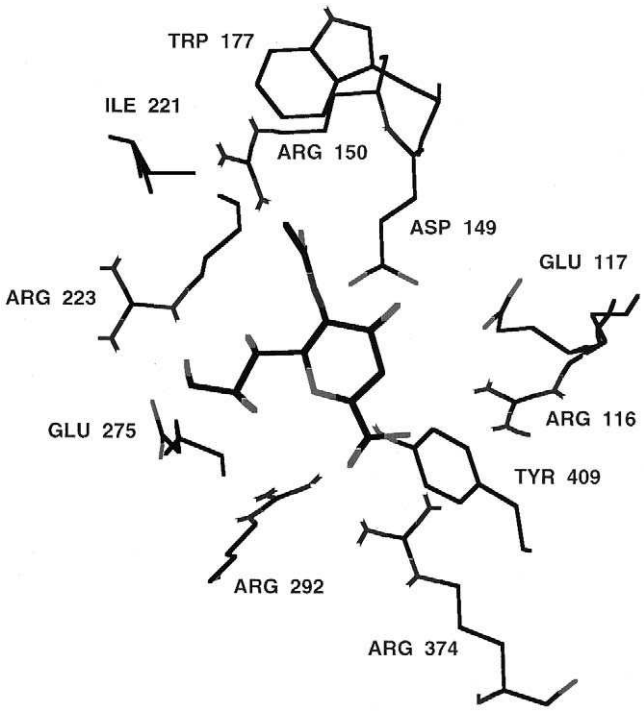

FIG 5. Schematic showing the interactions between NA of influenza virus B/Lee/40 and Neu5Ac2en (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid), a moderate inhibitor that mimics the transition state. Data from Ref. 48. Due to insertions or deletions, the conserved residues have different numbers in type B NA compared to type A. The corresponding numbers in type A (N2) NA of residues discussed in the text are Glu-119, Asp-151, Glu-275, and Tyr-406. (For color reproduction, see color section.)

a tyrosine approaches the sugar ring of the sialic acid from below, and a hydrophobic pocket accommodates the *N*-acetyl group of sialic acid.

III. STRUCTURE-BASED DRUG DESIGN

The rationale and procedures for designing antivirals based on knowledge of the structure have been reviewed (29) and will be summarized here.

The basic concepts of structure-based drug design were described more than 100 years ago. In 1894 Emil Fischer reported that glycolytic enzymes distinguish between stereoisomers of sugars and explained

the difference as a requirement for complementary shapes—the often-quoted "lock-and-key" metaphor (30). Paul Ehrlich, in his 1897 paper (31) on the quantitation of activity in diphtheria antiserum, described how the specificity and affinity of the antigen–antibody interaction could be explained by the laws of structural chemistry (32). Ehrlich later turned his attention from immunology to pharmacology, articulating in 1909 the concept that chemical treatment of disease relies on specificity, eliminating the invading microbe without damage to the host (21).

From these great insights, developed in the absence of any knowledge of macromolecular structure, comes the straightforward idea that it should be possible to design molecules that interact with other molecules. When the isomorphous replacement method was invented to allow macromolecular structures to be solved by X-ray crystallography (33,34), the concept could be tested. The first successful examples of *a priori* design of small molecules that could interact with a known protein were hemoglobin ligands, designed to fit into the diphosphoglycerate binding site (35). The first designed drug to reach the market was Captopril (36), an inhibitor of angiotensin converting enzyme developed with the use of knowledge of a related structure, carboxypeptidase, on which the active site of the target enzyme could be modeled.

The advent of powerful computers has allowed development of algorithms for systematic searching for complementary structures. The program DOCK (37) screens small molecule databases for molecules that fit the enzyme site. It is based on mapping the surface of a protein by rolling a water-sized sphere (radius 1.4 Å) over the exterior residues, resulting in a smooth description of the shape of the water-accessible surface (38). The database is screened for compounds that (a) fit the shape of the active site and (b) have electrostatic compatibility. The program was originally used to search the Cambridge Small Molecule structure database, but because small molecules are relatively easy to model with the use of programs such as SYBYL, DOCK is now also used to search databases such as all commercially available organic compounds, the Fine Chemicals Directory, or industrial collections.

To extend the search to novel small molecules, the program GRID was developed (39). GRID calculates the interaction energy for each of a variety of functional groups on a three-dimensional grid of the active site. The grid is then contoured by energy, the contours showing where a given functional group is predicted to bind. Model building is then used to connect these functional groups into a chemically feasible molecule. An alternative strategy to GRID is the multicopy simultaneous search (MCSS) method, which combines random placement with energy minimization/quenched dynamics techniques so that not only positions

but also orientations of functional groups in the binding site are determined (40). MCSS has been extended to link functional groups with molecular skeletons taken from a database to construct molecules containing the identified functional groups in the correct orientation, such as might bind in the HA sialic acid binding site (41).

A. Neuraminidase Inhibitors

To develop a rational basis for the design of effective antivirals targeted to the NA, it is important to understand the role of the conserved amino acids in enzyme catalysis so that inhibitors are designed to bind to essential amino acids that cannot mutate to resistance without losing function. Several kinetic analyses have been published, but there is no consensus on the mechanism of enzyme action (42). The consensus features of the reaction pathway are shown in Fig. 6. First, the α-linked sialoside binds to NA, leading to distortion of the pyranose ring of the bound sialic acid. A proton is donated from the solvent, leading to formation and subsequent stabilization of the endocyclic sialosyl cation transition state, followed by formation and release of sialic acid as the α anomer, which then rapidly converts to the β anomer in solution (43,44). Kinetic isotope effects showed that the rate-limiting steps in the reaction are steps leading to formation and breakdown of the enzyme:transition state complex, ET (k5 and k6, respectively, in Fig. 6) (45).

The determination of crystal structures with bound sialic acid (25,46), transition state analog (24,47), or transition state (48) has not provided clear insight into the mechanism, and several mechanisms have been proposed that differ with regard to how the glycosidic bond is cleaved and the force that stabilizes the transition state. It is clear that as the sialyl group of the substrate binds, it undergoes a ring distortion presumably mediated by the strong ionic interactions between the carboxylate of the substrate and the three guanidinium groups of Arg-118, Arg-292, and Arg-371, with the *N*-acetyl group bound firmly in a hydrophobic pocket at the other end of the active site. Varghese *et al.* (46) proposed that Asp-151 is a general acid catalyst of hydrolysis. However, the pH range for NA activity (4.5–9.0) is inconsistent with the pK_a of the solvent-exposed Asp-151, and mutagenesis studies have eliminated a proton donor role (42). Burmeister *et al.* (47) suggested that an unknown catalyst breaks the glycosidic bond to produce the oxocarbonium ion transition state, and ionization of the OH group of Tyr-406 is the major force for stabilization of the charged

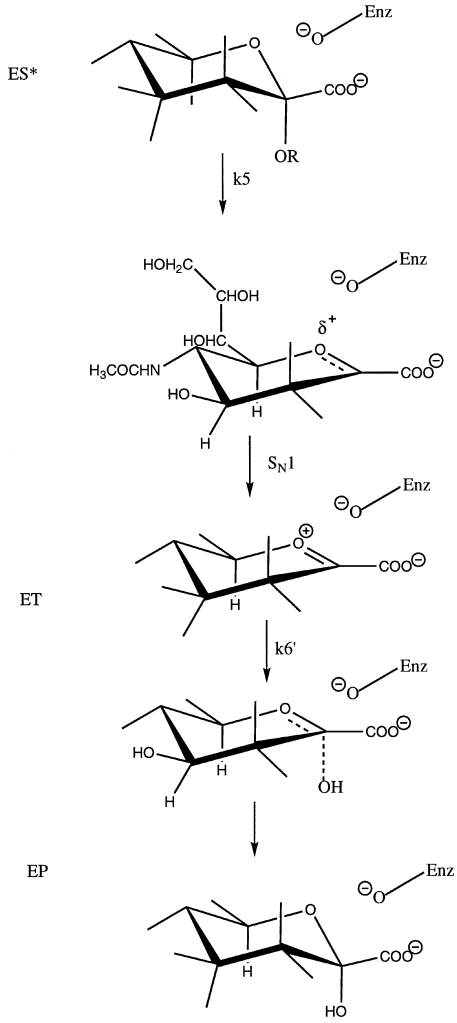

FIG 6. Mechanism of NA hydrolysis from kinetic studies. On binding, the α-linked sialic acid undergoes a conformational change, becoming planar at C-2 as the oxocarbonium ion intermediate (ET) forms and the glycosidic bond is cleaved. Addition of water then forms the free sialic acid, which is released as the α-anomer but rapidly converts to the more stable β-anomer. Adapted from Ref. 43.

transition state. Janakiraman *et al.* (48) argued that the conformational changes induced in the sialic acid when it binds to the rigid active site are sufficient to form the oxocarbonium ion, cleaving the glycosidic

bond. The force for the formation and stabilization of the transition state comes solely from the overall interaction of the substrate with the active site. Our kinetic analyses of site-directed mutants have shown the critical importance of Tyr-406 in the reaction (42), linking the two proposed mechanisms (25,48).

1. Sialic Acid-Based Inhibitors

More than 20 years ago, it was recognized that the oxocarbonium ion was the likely intermediate in NA hydrolysis, and hence analogs were synthesized as likely candidates for inhibitors. 2-Deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en) binds with micromolar affinity compared with millimolar affinity of sialic acid. The planar trigonal structure of Neu5Ac2en at the C-2 position (Fig. 7) fixes the sugar ring in a conformation that resembles the distorted boat conformation of sialic acid bound in the NA active site, as observed in the crystal structure (25,48). Derivatives of Neu5Ac2en were used in mechanistic studies (49) and evaluated as potential antivirals. The most potent inhibitor of influenza NA was the trifluoroacetyl derivative. It was effective in tissue culture (50,51) but failed to show an effect in animal models of influenza when given intravenously (52). It is now recognized that Neu5Ac derivatives have difficulty crossing biological membranes and are rapidly cleared from the circulation.

With the availability of the crystal structure of NA complexed with Neu5Ac2en (24,26,47), more potent derivatives of Neu5Ac2en were synthesized. The program GRID provided the lead to the most promising of these, 4-guanidino-2, 4-dideoxy-*N*-acetylneuraminic acid (53), now known as "zanamivir" or "GG167" (Fig. 7). The K_i has been variously reported due to the slow-binding nature of this inhibitor (54) but is of the order of 10^{-11} for the NA of A/Tokyo/67 (N2), 10^5-fold tighter than Neu5Ac2en. The compound is unavailable if delivered orally due to its high charge, and it is very rapidly cleared if given intravenously. However, when delivered intranasally, zanamivir is effective *in vitro* and in animal models (55). In human clinical trials it has been shown to ameliorate symptoms when given intranasally, either prophylactically or therapeutically, if delivered within 1–2 days of onset of symptoms (56).

There are some potential problems with zanamivir. It has several chiral centers and therefore is complex to synthesize, so it is expensive. The lack of oral effectiveness is generally considered an obstacle by the pharmaceutical industry. Resistance may be an issue; resistant virus variants have been selected in tissue culture studies but have

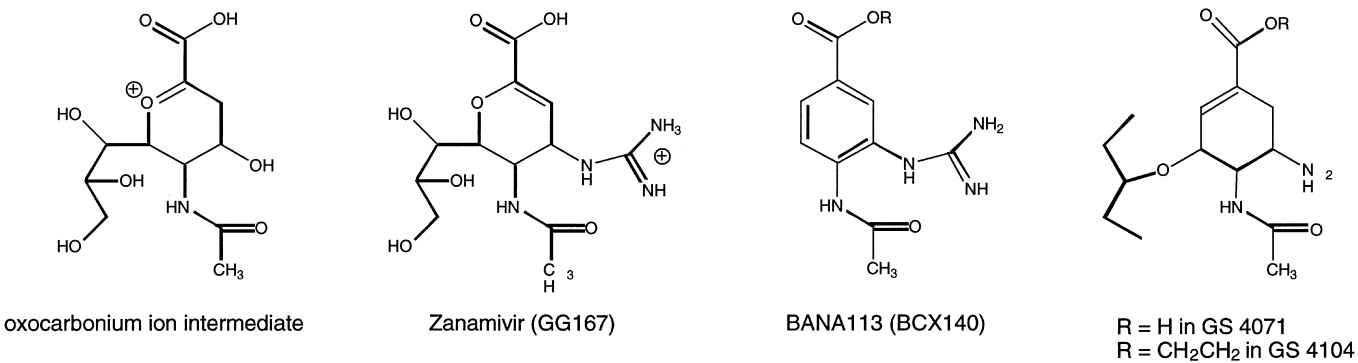

FIG 7. Inhibitors of NA.

not yet been reported in animal or clinical trials. These are discussed in Section III,B.

Other sialic acid derivatives that have been tested include phosphonate analogs (57), 9-amino- or 9-*N*-acyl-5-trifluoroacetylmethyl-α-ketosides (IC_{50} $10^{-6}M$) (58), and difluoromethylphenyl-α-ketosides (59), but none have proved to be effective in inhibiting influenza virus.

2. Carbocyclic Inhibitors of Influenza Neuraminidase

The second NA inhibitor to enter clinical trials is a transition state mimic, 4-acetamido-5-amino-3-[1-ethylpropoxyl]-1-cyclohexene-1-carboxylic acid, GS4071 (Fig. 7). Its design is based on the observation that there are no direct interactions involving the sugar ring between Neu5Ac2en and NA, implying that the position of atoms in the ring is not critical, and also on the presence of a hydrophobic cave near the glycerol side chain of Neu5Ac2en into which branched lipophilic side chains can be modeled using GRID or similar programs. Structure–activity relationships among compounds of this series have demonstrated very different inhibitory potency between influenza A and B NAs. It appears that the lipophilic side chains have quite different hydrophobic interactions with influenza A and B NAs despite the chemical and structural homology of the active sites. Influenza B NA does not accommodate as much steric bulkiness of inhibitors as does influenza A NA (60). GS4071 was selected for *in vivo* studies on the basis of equivalent inhibition of a broad range of type A and type B influenza NAs. The compound shows inhibitory activity *in vitro* similar to that of zanamivir (61). The ethyl ester, GS4104, is an effective prodrug when given orally. The compound has been shown to reach the bronchoalveolar fluids following oral administration (62). Oral administration of GS4104 protects mice and ferrets from disease (63) and effectively reduces all symptoms of influenza infection in mice even when treatment is delayed up to 60 hours postinfection. The compound is not toxic (64), and initial results from clinical trials are promising (65).

Following a similar line of reasoning, that the ring atoms are not important in binding the transition state, a series of compounds have been synthesized on the basis of a benzene ring (66). The most potent of these is 3-guanidino-4-(*N*-acetylamino)benzoic acid, BANA113 or BCX-140 (Fig. 7). The compound was designed to mimic zanamivir but to be more easily synthesized, and introduction of the guanidino group increased inhibitory activity, as expected (67). However, determination of the crystal structure showed that the compound is not bound to NA in the same way as zanamivir is, but is flipped 180° such that the guanidino group interacts with Glu-275 instead of with Glu-117 and

Asp-149, as shown in Fig. 8 (68). Addition of a second guanidino group to give 3,5-diguanidino-4-(*N*-acetylamino)benzoic acid resulted in lower inhibitory power even though the crystal structure showed that the compound bound as expected, with the guanidino groups interacting with Asp-149 and Glu-275. It has been proposed that desolvation energy as well as nonideal disposition of the guanidino groups may have reduced the interaction energy (68). Although the potency of BANA113 (BCX-140) is not high and although it failed to protect mice (69), the alternative binding of a guanidino group may be exploited in similar compounds with less potential for development of resistance compared to zanamivir (Section II,B).

3. Other Neuraminidase Inhibitors

Screening procedures have identified other molecules that inhibit influenza NA, but at this time none show particular promise. Plant flavonoids have been investigated for years, but they are not very potent (K_i 10^{-5} M) and their mechanism of action is still unclear (70). Thiacetazone inhibits the NA of WSN influenza virus by displacing Ca^{2+} (K_i 10^{-6} M) but it does not inhibit the NA of any other influenza strain tested (71). The acyl amino acids *N*-3-hydroxymyristoyl-D-cysteine and *N*-myristoyl-*O*-caproyl-D-serine inhibited the enzyme, but rather poorly (K_i 10^{-4} M) and in a noncompetitive manner, suggesting that they bind outside the active site, so that the inhibition may be restricted to certain strains of virus (72). Sodium 5-acetamido-2,6-anhydro-3,4,5-trideoxy-D-*manno*-non-2-enonate (73) was the best of a series of nonsialate transition state analogs (K_i 10^{-4}).

B. Will Resistance Develop?

A major concern in developing antivirals against a variable virus such as influenza is the possibility of the virus mutating under drug selection to become resistant. Amantadine has been found to induce resistance in a single passage *in vitro* (74) and from a single host *in vivo* (2). The active site of NA is highly conserved; there are 11 amino acid side chains directly in contact with substrate that never vary (24,25,75) and further conserved residues in a "second shell" that apparently serve to hold the inner shell in place (25). This high level of conservation may mean that drugs targeted to the active site cannot easily induce resistance because changes in the conserved residues to escape the drug are likely to be deleterious to enzyme activity.

In general, this appears to be the case; resistance against NA transition-state analog inhibitors develops only after several passages

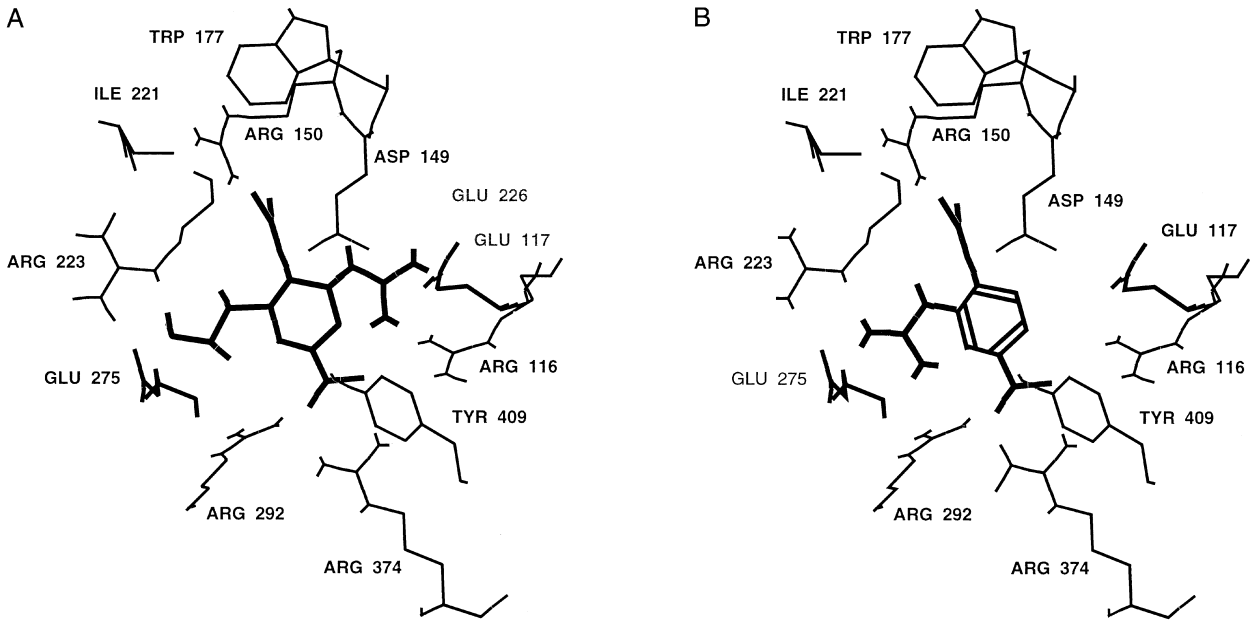

FIG 8. (A) Binding of GG167 (zanamivir) in the NA active site. The schematic representation is from data in Ref. 26. Oxygen atoms are red: nitrogen atoms are blue. (B) Binding of BANA113 (BCX-140) in the active site (68). Note that the molecule is flipped approximately 180° compared to zanamivir so that the guanidino group contacts Glu-275 instead of Glu-119. (For color reproductions, see color section.)

in the presence of the drug. When the resistant viruses are characterized, many have no change in the NA gene at all, and the NA activity remains sensitive to inhibitor. Instead, many viruses selected in MDCK cells with zanamivir (76,77) or BCX-140 (78) have changes only in the HA gene, usually causing amino acid changes somewhere near the sialic acid binding site. It was speculated that these viruses have lost the requirement for NA activity by reducing the affinity of the HA for sialic acid so that the virus can "shake itself" out of aggregates without the need for hydrolysis of sialic acid. The change in affinity has been demonstrated for some of the mutants (78,79). A mutant selected by zanamivir in MDCK cells with an HA change of R135G was found to be fully sensitive to the drug when used to infect mice, perhaps because the receptors for infection in mice are not the same as those in MDCK cells (80).

Of some concern has been the observation that replication of some clinical isolates in MDCK cells is not inhibited by either zanamivir or GS4071. However, the NA activity is inhibited by the drugs, and they are effective in reducing infection in the mouse model (63,81). Thus the same phenomenon is occurring as with resistance; the virus may be able to dispense with NA activity when grown in tissue culture cells but not *in vivo.* Indeed, clinical isolates that are resistant to the drugs in MDCK cells are sensitive when grown in human cell lines (82,83), again indicating that receptors on MDCK cells do not closely mimic those in the respiratory tract. However, it is interesting to note that the mutants of reassortant virus NWS-G70c (H1N9) that have no NA activity, due to deletion of most of the NA gene, are totally dependent on addition of exogenous sialidase for multicycle replication (15,84), and we have been unable to adapt them to growth without NA.

The changes reported in drug-resistant viruses are listed in Table I. The most commonly reported mutations in zanamivir-resistant viruses are in the HA gene, but several have shown changes in the NA active site, either in combination with HA changes or alone. The most frequent NA change is E119G, removing the charge on one of the two acidic side chains that bind the guanidino group of zanamivir (76,85,86). Changes to other amino acids at this site have also been found (76). The E119G mutant NA has reduced activity due to a population of monomers and dimers among the normal tetramers. The reason for the instability of the tetramer is not clear because E119 in the active site is nowhere near the subunit interface, and the crystal structure of the E119G mutant shows no changes except water molecules coming in to fill the void left by the missing Glu side chain (79,87).

TABLE I

SEQUENCE CHANGES IN HA AND NA GENES OF DRUG-RESISTANT MUTANTS

Drug	Virus	NA changes	HA changes	Reference
Zanamivir	H1N9 (1)	E119G	None	85
	(2)	None	R229S	77
	(3)	G90Q	V223I + R229I	77
	(4)	N346S	T155A	77
Zanamivir	H1N9	E119G	None	86
Zanamivir	B HK (HG)	E119G	N141S + N145S	86
Zanamivir	H2N2	None	G142D	80
Zanamivir	H4N2 (1)	E119G,A,D + R249K	HA2 G75E	76
Zanamivir	H4N2 (2)	R292K	?	90
6-Carboxamide 4-aminoNeu5Ac2en	H1N9	R292K	?	91
BCX-140	H2N2 (1)	G348R	R132Q	78
BCX-140	H2N2 (2)	None	R132Q	78
BCX-140	H2N2 (3)	S204T	A133T	78

The other amino acid side chain in contact with the guanidino group of zanamivir is E227, but no mutants have been reported at this site. When E227 was altered *in vitro* to 12 other amino acids, the mutant enzymes were defective, having less than 10% of the wild-type activity (88).

The only other mutation reported in the NA gene of drug-resistant mutants is R292K, at one of the three Arg residues that holds the carboxylate of the substrate firmly in place. This mutant has decreased activity due to decreased binding of substrate (89,90,91), but the reason for its resistance to drugs is not immediately clear. The R292K mutant shows much less resistance to zanamivir than to the 6-carboxamide derivative of 4-amino-Neu5Ac2en (92) or to GS 4071. The latter two both have hydrophobic substituents in place of the glycerol side chain of sialic acid or zanamivir, and their binding requires displacement of the side chain of Glu-276 (61,93). It has been proposed that in the R292K mutant the substituted Lys binds more firmly to Glu-276, preventing its displacement in order to bind the hydrophobic groups of GS4071 or the 6-carboxamide compound (91). Viruses with the change R292K are more than two logs less infectious than wild-type virus, suggesting that it is unlikely that this mutation will occur *in vivo* (90).

The NA active site mutations in drug-resistant mutants compromise the enzyme to varying extents, and it is clear that although the active

site residues are totally conserved in nature, some are more important than others. We have carried out a systematic study, making minimal changes in conserved residues and measuring the effect on kinetic parameters $K_{cat,}$ $K_{m,}$ and K_i. From the results, we conclude that resistance is least likely to develop if inhibitors are targeted to those residues at which a change has a drastic effect on k_{cat}. Changes at E119, R150, R223, and R374 caused less than a 10-fold reduction in k_{cat} and are not the best targets. However, changes at D149, E275, and Y409 had much more effect, and these are proposed to be the best targets for inhibitor design (42). For example, the change at E119G in viruses resistant to zanamivir is reasonably well tolerated with a k_{cat} of 20% of that of the wild type. However, the inhibitor BANA 113 (BCX-140) binds in the active site in the opposite way to zanamivir, with its guanidino group interacting with Glu-275 (68). In experiments to select resistant mutants we did not find a change at Glu-275 (78), and we predict that such a change would not be tolerated, as the k_{cat} for even a conservative change (E275D) is only 5% that of the wild type.

IV. Concluding Remarks

Structure-based drug design is rapidly becoming an important addition to the available methods to control disease. There is increasing understanding of the interactions that give rise to tight binding between a protein and a small molecule, and it is anticipated that the computational methods will become steadily more accurate and the design of inhibitors more reliable. Influenza NA is an excellent target for drug design in that its active site is highly conserved and very rigid. Inhibitors have been developed relatively quickly because organic synthesis concentrated on those compounds predicted to bind well. Resistance can probably be avoided by targeting interactions to the most essential amino acid side chains, but there is still a question of whether the need for NA activity can be circumvented *in vivo,* as has been demonstrated *in vitro.* We think not. Influenza is a highly variable virus, and if the NA function were not absolutely required, the gene would have been lost. Thus we are optimistic that there will soon be anti-influenza drugs related to some of the compounds discussed in this article.

Acknowledgments

Work in the authors' laboratory was supported in part by Grants Al-18203 and Al-31888 from NIH.

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ADVANCES IN VIRUS RESEARCH, VOL. 54

REPLICATION OF PARAMYXOVIRUSES

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I. Virion Structure and Genome Organization

The family *Paramyxoviridae* is part of a virus order, the Mononegavirales, which also includes the families *Rhabdoviridae* [e.g., vesicular stomatitis virus (VSV), rabies virus], *Filoviridae* (e.g., Ebola and Marburg viruses), and *Bornaviridae. Paramyxoviridae* contains two subfamilies: the *Paramyxovirinae* and the *Pneumovirinae.* The *Paramyxovirinae* contains three genera, *Respirovirus* [e.g., Sendai virus (SeV), human parainfluenza virus types 1 and 3 (hPIV1/3)], *Rubulavirus* [e.g., mumps virus, human parainfluenza virus types 2 and 4 (hPIV2/4)], and *Morbillivirus* [e.g., measles virus (MeV)]. These viruses contain a lipid envelope derived from the plasma membrane of the host cell. Positioned just under the envelope is a protein shell composed of the virally encoded matrix protein (M). The M protein appears to be a major determinant of virion morphology, due to its capacity to self-polymerize into two-dimensional crystal lattices. Such structures have been observed by freeze-fracture under the plasma membrane of infected cells (1). Although very pleomorphic in size (the diameter can vary from 1200 to 3250 Å), virions have a very regular spherical morphology when viewed by cryo electron microscopy (EM). Embedded within the envelope and extending 80 to 120 Å from the surface are

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glycoprotein spikes that are involved in viral entry and subsequent release from the host cell.

Inside the virion envelope lies the viral genome. The SeV genome is composed of a 15,384-nucleotide-long nonsegmented RNA of negative polarity (i.e., it does not serve as an mRNA) tightly associated with 2564 copies of the viral nucleocapsid protein (N) in the form of a ribonucleoprotein core. These nucleocapsids (or N:RNA) appear as regular left-handed helical coils 1 μm in length and about 200 Å in diameter when viewed in negative-stained electron micrographs (2–4). Image reconstruction of such images led Egelman and co-workers (5) to propose that each N protein subunit interacted with six nucleotides of the RNA, although the importance of this observation was not realized until later (see below), and that there were about 13 N subunits per turn of the helix. These structures are extremely stable, remaining intact (and active) after purification by CsCl density centrifugation. Transcriptionally active nucleocapsids contain two additional proteins, namely, the L and P proteins, which together constitute the viral RNA-dependent RNA polymerase (6,7).

The RNA within the nucleocapsid is resistant to nuclease attack at any salt concentration (8). It is quite likely that the paramyxovirus N:RNA template never disassembles during genome expression, which in turn raises the question of how the template bases are read by the polymerase during genome expression. An answer, at least in part, may come from the apparent elasticity of the N:RNA. The template can exist in a number of discrete pitch states (53 Å, 68 Å, and 375 Å). Transitions from the tightly coiled to the 375 Å state have been observed on the same nucleocapsid (5), which has led to the proposal that such transitions may be induced locally by the viral polymerase as it traverses the template. Such local distortion of the helical coil could make the template bases more accessible, and the coil would move along the template with the polymerase, rather like the bubble of melted DNA generated by cellular RNA polymerases as they transcribe double-stranded DNA.

Replication of paramyxoviruses requires a number of essential viral components (the "key players"), superimposed on which are a series of viral factors that either modulate the readout of the genome and/or modulate the virus–host interaction (which in turn may alter genome expression: the "supporting cast"). We will outline what is known about these components individually and then try to integrate this information into a model for viral replication.

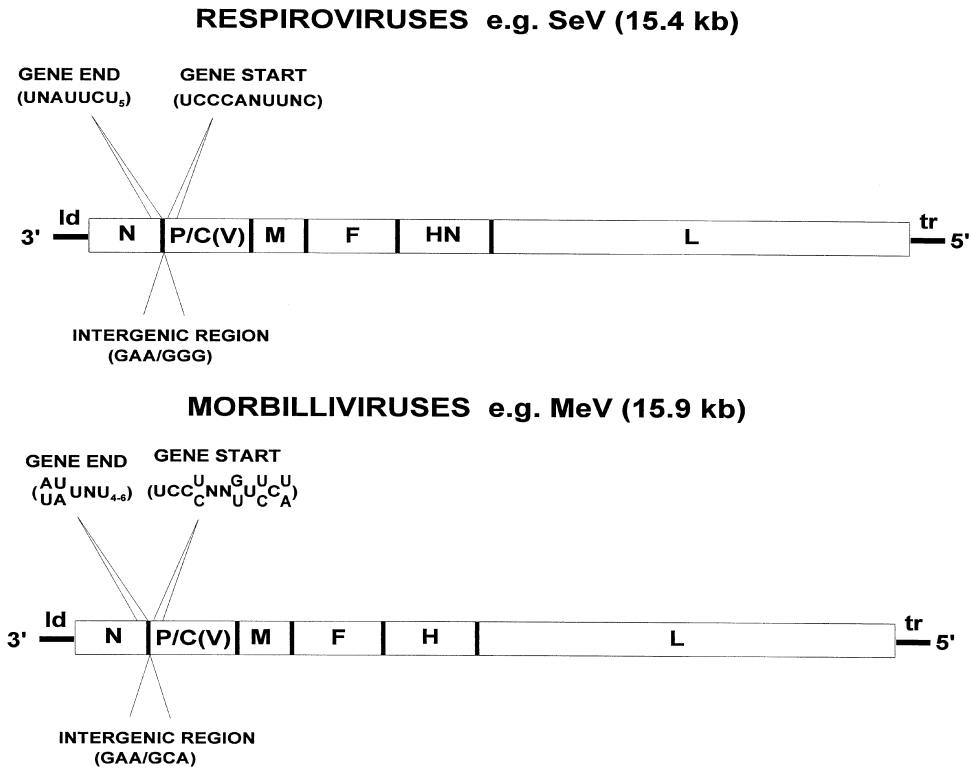
II. Key Players

A. RNA Genome

The complete nucleotide sequence is now known for a number of the *Paramyxoviridae,* including SeV, hPIV3, MeV, mumps virus, and respiratory syncytial virus (RS virus). Overall, the genetic organization is highly conserved within all the *Mononegavirales* (see Fig. 1), and it has been suggested that this gene order became frozen at an early stage during the evolution of this virus group due to the apparent absence of recombination (9). At the 3′ and 5′ extremities of the genome are short extragenic regions generally 40 to 60 nucleotides long, referred to as the "leader" and "trailer" [the trailer of RS virus is 155 nucleotides (nt) long], respectively. Approximately the first 12 nucleotides of the leader and last 12 nt of the trailer are complementary, probably because they each contain basic elements of the viral promoter (the genomic 5′ trailer sequences serve as the 3′ promoter on the antigenomic replication intermediate; see below). These terminal extragenic sequences flank 6 (for the respiroviruses and morbilliviruses), 7 (for a number of rubulaviruses) or 10 (RS virus) genes. At the beginning and end of each gene are conserved transcriptional control signals involved in initiation and termination/polyadenylation of the mRNAs (Fig. 1). Separating the genes are short intergenic regions that are not copied into mRNA. Their size and sequence vary among genera, with both respiroviruses and morbilliviruses having short (3 nt) and highly conserved intergenic regions, whereas the length and sequence of these regions in both rubulaviruses (1 to 47 nucleotides) and pneumoviruses (1 to 56 nucleotides) are variable. The function of this region and the consequences (if any) of its variability are now being elucidated. Studies performed with RS virus indicate that the size and sequence of the intergenic region play no role in modulating gene expression (10), whereas in the rhabdovirus VSV (which has a conserved intergenic dinucleotide), this region appears to form an integral part of the transcriptional termination/reinitiation signal (11). In VSV, the polymerase may actually scan the intergenic region in search of an appropriate start site (12).

It is now possible to rescue both virus minigenomes and infectious virus from cDNA clones (indeed, much of the work outlined above was performed with the use of this system; for a review on this technology, see Ref. 13). Studies initially performed on SeV revealed that minigenome templates were efficiently replicated only when the length of their RNA was a precise multiple of 6 nt (14), thereby providing a

functional basis for the earlier physical measurements of Egelman (see above). This observation, which was termed the "rule of six," was also strictly adhered too by MeV (15,16); by contrast, RS virus showed no particular integer rule (17), and the stringency of the rule of six for the rubulavirus simian virus 5 (SV5) was intermediate (18). It was proposed that each N subunit interacts with 6 nt and that templates are efficiently replicated only when their 3′ ends are precisely covered by the terminal N proteins. If one then assumes that the six nucleotide binding sites on each N subunit are not equivalent, this imposes a phasing on all the *cis*-acting sequences that regulate replication and transcription, i.e., the viral polymerase will read a linear RNA signal in the context of the N subunits with which it associates. Phase preferences have indeed been observed for the transcriptional stop/start signals at the intergenic junctions, as well as for the motif that regulates editing of the P gene mRNA (see below; 19) in the respiroviruses,



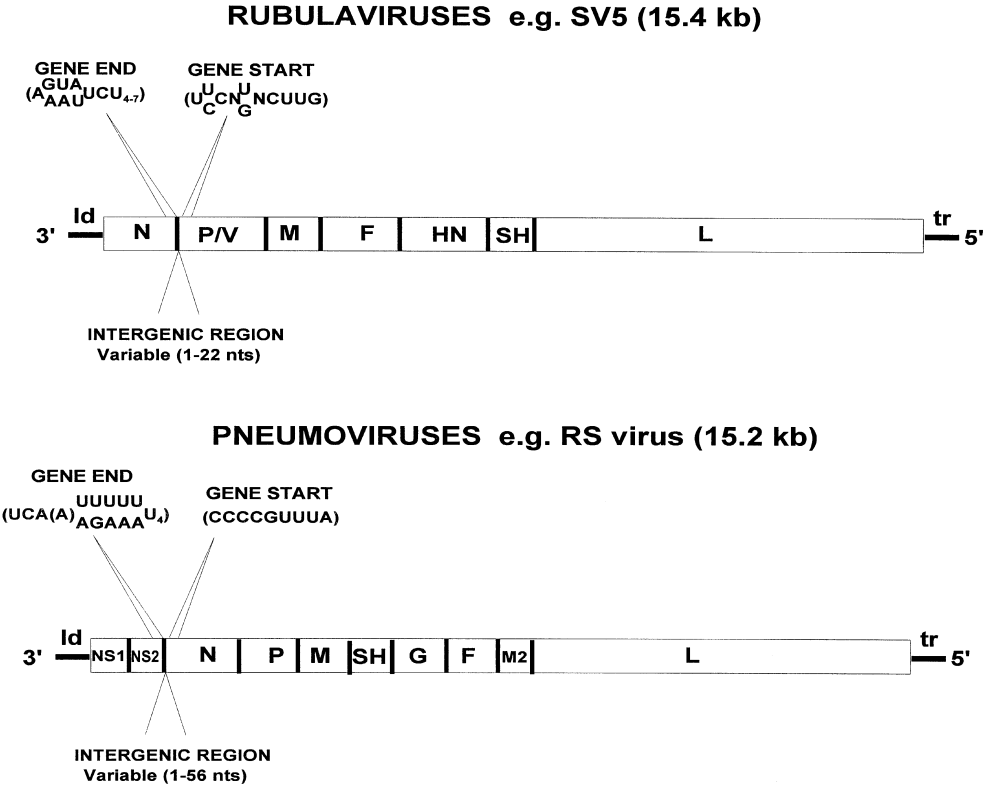


FIG 1. Genome organization within the *Paramyxoviridae*. A schematic representation (not to scale) of the various genera. The horizontal bars at the ends refer to the 3′ and 5′ extragenic regions, namely, the leader (ld) and trailer (tr), respectively. The rectangular boxes denote the various genes, and the letter within them indicates the protein(s) that is expressed. The consensus gene end and gene start signals of each genus are indicated above the map, and the consensus intergenic region is indicated below. The genome length of a prototypical member from each genus is also indicated. N, Nucleocapsid protein; P, phosphoprotein; M, matrix protein; F, fusion protein; G/H/HN, receptor binding protein; L, large protein; C, small basic protein; NS, nonstructural protein; V, cysteine-rich protein; SH, small hydrophobic protein; M2, small structural protein.

morbilliviruses, and rubulaviruses (viruses in which the rule of six appears to operate). Phasing is also a critical part of the BB box, an element of the paramyxovirus promoter (see below). This coupling of the *cis*-acting sequence and the N protein is consistent with the idea that the N protein is not locally displaced during reading of the template, but rather that it presents the bases to the polymerase.

The ability to manipulate paramyxovirus minigenomes as DNA has also permitted characterization of the viral promoters. It is believed that the viral polymerase obligatorily enters from the 3′ end of the template, and with the use of SeV as a model, it was shown that the first 31 nt from the 3′ end of the genome and antigenome constituted an important promoter element, containing sequences that appear to modulate promoter "strength" (20). However, in contrast to VSV, in which all the promoter elements reside within the leader/trailer sequences (21), the promoters of at least SeV and SV5 also contain a sequence element that is outside these extragenic regions. In SeV this additional element, referred to as the "BB box," contains the motif $(GNNNNN)_3$ (written in the negative sense, where G is the +1 position within the hexamer phase relative to the genomic 3′ end) spanning nucleotides 79 to 96 from the 3′ ends of both the genomic and antigenomic promoter (22). In SV5, a similar promoter element obeying hexamer phasing was also demonstrated, although its conserved sequence was somewhat different, i.e., $(CGNNNN)_3$ (23). This element was referred to as "conserved element II (CRII)" with reference to CRI, which represents the first 19 3′ nucleotides. Exactly how the CRII/BB element functions is unclear; however, with 13 N subunits per helical turn, these sequences are positioned on the same face of the nucleocapsid helix as the template 3′ end (or CRI region), which has led to the suggestion that these two regions together may constitute the polymerase entry site. In support of this model, insertions or deletions that alter the distance between the template 3′ end and the BB box, but that do not perturb the overall hexamer length of the template, have very detrimental effects on replication (24).

B. N Protein

The N protein must perform multiple functions during the viral life cycle. It encapsidates the genome RNA to generate the nucleocapsid template (this N:RNA is 97% N protein by mass). In turn, the nucleocapsid must interact with the viral polymerase (P–L) during genome expression and with the M protein during viral maturation and budding. As we will also see later, the unassembled N (N°) must also interact with the P protein to form a functional assembly complex (P–N°). The intracellular concentration of this P–N° complex is thought to be a major factor that regulates the relative rates of transcription and replication from the genome templates.

The paramyxovirus N proteins contain 489–553 amino acids. Somewhat surprisingly for a protein that is known to interact tightly with

RNA, the proteins contain no classical RNA binding motifs, nor can they be shown to bind RNA *in vitro* (using gel shifts of Northwestern blots). Alignment of the known N sequences suggests that the protein is composed of two domains. The N-terminal 80% is relatively well conserved within a genus, whereas the C-terminal 20%, although generally negatively charged, is much more divergent (25,26). This hypervariable C terminus appears to be a tail that extends from the globular N-terminal body of the assembled N protein (Fig. 2). In the nucleocapsids of both SeV and SV5, this tail is hypersensitive to trypsin attack, leaving a 48-kDa N-terminal core (27,28). This treatment does not affect either the overall nucleocapsid structure or the resistance of the RNA to nuclease attack, suggesting that the major determinants of nucleocapsid morphology (i.e., N:N subunit contacts and N:RNA contacts) must reside in the conserved N-terminal domain. Structure–function studies on the SeV N protein have also demonstrated that the C-terminal tail is dispensable for encapsidation, whereas deletions within the N-terminal domain abolish this activity (26,29,30). Mutational analysis indicates that a region spanning amino acids 258–357 is important for N–N and N–RNA interactions (31).

Although the C-terminal tail is not required for assembly, it is required for template function because SeV nucleocapsids assembled with tailless N protein are unable to serve as templates for further rounds of replication (26). The tail appears to mediate P protein and hence polymerase binding (Fig. 2; 29,32). This template function apparently does not reside exclusively in the C-terminal tail because N proteins carrying alanine substitution mutations between amino acids 114 to 129 were assembly competent, but these nucleocapsids were also unable to serve as templates for replication. In this case the defect did not reside in polymerase binding. One mutation tested, an N114 alanine substitution, generated a template with a cold-sensitive phenotype in that it was inactive at 30°C (the normal temperature at which the SeV polymerase reaction is performed) but was active at 37°C (33). The authors proposed that the defect was conformational, i.e., that the mutant N protein was unable to adopt an active conformation at the lower temperature, suggesting that this region of the protein may modulate changes within the template during RNA synthesis.

The structure–function studies outlined above have involved mainly the assembled form of the N protein. Like the assembled N, the unassembled N (N°) also interacts with P to form the assembly complex (P–N°). For SeV, only the N-terminal 80% of the N protein (not the C-terminal tail) is required for formation of a P–N° complex that is capable of assembling viral RNA (26). This suggests that the assembled and

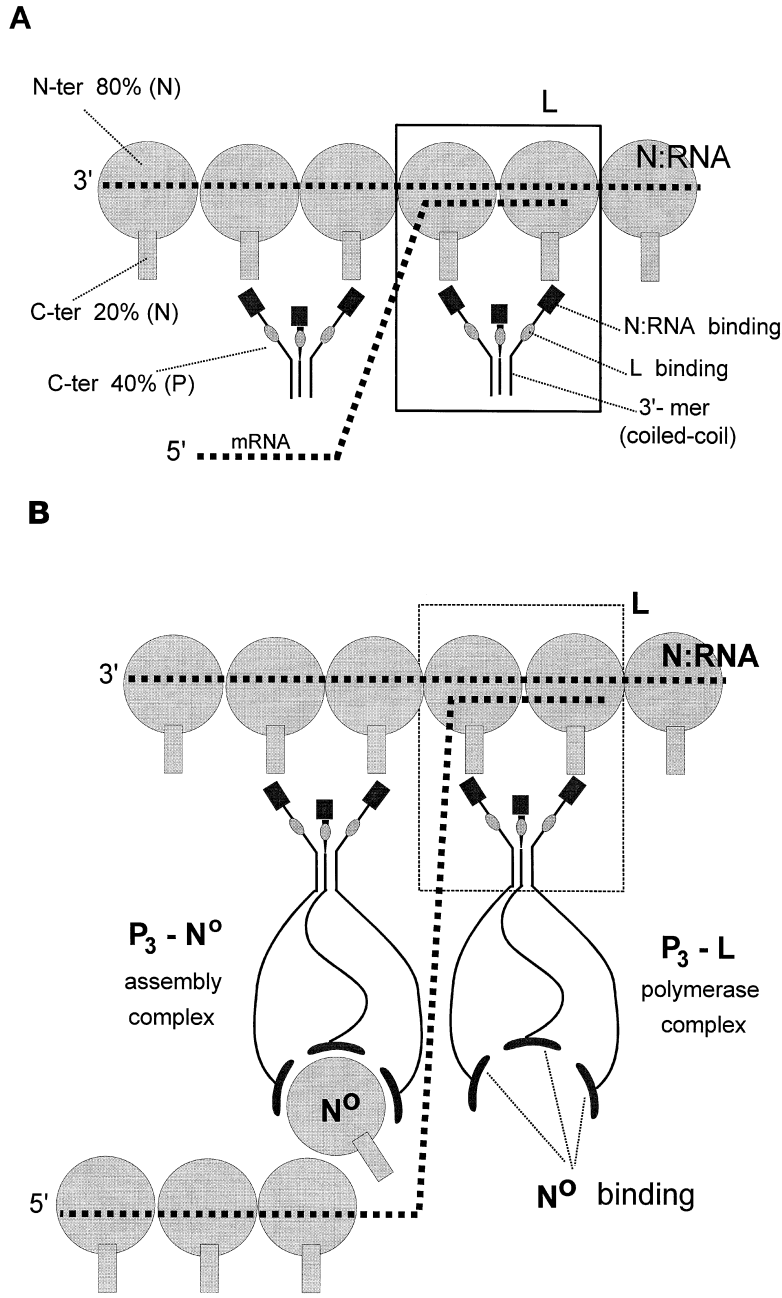


FIG 2. Schematic models for mRNA synthesis and replication. (A) mRNA synthesis (transcription). The N:RNA template is depicted as a string of beads (shaded circle), each bead corresponding to the globular N-terminal 80% of the N protein. The dotted lines represent the genomic RNA and mRNAs. The shaded rectangles protruding from

soluble forms of N may have different conformations (e.g., it is unknown if the C-terminal tail is exposed on N°) and thereby interact differently with P. Conformational changes have been observed in the MeV N protein prior to assembling into nucleocapsids (34). It is also known that different regions of the SeV P protein are involved in interacting with N° and the template (see later).

C. P Protein

The P protein is so named for its highly phosphorylated nature (35). The size of this protein varies considerably within the *Paramyxovirinae,* with the respiroviruses and morbilliviruses being 507–603 amino acids long and the rubulaviruses being 245–397 amino acids long. The P protein has been described as the central player in viral genome amplification because it forms complexes with both the N (assembly complex) and L proteins (the polymerase complex). It also has a supplemental role in RNA synthesis that requires, at least in part, binding to the template (Fig. 2;) (36,37).

The modular nature of P has made it particularly amenable to structure–function analysis. Such studies (largely performed on SeV) suggest that the protein is composed of N- and C-terminal regions separated by a hypervariable hinge (38). All the paramyxovirus P proteins

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the beads represent the exposed C-terminal tail of the assembled N. The C-terminal 40% of the P protein, which contains all the functional domains required for RNA synthesis, is depicted as a trimer. The strong L protein binding site on P is shown as a shaded oval. P is proposed to interact with the exposed tails of the template N protein via two of the C-terminal legs of the trimer (black boxes). A single P trimer has been positioned within the open rectangle representing the L protein to depict the polymerase complex (P_3–L). The supplemental P_3 that is required for RNA synthesis has been placed upstream of the P_3–L complex. However, the position of the supplemental P_3 relative to the polymerase is unclear, and it is not known if the supplemental P_3 and the P_3 of the polymerase complex exchange during RNA synthesis. (B) Genome synthesis. The schema is organized essentially as above, except that the full-length P_3 protein is depicted. The supplemental P_3 is shown carrying an unassembled N (the assembly complex P_3–N°) via its three N termini. It is not known, however, if the assembly complex interacts with the template as depicted or, indeed, if the P_3–N° complex can serve as the supplemental form of P. In addition, the N° is shown with the same conformation as the assembled N, although experimental evidence (at least for MeV) (34) suggests that this is unlikely. Release of N° from the assembly complex may be triggered by interactions between P_3–N° and P_3–L. This would ensure the release of N° close to the site of nascent RNA chain synthesis and may play an important role in ensuring that only virally expressed RNA is assembled.

studied are found as homotrimers, and oligomerization is probably mediated by a coiled-coil motif located within the C-terminal half of the protein (39). Unlike the VSV P protein, SeV P oligomerization is not facilitated by protein phosphorylation, and monomers within the trimers do not appear to exchange during RNA synthesis. The inherent stability of the paramyxovirus P_3 is further highlighted by the fact that it is the trimeric protein that forms both the assembly (P_3–N°) and polymerase complexes (P_3–L) (manuscript in preparation). Deletion of the coiled-coil motif in SeV prevents P protein from binding to both L and the template and severely impairs the interaction with N°. This would appear to preclude a role for a monomeric form of P in virus replication. For VSV, on the other hand, a monomer/oligomer equilibrium mediated by phosphorylation of the P protein may be an essential feature in the regulation of transcription/replication (40–42).

Mutants of the SeV P protein in which the entire N-terminal region has been deleted (i.e., Δ1–320) are transcriptionally active, indicating that all the domains required for RNA synthesis are contained within the C-terminal region (see Fig. 2a) (43). A binding site for L was mapped to amino acids 412–445 (indicated as an oval in Fig. 2) (43,44), and this was flanked by two discontinuous regions required for template binding (amino acids 344–411 and 479–568, respectively) (45). The upstream region contains the trimerization motif mentioned earlier, whereas the downstream region (block rectangle in Fig. 2) is predicted to form an α-helical bundle (39). It is through this latter motif that the P protein is thought to interact with the exposed C-terminal tail of the assembled N protein, this interaction then being stabilized by the simultaneous binding of two of these regions within the trimeric P (see Fig. 2). It has been proposed that the third leg of the trimer may be involved in the processivity of P protein on the template (46). In such a model, P is envisaged to "walk or cartwheel" on the N:RNA via the simultaneous breaking and reforming of contacts between the legs of the P trimer and the exposed C-terminal tails of the assembled N protein (Fig. 2). This movement of P may be required to induce a localized distortion of the helical coil so that the bases can be read. All these C-terminal domains are important for RNA synthesis because deletions here render the protein nonfunctional.

The N-terminal region of the P protein tends to be acidic in both the respiroviruses and morbilliviruses but is generally more basic in the rubulaviruses. In SeV, a region mapping to the N-terminal 77 amino acids was shown to be important for the formation of an active assembly complex with N° (47). Deletion of this region resulted in the nonspecific aggregation of the coexpressed N protein, whereas deletion of the re-

mainder of the N-terminal region (amino acids 78 to 325: this deletion also removes the major sites of protein phosphorylation) had only a marginal effect on the ability of the protein to support genome replication.

D. L Protein

The L protein is the largest (about 240 kDa) and the least abundant viral protein, consistent with the 5′ promoter distal position of the gene on the transcriptional map (see below; Fig. 1). Largely on the basis of an analogy with VSV, it is thought that the L protein contains all the catalytic activities associated with the viral polymerase. The protein is never found free in infected cells (when expressed alone, the L protein is highly unstable) but forms a complex with the viral P protein (6,7). This complex constitutes the viral polymerase, and L is thought to engage the template via interactions between P and the nucleocapsid protein of the N:RNA (Fig. 2) (48). Attempts to map the region on L that interacts with P suggest that the N-terminal part of the protein is important, although the extent of the sequences involved varies among viruses (49,50). Alignment of the L protein sequences indicates that there are six conserved regions that are thought to represent common structural features (51). Structure–function analyses of these regions are limited, and directed mutations frequently inactivate the protein or give no discernible phenotype. However, mutations in the SeV conserved domain I (amino acids 348–379) have been reported to uncouple transcription and replication, indicating that this may be a region that interacts with the assembly complex (52).

III. Supporting Cast

This group of proteins are not found in all the paramyxoviruses, but appear to modulate genome expression and virus–host interactions.

A. C Proteins

These are a group of small (180 to 204 amino acids) basic proteins expressed by the respiroviruses and morbilliviruses. The C proteins are expressed from the P gene using an open reading frame (ORF) that overlaps the 5′ portion of the P mRNA in the +1 frame relative to the P ORF. The proteins are underrepresented in purified virions relative

to their levels in infected cells, which led to their initial characterization as nonstructural. However, small amounts are present on the nucleocapsid (53). Both SeV and hPIV1 express a C-terminal nested set of four C proteins, designated C′, C, Y1, and Y2, by a mechanism of ribosomal choice that involves the use of non-AUG start codons, leaky scanning, and a ribosomal shunt (54,55). In the respiroviruses, the C proteins are a major translational product of the P gene, whereas their level of expression within the morbilliviruses is considerably lower (56).

The C proteins are not required for mRNA synthesis or genome replication, at least in cell culture. Indeed, the first function ascribed to the SeV C proteins was that they inhibited RNA synthesis *in vitro,* but only when coexpressed with the viral polymerase (57). This negative effect was also subsequently demonstrated *in vivo* (58). In both instances, the inhibitory effect of C could be relieved by overexpressing P but not L. However, it has not been possible to show a direct interaction between the P and C proteins in either SeV, PIV3, or MeV (59–61), although a glutathione *S*-transferase (GST)-tagged SeV C protein was capable of coselecting L (59). The SeV C proteins, therefore, appear to be modulating the function of the viral polymerase, although the mechanism through which they operate remains unclear. The C proteins may also serve as replication fidelity factors (58). This idea arose from the observation that the amplification of SeV cDNA templates that were not a multiple of 6 was more sensitive to the presence of the C proteins than were similar templates corrected for the hexamer rule. Such a function would ensure genetic stability because nucleocapsids containing insertions or deletions would be selected against during viral amplification. Once again, the C protein would mediate this effect through the viral polymerase, possibly at the initiation step of RNA synthesis.

Apart from their intracellular concentration during viral Infection, the respirovirus and morbillivirus C proteins have other properties that suggest that there may be functional differences between the two. Most noticeably, whereas recombinant C^- MeV are apparently viable and grow to titers equivalent to those of the recombinant wild-type virus (62), recombinant SeV in which C protein expression has been reduced or nearly ablated are severely debilitated for growth in the egg allantoic cavity and are nonpathogenic in a natural host, the laboratory mouse (63,64). Consistent with the negative effects already described for the SeV C proteins, these recombinant viruses overtranscribe. Interestingly, the recombinant virus that does not express C and C′ shows a delay in accumulation of viral RNA, hinting that these proteins may exert a positive function at some early event during virus growth (63).

B. V Proteins

The transcripts of the P gene (except for the pneumoviruses and hPIV1) are cotranscriptionally edited downstream of the C ORF. This involves the pseudotemplated addition of either +1G [in the case of the respiroviruses, morbilliviruses, and Newcastle disease virus (NDV)] or +2G nucleotides (for the rest of the rubulaviruses) within a G run (65). This addition is regulated by a *cis*-acting template sequence. Editing in those viruses that add +1G fuses the N terminal ORF of the P protein to an internal V protein ORF (in the −1 frame relative to P) that is rich in cysteine. In those viruses that add +2G the nonedited mRNA makes the V protein, and it is the N- and C-terminal ORFs of the P protein that are fused by editing. The V domain can bind two molecules of Zn^{2+}, presumably to form a specific structure (66,67). Because they lack the C-terminal region of the P protein, the V proteins are unable to form stable complexes with L and the nucleocapsid template. However, the SeV V protein was found to interfere with replication but not transcription, although this effect was not due to the cysteine-rich V domain because a similar result was observed when the N-terminal domain of P was tested (38). Because these proteins retained the region of P that interacts with N°, it was speculated that this inhibition was either due to competition between V and P for N° (the V–N° complex then being inactive for assembly), or that the presence of V somehow interfered with the utilization of the P–N° assembly complex (68). However, more recent results indicate that the monomeric V protein is unable to compete with the trimeric P protein for N°, suggesting that it is the utilization of the P–N° complex that is impaired.

Recombinant viruses unable to express the V protein have been generated for both SeV and MeV. These V^- viruses were not impaired for multiplication in tissue culture but were attenuated in animal models (69,70). At least for SeV, this attenuation correlated with a rapid clearance of the virus from the lung, suggesting that the V protein may be involved in protecting the virus from an early host immune response.

C. M1/M2

The RS virus genome contains 10 genes (3 or 4 more than are found in the other families). One of these additional genes, M2, lies between the fusion protein gene (F) and L. The mRNA of this gene contains two ORFs that code for the M2 protein (a virion structural protein) and M1. Coexpression of the M1 protein with the viral polymerase

interferes with RNA synthesis (71). M2, however, functions as a processivity factor for mRNA transcription because in the absence of this protein the greater part of mRNA synthesis terminated prematurely. It also acts as a transcriptional antiterminator that enhances transcriptional readthrough at the intergenic junctions (71,72). However, M2 is not required for processivity during genome replication, leading to the speculation that it may play a role in regulating the balance between these two processes.

IV. Genome Expression

The naked RNA of *Mononegavirales* is noninfectious for two reasons: (i) the active template is a ribonucleoprotein complex (N:RNA) and (ii) the genomic RNA cannot serve as a messenger, but must be transcribed by a virally encoded polymerase (the P-L complex). Holonucleocapsids carrying 50 molecules of L and 300 of P (i.e., 100 P trimers) can initiate an infection on entry into the cell cytoplasm (73). The polymerase is thought to enter the genome obligatorily from the 3′ end, synthesize the short +ve sense leader, terminate, and then reinitiate at the beginning of the first gene. Reinitiation at this first junction is critical because it locks the polymerase into a transcriptive (i.e., it will make mRNAs) rather than a replicative (it will copy the entire genome, ignoring the intergenic signals) mode. Polymerases that read through this junction without concurrently assembling the nascent chain are nonprocessive and never reach the end of the first gene.

Reading through this first junction is generally a rare event, however. In the polR mutant of VSV and strain Z of SeV, the percentage of readthrough is about 20% (74,75). In both of these examples the phenotype maps not to the viral polymerase but to the N protein of the nucleocapsid, highlighting the fact that this protein is an integral part of the template. This early event in the replication cycle is termed "primary transcription," and it is independent of *de novo* protein synthesis. It results in the accumulation of 5′ capped and 3′ polyadenylated viral mRNAs derived from the infecting templates (Fig. 2). Because these viruses replicate exclusively in the cytoplasm, the capping and polyadenylation activities are thought to reside on the L protein. The poly(A) addition occurs due to reiterative copying of an oligo(U) stretch that proceeds the intergenic junctions and forms part of the transcriptional termination signal. Capping of the viral RNAs is also tightly coupled to stopping/restarting at the intergenic boundaries, and caps are never found on the extragenic leader RNAs (they contain a 5′-triphosphate). Not all the polymerases that terminate at a junction

restart at the next gene. This in turn leads to transcriptional attenuation, with genes positioned 3′ on the genome being expressed at higher levels than those located more 5′. Moreover, in MeV this transcriptional gradient is steeper in neuronal cell infections, in which the virus grows slowly and develops toward persistence, than in cell lines in which the infection is lytic (76). This suggests that reinitiation at the intergenic boundaries may be modulated by cellular factors.

Translation of the primary transcrips results in an intracellular accumulation of viral proteins. This in turn is believed to trigger a switch from transcription to replication. Here ostensibly the same viral polymerase enters the template from the 3′ end but now ignores all the intergenic signals (and editing sites) to synthesize an exact complementary copy. This antigenome serves as a replication intermediate. Replication and genome assembly are tightly coupled, and it is believed that this coupling somehow permits the polymerase to ignore the *cis*-acting transcriptional signals. The major regulator of the switch is the intracellular concentration of unassembled N (or, more precisely, the concentration of the assembly complex, P-N°; Fig. 2). This creates a self-regulatory mechanism in which, when the P–N° concentrations are high, replication is promoted, which in turn reduces the N° pool, thereby promoting transcription. By analogy with VSV, approximately the first 12 nt of the leader RNAs are believed to contain an encapsidation signal that promotes assembly of the nascent chain. It remains unclear, however, at what point during leader synthesis that assembly begins (and, indeed, if it begins at the 5′ end of the nascent strand) and at what point the polymerase becomes committed to replication.

Release of N° from the assembly complex is also regulated by interactions between the P–N° complex and the viral polymerase, as mutations in the SeV L protein have been reported that continue to make RNA but are defective in replication (52). In addition, rather than promoting the switch, increased expression of the N protein of RS virus, in a minigenome replicon assay, increased both replication and transcription (77). Results in our laboratory with SeV indicate that low concentrations of P–N° enhance transcription rather than promote replication (78). This suggests that the switch (if it exists), although controlled principally by the level of N°, may be amenable to regulation at other levels.

V. Summary

Molecular studies on the replication of paramyxoviruses have undergone a revolution in recent years due to the development of techniques

that permit the manipulation of their genomes as cDNA. This has led to new information on the structure–function organization of the viral proteins involved in genome expression, as well as dissection of the *cis*-acting template sequences that regulate transcription and replication. Studies using recombinant viruses have also provided new insights into the role of the accessory proteins (V, C, M1/M2) in both for virus growth in cultured cells and pathogenesis in animals.

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RATIONAL DESIGN OF LIVE-ATTENUATED RECOMBINANT VACCINE VIRUS FOR HUMAN RESPIRATORY SYNCYTIAL VIRUS BY REVERSE GENETICS

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I. Introduction

Human respiratory syncytial virus (RSV) is the most important viral agent of pediatric respiratory tract disease, and is responsible for a huge burden of illness worldwide as well as significant mortality (4,7,12,16,25,36). It is also a suspected factor in the development or exacerbation of asthma (38). RSV infects essentially everyone, usually by age 2 years. The peak of serious disease is between 6 weeks and 6 months of life. RSV also causes serious disease in the elderly and in immunosuppressed individuals.

The single available therapy, the nucleoside analog ribavirin, administered by ventilated aerosol, has uncertain efficacy (8). Serum immunoglobulin was shown to be efficacious when administered as immunoprophylaxis to children who are at high risk of serious RSV disease due to bronchopulmonary dysplasia or premature birth (39). These therapies notwithstanding, the history of the control of major viral and bacterial

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diseases illustrates the primary, essential role of vaccines (19). Despite the clear need, a vaccine for RSV is not currently available.

"Reverse genetics" is the reconstitution of biological components from cDNA. It is now possible to produce infectious recombinant (r) RSV entirely from cDNA (5). Thus, one can introduce predetermined changes into RSV through the cDNA intermediate (5,11). This provides a powerful tool for studies of molecular biology, viral pathogenesis, and the host immune response, as well as a method for the rational design of live-attenuated viral vaccine candidates. This article will describe the use of reverse genetics to prepare and characterize live-attenuated recombinant versions of the RSV A2 strain (antigenic subgroup A).

II. Respiratory Syncytial Virus

RSV is an enveloped virus with a nonsegmented, single-stranded, negative-sense RNA genome of 15,222 nucleotides for strain A2 (Fig. 1) (7). It is classified within the *Paramyxoviridae* family (Table I; refs. 7 and 40). RSV encodes 10 subgenomic mRNAs that are capped and polyadenylated. Each mRNA encodes a single major protein except for the M2 mRNA, which contains two overlapping translational open reading frames (ORFs), each of which encodes a separate protein, M2-1 and M2-2 (6). Thus, there is a total of 11 different RSV proteins (Figs. 1 and 2 and Table II).

The virion consists of a nucleocapsid packaged within a lipid envelope (Fig. 2). The nucleocapsid contains the RNA genome in close association with the nucleocapsid N protein, which confers nuclease resistance. Other proteins of the nucleocapsid include the phosphoprotein P, the major polymerase subunit L, and the transcription elongation factor M2-1. Together with the RNA-N protein template, the P and L proteins are necessary and sufficient for RNA replication and for a poorly processive form of transcription, and the M2-1 protein in addition is necessary for fully processive transcription (6,24,26,49).

The envelope is derived from the host cell plasma membrane and contains spike-like projections, each being a homo-oligomer of one of three transmembrane viral envelope proteins: the attachment protein G, the fusion protein F involved in viral penetration, and the small hydrophobic protein SH of unknown function. The G protein is unusual among paramyxoviruses because it has a high content of O-linked sugars, perhaps 20 or more chains (7). The ectodomain has a high content of proline, serine, and threonine residues that, together with the many carbohydrate side chains, are thought to form an extended

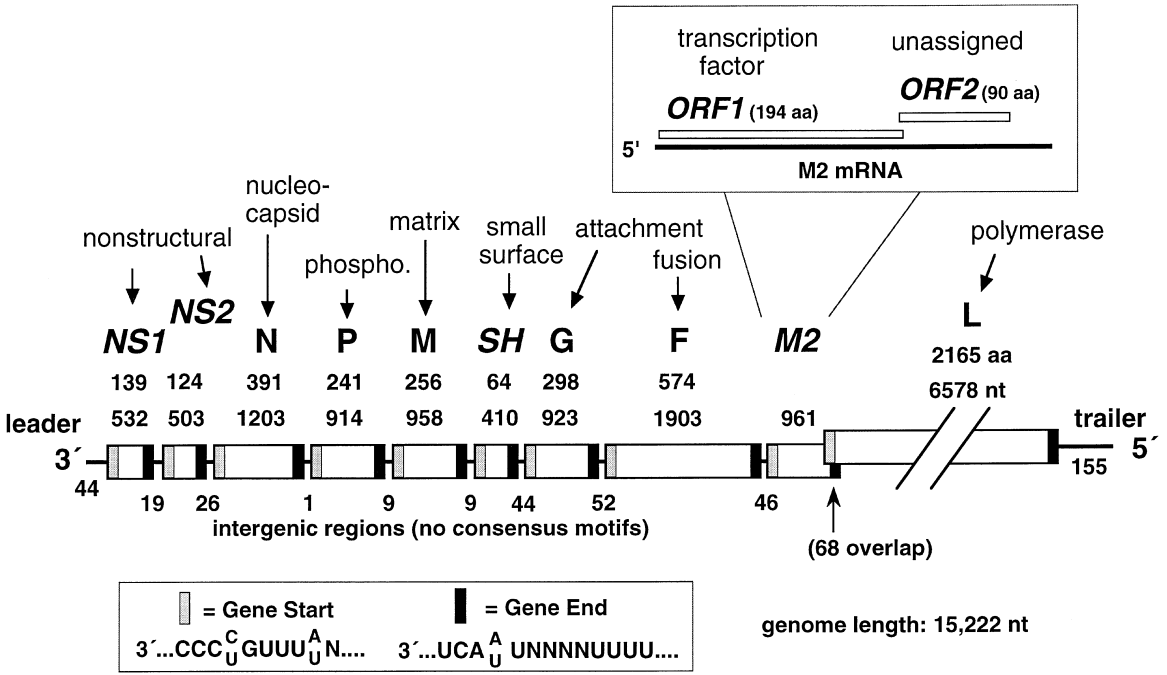


FIG 1. Map (not to scale, 3′ to 5′) of negative-sense genomic RNA of RSV strain A2. Each long rectangle, including the shaded and filled regions at either end, corresponds to an encoded mRNA. The shaded and filled regions identify the gene-start and gene-end signals, respectively. The nucleotide length of each mRNA (exclusive of nontemplated polyadenylate) is indicated immediately over its box, and the amino acid length of the encoded protein is indicated above that. An expanded drawing of the M2 mRNA illustrates the two ORFs, shown as open rectangles over the line representing the mRNA. The extragenic leader, intergenic, and trailer regions are drawn as thin lines, with nucleotide lengths shown underneath. Only the F and L proteins have unambiguous, albeit low, sequence relatedness to their counterparts in other genera of *Paramyxoviridae;* the NS1, NS2, and M2 genes do not have known counterparts in other paramyxoviruses, and the SH gene has a counterpart only in simian virus type 5 and mumps virus (7,40).

TABLE I

CLASSIFICATION OF RSV WITHIN FAMILY *PARAMYXOVIRIDAE*[a] AND SELECTED MEMBERS OF TAXON

Classification	Member
Subfamily *Paramyxovirinae*	
Genus *Respirovirus*	Sendai virus, human parainfluenza virus types 1 and 3
Genus *Morbillivirus*	Measles virus
Genus *Rubulavirus*	Mumps virus, simian virus 5, human parainfluenza virus type 2
Subfamily *Pneumovirinae*	
Genus *Pneumovirus*[b]	Respiratory syncytial virus
Avian pneumovirus[c]	Turkey rhinotracheitis virus

[a] *Paramyxoviridae* is one of four families that make up the order *Mononegavirales,* the nonsegmented, negative-strand RNA viruses; the others are *Rhabdoviridae, Filoviridae,* and *Bornaviridae.*

[b] The genus includes bovine, ovine, and caprine RSV and pneumonia virus of mice.

[c] Assignment as a separate genus, yet to be named, is planned.

mucin-like structure. The high content of carbohydrate, being host-specified and possibly exhibiting variable usage of potential acceptor sites, might shield the virally encoded protein from host immune recognition. There is a central conserved, disulfide-bonded domain that is thought to be involved in receptor binding. The G protein is expressed in both a membrane-bound form and an amino terminally truncated, secreted form (41). The latter might serve as a decoy to spare virus from antibody-mediated neutralization and also has been suggested to influence the nature of the immune response (see below and Ref. 27).

The matrix M protein is an internal protein that is essential for virion assembly (46). There are two proteins that are provisionally considered to be nonstructural: NS1 and NS2. It is not known whether the M2-2 protein is a virion component. This species is much less abundant intracellularly than the other proteins, due either to instability or inefficient translation of its overlapped ORF.

RSV exists as a single serotype based on virus neutralization, but protein-specific antigenic analysis and nucleotide sequencing identified two antigenic subgroups, A and B. The two subgroups exhibit significant sequence divergence throughout the genome (and thus represent two distinct divergent branches) but have the greatest differences in the ectodomains of the G and SH glycoproteins (44% and 43% amino acid sequence identity between subgroups, respectively). The question of whether significant antigenic drift occurs remains open: progressive

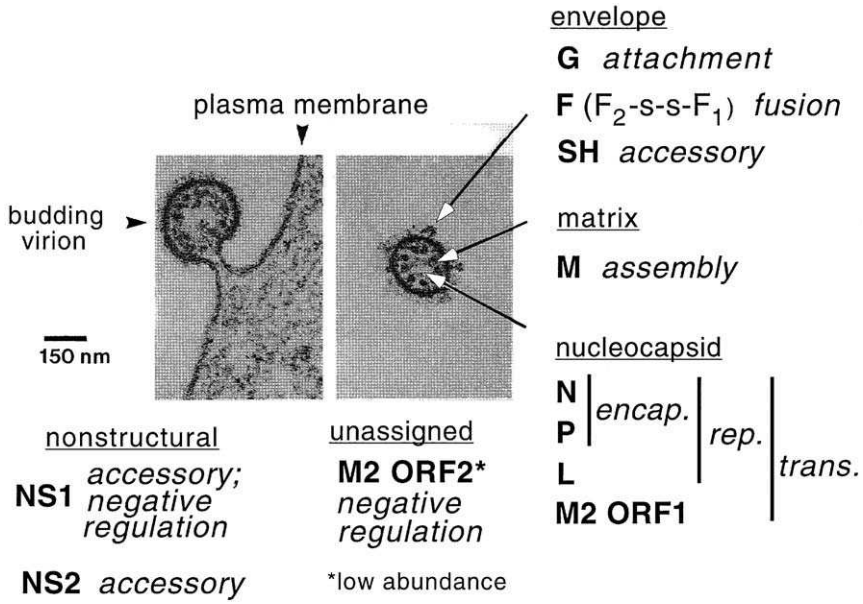


FIG 2. Locations and functions of the RSV proteins. Shown are electron micrographs of thin-layer sections of (*left*) an RSV virion in the final stage of budding from the plasma membrane of an infected cell and (*right*) a free virion (adapted from Ref. 29). N and P are sufficient for encapsidation of minireplicon RNA (1); N, P, and L are the proteins necessary for replication (6,24,49); N, P, L, and M2-1 are the proteins necessary for fully processive transcription (6); NS1 and M2-2 inhibit minireplicon transcription and RNA replication and thus are potential negative regulatory proteins (1,6); G, F, M, N, and P are sufficient to direct packaging and passage of a minireplicon (46); SH, NS1, and NS2 are accessory proteins because they do not have an established function and can be deleted without ablating the ability of RSV to grow in cell culture (3; 46a, M. N. Teng and P. L. Collins, unpublished data).

nucleotide changes in circulating virus have been noted, but it is unclear whether this has been accompanied by significant antigenic changes.

RSV binds to an unidentified cellular receptor(s). The nucleocapsid enters the cytoplasm by fusion at the cell surface. Viral transcription occurs from the 3′ end by a sequential stop-start mechanism directed by the gene-start (GS) and gene-end (GE) transcription signals that flank the viral genes (Fig. 1) (33–35). Replication involves the synthesis of a full-length, positive-sense copy called the "antigenome." The antigenome also is found only in nucleocapsid form and is the template for the synthesis of progeny genome. Virus transcription and RNA replication occur in the cytoplasm, and virions form by budding at the plasma membrane.

TABLE II

CHARACTERISTICS OF PROTEINS OF RSV

Protein	Characteristics
Nucleocapsid-associated	
N	Major nucleocapsid protein. 43.4 kDa. Binds tightly to genomic and antigenomic RNA, forming the ribonucleoprotein template for RNA synthesis.
P	Phosphoprotein. 27.2 kDa, but migrates anomalously slowly in SDS–polyacrylamide gel electrophoresis. Thought to associate with free N and L proteins to maintain them in soluble form prior to their assembly into or interaction with the nucleocapsid. N and P are required for encapsidation of minireplicon RNA. P is thought to be a polymerase cofactor.
L	Large protein. 250.2 kDa. Major polymerase subunit. Contains conserved polymerase motifs that presumably represent catalytic domains. N, P, and L are necessary and sufficient for RNA replication and a poorly processive form of transcription (6,24,49).
M2-1	Major product of the M2 mRNA, encoded by 5′-proximal ORF. 22.2 kDa. Promotes transcription elongation (6) and increases the frequency of readthrough of GE signals (26). The N, P, L, and M2-1 proteins are necessary and sufficient for fully processive transcription and as support proteins to direct the production of infectious virus from cDNA (5,6).
Envelope-associated	
G	Most heavily glycosylated RSV protein. Transmembrane surface attachment protein. Major neutralization and protective antigen. Antigenic and sequence divergence in G defines the two antigenic subgroups (A and B). Contains several N-linked sugar side chains and extensive O-linked glycosylation; the Mr of the unglycosylated form is 32.5 kDa and that of the fully glycosylated form is 90 kDa. Translational initiation at the second methionine in the ORF yields an N-terminally truncated form that, following additional N-terminal proteolytic trimming, is secreted (41). A virus in which the G gene has been deleted is viable in cell culture, implying the existence of a second attachment activity (31; M. N. Teng and P. L. Collins, unpublished results).

F Fusion protein. Transmembrane surface protein. Mediates viral penetration and syncytium formation. Major neutralization and protective antigen. Synthesized as an N-glycosylated, 70-kDa precursor, F_0, which is cleaved by a furin-type intracellular protease into two disulfide-linked subunits, F_2 (amino acids 1–130) and F_1 (amino acids 137–574).

SH Small hydrophobic protein. Transmembrane surface protein. Function unknown. Translational initiation at first and second methionines of the ORF yields full-length (SH_0, 7.5 kDa) and N-terminally truncated (SHt) forms. A fraction of SH_0 is modified with an N-linked sugar side chain to form SHg, and some of SHg is modified by the addition of polylactosaminoglycan to the N-linked sugar to form SHp. A recombinant virus in which the SH gene has been deleted is fully viable in cell culture (3).

M Matrix protein. 28.7 kDa. Unglycosylated internal protein. Mediates virion assembly (46).

Nonstructural

NS1 Nonstructural protein 1. 15.6 kDa. Putative negative regulatory factor for replication and transcription (1). A recombinant virus in which the NS1 gene has been deleted is viable in cell culture (M. N. Teng and P. L. Collins, unpublished results).

NS2 Nonstructural protein 2. 14.7 kDa but migrates more slowly than NS1 in SDS–polyacrylamide gel electrophoresis. Function unknown. A recombinant virus in which the NS2 gene has been deleted is viable in cell culture (46a, 48a).

Unassigned

M2-2 Second product of the M2 mRNA, encoded by the second, internal ORF. Putative negative regulatory factor for replication and transcription (6). Much less abundant than the other viral proteins.

III. RSV Disease

RSV replicates in the surface epithelium of the respiratory tract and generally is restricted to that site by host immunity, as evidenced by its ability to spread to other tissues in situations of immune deficiency. It causes an acute infection that is cleared by host immunity. Disease can range from rhinitis to severe bronchiolitis and pneumonia. A major part of RSV disease is due to virus-mediated destruction of the epithelium and thus is determined by the extent of viral replication. It also is likely that during natural infection some components of RSV disease are due to the host immune response (10,22,23,27,38,44). However, the fact that RSV can be fatal in severely immunocompromised individuals shows that severe disease is not dependent on a vigorous host immune response.

An example of an immune-mediated component of RSV disease, albeit one that does not involve natural infection alone, is illustrated by the experience in the 1960s, when a formalin-inactivated, alum-precipitated RSV vaccine was administered intramuscularly to infants and young children (4,7,22,30,37). The vaccine failed to induce protection against natural infection and, paradoxically, RSV disease was exacerbated. Retrospective serology and studies in experimental animals indicated that the failure to induce protection was associated with a weak neutralizing antibody response, probably due to denaturation of critical antigen epitopes, and with a lack of a significant cytotoxic T-cell response, probably due to the nonreplicating nature of the antigen. The formalin-RSV recipients manifested an augmented peripheral blood lymphoproliferative response to RSV antigens suggestive of an exaggerated CD4+ lymphocyte response.

More recently, studies in rodents have shown that formalin-treated RSV stimulates a CD4+ T-cell response which is biased toward the Th2-like subset [for which interleukin-4 (IL-4) is a marker cytokine] and is associated with augmented lung histopathology on subsequent RSV infection (10,22,23,38,44). In contrast, intranasal infection of rodents with RSV stimulates a Th1-biased response (for which γ interferon is a marker cytokine), and subsequent reinfection is highly restricted. Augmentation of disease in humans or rodents is not a feature of reinfection with RSV. Interestingly, the effect seen with formalin-treated RSV also was observed in seronegative rodents with purified F and G antigen (37), which indicates that vaccines for seronegative subjects based on purified antigen or peptides should be viewed with caution until more information is available. The risk of priming for enhanced disease appears to be limited to the first exposure in life to

RSV antigen (23). A vaccine based on purified RSV glycoproteins might be useful in the elderly, a seropositive population that can experience severe RSV disease.

Another line of experimentation showed that expression of the G protein alone in BALB/c mice, and in particular its secreted form, stimulated the expression of IL-5 and resulted in increased lung eosinophilia on subsequent RSV infection (27). This might be the aspect of the immune response that is associated with subsequent allergic disease. However, this effect was not observed when the G protein was expressed together with a fragment of the M2-1 protein that strongly induces CD8+ cytotoxic T cells (44). This finding hints at a role for CD8+ cells in regulating the immune response and is a further argument in favor of immunization with a live-attenuated virus rather than a subset of viral antigens. These various clues suggest that the nature of the lymphocyte response can be an important determinant of RSV disease and vaccine efficacy and safety, but our understanding is at a preliminary stage.

IV. Immunity to RSV

Resistance to reinfection with RSV is mediated primarily by RSV-neutralizing secretory and serum antibodies (4,7,12,16). The G and F glycoproteins are the only RSV antigens that induce neutralizing antibodies, and have been identified as the major protective antigens. In BALB/c mice, the M2-1 protein elicited protective immunity that was mediated by cytotoxic T cells and was very short-lived. This cellular arm of the immune response is thought to play a lesser role in long-term resistance to reinfection.

Human newborns are spared serious RSV disease because virus replication is partially restricted by the presence of transplacentally derived, serum RSV-neutralizing antibodies. However, this immunity wanes within the first weeks to months of life. The incomplete, short-lived nature of this protection in the case of RSV is unusual and is thought to reflect, in part, the highly infectious and virulent nature of the virus.

Immunity to RSV acquired by natural infection is incomplete, and reinfection can occur throughout life. However, serious disease is associated only with the first or, at a greatly decreased rate, the second RSV infection of life. This is because induced host immunity partially restricts subsequent infections. This has been demonstrated in numerous studies in experimental animals, in which prior infection or the passive transfer of immune effectors such as RSV-neutralizing serum

antibodies confers significant resistance to RSV challenge. Another observation indicating the importance of intact immunity for protection is that older children and adults whose immunity is compromised by chemotherapy or irradiation experience life-threatening RSV infection at high frequency.

Reasons for frequent reinfection with RSV include its highly infectious nature; its antigenic diversity; the less effective and shorter-lived immunity operative at its mucosal site of replication; resistance to efficient neutralization *in vivo,* possibly mediated by the unique structure of the G glycoprotein; and the decreased immune response following RSV infection in the neonatal period due to immunological immaturity, as well as immunosuppression mediated by maternally acquired antibodies.

V. Obstacles to Development of RSV Vaccine

An RSV vaccine based on the intransal administration of a live-attenuated virus should be able to overcome some of the immunological obstacles indicated above. Intranasally administered RSV, either wild-type (*wt*) or attenuated, induces secretory and serum antibodies and a balanced cellular immune response, and does not prime for enhanced disease upon subsequent RSV reinfection. The replication of *wt* or attenuated RSV in the respiratory tract is only partially restricted by the presence of passively derived serum antibodies and, in contrast to parenterally administered vaccines, induces protective immunity in the presence of such passively transferred antibodies. This was demonstrated with an attenuated RSV vaccine candidate administered to the respiratory tract of chimpanzees that had been infused with RSV-neutralizing serum antibodies sufficient to mimic the high level of maternal antibodies seen in neonates (15). The replication of the attenuated virus was slightly reduced, but it was able to confer a high level of resistance to subsequent challenge with *wt* RSV. An RSV vaccine will have to be given within the first weeks of life, when the magnitude of the immune response is expected to be low for the reasons given above. To compensate partially for this deficiency in immunogenicity in the target population, a live RSV vaccine will have to be given in a multidose schedule similar to that of the live poliovirus and rotavirus vaccines to achieve a satisfactory level of protection.

Both antigenic subgroups A and B should be represented in an RSV vaccine, and it will be necessary to develop two live-attenuated RSV vaccines or, alternatively, express protective antigens for both sub-

groups from a single recombinant virus. The recombinant DNA methodology outlined below can expedite development of a bivalent vaccine.

The poor growth of RSV in cell culture and the instability of infectivity have been long-standing obstacles to basic virologic studies. However, the *in vivo* infectivity of candidate vaccines studied to date is high, and poor virus growth has been partially overcome by optimized cell culture methods.

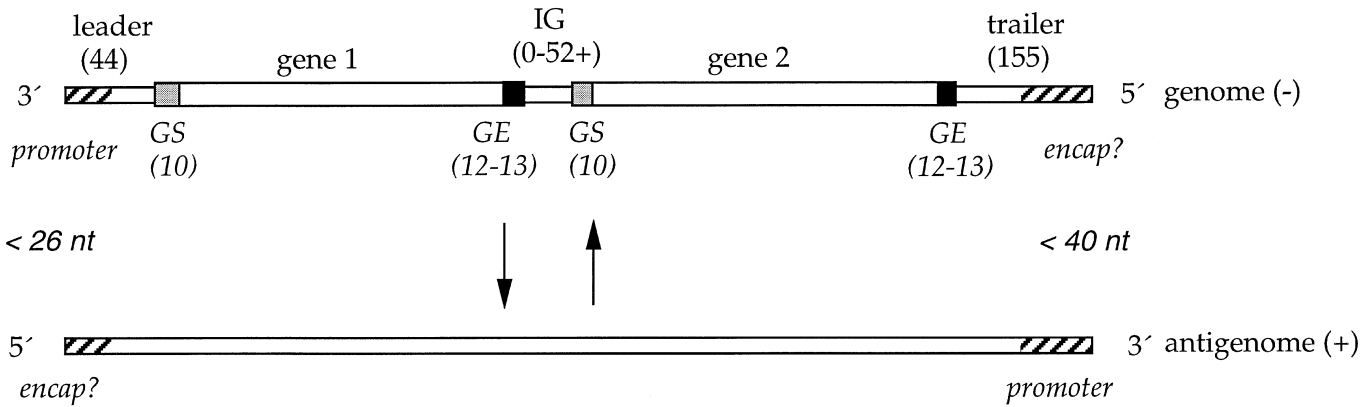
Only chimpanzees and humans support permissive RSV replication and exhibit authentic RSV disease. Convenient small experimental animal models like mice are only semipermissive for RSV replication, such that relatively few cells in the lung are infected. Vaccine studies under these conditions, where the virus is already severly restricted, yield falsely optimistic results. Therefore, accurate data on vaccine safety, immunogenicity, and efficacy depend on clinical trials, which begin in seropositive adults and proceed stepwise to seropositive children and infants, and then to seronegative children and infants of decreasing age (for example, see Refs. 31 and 32).

Other problems in making a live-attenuated RSV vaccine are to find the appropriate balance between attenuation and immunogenicity and to produce a virus that is genetically stable, i.e., that maintains the attenuation phenotype following replication in humans. The recombinant cDNA technology described in the next section is uniquely suited to achieve these goals.

VI. RSV Reverse Genetics: Antigenic Subgroup A

The strategy for introducing predetermined changes into infectious RSV is to do the genetic engineering in a complete cDNA copy of the genome or antigenome, copy this into RNA, and produce rRSV (5,11). This is complicated by the fact that neither the nonsegmented, negative-strand genome nor its antigenome is infectious alone. Rather, the minimum unit of infectivity is a nucleocapsid competent for transcription and RNA replication. Therefore, the strategy is to coexpress genomic or antigenomic RNA intracellularly in the presence of plasmids expressing the subset of proteins needed to assemble an "infectious" nucleocapsid. Once a functional nucleocapsid is assembled, the reconstituted RSV polymerase will express all of the RSV genes and launch a productive infection.

This strategy was first developed with helper-dependent minireplicons, cDNA-encoded versions of genomic or antigenomic RNA in which the viral genes have been deleted and replaced by a marker gene such as bacterial chloramphenicol acetyltransferase (CAT; Fig. 3) (1,6,24,


leader
(44)
gene 1
IG
(0-52+)
gene 2
trailer
(155)
3´
5´ genome (-)
promoter
GS
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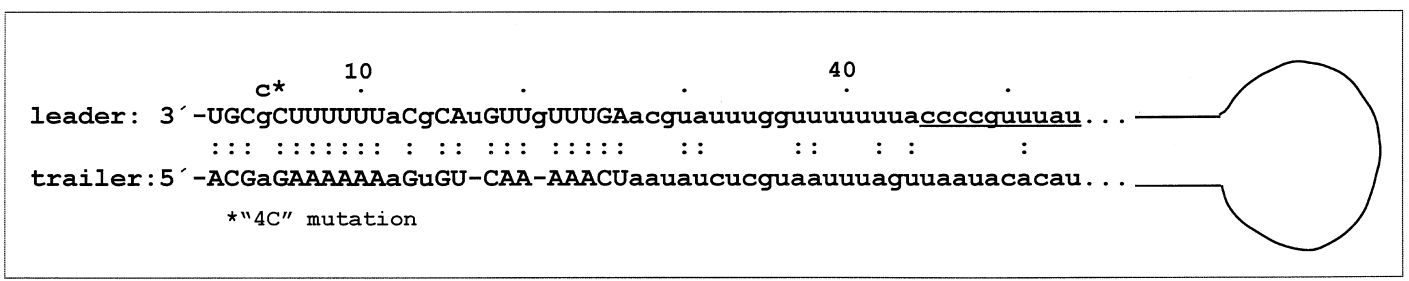
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*"4C" mutation

33–35). These studies showed that RSV transcription and RNA replication indeed can be reconstituted from a minireplicon and proteins supplied from cotransfected plasmids, where expression is driven by T7 RNA polymerase supplied by a recombinant vaccinia virus. Importantly, this showed that, together with the RNA-N protein template, the P and L proteins are sufficient to reconstitute the RNA replicase and that the further addition of the M2-1 protein reconstitutes the transcriptase (6,24). This identified the proteins needed to support virus recovery.

Coexpression of the M2-2 or NS1 protein along with a minireplicon and the N, P, and L proteins (with or without the M2-1 protein) drastically inhibited both transcription and RNA replication, suggesting that each protein might have negative regulatory activity (1,6). Coexpression of the M, F and G proteins along with the minigenome and the N and P proteins was sufficient to direct the production of particles capable of passaging the minigenome to fresh cells (46). The SH, NS1, or NS2 protein did not appear to play any detectable role in the assembly and passage of these helper-dependent particles. Coexpression of the M2-2 protein increased the efficiency of passage, suggesting that its inhibitory role might function at the level of virion morphogenesis,

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FIG 3. The *cis*-acting elements of the RSV genome illustrated with a dicistronic minigenome (top diagram) and its antigenome (middle diagram; RNA polarity is indicated in parentheses). The signals involved in RNA replication (crosshatching) have not yet been precisely defined but appear to be located within the 26 nucleotides at the 3′ end of the genome and the 40 nucleotides at the 5′ end (33–35, R. Fearns, M. Peeples, J. Cristina, P. Atreya, S. Samal, and P. L. Collins, unpublished data). The gene-start (GS) and gene-end (GE) motifs (filled gray and black boxes, respectively) are transcription signals that direct initiation of mRNA synthesis and polyadenylation/termination, respectively. The intergenic regions and the remainder of the genome appear to play little or no role in transcription and replication other than being the template. Thus, the *cis*-acting signals of RSV consist of short, circumscribed, self-contained sequences that in aggregate are fewer than 300 nucleotides (<2% of the genome). The complementarity between the 3′ and 5′ ends is illustrated at the bottom: complementary nucleotides are in capital letters, and the dashes indicate single-nucleotide gaps introduced to maximize the alignment. These sequences are 81% complementary, after which the degree of relatedness is insignificant. These sequences are drawn as a panhandle to illustrate the sequence relationships: it seems unlikely that such a panhandle forms in nature, and the terminal complementarity probably reflects the conservation of sequence identity of the promoters at the 3′ ends of the genome and antigenome. The "4C" mutation in the leader region is associated with enhanced minireplicon RNA replication (M. E. Peeples and P. L. Collins, unpublished data) and has been found in some biologically derived vaccine candidates (20).

perhaps to render the nucleocapsids quiescent with regard to RNA synthesis.

Minireplicon studies also have mapped the *cis*-acting signals of the genome (Fig. 3), although detailed mapping and characterization are still in progress (33–35; P. L. Collins, R. Fearns, P. Atreya, J. Cristina, S. Samal, and M. Peeples, unpublished data). This showed that the sequences required for RNA replication are contained within the 26 or 40 nucleotides at the 3′ or 5′ genome ends, respectively. The 3′ ends of genome and antigenome presumably each contain a promoter, and the 5′ ends might each contain an origin of encapsidation. Interestingly, the substitution of a C residue for G (negative sense) at position 4 in the leader region resulted in an increase in RNA replication (M. E. Peeples and P. L. Collins, unpublished data). This substitution is of note because it also has been observed in some biologically derived candidate vaccine viruses (20), and it has been included in the backbone used to generate recombinant vaccine viruses described below.

Other studies confirmed that the 10-nucleotide GS and the 12- to 13-nucleotide GE motifs are self-contained transcription signals (34,35). In contrast, the naturally occurring intergenic regions did not appear to play a role in RNA synthesis (33). Knowledge of the location and nature of the *cis*-acting sequences in the RSV genome has been helpful in engineering complete infectious virus and for interpreting effects of mutations in biological or recombinant viruses. Detailed analysis of the individual *cis*-acting signals is in progress and should identify potential attenuation mutations for evaluation in recombinant virus. Unlike other paramyxoviruses, replication of RSV does not obey the "rule of six," which is a requirement in other paramyxoviruses that the nucleotide length of the genome be an even multiple of 6 for efficient genome replication (42).

Complete infectious virus was produced by coexpression of a complete cDNA-encoded antigenome RNA supported by the N, P, L, and M2-1 proteins expressed from cotransfected plasmids (Fig. 4) (5). The genome contained several nucleotide changes introduced as markers for identification (5,47). Recovery is low, from 1 to 150 infectious recombinant viruses per 1.5×10^6 cells. A similar low yield has been observed for most mononegaviruses. Despite the low efficiency, even severely debilitated viruses have been successfully recovered, indicating the utility of the system. The recovered rRSV, whose cDNA had been constructed to be the consensus *wt* sequence, was confirmed to be *wt* on the basis of growth in cell culture and the ability to replicate to high titer and cause disease in chimpanzees.

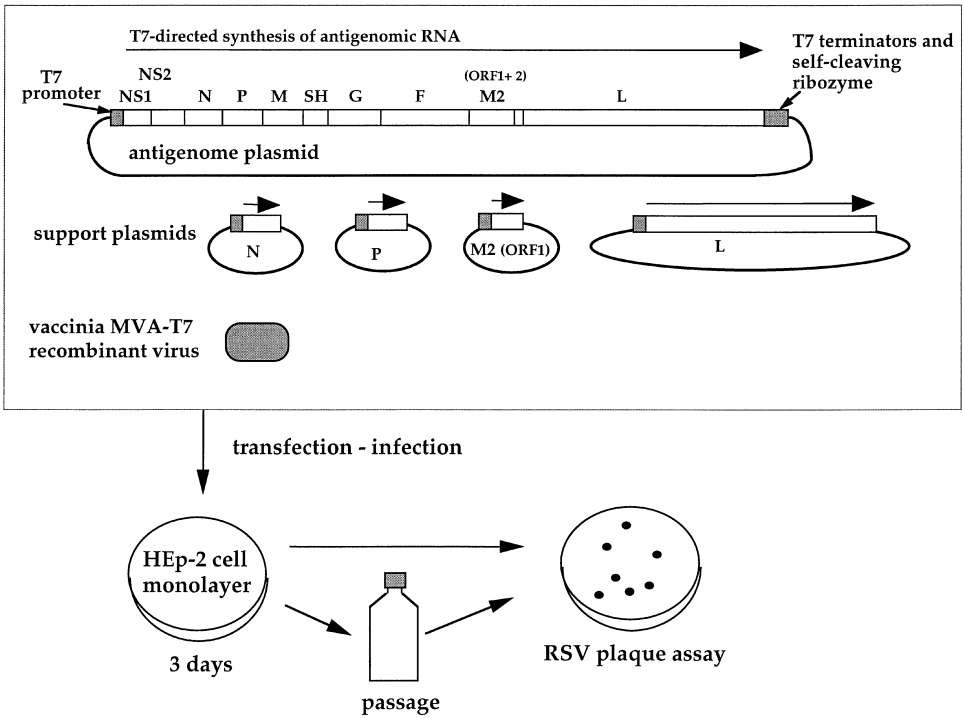

FIG 4. Recovery of infectious rRSV from cDNA. Plasmids encoding a complete antigenome and the N, P, L, and M2-1 proteins were transfected into cells that were simultaneously infected with a recombinant vaccinia virus expressing T7 RNA polymerase (5 and references therein). The vaccinia virus recombinant is based on the MVA strain, which is severely restricted in most mammalian cells for the formation of progeny virions. By this strategy, the antigenomic RNA and the N, P, L, and M2-1 proteins produced by the transfected plasmids assemble into antigenomic nucleocapsids. These, in turn, are replicated into genomic nucleocapsids, which are transcribed such that all of the RSV genes are expressed and a productive infection is launched. The purpose of expressing the plasmid-encoded RSV RNA in antigenome sense rather than genome sense was to preclude hybridization with mRNAs encoded by the support plasmids, although the successful rescue of parainfluenza virus (PIV) from genomic plasmids indicates that this consideration is not critical (16 and references therein). After 3 days, the medium supernatants were analyzed directly by plaque assay or were subjected to one passage before plaque assay.

VII. EXPRESSION OF FOREIGN GENES IN RECOMBINANT RSV

Three marker genes were engineered to be flanked by GS and GE transcription signals and were inserted individually into recombinant

RSV, namely, the genes encoding CAT, firefly luciferase (LUC), and jellyfish green fluorescent protein (GFP) (Fig. 5) (2; A. Bukreyev, M. Peeples, and P. L. Collins, unpublished data). The insertion sites were the leader-NS1, SH-G, or G-F junctions. In each case, the resulting rRSV expressed the additional gene as a separate mRNA, and the foreign protein was expressed at a high level comparable to that of the other viral proteins. The foreign gene was surprisingly stable genetically, such that each of 25 plaque-purified viruses isolated after 10 passages expressed the marker protein. These characteristics of high expression and stability have also been observed with other recombinant mononegaviruses. However, RSV is unusual in that the foreign gene has a strongly attenuating effect in cell culture that is proportional to its length, such that the CAT gene attenuates growth 20-fold, and virus containing the longer LUC gene is barely viable. Thus, there are limitations to the use of RSV as a vector for additional foreign antigens.

VIII. Gene Knockouts

It has been possible to prepare rRSV in which one of the following genes was deleted: NS1, NS2, SH, or G (3, 46a, and M. N. Teng and P. L. Collins, unpublished data). That four genes can be individually deleted without ablating growth in cell culture is in itself remarkable.

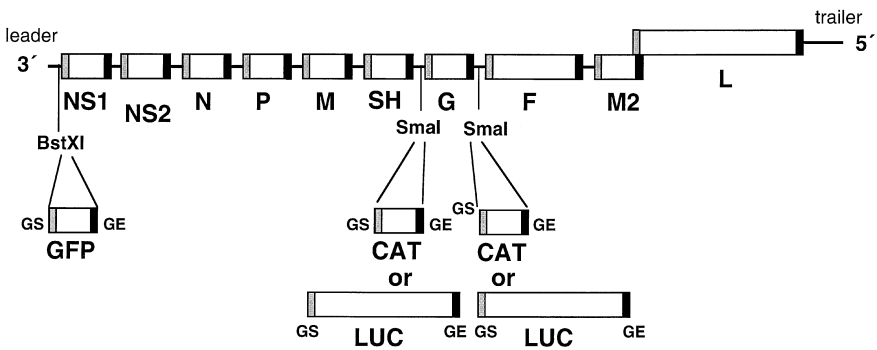

Fig 5. Expression of foreign genes from rRSV. Three different marker genes, encoding bacterial CAT, LUC, or GFP, were modified by the addition of flanking RSV GS (shaded box) and GE (filled box) signals (2; A. Bukreyev, M. Peeples, and P. L. Collins, unpublished data). The chimeric transcription cassettes were inserted into the leader-NS1, SH-G, or F-M2 junction, as illustrated. Each rRSV that was made contained a single foreign gene. The nucleotide length (in kilobases) of the GFP, CAT, or LUC insert is approximately 670, 1720, and 720, respectively.

In each case, the entire mRNA-coding region was deleted, yielding a virus whose genome was reduced in length and encoded one fewer mRNA. Virus in which the NS1, NS2, or G gene has been deleted (ΔNS1, ΔNS2, or ΔG) grows less efficiently in cell culture, and further characterization of these viruses is in progress. The ability of rRSV-ΔG to grow is remarkable and implies that the loss of the attachment function of G is accommodated by an auxiliary attachment function in another surface protein. The description of a viable biologically derived subgroup B virus in which the SH and G genes were spontaneously deleted implies that the alternative activity is in F (31). Of course, deletion of the G gene probably would not be appropriate for vaccine purposes because G and F are the two major neutralization and protective RSV antigens. The ΔSH virus forms larger plaques and grows somewhat better than *wt* virus in some, but not all, cell types. It is marginally attenuated in mice and chimpanzees, but in the latter animal it is associated with greatly reduced upper respiratory tract disease (3,48a). This would be highly desirable for a vaccine virus. The NS1, NS2, and SH proteins might be dispensable because they encode nonessential viral functions or because they have functions involved with pathogenesis or the host immune system. Depending on what effects are observed on attenuation, pathogenicity, and immunogenicity, these gene knockout viruses have the potential to serve as stand-alone vaccine viruses or, if incompletely attenuated, one or more gene knockouts can serve as additions to an rRSV vaccine candidate containing other attenuation mutations.

IX. Biologically Derived RSV Subgroup A Candidate Vaccine Viruses

Many years ago, a low-passage preparation of strain A2 called HEK-5 was subjected to 52 passages in cell culture at progressively lower temperatures (Fig. 6). The resulting cold-passaged (*cp*) RSV was not detectably cold-adapted or temperature-sensitive (*ts*) in cell culture, and was not attenuated in mice, but was moderately attenuated in chimpanzees (9,21,47). This phenotype was designated a non-*ts* host range (*hr*) restriction. More recently, *cp*RSV was then mutagenized with 5-fluorouracil, and *ts* mutants were selected (14,17,28). Two of these, *cpts*248 and *cpts*530, were subjected to a second round of mutagenesis and viruses of greater temperature sensitivity were isolated, including *cpts*248/955, *cpts*248/404, *cpts*530/1009, and *cpts*530/1030 (Fig. 6) (13,20,32,48). Each *cpts* virus was attenuated in mice and

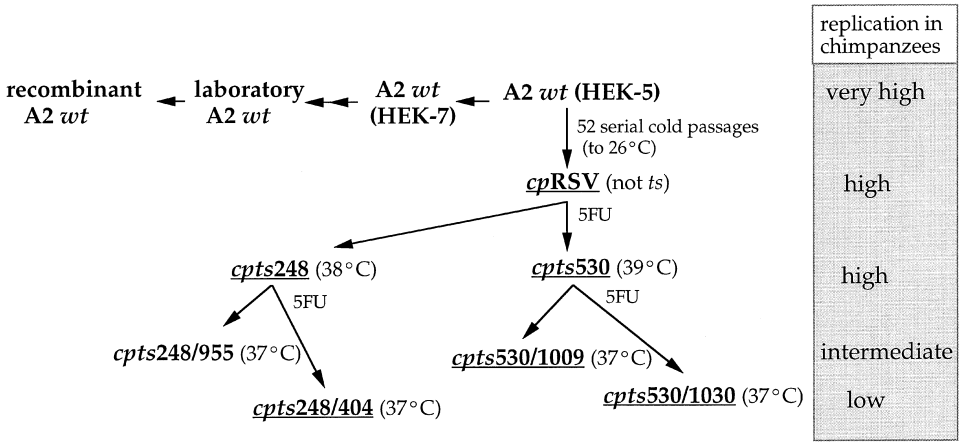

FIG 6. Passage history of RSV preparations and approximate level of replication in the respiratory tract of chimpanzees. All viruses are biologically derived except for recombinant A2 *wt*. *Viruses:* the strain A2 *wt* HEK-5 virus was five passages removed from the original strain A2 clinical isolate of 1961. It was subjected to 52 passages at suboptimal temperatures to yield *cp*RSV (21). *cp*RSV was mutagenized with 5-fluorouracil (5FU), and *ts* derivatives were isolated, two of which are shown: *cpts*248 and *cpts*530 (14,17,28). Each of these viruses was mutagenized again with 5FU, and several of the viruses of greater temperature sensitivity that were isolated are shown (13,20,32,48). The shut-off temperature of each *ts* virus, which is the lowest restrictive temperature at which a 100-fold reduction in plaque formation occurs, is shown in parentheses. The HEK-7 virus is two passages removed from HEK-5, and current laboratory strains of A2 used to construct rRSV were derived from HEK-7 through many passages and plaque isolations over more than 30 years. *Sequence analysis:* the *cp* and *cpts* viruses that are underlined were sequenced completely (9,17,20,28,28a,47,48,48b). The sequence for present-day laboratory A2 *wt* virus was compiled from two different laboratories with the use of different virus preparations (7). The cDNA encoding the recombinant A2 *wt* virus was sequenced in its entirety following its construction, and the recovered rRSV is presumed to be identical.

chimpanzees, with the magnitude of the attenuation phenotype being roughly proportional to the level of temperature sensitivity. Thus, *in vitro* passage at low temperature followed by sequential steps of mutagenesis did yield progressively more attenuated RSVs, but the process was hit or miss, labor intensive, and very lengthy, and in the end did not produce a sufficiently broad spectrum of attenuated vaccine candidates.

Clinical evaluation of these viruses is still in progress, but the available results indicate that the development of further-attenuated derivatives would be desirable, although the *cpts*248/404 virus appears to be close to the desired level of attenuation. Attempts at further attenuation

by an additional cycle of mutagenesis failed to produce the desired mutants and, as a consequence, the recombinant method was chosen as an alternative approach, as described below. It was recognized, however, that analysis of this panel of attenuated viruses would provide a set of mutations from which to create a recombinant vaccine virus and that the *cpts*248/404 virus, if recreated in recombinant form, would be an appropriate backbone on which to create a further-attenuated virus.

The *cp*RSV virus was sequenced in its entirety and compared with the published sequence for strain A2, which is a composite sequence from two laboratories using different current virus preparations that were many passages removed from HEK-5. Sixteen single nucleotide differences were found (9,17), which is a remarkably low number considering the divergence in passage histories. The HEK-5 progenitor of *cp*RSV was no longer available for comparison, but each of the 16 positions was examined in a virus preparation, HEK-7, which differed by two additional passage levels (Fig. 6). This analysis showed that *cp*RSV and HEK-7 have only five nucleotide differences at these positions, each of which encodes a single amino acid change in either the N, F, or L protein (Table III).

Construction of the antigenomic cDNA involved extensive recloning from RNA, and the resulting cDNA would not necessarily be identical to published strain A2 *wt* sequence. Sequence analysis of the completed antigenomic cDNA showed that it differed from the HEK-7 virus by only nine nucleotide point mutations (not shown), discounting seven other changes that had been deliberately introduced into the antigenomic cDNA as markers. The close similarity of present-day RSV A2, represented by the cDNA, to its progenitor of more than 30 years ago attests to a remarkable genetic stability through many passages and plaque purifications. Only two of these nine nucleotide differences resulted in amino acid changes, and both were in the F protein (Lys-66-Glu and Gln-101-Pro, with the HEK assignment given last) (47); the others were silent nucleotide substitutions in the F and L ORFs. The rRSV antigenomic cDNA was modified to make the two amino acid positions in F have the HEK assignment, and thus the recombinant *wt* virus is identical at the amino acid level to the HEK virus and is a suitable substrate for examining individual mutations and reconstructing vaccine virus in recombinant form.

Insertion of the five *"cp"* changes (Table III) into rRSV conferred the *hr* phenotype of restricted replication in the chimpanzee (47). The five changes have not been analyzed individually because the phenotype is

TABLE III

DETECTION OF MUTATIONS IN BIOLOGICALLY DERIVED *cp* AND *cpts* RSV MUTANTS BY SEQUENCE ANALYSIS AND CHARACTERIZATION BY THEIR INDIVIDUAL INTRODUCTION INTO *wt* rRSV

Mutation[a]	Nature and location of mutation, deduced by sequence analysis		Phenotype conferred by introduction of indicated mutation into rRSV	
	Amino acid substitution (*wt* to mutant)	Nt substitution in *cis*-acting element (*wt* to mutant)	*ts*[b]	Attenuation[c]
cp[d]	Val-267-lle (N) Glu-218-Ala (F) Thr-523-lle (F) Cys-319-Tyr (L) His-1690-Tyr (L)		No	Yes (chimps)
248	Gin-831-Leu (L)		Yes	Yes
404-L	Asp-1183-Glu (L)		No	No
404-M2		9A > G[e] (M2 gene-start)[f]	Yes	Yes
530	Phe-521-Leu (L)		Yes	Yes
1009	Met-1169-Val (L)		Yes	Yes
1030	Tyr-1321-Asn (L)		Yes	Yes

[a] Mutations are named according to the step in mutagenesis at which they were acquired (see Fig. 6).

[b] The *ts* phenotype was evaluted by efficiency of plaque formation at temperatures between 32°C and 41°C.

[c] The attenuation phenotype was based on reduced replication in mice or, where indicated, in chimpanzees.

[d] The *cp* mutations are employed as a set.

[e] Negative-sense.

[f] The most potent single *ts* attenuation mutation identified to date.

observed only in the chimpanzee model, and a limited number of such studies is possible.

Five *cpts* viruses were sequenced completely (Fig. 6, Table III) (17,20,28,28a,47,48,48b). Four of these viruses (*cpts*248, *cpts*530, *cpts*530/1009, and *cpts*530/1030) had a single nucleotide substitution compared to their immediate progenitor, and in each case the substitution was in the L gene and encoded an amino acid substitution (Table III). Each of these mutations has been confirmed to be an independent *ts* and attenuation mutation on the basis of their individual characterization by insertion into rRSV (Table III). The *cpts*248/404 virus had

two single nucleotide changes relative to its immediate progenitor *cpts*248: a substitution in the L ORF that encoded an amino acid substitution and a point mutation in a *cis*-acting RNA sequence, the GS signal of the M2 gene (Table III). Analysis of these two mutations in rRSV indicated that the amino acid substitution in L conferred neither the *ts* nor the attenuation phenotype, whereas that in M2 conferred both phenotypes and indeed is the most attenuating *ts* mutation identified to date (48). It is remarkable that the *ts* phenotype is specified by a mutation in a transcription signal, a sequence that is not thought to be involved in secondary structure due both to its very short length and to the encapsidated nature of the genome.

Thus, analysis of the biologically derived viruses identified three types of mutations: (i) the five amino acid substitutions from *cp*RSV that as a set confer the *hr* attenuation phenotype, (ii) four amino acid substitutions in the L protein that individually confer the *ts* and attenuation phenotypes, and (iii) a point mutation in a GS signal that independently confers the *ts* and attenuation phenotypes. These mutations are now being analyzed in detail with the use of minireplicons as well as recombinant virus.

X. Rational Design of Recombinant RSV Subgroup A Vaccine

The design of our first-generation live-attenuated subgroup A rRSV vaccine involves the following considerations: (i) a recombinant form of the *cpts*248/404 virus will be used as the backbone; (ii) attenuation mutations of the *ts* variety will be supplemented with non-*ts* ones because in influenza virus the latter appear to stabilize the former; (iii) mutations will be selected to involve proteins (e.g., L protein mutations) and RNA signals (e.g., the M2 GS signal) and to involve as many different gene loci as possible (e.g., amino acid point mutations in several proteins rather than solely in L); (iv) as many attenuation mutations will be included as possible to maximize genetic and phenotypic stability; (v) where possible, amino acid changes from wild type will be made with the use of more than one nucleotide change to reduce the possibility of same-site reversion; (vi) at least one gene deletion, namely, SH, will be used because reversion of its genotype should be precluded; and (vii) viruses will be made and analyzed that span a range of level of attenuation so that the most appropriate level of attenuation can be found.

To date, recombinant versions of the *cp*RSV, *cpts*530, *cpts*248, *cpts*530/1009, *cpts*530/1030, and *cpts*248/404 viruses have been recon-

structed and shown to be indistinguishable from their biological counterparts in cell culture, in rodents, and, in some cases, in chimpanzees (28,28a,47,48,48b). As an example, Table IV shows analysis in cell culture and mice of rRSVs containing the various mutations of *cpts*248/404. This particular example showed that (i) biological and recombinant *cp*RSV (groups 2 and 3) were indistinguishable by these assays; (ii) biological and recombinant *cpts*248 were indistinguishable (groups 4 and 5); (iii) the M2 GS mutation of *cpts*248/404 is a *ts* and attenuation mutation (group 6), whereas the L mutation is not (group 7); (iv) biological and recombinant *cpts*248/404 were indistinguishable (groups 8 and 9); and (v) recombinant *cpts*248/404 retained its biological characteristics when the irrelevant L mutation was removed (group 10). We also are in the process of making viruses based on *cpts*248/404 to which additional mutations have been added, such as the ΔSH mutation and point mutations from the other biologically derived viruses. It is anticipated that these additional mutations will yield a graded series of attenuation phenotypes from which the appropriate one can be identified.

XI. Advantages of Recombinant DNA Method

Application of recombinant DNA methods to produce an RSV vaccine virus has a number of advantages, some of which have been mentioned above. Mutations found in separate viruses can be combined, and the level of attenuation can be fine-tuned by the progressive addition of mutations. Mutations not found in nature, such as the ΔSH mutation, can be used. This particular mutation had the unexpected benefits of slightly better growth in cell culture and reduced pathogenicity in chimpanzees, as well as the greater genetic stability that should be associated with a gene deletion compared to point mutations. Further analysis of this and the other gene deletion viruses might reveal additional beneficial phenotypes associated with these deletions. The final recombinant vaccine virus will consist of mutations that have been directly identified and characterized both individually and in combination. Thus, the vaccine virus will be highly characterized, and the production of vaccine lots could therefore be specifically monitored. Its genetic stability will be improved through the use of multiple attenuating mutations, by the stabilization of individual codon changes (48), and through the creation of one or more gene deletions. The ability to regenerate virus from cDNA means that a valuable virus will never be lost. An existing vaccine virus can be modified as needed, for example

TABLE IV

CHARACTERIZATION IN CELL CULTURE AND IN MICE OF RECOMBINANT RSVs[a]

Group	Virus	Mutations				Means virus titer (log_{10} pfu/ml) at indicated temperature (°C)						Shut-off temperature[c]	Mean virus titer in mice[b] (log_{10} pfu/g tissue)	
		cp	248	404-M2	404-L	32	36	37	38	39	40		Nasal turbinates	Lung
1	A2 HEK-7[d]					5.9	5.9	5.8	6.0	5.8	5.7	>40	4.4	4.9
2	*cp*RSV[d]	X				5.8	5.8	5.8	5.7	5.6	5.4	>40	4.3	4.4
3	r*cp*RSV	X				5.8	5.5	5.5	5.2	5.1	5.0	>40	4.0	3.8
4	*cpts*248[d]	X	X			6.1	5.7	4.4[e]	**3.4**[e]	<0.7	<0.7	38	3.4	3.1
5	r*cpts*248	X	X			6.0	5.6	4.7[e]	**2.5**[e]	<0.7	<0.7	38	2.8	3.2
6	r*cpts*404-M2	X		X		6.1	5.5[4]	**<0.7**	<0.7	<0.7	<0.7	37	2.2	2.3
7	r*cpts*404-L	X			X	5.9	5.9	5.8	5.8	5.5	5.4	>40	3.6	4.4
8	*cpts*248/404[d]	X	X	X	X	5.5	4.2[e]	**<0.7**	<0.7	<0.7	<0.7	37	2.4	2.2
9	r*cpts*248/404	X	X	X	X	5.7	4.6[e]	**<0.7**	<0.7	<0.7	<0.7	37	2.2	1.8
10	r*cpts*248/404-M2	X	X	X		5.7	4.5[e]	**<0.7**	<0.7	<0.7	<0.7	37	2.2	1.8

[a] Bearing the set of five *cp* mutations, the 248 mutation, the 404-M2 mutation, the 404-L mutation, or combinations thereof.
[b] Mice were administered 10^6 pfu of the indicated virus intranasally on day 0 and sacrificed on day 4. $n = 5$.
[c] Shut-off temperature is the lowest restrictive temperature at which a 100-fold or greater reduction occurs (boldfaced numbers).
[d] Biologically derived viruses are underlined; the others are recombinant.
[e] Pinpoint plaque size.

to accommodate genetic drift in circulating virus should that prove to be a problem. Many other possible improvements for a vaccine virus also can be imagined and will be explored, such as ablating expression of the secreted form of the G protein or altering codon usage to achieve increased antigen expression.

XII. Current Directions

These methods also are being used to develop recombinant live-attenuated vaccine viruses for RSV antigenic subgroup B and parainfluenza viruses types (PIV) 1, 2, and 3. For example, rPIV3 has been recovered from cDNA (18), and a program comparable to that described above for RSV is underway. This is based in part on the molecular characterization and reconstitution in recombinant form of a live-attenuated, biologically derived PIV3 candidate, *cp*45. Biologically derived *cp*45 is currently under clinical evaluation and, should it prove necessary, the recombinant version would be the starting point for further attenuation. Toward this end, Skiadopoulos *et al.* have identified three point mutations in the *cp*45 L protein that are independent *ts* and attenuation mutations (43).

rPIV, including viruses of animals such as Sendai virus as well as the human PIVs, also provides an experimental system for developing additional attenuation mutations, such as gene knockouts. Tao *et al.* also showed that the HN and F glycoproteins of rPIV3, which are the major neutralization and protective PIV antigens, can be replaced by their counterparts from PIV1, and that the resulting chimeric virus (rPIV3-1) is as viable in cell culture and rodents as is either parent (45). It therefore represents the starting point for a PIV1 vaccine. Specifically, this chimeric virus bears the major PIV1 protective antigens in a PIV3-specific backbone that can be attenuated by introducing mutations identified in *cp*45. The idea of using glycoprotein swapping to confer new antigenic specificity to an attenuated backbone can also be applied to making live-attenuated chimeric viruses for RSV subgroup B, based on the rRSV subgroup A backbone, and PIV2, based on the rPIV3 backbone. Finally, the ability to generate defined recombinant viruses for studies of viral pathogenesis and host immunity should increase our understanding of issues in paramyxovirus vaccinology and might result in improvements leading to second-generation vaccines.

XIII. Summary

RSV is a major cause of pediatric respiratory tract disease worldwide, but a vaccine is not yet available. It is now possible to prepare live

infectious RSV completely from cDNA. This provides a method for introducing defined mutations into infectious virus, making possible the rational design of a live-attenuated vaccine virus for intranasal administration. This is particularly important for RSV, for which achieving the appropriate balance between attenuation and immunogenicity by conventional methods has proven elusive. We took advantage of the existence of a panel of biologically derived vaccine candidate viruses that were incompletely attenuated but well characterized biologically. The mutations in these viruses were identified by sequence analysis and characterized by insertion into recombinant virus, thereby providing a menu of known attenuating mutations. These included a series of amino acid point mutations, mostly in the L polymerase, and a nucleotide substitution in a transcription gene-start signal, a *cis*-acting RNA element. The second source of mutations was from experimental mutational analysis of recombinant virus and involves deletion of the NS1, NS2, or SH gene. We have reconstructed a previously tested, biologically derived attenuated virus, *cpts*248/404, in recombinant form and are now proceeding to introduce additional mutations from the menu to achieve stepwise increases in attenuation. The ability to modify the attenuation phenotype incrementally in a directed manner should result in an appropriate vaccine virus.

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ADVANCES IN VIRUS RESEARCH, VOL. 54

CLINICAL VIROLOGY OF RHINOVIRUSES

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I. Introduction

Rhinoviruses are responsible for more episodes of human illness than any other infectious agent. Both children and adults have one illness due to rhinovirus every 1 to 2 years (0.5–1 illness per person-year). Rhinoviruses are the predominant cause of the common cold, a complex of subjective symptoms well known to everyone. The clinical illness is characterized by sore/scratchy throat, nasal obstruction, rhinorrhea, and malaise. Coughing and/or sneezing may be present. Young children may have fever early in the illness, but adults usually do not. Although symptoms and signs may persist for 10–14 days in children, in adults the illness subsides by 5–7 days after onset. Rhinovirus infections commonly precipitate exacerbations of asthma or chronic bronchitis and may predispose to secondary bacterial infections of the paranasal sinuses and middle ear in healthy children and adults.

This article is not intended to review all aspects of the virus and disease. Instead, three areas that may be of interest to the clinical virologist will be discussed. First, some attributes of the virus and epidemiology of disease that seem to have clinical relevance will be highlighted. Second, the efficiency of methods for detecting the presence of the virus in the human respiratory tract will be analyzed. Third, the means by which symptomatic illness is produced by rhinovirus infection of the respiratory tract will be discussed.

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II. Attributes of Virus and Epidemiology of Disease

Rhinovirus is a member of the picornavirus family. The virus contains single-stranded RNA within a capsid with icosahedral symmetry that is composed of 60 copies of each of four polypeptides (VP1–VP4). The viral particle is 30 nm in diameter and appears as a nondescript dot by electron microscopy. Infectivity of the virus is destroyed by acid treatment (pH 3–5), which differentiates it from the enteroviruses. The surface of rhinovirus is notable for the presence of depressions (canyons) at the base of which are the sites for attachment to receptors on the surface of susceptible target cells. The majority of the rhinovirus immunotypes (91 of the recognized 100 types) bind to the intercellular adhesion molecule-1 (ICAM-1) receptors on host cells; the receptor for the remaining immunotypes is not known. Neutralization testing with animal hyperimmune sera has been used to identify 100 different immunotypes, which have been given numbers.

Neutralization of virus infectivity by antibody occurs when IgG binds to the viral surface so that access of the host cell receptor to the canyon is blocked (1). The receptor binding site in the base of the canyon is inaccessible to antibody and is highly conserved across immunotypes (2).

Rhinovirus infects human respiratory epithelium. There is no evidence that cells in the submucosa of the respiratory tract are infected; virus has not been detected in blood. The optimal temperature range for growth of virus *in vitro* is 33°–35°C, which is the temperature range of the normal nasal mucosa. The nose and nasopharynx are the primary sites for viral replication in humans. Whether virus replicates in lung/bronchial epithelium during rhinovirus infection has not been definitively settled due to the difficulty of obtaining material from the lower airway uncontaminated by nasal secretions (3). During experimental infection, rhinovirus has been detected most frequently in the nasopharynx (Fig. 1). In one study (4), rhinovirus was inoculated onto the conjunctival surface of susceptible volunteers and was delivered to the nose below the inferior turbinate at the point where the nasolacrimal duct enters. Four sites in the nose (anterior and posterior parts of both inferior turbinates) and one site on the nasopharyngeal wall were sampled daily with a small cytology brush. The brush samples were cultured for rhinovirus. The viral recovery rate from the nasopharynx was consistently higher than that from the combined intranasal sites (Fig. 1). Virus was detected earlier in the nasopharynx and persisted until rapid decline occurred 16 days after inoculation. The authors (4) suggested that viral replication was occurring in cells at each virus-

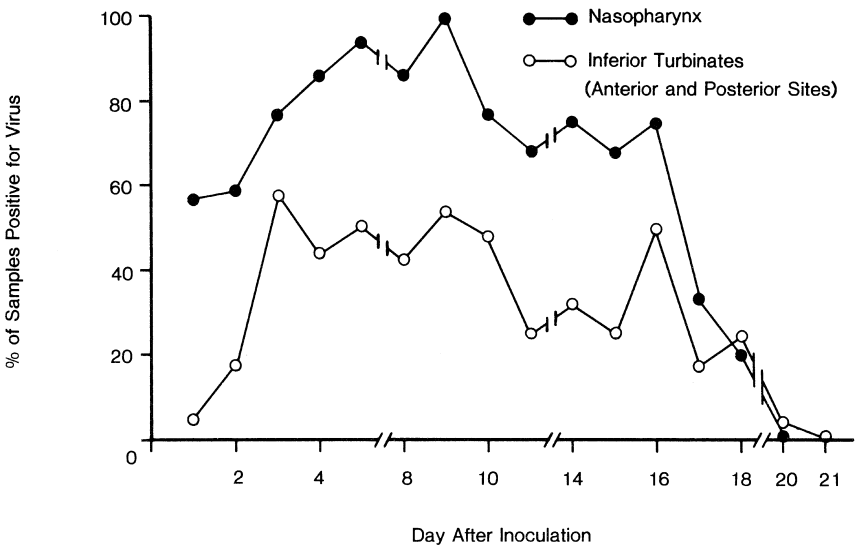

FIG 1. Rhinovirus recovery rates from brush biopsy specimens from nasopharynx and inferior turbinates of infected volunteers inoculated by way of the eye. Reprinted with permission from Ref. 4.

positive site, but the recovered virus may have been present only in the mucus overlying the site. The higher viral recovery rate in the nasopharynx may simply reflect the fact that this site is the endpoint of the mucociliary clearance system of the nasal cavity, paranasal sinuses, and middle ear (5).

Rhinovirus colds occur year round. However, a sharp rise in rhinovirus infections occurs every September. This rhinovirus peak initiates the yearly epidemic of colds in temperate climates that results from successive waves of different viruses moving through the population (6). Colds during the summertime are infrequent, but the proportion of summer and early fall colds that are due to rhinovirus is very high. Illness appears 1 to 2 days after rhinovirus is inoculated onto the nasal mucosa; oral inoculation of virus is an ineffective means of initiating infection. The home is the environment most conducive to transmission of rhinovirus infection (7). Rhinovirus may be transferred from an ill person to the nasal mucosa of a susceptible person by way of one (or more) transmission route(s): small-particle aerosol, large-particle aerosol, or direct contact of virus-contaminated hands with conjunctival or nasal mucosa (hand/self-inoculation route). The routes of transmission by which rhinovirus spreads under natural conditions have not

been established (8), but frequent hand washing to remove virus may reduce the likelihood of infection acquired by self-inoculation.

Immunity to reinfection with each immunotype of rhinovirus correlates with, but is not necessarily mediated by, neutralizing antibody in serum. Serum antibody appears to persist for years, but immunity to one immunotype does not provide protection against the other 99 types. As a consequence, a rhinovirus vaccine is not a practical prospect.

III. Detection of Rhinovirus

Rhinoviruses have long been known to be the major etiologic agent in common colds. In studies by several investigators using samples of nasal secretions inoculated prior to freezing into either HeLa cells or human embryonic lung fibroblasts (WI-38, MRC-5), 25–33% of secretions were shown to be rhinovirus positive. Sampling of nasopharyngeal secretions by a swab or nasal wash, use of cells with optimal sensitivity for rhinovirus, and incubation of cell cultures at 33°–35°C under conditions of motion were important requisites to achieve these results. More recently, with refinements in culture diagnosis and the use of polymerase chain reaction (PCR) technology, about 50% of common colds can be shown to be due to rhinovirus (9). In this section, information on the most efficient means to detect rhinovirus in the upper respiratory tract that may be helpful to the clinical virologist will be reviewed.

A. Sampling and Cell Culture

During infection, rhinovirus is present most frequently in the nasopharynx (4). Secretions from the nasopharynx can be obtained by one of three methods: an intranasal swab inserted far enough in the nose to reach the posterior nasopharyngeal wall; aspiration of nasopharyngeal mucus by a thin tube inserted through the nose; or a nasal wash with 10 ml of physiologic salt solution. If subjects will tolerate aspiration and if mucus is present in the nasopharynx, this method is expected to be efficient. The relative efficiency of the nasal swab and the nasal wash has been compared in two studies. In 1964, Cate and co-workers (10) compared detection rates of rhinovirus with nasal wash, nasal swab, and pharyngeal swab in experimentally infected volunteers during the 7 days after inoculation. Virus was detected in 76% of nasal washes, 36% of nasal swabs, and 35% of pharyngeal swabs. Virus was

detected in 50% of samples if results of nasal and pharyngeal swabs were combined. Arruda *et al.* (11) compared the isolation rate of rhinovirus from a nasal wash to a "vigorous deep nasal swab sample through both nostrils" combined with a pharyngeal swab in 100 subjects with naturally acquired common colds in the fall. Virus was grown in WI-38 cells from one supplier in 53% of nasal washes compared to 40% of the nose and throat swab samples. The findings in these two studies suggest that the nasal wash is more efficient than the nasal swab for detection of rhinovirus.

For transport, nasopharyngeal secretions are dispersed in medium containing protein. If immediate inoculation into cell culture is not convenient, the samples can be stored frozen at −70°C. One freeze-thaw cycle prior to inoculation does not appear to reduce the recovery rate of rhinovirus (11). Arruda *et al.* (11) emphasized that the efficiency of detection of rhinovirus in cell culture is increased by the use of more than one sensitive cell type. They compared recovery rates of rhinovirus from nasal washings from subjects with natural colds in several cell types. The WI-38 strain of human embryonic lung fibroblasts and a rhinovirus-susceptible clone of HeLa cells were the most sensitive types used. However, neither cell type detected all the rhinovirus isolates from the subjects; each type missed 20–35% of the positive samples. The authors concluded that samples should be inoculated into both WI-38 cells and susceptible HeLa cells in order to have maximum sensitivity for culture detection of rhinovirus. This strategy of inoculating samples into both fibroblasts and HeLa cells rather than into a single type was also used by Mäkelä *et al.* (9).

In summary, the most efficient method for diagnosis of rhinovirus infection by culture would include sampling nasopharyngeal secretions by nasal wash or nasopharyngeal aspiration, storing the sample at −70°C dispersed in protein-containing medium, and inoculating the sample into both fibroblasts and susceptible HeLa cells.

B. Polymerase Chain Reaction

The RNA of the picornavirus genome contains 7.2–7.5 kilobases. There is a single open reading frame preceded by a 5′ noncoding region containing short, highly conserved sequences (12). The nucleotide sequences of a number of human picornaviruses have been delineated. Synthetic oligonucleotide probes have been used in hybridization assays to detect picornaviruses. However, the usefulness of direct hybridization for detection of virus in respiratory samples is limited be-

cause $\geq 10^2$ copies of the viral genome are required for a positive result. Beginning in the late 1980s, conserved sequences were employed to prime reverse transcription (RT) of segments of viral RNA to produce cDNA for amplification in PCR (RT-PCR). Refinement of RT-PCR has resulted in formats that allow detection of one viral genome in samples from the upper respiratory tract.

For detection of rhinovirus in respiratory secretions with RT-PCR, several things should be noted. Nasal mucus may contain RNases or inhibitors of transcriptase/polymerase enzymatic activity. In order to have an assay sensitive enough to detect one genome, either nested PCR with detection of amplimers on a gel with ethidium bromide staining (13) or standard PCR with detection of amplimers by hybridization with labeled oligonucleotide probes must be used. Finally, identification of picornavirus genomic material in respiratory samples may indicate infection with either rhinovirus or enterovirus. The two viruses have been differentiated by the use of primers that amplify a region of picornavirus genome in which the number of nucleotide bases in rhinovirus RNA differs from that in enterovirus RNA so that amplimers can be distinguished on gel electrophoresis (13,14). Alternatively, the product of PCR amplification of a conserved sequence may be detected with an oligonucleotide probe that is specific for either rhinovirus or enterovirus genomes (9,15,16).

Four epidemiologic studies published since 1992 have utilized both RT-PCR and cell culture for detection of rhinoviruses in respiratory tract samples from adults or children with naturally acquired colds (9,14,16,17). These studies illustrate the variation in RT-PCR formats developed for detection of rhinovirus in clinical samples. In addition, they allow comparison of the efficiency of RT-PCR and cell culture for rhinovirus diagnosis. Highlights of the features of the RT-PCR protocols are shown in abbreviated, telegraphic form in Table I; full descriptions of the protocols are provided in the referenced publications. In all four studies, samples of respiratory secretions to be tested with RT-PCR (and to be inoculated into cell cultures) were stored frozen at $-70°C$ without additives. RNA was extracted from thawed samples in three of the four with proteinase K treatment followed by phenol extraction and ethanol precipitation. In the fourth sample the RNA was recovered with the use of a commercially available matrix affinity chromatography column. The primers used for RT and PCR in all four studies were from the 5′ noncoding region of the picornavirus genome except for one primer from the VP2 coding region in Method 1 (18) used by Mäkelä *et al*. (9). In three of the studies, the PCR product was detected with the use of labeled oligonucleotide probes; in the fourth, the amplimers

TABLE I

ATTRIBUTES OF RT-PCR ASSAYS FOR RHINOVIRUS EMPLOYED IN EPIDEMIOLOGIC STUDIES OF RESPIRATORY ILLNESS

RNA extraction	RT method	PCR design	Detection	Ref.
Proteinase K–phenol/ chloroform extraction–ethanol precipitation	Primer complementary to sense RNA sequence between 548 and 563 of HRV-14	"Touchdown" primary PCR with addition of primer of nucleotides between 183 and 198 of HRV-14. Seminested secondary PCR with 370–384 primer in addition to 548–563 primer (HRV-14)	Agarose/ethidium bromide detection of 202-bp product of seminested PCR	13, 14
Proteinase K–phenol/ chloroform/isoamyl alcohol extraction–ethanol precipitation	Primer complementary to sense cDNA sequence 547–562 on HRV-14	Second primer complementary to antisense sequence between 182 and 197 of HRV-14	PCR product applied to nitrocellulose membranes by slot blot manifold. Hybridized with ^{32}P-labeled probe complementary to 452–468 nucleotide sequence of HRV-14	16
Matrix affinity chromatography of diluted sample	Primer complementary to sense RNA sequence between nucleotides 540 and 555 of HRV-89	HotStart-5′ biotinylated primer complementary to antisense cDNA between base pairs 168 and 183 of HRV-89; 35 cycles	Amplimers separated from unreacted reagents; hybridization with digoxigenin dUTP-labeled probe complementary to sense viral RNA between nucleotides 451 and 467 of HRV-14; capture on streptavidin-coated plate for detection of labeled amplimers	17
Proteinase K–phenol extraction–ethanol precipitation	Two methods for RT-PCR *Method 1:* primers from 5′ noncoding region and from VP2 capsid protein coding region of enterovirus genome *Method 2:* both primers from 5′ noncoding region		Detection method not discussed	9

from nested PCR were detected by ethidium bromide staining after electrophoretic separation on an agarose gel. Differentiation of rhinovirus from enterovirus was based on the size of the amplimers (13) or on hybridization with a rhinovirus-specific probe (16); differentiation was not done in the study of samples collected in the fall (17).

In all four studies, 200 or more respiratory tract samples from people with colds were tested for rhinovirus by both cell culture inoculation and RT-PCR (Table II). Samples were obtained year round in three studies; in the fourth, they were obtained in the fall at the time of highest rhinovirus prevalence. Appropriate samples of nasopharyngeal mucus were obtained in three of the four studies. Anterior nasal swab culture in the fourth study (14) would not be expected to provide maximum sensitivity because nasopharyngeal secretions were not sampled. In accord with this expectation, the detection rate for rhinovirus in this study was only 33%. Rhinoviruses were detected in 50% of colds during year-round surveillance when RT-PCR results were combined with cell culture detection (9,16); 80% of illnesses in the fall were associated with rhinovirus (17).

The relative sensitivity of detection of rhinovirus with cell culture inoculation could be compared to that of RT-PCR in the three studies in which nasopharyngeal secretions were obtained. Only one sensitive cell line was inoculated in one study (16); only 32% of the rhinovirus positives were detected with cell culture, whereas RT-PCR detected 99%. In contrast, in the two studies in which two sensitive cell lines were inoculated (9,17), rhinovirus was grown in cell culture in 76–82% of the positive samples and RT-PCR was positive in 98–100%. Although RT-PCR was more sensitive, properly done cell culture inoculation using two cell systems detected three of every four positive samples.

Appropriate precautions to prevent false-positive results were employed in the PCR protocols in these studies. The consistency of the results and the cell culture detection of virus in at least three quarters indicate that the RT-PCR results were true positives. It is reasonable to conclude that (1) nasopharyngeal mucus must be sampled for reliable detection of rhinovirus; (2) RT-PCR is the most sensitive way to detect virus; (3) cell culture with two sensitive cell lines is 75–80% sensitive; and (4) fully 50% of colds during a year in children and adults are due to rhinovirus.

IV. Pathogenesis of Symptoms

In three reports, the published information on symptom pathogenesis in rhinovirus infection has been reviewed and referenced (5,6,19).

TABLE II

SENSITIVITY OF CELL CULTURE COMPARED TO RT-PCR FOR DETECTION OF RHINOVIRUS IN EPIDEMIOLOGIC STUDIES OF RESPIRATORY ILLNESS[a]

					Rhinovirus			
Population	Season	Sample	RV Infections/no. of samples tested	Cells used for culture	Culture no. positive	PCR no. positive	Culture no. positive/sample no. positive	Ref.
Adults with asthma	2 years	Anterior nasal swab plus throat swab	76/229 (33%)	Ohio HeLa and MRC-5	14	76	14/76 (18%)	14
Children	1 year	NPA	147/292 (50%)	Ohio HeLa	47	146	47/147 (32%)	16
Adults	Fall	NW	283/346 (82%)	HeLa I and WI-38	231	283 (PCR not done on all culture-positive samples)	231/283 (82%)	17
Adults	1 year	NPA	105/200 (53%)	Ohio HeLa and foreskin fibroblasts	80	103	80/105 (76%)	9

[a] NPA, Nasopharyngeal aspirate; NW, nasal wash.

In this article, selected features of current knowledge of symptom pathogenesis will be emphasized as a background for speculation on how the viral infection, the host response to the viral infection, and the symptoms perceived by the host may fit together.

Five features of rhinovirus infections of the human nose deserve emphasis: (1) There is no discernible destruction of the nasal epithelium by the virus during rhinovirus infection either *in vivo* or *in vitro*. This feature was illustrated in a study in which nasal epithelium in culture was infected with four different respiratory viruses (20). Rhinovirus and coronavirus produced no apparent cytopathic effect in the epithelial monolayer, whereas adenovirus and influenza virus had a profound effect (Fig. 2). This picture was obviously different from that produced by rhinovirus infection of susceptible fibroblasts in culture, in which

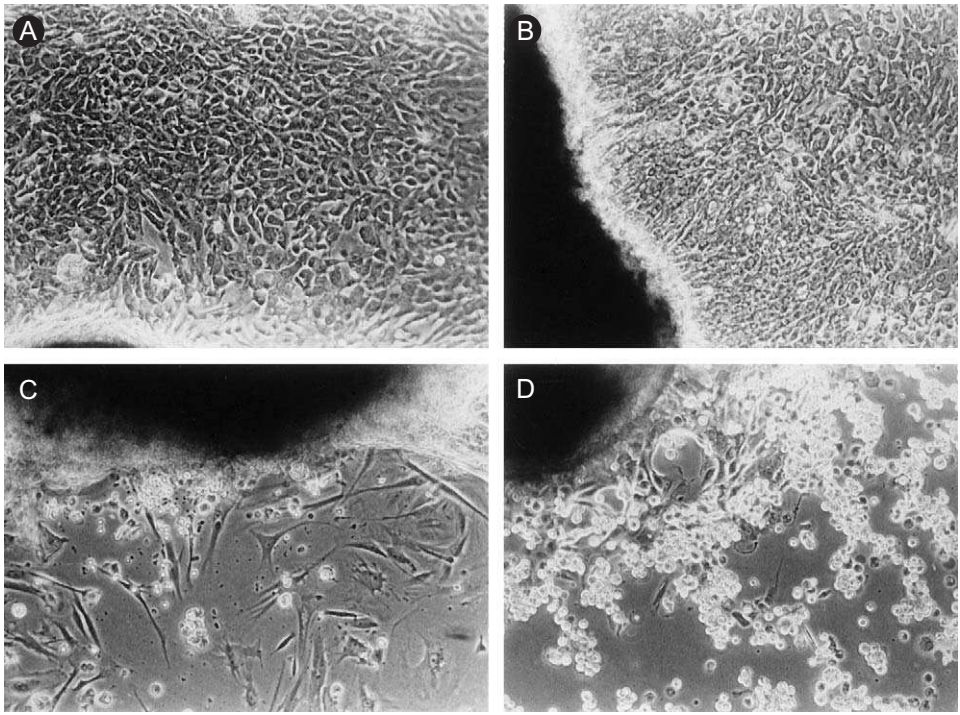

FIG 2. Outgrowth of epithelial cell monolayer from fragments of nasal mucosa 96 hours after exposure to virus (phase contrast; magnification: × 100). (A) Rhinovirus, (B) Coronavirus. (C) Influenza type A. (D) Adenovirus. Reprinted with permission from Ref. 20.

distinct cytopathic changes were apparent (20). (2) Very few cells in the nasal epithelium of volunteers are infected at any point during symptomatic rhinovirus infection. Two different groups of investigators have demonstrated this using *in situ* hybridization. (3) An epithelial cell can elaborate cytokines in response to infection with rhinovirus. This important observation may be central to symptom pathogenesis. (4) Symptoms in volunteers with experimental rhinovirus infections parallel the presence of markers of inflammation and the concentration of virus in nasal secretions (Fig. 3). Volunteers who are infected but not ill do not have elevated levels of inflammatory markers in their nasal washes. In the volunteer model, symptom severity, viral titers, and concentrations of serum albumin, polymorphonuclear neutrophils (PMNs), interleukin-8 (IL-8), and kinins all peak 48 hours after inoculation, and rise and fall in parallel. (5) Symptomatic illness subsides in adults after 5–7 days in spite of the fact that virus does not disappear from the nasopharynx until 16 to 18 days after inoculation (Fig. 1). Persistence of virus in the absence of symptoms was seen in the study from which Fig. 1 was taken (4).

With the above features in mind, one can speculate on the mechanism by which symptoms are produced during rhinovirus infection of nasal

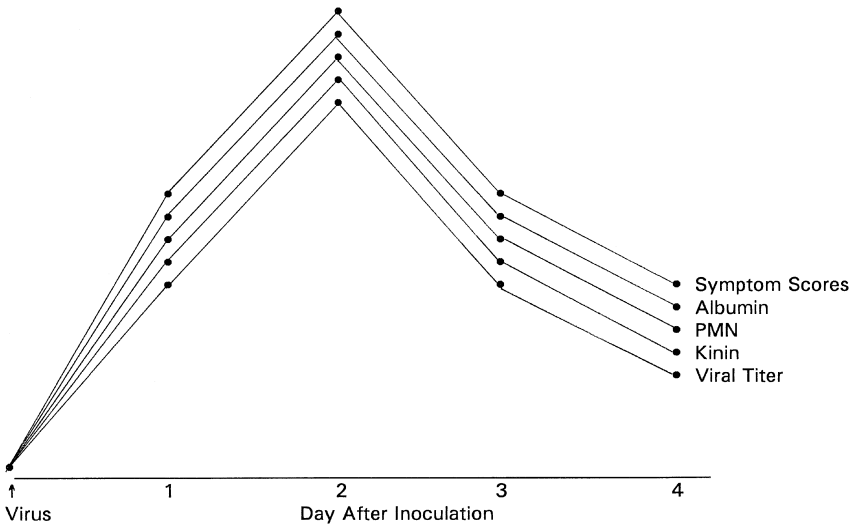

FIG 3. Schematic composite of average symptom scores compared to concentrations of markers of inflammation and virus in nasal washes following intranasal inoculation of rhinovirus.

mucosa. Following inoculation of virus into the nose, single epithelial cells that are infected elaborate cytokines (and chemokines) as "distress signals." Cytokine release results in a localized vascular leak with extravasation of albumin and other serum proteins and an influx of PMNs into mucosa from the IL-8 effect (21). Appearance of kinins in nasal secretions is a side effect of the vascular leakage of kininogen. If enough epithelial cells are infected and elaborate cytokines, the inflammatory response produces symptomatic illness. At the same time, the localized inflammatory response is effective in limiting viral replication, perhaps by washing extruded infected cells away from the epithelial surface before the virus spreads to neighboring cells. This results in a decline in viral titer after the peak at 48 hours postinoculation. As fewer cells are infected, the extent of the inflammatory response and the symptoms diminish. Finally, between the second and third weeks after inoculation, sufficient neutralizing antibody becomes available to shut down viral replication and the infection ends. The antibody may be secretory IgA or serum IgG that has leaked into the mucosa.

In brief, the symptoms during rhinovirus infection of the nose appear to be caused by the host response, not by the virus *per se*. This introduces the notion that the virus need not be killed to ameliorate the illness if the host response can be altered. However, it should be noted that the use of oral prednisone, a broad and potent anti-inflammatory agent, by infected volunteers resulted in increased titers of rhinovirus in nasal washings (22). This finding is consistent with the speculation that the localized inflammatory response to rhinovirus infection of epithelial cells is instrumental in limiting viral replication in the nasal mucosa. Although theoretically there is no need to kill the virus in order to reduce the symptoms, an effective antiviral may be required in conjunction with anti-inflammatory treatment in order to alter the illness in view of this demonstrated enhancement of viral replication by steroid.

V. Summary

Rhinoviruses cause more infections in humans than any other microorganism. These acid-sensitive picornaviruses infect epithelial cells following inoculation onto the nasal mucosa and are detected reliably in nasopharyngeal secretions. Rhinovirus colds occur year round, with a peak of illness in the fall. Type-specific serum antibody correlates with protection against infection. The fact that there are at least 100 different immunotypes makes development of an effective vaccine unlikely.

Nasopharyngeal secretions must be sampled for detection of rhinovirus by culture or RT-PCR. Efficient isolation of virus requires inoculation into two different types of sensitive cell cultures (i.e., fibroblasts and HeLa cells). RT of conserved sequences in the 5′ noncoding region of the viral RNA to produce cDNA for PCR amplification has been coupled with detection of amplimers either by gel electrophoresis after nested PCR or by hybridization with labeled oligonucleotide probes to detect one viral genome in samples. In two studies in which both RT-PCR and cell cultures were used, virtually all of the positives were identified with RT-PCR; culture in two cell lines identified 75–80% of the positives. In year-round surveillance, 50% of colds in adults and children were rhinovirus positive.

The symptoms occurring during rhinovirus colds are caused by the host's response to the virus, not by the virus itself. Elaboration of cytokines by infected epithelial cells is central to symptom pathogenesis.

Acknowledgments

I appreciate the helpful discussions with Drs. Ron Eccles, Birgit Winther, Jack M. Gwaltney, Jr., and Frederick G. Hayden.

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