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Experimental Investigation of Herpes Simplex Virus Latency†

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† This review is dedicated to an appreciation of the career of J. G. Stevens, who recently retired from his position at UCLA.

INTRODUCTION

One of the great triumphs of modern molecular biology has been the application of biochemistry and genetics to the understanding of various diseases, primarily but not exclusively those of humans. Although many details remain to be elucidated, the general outlines of the replication and association of herpes simplex viruses (HSVs) with their hosts are well established. The present review is designed to correlate our knowledge of both their clinical and laboratory behavior. Due to the admitted bias of the authors, the molecular biology of the process of HSV replication and latency is emphasized, but, hopefully, this review will also be of some value to more clinically oriented readers.

Herpesviruses

Classification and general properties. Herpesviruses are nuclearly replicating, icosahedral, enveloped DNA viruses. They infect members of all groups of vertebrates, and, indeed, the same host can be infected with multiple distinct and unique types. Herpesviruses have been typically classified into three groups based upon details of tissue tropism, pathogenicity, and behavior under conditions of culture in the laboratory (235): the alphaherpesviruses are neurotropic with a rapid replication cycle and (usually) a broad host and cell range; and the beta- and gammaherpesviruses differ in genome size and structure but both replicate more slowly and in a much more restricted range of cells of glandular and/or lymphatic origin.

An understanding of the molecular phylogeny of the herpesviruses has been facilitated by the large amount of nucleotide sequence data available. The most sophisticated analyses have been carried out by McGeoch et al. (170, 171), and several conclusions can be drawn. First, the herpesviruses are ancient in an evolutionary sense and have undergone extensive cospeciation with their hosts; second, the division of the viruses into the three major groups is of ancient origin, and this classification originally based upon laboratory and pathological phenomena can be supported by the molecular data available.

To date, eight discrete human herpesviruses have been described; each causes a characteristic disease. HSV-1 and HSV-2 are the primary agents of recurrent facial and genital herpetic lesions, respectively (310), while varicella-zoster virus (VZV) is the causative agent of chicken pox and shingles (92, 141, 308). Infections with human cytomegalovirus—the prototype of the betaherpesviruses—are linked both to a form of infectious mononucleosis and to congenital infections of the nervous system (262). In contrast, infections with two other lymphotropic herpesviruses, the closely related betaherpesviruses human herpesviruses 6 and 7 (HHV-6 and HHV-7) are generally mild early childhood diseases (exanthem subitum) (84, 200, 210, 285).

Infections with two human herpesviruses, the gammaherpesvirus prototype Epstein-Barr virus (EBV) and the recently described Kaposi's sarcoma herpesvirus or HHV-8, are convincingly linked to human cancers (1, 75, 135, 155, 203, 246). In the case of EBV, despite its high frequency in the general population, carcinogenesis is linked to specific environmental agents (105). In contrast to the ubiquitous occurrence of HCMV, EBV, HHV-6, and HHV-7, HHV-8 may be associated with populations at high risk for sexually transmitted diseases, which would correlate well with a causative role in Kaposi's sarcoma, although there is controversy on this point (43, 58).

Unlike adenoviruses, which all share a basic genomic structure as well as their general architecture, a comparative survey of the genomic structure of the various herpesviruses displays a staggering array of individual variations upon a general theme. Still, within this variation, gene order is generally maintained within large blocks of the genome, and, as noted above, various degrees of genetic homology are clearly evident. Briefly, the genomic structure is complex, containing significant regions of inverted repeat sequences with wide variations both in total genomic size (100,000 to 240,000 bp) and base composition (>45% to <70% G+C).

Patterns of herpesvirus replication. Despite great variation in the details of genome arrangement, herpesvirus genomes are promoter rich. This means that, generally, the expression of a given protein is mediated by a specific promoter mapping at that gene. Thus, extensive transcription units expressing long multigene transcripts that must be processed into a variety of mRNAs are the exception rather than the rule. This means that there need be no strict constraint on the precise genomic order of genes or genomic organization, only on genomic content and coregulation of essential functions. In addition to the

relatively large number of primary transcription units expressing viral genes, all herpesviruses share two basic features that define the group as a whole—(i) patterns of productive cycle gene expression and (ii) the ability to establish and maintain latent infections.

All herpesviruses share general patterns of gene expression during the productive cycle. Gene expression is characterized by a progressive cascade of increasing complexity, where the earliest genes expressed are important in "priming" the cell for further viral gene expression and in mobilizing cellular transcriptional machinery. This phase is followed by the expression of a number of genes that are either directly or indirectly involved in viral genome replication. Finally, upon genome replication, viral structural proteins are expressed in high abundance. General aspects of this productive cascade are covered in somewhat more detail for HSV below.

The second general feature unifying the herpesviruses is that they all are able to establish and maintain a latent state of infection at a specific physiological site within an immunocompetent host. A general description of the general properties of latent phase transcription is now available for a number of human and animal herpesviruses (302). In the latent phase of infection, only a limited group of viral genes are expressed, yet productive infection can be readily induced to provide a continuing reservoir of virus from any immunocompetent individual. Despite the similarity in the phenomenology of latency, the actual molecular details and mechanisms of latency and (probably) reactivation are quite different in the various groups of herpesviruses. Understanding of the switches between productive and latent infection requires knowledge of the specific details of viral tissue tropism and pathogenesis, as well as a thorough description of the details of viral gene expression.

Herpes Simplex Virus

General. HSV-1 and HSV-2 appear to share all or most features of their replication; however, HSV-1 is the prototype and best-studied representative of the alphaherpesvirus group, and its replication will serve as a model for both. HSV-1 is neurotropic and establishes latent infections in sensory and autonomic neurons. It is characterized by an extremely rapid productive replication cycle compared to many other types of herpesviruses as well as smaller nucleus-replicating DNA viruses such as adenoviruses and papovaviruses. Furthermore, both types of HSV are able to replicate in a wide selection of animals, tissues, and cultured cells. The virion contains two important host-modifying proteins: the α-*trans*-inducing factor (α-TIF; also known as VP16, $V_{MW}65$, U_L48, or the virion-stimulatory protein and U_L41 (virion host shutoff protein); the functions of these two proteins are discussed briefly below.

Productive infection. The specifics of productive infection of a cell by HSV have been established in cultured cells. Although there may be specific and/or minor differences in the process in differentiated neuronal cells in vivo, no obvious differences are seen in infections in cultured neuronal cell lines. Replication involves a number of stages representing different levels of viral gene expression and interaction of viral gene products with host machinery—this process has been recently reviewed in some detail (301). Virus entry requires sequential interaction between specific viral membrane glycoproteins and cellular receptors; upon entry, the nucleocapsid is transported to the nuclear pores, where viral DNA is released into the nucleus. The viral genome is accompanied by the α-TIF protein, which functions in enhancing immediate-early viral transcription via cellular transcription factors. U_L41 appears to remain in the cytoplasm, where it causes the disaggregation of polyribosomes and degradation of cellular and viral RNA.

Five HSV genes (α4 [ICP4], α0 [ICP0], α27 [ICP27/U_L54], α22 [ICP22/U_S1], and α47 [ICP47/U_S12]) are expressed and function in the earliest stages of the productive infection cycle. This stage of infection is termed the immediate-early or α phase of gene expression and is mediated by the action of α-TIF through its interaction with cellular transcription factors at specific enhancer elements associated with the individual transcript promoters. In the absence of virus-encoded protein synthesis, only α transcripts are expressed. Since promoters controlling the expression of all kinetic classes of HSV transcripts have features of cellular promoters and can be expressed by unmodified cellular transcription systems, the restriction of viral transcription in the absence of virus-induced protein synthesis is, in itself, sufficient to imply that the nature of the viral genome as a transcription template plays a critical role in subsequent viral gene expression.

Proteins encoded by the α4, α0, and α27 transcripts play clear roles in the regulation of viral gene expression at the level of transcription or, at least, mRNA expression. They functionally interact to form nuclear complexes with viral genomes (183, 217, 328), and the role of these interactions in the global aspects of HSV transcription is a question of critical interest. Surprisingly, only two (α4 and α27) have extensive areas of sequence similarity among a large number of alphaherpesviruses, and only amino acid sequences in α27 appear to be extensively conserved among the more distantly related beta- and gammaherpesviruses.

Less is known about the function of the two other α proteins, α22 and α47. Both are dispensable for virus replication in many types of cultured cells, but α22 is required for HSV replication in others and may play a role in maintaining the ability of the virus to replicate in a broad range of cells in the host—perhaps by mediating the expression of a set of late transcripts (29, 153, 212, 216). The α47 protein has recently been inferred to play a role in modulating host response to infection by specifically interfering with the presentation of viral antigens on the surface of infected cells by MHC-I (292, 323).

Activation of the host cell transcriptional machinery by the action of α gene products results in the expression of the early or β genes. Seven of these are necessary and sufficient for viral replication under all conditions: DNA polymerase (U_L30), DNA binding proteins (U_L42 and U_L29 or ICP8), ORI binding protein (U_L9), and the helicase/primase complex (U_L5, U_L8, and U_L52) (195, 307, 319). When sufficient levels of these proteins have accumulated within the infected cell, viral DNA replication ensues. Other early proteins are involved in increasing the deoxyribonucleotide pools of the infected cells, while still others appear to function as repair enzymes for the newly synthesized viral genomes. These accessory proteins are "nonessential"for virus replication in that cellular products can substitute for their function in one or another cell type or upon replication of previously quiescent cells; however, disruptions of such genes often have a profound effect upon viral pathogenesis and/or ability to replicate in specific cells (24, 121, 154, 255, 288). Thus, any deficiencies in these genes greatly impair virus replication in the natural host.

The vegetative replication of viral DNA represents a critical and central event in the viral replication cycle. High levels of DNA replication irreversibly commit a cell to producing virus, which eventually results in cell destruction. DNA replication also has a significant influence on viral gene expression. Early expression is significantly reduced or shut off following the start of DNA replication, while late genes begin to be ex-

pressed at high levels. These late genes can be divided into two subclasses: "leaky-late" (βγ) and "strict late" (γ). The βγ transcripts are expressed at low levels prior to DNA replication but reach maximum expression after viral DNA replication has been initiated. In contrast, γ transcripts are difficult to detect until the onset of viral DNA replication.

Immunofluorescence studies show that DNA replication occurs at discrete sites, or "replication compartments," in the nucleus (53, 133). Prior to DNA replication, the α4 protein and the β single-stranded DNA binding protein ICP8 (U_L29) are distributed diffusely throughout the nucleus; concomitant with viral DNA replication, the distribution of these proteins changes to a punctate pattern. In the case of α4, this change involves interaction with α0 and α27. These observations have two important implications. First, the virus can cause a restructuring of the nucleus and in effect create its own organelle-like structures. Second, transcription of early and late genes is likely to occur in two distinct environments. The transcriptional environment within replication compartments may lead to preferential transcription of late genes.

More than 30 HSV-1 gene products are structural components of the virion, and all are expressed with late kinetics; however (and as noted above), some are readily detectable prior to viral genome replication (βγ) while others are only detectable following this watershed event (γ). It is not clear whether the kinetic distinction between βγ and γ transcripts and the proteins they encode is of a functional significance or merely represents a conveniently measurable experimental discrimination. For example, while expression of the major capsid protein, VP5, with βγ kinetics and the penton protein, U_L38, with γ kinetics might be important in achieving maximum virus yields, there is no experimental evidence which suggests that this is indeed the case.

Viral capsids assemble in the nucleus and bud through the nuclear membrane, which contains the viral glycoproteins. During the maturation process, the capsids are surrounded by tegument proteins including α-TIF and U_L41, which may functionally interact to aid in envelopment (264). Enveloped infectious virions either can remain cell associated and spread to other cells via virus-mediated fusion or can be released from the cell for reinfection. Obviously, in the latter case, the virion itself is subject to immune surveillance and host-mediated immune clearance. The replication cycle is quite rapid for nucleus-replicating DNA viruses, with mature virions being formed in as little as 8 h in some cell culture systems.

Latent Infections by Herpesviruses

As the major subject of this review, HSV latency is extensively covered in the following sections. However, to "set the stage," it is well to consider the following general points. Virus persistence restricted to specific tissues of the mammalian host is a common phenomenon of the natural course of infection of nucleus-replicating DNA viruses. Despite this universality, latency—the hallmark of herpesvirus infections—appears to be biologically unique. In such a "true" latent infection, the viral genome is maintained intact and genetically equivalent to that present in a viral particle, but the highly regulated productive cycle cascade of gene expression, also so characteristic of herpesvirus infections, does not occur. As a consequence, any transcription during latent infection with most herpesviruses is from a very restricted portion of the viral genome, and this transcription is important in some aspect of the process itself. In a very general sense, then, herpesvirus latency is comparable to the lysogenic phase of infection engendered by bacteriophage lambda, but the parallel stops there. The molecular and physiological details of the latent phase of infection by specific herpesviruses are quite varied and divergent, and, indeed, the only common denominator appears to be latency itself.

Any latent infection with a herpesvirus can be viewed as having three separable phases: establishment, maintenance, and reactivation. In the establishment phase, the virus must enter the appropriate cell, and following entry, there must be a profound restriction of viral gene expression so that the cytopathic results of productive infection do not occur. Thus, most (or many) productive cycle genes will be transcriptionally and functionally quiescent and only a limited number of viral genes should be expressed; indeed, expression of such latent phase genes could well be limited to the latent phase of infection. Where investigated, the establishment of latent herpesvirus infections can be essentially viewed as the result of failure of the productive cascade, and this restriction is clearly mediated by features of the specific cell and cell type in which the latent infection is established.

As to maintenance of the latent infection, operationally, viral genomes persisting in latently infected cells must provide a reservoir of potential infectious virus upon reactivation. The two best-characterized herpesvirus latency systems, HSV in neuronal cells and EBV in lymphatic cells, illustrate a fundamental division which reflects the capacity of the latently infected cell to replicate and thus to place different stringencies upon the maintenance of viral genomes. Much of the gene expression seen in latent EBV infections is functionally directed at ensuring the maintenance of viral genomes in dividing cells, mediating virus-directed immune evasion, and ensuring that the cells do not enter apoptosis (258, 284). In contrast, latent HSV genomes are harbored within the nucleus of a nondividing sensory neuron and do not face such a challenge. Here, the challenge arises from the need for the virus to reactivate from a transcriptionally quiescent, nonreplicating cell. The maintenance of the HSV genome in latently infected neurons appears to be entirely passive; i.e., it requires no viral gene expression or gene product at all. However, HSV DNA is maintained as a nucleosomal, circular episome in latent infections (54, 229), and low levels of genome replication could occur or be necessary for the establishment or maintenance of a latent infection from which virus can be efficiently reactivated.

Successful reactivation of a herpesvirus results in the appearance of infectious virus in an immune host. Despite the contrasts between neurotropic and lymphotropic herpesviruses in the genetic requirements for the maintenance of latency, the process of reactivation can be envisioned to follow essentially equivalent routes. Transcriptional stimulation of EBV-immortalized B lymphocytes leads to the productive cascade, although this lytic phase may be aborted before infectious virus is recovered (104, 284). Thus, in the host, the sporadic induction of cytocidal replication in a B lymphocyte could be a continuing reservoir of infectious virus without compromising the reservoir or the function of the tissue in which the latent phase is established, and one could envision reactivation being due to one of a number of unspecified physiological stresses to the immune system that lead to generalized transcriptional activation of lymphatic cells and result in the production of virus and cell death in a few of them.

The process of reactivation from latency by neurotropic herpesviruses may not be so generally cytopathic. While reactivation is triggered by stress as well as other signals which transiently lead to increased transcriptional activity in the harboring neuron, the sensory nerve ganglia must survive repeated bouts of reactivation without losing function. While this could happen if only one or a few neurons were destroyed in

each reactivation cycle, the high frequency of subclinical reactivation seen in many latently infected humans with no neurological sequelae makes a process which involves significant virus replication and cytopathology difficult to envision (45). A more believable scenario might involve the ability of one or several latently infected neurons to replicate only a few viral genomes and generate only a few infectious virions during the initial reactivation event. This might happen without the extensive cytopathology associated with normal vegetative viral replication or from the death of only a very few cells. This process may be augmented by viral genes shown to interfere with apoptosis, such as ICP34.5, which act to prevent neuronal death during reactivation when limited replication occurs (33).

CLINICAL MANIFESTATIONS OF HERPES SIMPLEX VIRUS INFECTION IN HUMANS

Primary Infection and Recrudescence

Generally, in an immunocompetent human, primary infection with either HSV-1 or HSV-2 in the facial or genital area is unremarkable. Virus infection in peripheral tissue is initiated from an active (either primary or recurrent) infection of another individual, and limited replication occurs with ensuing tissue-associated cytopathologic changes and formation of virus-filled vesicles in the immediate vicinity of the site of original infection. The primary infection resolves within 2 to 3 weeks concomitant with seroconversion, and no evidence of virus infection is seen. Clearly, during this primary phase of infection, productive replication proceeds following the patterns established by investigations of viral replication in cell culture until the immune system of the host is able to mount an effective counter. As might be expected, during this initial (and subsequent reactivation) phase of virus replication, some viral gene products are expressed which both modulate and help evade the immune response (65, 85, 323).

Following primary infection and recovery, many individuals manifest recrudescence of infectious virus upon exposure to one of several types of stress including high levels of UV light, menses, lactation, malnutrition, extensive fatigue, anxiety, and mild immunosuppressive infections with other viruses. In clinical reactivation, there may be evidence of active virus infection and infectious virus can be recovered from vesicles at the site of primary infection, but episodic reactivations normally involve a much more limited extent of affected tissue, and recovery times are shorter than in the case with a primary infection, presumably due to the modulation of the process by host immunity. Again, the general rate of formation of reactivated lesions and cytopathologic abnormalities are consistent with rapid productive virus replication in cell culture. It is important to keep in mind that the clinical patterns seen in humans may only generally correspond to molecular patterns of reactivation seen in animal models.

Extensive reviews of the course of human infections from both a clinical and historical perspective are available (276, 311), and detailed coverage is out of the scope of this review. Despite this, the fact that all laboratory models require some correspondence to the clinical course of infection means that a brief outline of salient features is germane and is covered in the following portions of this section.

Clinical differences between HSV-1 and HSV-2 infections. The course of primary infection and recurrence is generally similar for HSV-1 and HSV-2. Primary infections with HSV-1 usually occur in childhood, while the frequency of primary infection with HSV-2, a sexually transmitted disease (44), corresponds to the age of onset of extensive sexual activity. There are significant epidemiological data indicating that prior HSV-1 infection and subsequent immunity have a partially protective and/or ameliorating effect upon HSV-2 infection and reactivation. Such limited cross-protection does not, however, militate against individuals being infected with both types of viruses and even (although more rarely) distinct strains of the same virus type.

Despite the general similarities of the course of infection, there is growing evidence that HSV-2 infections in the genital area tend to reactivate more frequently than do HSV-1 infections in the facial regions, although recurrences of genital HSV-1 or HSV-2 infections occur at essentially identical rates following primary infection (11, 63, 223). Not all or even most reactivations need be accompanied by obvious clinical symptoms, and the most recent detailed analyses of reactivation by PCR analysis indicate that reactivation episodes can occur as frequently as monthly for genital infections (11, 38, 45). This finding has tremendous public health implications, of course, but such high rates of reactivation from nonreplicating neurons suggests that the reactivation event itself may not be obligately cytocidal.

Role of the immune system in HSV infection. Myriad studies in animals have and continue to document the importance of both specific and non-specific portions of the immune response in HSV pathogenesis (cf references 127 and 177), but there is also ample evidence that specific viral genes and the overall genetic makeup of various virus strains play a significant role (18, 32, 87, 107, 278, 295). Of course, no consideration of HSV latency can truly be complete without a discussion of the immunological factors which affect the processes of latency and reactivation; however, an in-depth coverage of immunology is beyond the scope of this review, and the reader is referred to other works on the subject (185, 188, 250, 320). Both the cellular and humoral arms of the immune response play a key role in limiting the severity of the HSV acute infection (250, 260, 320). In the periphery, $CD4^+$ T cells seem to be particularly important in recruiting and activating macrophages (188), and in bulk cultures of human lymphocytes, $CD4^+$ cells are the predominant cytotoxic lymphocytes (CTLs) that respond upon stimulation with HSV antigen (249, 293). In the murine nervous system, in contrast, $CD8^+$ T-cell responses (187, 261) and immunoglobulin G (128, 259) seem to play a key role in mediating viral clearance and in blocking the spread of the virus at synaptic junctions within the peripheral nervous system. These virus-specific responses mount rapidly (detectably within 4 to 6 days postinfection) at local sites within the peripheral ganglia long before a general host response, as often measured by antibody levels in serum, appears (127, 186).

It has been established that while both $CD4^+$ and $CD8^+$ cells are present within HSV-infected nervous tissue, there is no CTL response directed toward infected neurons and it is likely that the targets of these cells are nonneuronal cells. Perhaps an even more important role of CTLs is the secretion of cytokines. Alpha/beta interferon (IFN-α/β) is detectable within the first few days of HSV infections and acts as an inhibitor of viral replication as well as an activator of macrophages (126). In addition, activated $CD4^+$ cells produce IFN-γ, which also is a strong inducer of macrophages (253). In turn, activated macrophages (as well as cells of the nervous system such as microglia and oligodendrocytes) produce cytokines such as tumor necrosis factor alpha and interleukin-1, which have global effects on both other immune cells as well as on the transcriptional status of neurons. IFN-γ has been shown to specifically facilitate the clearance of viral infection from HSV-infected mice (78, 142). In addition to their role in limiting acute viral infections, it is intriguing to consider possible

influences on the permissivity of neurons for establishment of latency, given the ability of cytokines to alter the transcriptional status of neurons.

Currently, it is unclear to what degree the immune system affects (either positively or negatively) the establishment of the latent infection. The demonstration that following footpad inoculation of the mouse, a replication-defective virus can establish a latent infection in spinal ganglia in a matter of hours (252) argues that it would be difficult for the immune system to completely block the establishment of a latent infection. However, as outlined above, the immune system does act to limit the acute infection of HSV, and this probably reduces the amount of virus available for the establishment of latent infection, in distal sites at least. While it is possible that the immune response, especially cytokines, play a role in promoting a latent infection by suppressing the replication of HSV within neurons, there is little evidence for this. In fact, a large number of studies of immunosuppressed mice indicate that latent infections are still established in animals with restricted cellular responses, although the animals often succumb to uncontrolled acute infections (90, 297). This does not, of course, rule out possible effects of cytokines produced by cells other than lymphocytes, as discussed above.

In contrast to the situation with establishment of latent infections, the immune response undoubtedly plays an important role in determining whether virus resulting from the reactivation of an individual neuron will ultimately produce an infectious lesion at the periphery. Indeed, it has been demonstrated that T-cell immunity plays an important role in limiting the degree of replication or reactivation at peripheral sites and also, to some extent, at the level of the reactivating neuron itself (188, 317). Therefore, what we regard as the basis of "productive" or clinical reactivation may be determined largely not by the molecular events of reactivation within individual neurons but by how efficiently the host can limit the replication and spread of the reactivating virus. This line of thinking is reinforced by data which suggest that the immune makeup of an individual is more important than the viral strain in determining whether he or she experiences frequent reactivations (236). It is interesting that the stress associated with reactivations could play a dual role: first in triggering reactivation, and second in suppressing the existing immune response, which could limit viral spread back to the periphery.

The effect of the immune response on *wt* (wild-type) HSV (i.e., nonlaboratory strains) replication and spread throughout the host is also manifest in clinical complications arising from infection and reactivation. Thus, the generally benign course of primary infection and reactivation when an immunocompetent host is exposed to HSV is in distinct contrast to the patterns of disseminated infections described when an immunosuppressed or immune incompetent individual is infected. Among notable clinical manifestations are neonatal HSV infections (usually caused by exposure of the neonate to a primary HSV infection in the mother), increased risk of atypical HSV infections during pregnancy, zosteriform HSV infections in organ transplant recipients, and HSV pneumonia and other complications in patients with AIDS (86, 106, 118, 172, 184, 202, 272).

Herpes encephalitis. Although severe immunocompromise is usually associated with atypical disseminated HSV infections, sporadic HSV encephalitis need not be correlated with obvious immune distress at the onset of the disease (196). For example, in a recent survey of 43 Swedish patients, a maximum of 12 had clear signs of primary disease while the remainder had recurrent infections (263). Exactly what features of viral infection and/or reactivation lead to encephalitis are unknown, but a transitory crisis in immunity could be a major factor. Although clinical isolates of HSV are often high in neurovirulence and neuroinvasiveness indices when tested in laboratory animals, there is no evidence that the viruses recovered from patients with herpes encephalitis are any more virulent than are those isolated from patients with labial or genital infections. Furthermore, there has never been any confirmed epidemiological pattern of the occurrence of herpes encephalitis, which would suggest that a specific virus genotype plays a significant role in its etiology (91).

Sites of Latent HSV in Humans

Although the vast majority of the molecular analyses of HSV latency have depended upon animal and cell culture studies, the results hopefully reflect the situation with human infections. Despite inevitable and expected differences in detail, correspondence between clinical and autopsy studies in humans and laboratory investigation is excellent. As elegantly reviewed by Stevens (276), careful clinical observations coupled with insightful interpretation of the results of surgical destruction of the trigeminal nerve (trigeminal neurectomy) and other manipulations of sensory neurons in humans and early animal models led to the suggestion that HSV is able to establish latent infections in sensory nerve (and particularly trigeminal) ganglia in humans well before the availability of the appropriate tools and animal models for the study of the phenomenon. Goodpasture is generally credited with generating the most complete synthesis of clinical observations along with those on experimental infections in animal models (95), but many other contributors played major roles.

Of course, despite this head start, the definitive demonstration of the presence of latent virus in human nervous tissue and mechanistic studies has required the stimulus of appropriate observations in carefully controlled animal models. For example, only after Stevens and Cook demonstrated the ability to recover infectious HSV following cocultivation of latently infected murine dorsal root ganglia (279) were reliable recoveries of infectious virus from human trigeminal and sacral ganglia at autopsy reported (3, 5, 6, 173, 234). Clear evidence of nonlinear viral genomes was provided by Rock and Fraser by using restriction endonuclease and Southern blot analysis of latently infected mouse neuronal tissue (228, 229) well before similar results were reported for human tissue (69). Furthermore, it was determined that limited transcription of the viral genome occurs during latency (227). Subsequent studies revealed that a single transcript, the latency-associated transcript (LAT), could be detected by in situ and Northern blot hybridization of RNA first isolated from latently infected mouse spinal ganglia (281) and then isolated from human trigeminal ganglia (47, 282). It was ultimately revealed that the form of LAT that was detected was a stable intron antisense to the $\alpha 0$ transcript and processed from a larger primary transcript (281).

While the correspondence between human infections and those of animals is not complete—nor should it be expected to be—many of the tissues shown to be available for latent and/or inapparent HSV infection in animals are also available in humans. HSV DNA can readily be isolated from the brains of mice and other animals following corneal infection, and this recovery does not necessarily depend upon the appearance of active infection or encephalitis in the central nervous system (CNS) (27, 52, 64, 100, 175, 287); a similar situation appears to obtain in humans, although there is some controversy in the literature (4, 123, 192).

It is also clear that HSV genomes can be detected in other neuronal nuclei in both humans and laboratory animals, al-

though at a frequency much lower than that observed for the nominal site of latency, the trigeminal ganglia (42, 89, 97, 114, 130, 182, 199, 222, 242). One such site is the nodose ganglion of the vagus or 10th cranial nerve, which innervates the gastrointestinal tract. A number of years ago, latent HSV was detected within the nodose ganglion of humans (303), and it has been postulated that primary HSV-1 infections (or reactivations) of the oral mucosa could seed the esophagus or stomach and lead to an infection of these ganglia. Recently, Gesser et al. demonstrated that one can experimentally infect the nodose ganglia following oral infections of mice and that latent infections are readily established there (88, 89). In addition, HSV was shown to establish a latent infection in the nodose ganglia and produce gastric ulcers within the stomach in SCID mice (28). These observations shed some light on the etiology of esophageal mucosal disease that is seen in humans with AIDS (316), and it is possible that further studies will demonstrate that HSV plays a role as a direct or underlying factor in other diseases of the gastrointestinal tract.

Different Pattern of Infection, Latency, and Reactivation—Varicella-Zoster Virus

In contrast to the situation with HSV-1 and HSV-2, where numerous animal and cell culture models and systems exist for both productive and latent infection, VZV, a closely related alphaherpesvirus based upon sequence comparisons, has no good animal model and is not particularly convenient to work with in cultured cells. Despite this, the availability of comparative sequence data and the close correspondence of many of the genes of the two viruses (49, 50, 79, 169), along with the contrasts in the clinical course of infection and reactivation (92, 141, 277, 308), make it instructive to consider comparative features.

The contrasts between HSV and VZV are illustrated dramatically by the differences between the course of their acute infections. While HSV is spread by direct contact, forms local lesions, and rarely disseminates, VZV can be spread by aerosols, making it more highly contagious. In addition, VZV readily disseminates to multiple organ systems during the primary infection. Although cell-free infectious VZV cannot be readily isolated, it is evident that gene expression and genome replication during productive infection follow a course similar to that described for HSV; only the final stages of morphogenesis and lack of release of infectious virus from the infected cell distinguish them (201, 239). Furthermore, the gross pathologic findings and the appearance of virus-filled vesicles formed during primary infection are similar; despite this, primary VZV infection is disseminated throughout the dermatome, in contrast to the highly localized nature of primary HSV infection.

This difference may correlate with differences in the efficiency of virus spread through viremia and (perhaps) in the precise mode of host immune modulation of the infection. Like HSV, VZV is markedly neurotropic and can establish latent infections in sensory neurons following primary infection. It is reasonable to suppose that the migration of virus up the axon and establishment of latent infection via cell-specific restriction of productive virus replication are similar between the two viruses also. Again, since the maintenance of alphaherpesvirus genomes in latently infected tissue is essentially a passive phenomenon, there does not appear to be any marked difference between the two.

Based upon autopsy data, VZV genomes can be detected in a number of sensory ganglias throughout the body, including the trigeminal ganglia and CNS (66, 93, 165), but while latent HSV genomes tend to predominate in the trigeminal ganglia, VZV genomes are more frequently found in somatic nerve ganglia—in keeping with the fact that VZV primary infection is generally disseminated. In contrast to the restricted expression of the LATs marking HSV latency, which will be covered in detail in following sections, no corresponding transcription unit has been identified in the VZV genome, and no latency-specific genes have been described. Instead, based upon analysis of neurons obtained at autopsy and with the proviso that traumatic reactivation in such material can never be rigorously excluded, VZV latency appears to involve the sporadic and low-level expression of one or another productive-phase viral transcripts, although there is some controversy about whether this gene expression takes place in sensory nerve ganglia, satellite cells, or both (36, 37, 48).

Another major contrast between HSV and VZV is the course of reactivation and the fate of the neuron harboring the reactivating virus. Although HSV reactivation is induced by stress periodically during the life of the infected individual, VZV reactivation is common only at an age of the individual that correlates with a general decline in immunity. As outlined above, HSV reactivation is generally not destructive to the site of latent infection, and repeated recurrences have no obvious deleterious effect upon the function or physiology of the trigeminal ganglia. For VZV, however, the situation is quite different, and reactivation leads to zosteriform shingles, i.e., necrotic lesions marking the dermatome served by the sensory neurons in which the VZV reactivated. Furthermore, the postherpetic neuralgia so characteristic of the course of VZV reactivation may reflect destruction of neural tissue.

It is tempting to speculate that at least part of the difference seen in the behavior during latency and reactivation with the two viruses is the result of the lack of an LAT unit in the genome of VZV. Of course, it must be acknowledged that other differences in virus-mediated pathologic changes and virulence may play a prominent role in the differences in the course of VZV and HSV infections and reactivation. Still, the fact that the LAT unit is important in efficient reactivation in HSV latency could correlate with such a difference. It will be seen that it is not easy to devise a simple model that can clearly explain how such a difference might operate; nevertheless, the contrast between these two representatives of human alphaherpesviruses makes such speculations extremely interesting.

ANIMAL MODELS FOR THE STUDY OF HSV LATENCY

As noted above, the broad host range of HSV has allowed the use of animal models for the study of viral pathogenesis, neuropathology, neuroinvasiveness, and latency. An ideal animal model would be able to re-create all aspects of the human disease, but, obviously, this is not attainable. Still, in terms of the ability of HSV to establish a localized initial infection followed by neuronal spread and establishment of latency, a number of very useful models exist and, again as noted above, have provided the basic tools for our understanding of the disease in humans. In contrast, the lack of corresponding models for VZV has resulted in significant gaps in our comparative knowledge of the process of infection, latency, and reactivation in this related pathogen.

The most appropriate model for latency must allow virus reactivation, and this reactivation should be similar to that seen in humans. Thus, in an immunocompetent animal, it should occur spontaneously and be inducible by stress, and recovery from recrudescence should be complete. Also, the initial infection should be mild enough to ensure that all or most experimental subjects survive with no sequelae. Two animal models, the rabbit and the guinea pig, approximate this

ideal situation, although both suffer from limitations and both involve considerable expense. A third model animal, the mouse (the most reasonable in terms of cost), is being used extensively but suffers from the lack of efficient in vivo reactivation of virus. These three animal systems have provided the means for generating the vast majority of data now available concerning HSV latency and reactivation, but other models also exist. These are described in the following sections.

It should be noted at the outset, however, that all these models have significant problems when direct correlations with human disease are attempted. First, as noted above, many clinical isolates of HSV are extremely neurovirulent and pathogenic in animals. Thus, the number of allowable genotypes, serotypes, and phenotypes available for study is limited, especially when compared with the panoply of naturally occurring ones. Furthermore, the level of neurovirulence and other aspects of pathogenesis, including the ability of the virus to replicate at low titers in specific tissues, can often be altered by the very mutations and/or manipulations of the viral genome carried out to understand the interrelationship between viral genes.

Finally, the specific pattern of virus infection, latency, and reactivation is often quite strain specific, so that even though interesting mutations of viral genes may be available for study, they may well not be available in the appropriate viral strain, and random mutations occurring in the viral genome, especially under the mutagenic processes of generating recombinant viral genomes, can significantly complicate the interpretation of data. Only a few examples need be documented. (i) The sequence of the 17*syn*$^+$ strain of HSV-1 is the standard for the virus and is thus is the most appropriate strain for generating mutations in critical genes, but it is much more virulent in mice than is the KOS(M) strain, which is often more useful for biological studies (291). (ii) HSV-2 is not able to reactivate in rabbits; this is also the case for many strains of HSV-1 with potentially interesting properties for study (87, 112). (iii) Strain-specific alterations in the glycoproteins as well as other proteins of HSV-1 can profoundly affect the ability of the virus to spread through the nervous system and potentially alter important parameters of latency (18, 120, 124, 177, 327). (iv) Alterations in other HSV genes can profoundly affect the course of virus infection in animal models; thus, for example, mutations in the thymidine kinase gene affect many aspects of neurovirulence and pathogenesis, even though the gene is "nonessential" in cell culture (80).

Murine Models of Latency and Reactivation

Footpad/dorsal root ganglion model. Direct demonstration of the ability of HSV to establish and maintain a latent infection in neuronal cells was accomplished by mouse footpad infection, which is followed by latent infection of spinal ganglia (279). This model system is analogous to genital infection of HSV in humans and has been central to describing many of the parameters of latent HSV infection including the identification of the neuron as the site of latent infection, axonal transport of virus through the sciatic nerve, ability of nonreplicating virus to establish latent infections, and characterization of restricted transcription of the latency-specific transcription unit during the latent phase of infection (39, 40, 60, 140, 144, 173, 282, 299, 300). Furthermore, the model is useful with HSV-2, despite the greater neuropathology of this virus (34).

Following infection of the footpad, local pathologic changes are observed, with clear evidence of involvement of the CNS; the mice that recover are evidently physiologically normal. When dorsal root ganglia are dissected and cultured (either whole or following disruption) on feeder cells, HSV-induced cytopathologic changes can be detected within 4 to 12 days (131, 279). This explant recovery of HSV from such latently infected spinal ganglia has been an extremely useful and relatively inexpensive means of assaying the presence of viral genomes within the tissue in question. It has been termed reactivation, but this term should strictly be reserved for the process in animals in which virus can be recovered from peripheral tissue, not from nervous tissue itself. Just how accurate this explant recovery model is for studying reactivation is a matter of continuing dispute as well as occasional acrimony.

Mouse eye/trigeminal ganglion model. A second murine model for HSV-1 and HSV-2 latency involves the infection of the cornea followed by virus latency in the trigeminal ganglia (150, 161, 178, 244, 269). As in the footpad model, latent HSV genomes express LAT in a portion of the neurons maintaining them, and virus can be recovered by cocultivation of explanted ganglia.

An interesting variation on this method which comes closer to an in vivo method has been developed by Sawtell and Thompson (244, 245). Here, latently infected mice are transiently exposed to hyperthermia, and then trigeminal ganglia are excised, sectioned, and assayed for the presence of observable virus by immunohistochemical methods or, if recombinant virus in which an expressible marker has been included in the genome by genetic engineering methods is used, by localization of such reporter gene activity.

Although such a model is not equivalent to recovering virus at the site of initial infection and implicitly assumes that virus recovery in the nerve ganglion is equivalent to reactivation as assayed at a peripheral site, it does provide a second method for modeling reactivation in the mouse. As discussed below, some of the results obtained by this approach are at some variance with those obtained by the explant techniques.

A related method involving Cd^{2+} induction of latent virus in murine trigeminal ganglia was reported by Fawl and Roizman to result in high efficiency of recovery of infectious virus (77). Unfortunately, a recent follow-up of this work by Fawl et al. reported that the approach was very mouse strain dependent and that the original levels of reactivation reported for CBA mice could not be readily replicated (76).

Other murine latency models. An accurate model of reactivation as assayed by virus recovery at a peripheral site following a stress stimulus would be an extremely useful addition to our experimental repertoire of methods for studying HSV reactivation by using the attractive features of murine systems. Other methods have been reported but have not (at least to date) provided much promise of real advance because of the difficulty in reproducing results over time or between laboratories. Hill et al. (114) reported that after establishing a latent infection in mouse trigeminal ganglia following infection of the ear, they were able to recover virus from the site following mild abrasion with adhesive tape. UV irradiation and ocular iontophoresis of epinephrine have also been reported to result in ocular virus shedding following latency in the trigeminal ganglia (149, 318), but extensive use of either method has not been seen.

Finally, some of the earliest sources of latently infected neurons were tissues of the CNS (228, 229). However, the relative amount of viral genomes in this tissue is not large, and CNS tissue does not rapidly yield virus upon cocultivation on feeder cells (134). Thus, the general use of CNS tissue for the study of latency and reactivation has not been reported.

Latency and Reactivation in Rabbits

As reported in an earlier section, some of the earliest studies of HSV pathogenesis leading to the inference of axonal transport were based upon studies in rabbits (see reference 276 for a review). Infection of rabbit eyes and the ability to sporadically recover virus following periods of latency were originally reported by Nesburn et al. (191), and this was subsequently shown to involve latent infection of the trigeminal ganglion of affected rabbits (190). The fact that no infectious virus was recoverable from latently infected trigeminal ganglia until cultivation with feeder cells suggested that this model system could mimic human infections in broad detail. Despite the expense involved, the rabbit eye model has gained considerable currency due to the investigations by Hill and his colleagues showing that efficient reactivation can be induced by iontophoresis of epinephrine and other adrenergic agents (13, 108, 110, 112, 143, 144).

Although not strictly necessary for investigation of the genetic requirements for reactivation by using mutant viruses or for general characterization of LATs (96, 205, 207, 208, 304, 305), the ability to efficiently induce reactivation is of great value for analyzing the nature of the changes seen in neural tissue during reactivation and for analyzing the function of critical portions of the viral genome on reactivation itself (16, 17, 109, 294). Despite these advantages, efficient establishment of latency and reactivation is confined to HSV-1 in rabbits and is highly strain dependent (87, 112).

Latency and Reactivation in Guinea Pigs

Vaginal inoculation of female guinea pigs with HSV-1 or HSV-2 results in obvious primary infection with some mortality. Following recovery, the survivors of primary infection periodically display vesicular recrudescence in the vaginal area from which infectious virus and/or viral DNA can be recovered (81, 148, 273, 290). Although reactivation cannot be reliably induced (25), the fact that HSV-2 spontaneously reactivates with much higher frequency than HSV-1 makes this a very attractive system for comparative analysis of the influence of viral genes on reactivation, and HSV-1 × HSV-2 recombinant viruses are being investigated with an view to attempting to identify features important in this difference (139, 324, 325). The value of guinea pigs in studying vaccine efficiency and in other aspects of experimental pathogenesis makes this an extremely valuable and promising system.

Other Animal Models

Other animal models have been used in the investigation of HSV infection and latency, but specific factors make any particular one somewhat less attractive than those discussed above. HSV infects laboratory rats and establishes latent infections, but reactivation or explant recovery of virus has not been readily accomplished (12, 139, 194, 219, 256, 257, 325). HSV infection of primates also results in latent infection, which might be useful in reactivation analysis (237, 298), but the high expense of working with such models has not allowed deep exploitation of their potential.

Animal Models Demonstrate Latent Viral Genomes in Corneal Cells

The overwhelming majority of models involving HSV latency and reactivation posit the neuron as the site of latent virus genomes. Despite this, animal models suggest that viral genomes can be maintained in a stable form in corneal cells of latently infected rabbits (41, 42, 199). Although it is a debatable point whether the virus is present as a persistent or "true" latent infection, the fact that LATs can be detected and virus can be recovered by explant cocultivation makes the distinction rather moot. The proportion of corneas in which virus or virus transcripts can be detected in latently infected animals is considerably lower than is the proportion of trigeminal ganglia. Still, the fact that viral genomes are present makes it very important to carefully assess reactivation data, especially low-level reactivation, particularly in terms of viral genomic function. Practically, the ability to isolate virus from human corneas is a very important factor in assessing risks associated with corneal transplants and traumatic eye surgery (97, 130, 182).

Other peripheral sites for maintenance of viral genomes by virtue of an atypical latent or low-level persistent infection have been suggested as playing a role in the epidemiology of asymptomatic shedding and viral transmission. For example, HSV has been isolated from the skin of latently infected mice (114); however, a thorough, quantitative analysis of virus recovery has not been reported.

In Vitro Models of Latency

Despite the value of and progress made with animal models of latency, many questions concerning specific features of latent phase gene expression, the state of latent viral genomes, etc., can best be investigated in a cell culture model. Indeed, the ability to reduce all biological phenomena to cell culture manipulation is a "holy grail" of molecular biologists. A number of cell culture systems in which viral genomes are stably maintained and from which virus can be recovered under appropriate conditions of "reactivation" have been reported in the past 20 years. Although none have been extensively employed or have been used to demonstrate features of latency unknown from animal studies, such systems do hold great promise.

The earliest attempts to set up a latency system in vitro were developed by Rapp and coworkers and involved low-multiplicity infections of cultured cells in the presence of IFN, elevated temperature, and metabolic inhibitors of viral genome replication (197, 198, 312–314). Such treatment yields "latently" infected cells in which viral genomes can be detected for long periods and from which virus can be recovered by removing inhibitors. Despite its promise, the system has not yielded results suggestive of a true latent infection; thus, the viral genomes present were determined to be linear, not circular, and expression of LAT was not observed (248).

Despite these problems, Preston and colleagues adapted this approach to establish latent HSV infections in cultured human fibroblasts. They used the system to establish such infections with replication-impaired viral mutants; in this system, viral genomes have been shown to be maintained as circular molecules, and the predominant viral transcript expressed is the LAT (68, 103, 122, 213). The model has been exploited to investigate the role of specific viral regulatory genes on the reactivation process, and one report has concluded that the $\alpha 0$ gene product is all that is required to induce productive infection (102). Since this gene product is known to be important in efficient virus replication at low multiplicity of infection (70, 71, 240, 241, 283), this result is not surprising. However, it is clear that in animal models, a genetic element(s) within the LAT is critically involved in reactivation (see below and references 16, 17, and 113); furthermore, many other viral genes play at least an accessory role in efficient reactivation, although this role may not be critical in the primary event leading to reactivation.

Several laboratories have reported the use of cultured neu-

ronal systems for modeling latent infections and reactivation in vitro (62, 68, 131, 147, 312, 313, 315, 329). Such cultures, in theory at least, would provide some real advantages, and the one described by Wilcox and colleagues in which acyclovir is used to establish latent infections and nerve growth factor is used to maintain latency in both peripheral sympathetic and sensory neurons holds promise. Using such cultures, these workers have suggested that LAT is expressed and that activation of second-messenger pathways reactivates HSV—a result well in keeping with observations in vivo (265, 315).

These authors have also reported the expression of a protein encoded by a portion of the viral DNA encoded by LAT in such latently infected cultured neurons (61). This antigen is expressed during productive infection (224); however, it is encoded by a portion of the transcription unit that is processed as a stable intron during latent infection. This portion of LAT is not involved in the efficient reactivation phenotype seen with expression of the transcript (73, 211). Thus, the significance of detecting the antigen in latently infected cultured neurons is unclear.

More recently another in vitro model has been developed to study reactivation. This model, developed by Moriya et al., makes use of latently infected mouse trigeminal ganglia that are dissociated and maintained in a latent state by the use of BVaraU, an inhibitor of HSV replication (181). The authors have demonstrated that these cultured trigeminal ganglia can be induced to reactivate by heat shock, much akin to the in vivo heat shock system of Sawtell and Thompson discussed above (244, 245). Halford et al. have shown that the cells in this culture system synthesize at least part of the LAT and that HSV can be induced to reactivate by treating the cells with dexamethasone (101). While this model clearly has promise in the study of a number of molecular and biochemical parameters, it suffers from the requirement that an inhibitor of viral replication be used.

Correspondence among Various Animal Models of Latency

Clearly, the animal models described above provide generally complementary and equivalent data in terms of the site, state, and expression of the viral genome during latency. Differences between them in terms of the ability to generate a type of reactivation physiologically similar to that seen in humans have been outlined. These differences are also reflected in the fact that viral genetic elements expressed during latency may have slightly different effects upon the establishment of latency and the precise mode of reactivation seen in mice as compared to guinea pigs and rabbits. Furthermore, the precise location of the genetic elements important in induced, and spontaneous reactivation in these last two models does not precisely correspond to that identified as important in explant recovery (see below). Despite this, the great convenience of murine models, along with their great wealth of genotypes and the availability of immunological reagents, makes such systems of continuing utility. It is important to remember, however, that precise details of actual modes of reactivation may well be model specific.

STATE OF THE HSV GENOME IN THE ESTABLISHMENT AND MAINTENANCE OF LATENT INFECTIONS IN NEURONS

Establishment of Latent Infections in Sensory Neurons

The establishment of latent infection is a process which can be readily examined only in animal models, and most studies have been carried out with various routes of infection in mice. The establishment of latency in neurons is essentially or completely a passive phenomenon; no viral gene product is involved in the process.

Margolis et al. used a recombinant HSV-1 expressing the *lacZ* (β-galactosidase) gene under the control of the LAT promoter and compared viral gene expression in various tissues in mice following peripheral infection (168); they concluded that neurons in which latent infection was established were infected as rapidly as those undergoing productive virus replication. Furthermore, they determined that of at least four classes of neurons in the ophthalmic division of the trigeminal ganglion that are susceptible to HSV infection (substance P^+, $CGRP^+$, $LD2^+$, and $SSEA\text{-}3^+$), the major group of neurons in which latent (i.e., nonproductive) infection was occurring were those with the SSEA-3 surface marker. This marker (SSEA-3 [stage-specific embryonic antigen 3]) is a cell surface marker of primitive and visceral endoderm that is present on 40% of the ophthalmic division of the mature trigeminal ganglion neurons (167). To a lesser extent, latent infection was also observed in $CGRP^+$ (calcitonin gene-related peptide) neurons; however this group has significant overlap with the $SSEA\text{-}3^+$ group (168). Their conclusion that latent infection occurs as an early event in infection and thus defines a restricted group of neurons is consistent with the conclusions of Speck and Simmons (268).

Latchman and colleagues carried out an extensive analysis of potential mechanisms to account for the failure of the HSV productive cycle in the establishment of latency, and they concluded that specific transcription factors important in the expression of the immediate-early regulatory proteins of the virus are either altered or absent in cultured neuronal cells compared to nonneuronal ones. They suggested that such differential populations of transcription factors could play a major role in the restriction of productive infection in certain classes of neuronal cells, notably by interfering with the first activation via α-TIF (117, 156, 157, 309).

The greatest support for the idea that latent infection is the result of a failure of the productive cascade comes from numerous studies on replication-impaired and replication-deficient HSV mutants. In all cases studied, there is no evidence that any gene expression is required. Most convincingly, a number of studies have shown that viral mutants lacking functional α4 or α-TIF genes and which can express few if any productive-cycle proteins are readily able to establish latent infections (59, 103, 129, 252, 274, 296). These results also demonstrate that virus replication is not a requirement for the establishment of latent infection, and Margolis et al. directly established that virus replication does not occur in neurons fated to be latently infected with replication-competent virus (168).

There are many reports of efficient establishment of latent infections in numerous animal models with virus mutants which do not express LAT (113, 125, 139, 150, 251, 275). Where carefully studied, such mutants have been shown to have no, or only a very limited, effect on the actual number of latently infected neurons in such animals (16, 56, 119, 151, 245, 251). Thus, it is clear that LAT expression is not a requisite for establishment of latency per se.

Viral Genomes in Latently Infected Neurons

Viral DNA can be readily detected in peripheral neurons and CNS tissue of latently infected animals and in human autopsy material. Rock and Fraser demonstrated that viral genomes were deficient in free ends and concluded that they

are either episomal or concatenated in animal neurons (228, 229); this observation was confirmed and also shown to be the case in human neurons by Efstathiou et al. (69). A more complete demonstration of an episomal state for viral DNA in mouse brains was subsequently presented by Mellerick and Fraser (175).

The number of viral genomes per latently infected neuron has been calculated to be in the range of 10 to 100 (27, 69, 109, 228), with the smaller numbers corresponding to neurons isolated from the CNS. This range of values is within the detection limits of standard in situ hybridization methods, but with one exception (254), there has been a general failure to detect viral genomes by the technique. This failure may reflect some aspect of the physical state of the viral genome in latently infected cells, and only PCR amplification in situ has provided positive results in both animal and human tissue (100, 174, 176, 192).

Most Latent HSV Genomes in Neurons Are Not Transcriptionally Active

The ability to detect LATs expressed from the HSV genome during latent infections in neurons provided a great impetus to the study of HSV gene function during latency and reactivation. Indeed, the abundance of the stable intron processed from LAT makes it a reliable marker for assaying latent infections, and the fact that it is expressed at a very low level compared to productive-cycle transcripts during productive infection makes its presence a useful marker for the study of the establishment of latent infections outlined above.

Despite the convenience of LAT as a marker of latently infected cells, in mice and rabbits the number of LAT-expressing neurons ranges between 30 and 10% or less of the total latently infected neurons containing viral genomes (67, 68, 100, 109, 164, 174). Since this transcription unit is correlated with efficient reactivation (see below), it will be important to devise methods of correlating HSV transcription during latency with reactivation per se.

It should be noted in passing that Ramakrishnan and colleagues have found a much higher proportion of latently infected neurons expressing LAT in rat brains latently infected with an HSV-1 mutant lacking the essential ribonucleotide reductase gene (220, 221). Whether this difference reflects the animal or the viral mutant used is unclear.

HSV Genomes Are Stably Maintained in Latently Infected Neurons

As is the case with the establishment of latent infection by HSV, the maintenance of viral genomes appears to be a passive phenomenon; i.e., it requires no viral gene expression. This is in keeping with the stability of the neuronal population itself. The number of viral genomes present and the number of neurons expressing the stable intron processed from the LAT do not change with time following establishment of latent infection (136, 228, 300). In an investigation of any possible role for latency-associated transcription in maintaining viral genomes, Sedarati et al. did a very careful quantitative analysis of viral DNA recoverable from 1 to 11 months following the establishment of latent infection in murine dorsal root ganglia by the footpad route of infection. Following an initial decline in genomes concomitant with the cessation of productive infection, no changes were seen (251). Furthermore, these workers showed that the genome of a replication-deficient mutant was fully stable over time in latently infected neurons (252). Indeed, there are no reports in the literature to suggest that any replication-impaired or -deficient mutants studied for possible viral gene functions required for the establishment of latent infections evidenced a time-correlated loss of latently infected neurons.

HSV GENE EXPRESSION DURING LATENT INFECTION IN NEURONS

The DNA encompassing the HSV latent phase transcription unit is situated in the long repeat regions of the viral genome and thus is diploid. It is colinear with a number of transcripts of both known and unknown functions expressed both in the same orientation and in the antisense orientation expressed during productive infection. Our current understanding of the complex pattern of transcription seen in the repeat regions of the HSV-1 genome is shown in Fig. 1, and all current evidence indicates that this map is essentially the same for HSV-2.

In Situ Hybridization Analysis

Although difficult to quantitate, the sensitivity of in situ hybridization has been of great value in the detection and mapping of latency-associated transcription by HSV in neuronal tissue (2, 46–48, 51, 52, 60, 137, 178–180, 209, 230, 232, 269, 279, 281, 289, 299). The following is a summary of data reported, and this is indicated schematically in Fig. 1. (i) Both neuronal and nonneuronal cells in acutely infected ganglia yield nuclear and cytoplasmic signals when hybridized with probes covering any region of the viral genome. (ii) In contrast, hybridization of latently infected tissues with defined probes covering 85% or more of the genome demonstrates no signal at all while hybridization signals confined to neuronal nuclei are readily detectable with probes complementary to a region subsequently shown to encompass the DNA encoding the stable LAT intron and partially overlapping the α0 regulatory gene. (iii) Hybridization signals with intensities equivalent to those seen for acutely infected tissue are seen only with probes corresponding to DNA sequences within a limited region of the total hybridization positive region. (iv) A significantly weaker signal in fewer cells is seen with probes extending both 3′ and 5′ of the region exhibiting strong hybridization signals, and this signal localizes in different regions of the nucleus.

Although such data are consistent with a number of different schemes, the data discussed below demonstrate that this difference in hybridization intensity corresponds to different abundances of RNA species processed from the primary LAT unit.

Detailed Characterization of LATs

Much of our picture of how latent phase transcripts are expressed and how they may function in reactivation is based upon the detailed characterization of these transcripts by Northern blot, S1, and RNase resistance analysis of RNA-DNA and RNA-RNA hybrids (52, 57, 60, 137, 178, 179, 270, 271, 282, 286, 299, 300, 304, 305, 331, 332). For this review, all our mapping data have been correlated with the sequence of the 17*syn*$^+$ strain, and minor differences in sequence between strains noted in the primary references have no impact upon the general picture outlined below.

The most abundant forms of HSV LATs are stable introns. Northern blots of RNA from latently infected ganglia hybridized with defined DNA and short oligonucleotide probes demonstrate the presence of at least two partially colinear, relatively abundant poly(A)$^-$ RNA species mapping within the limits of the strong in situ hybridization signal noted above. The most abundant RNA species is approximately 2.0 kb, and there is a less abundant one of 1.5 to 1.4 kb.

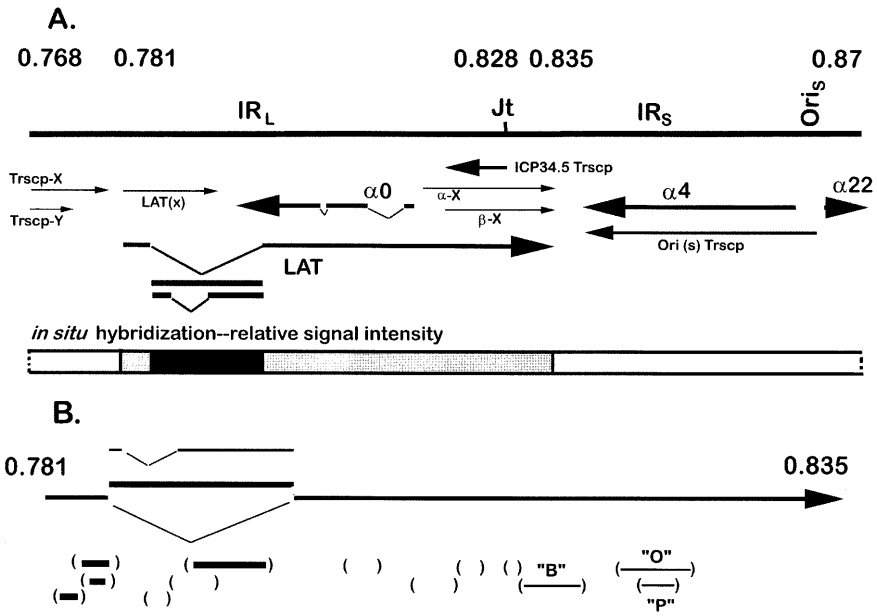

FIG. 1. Transcription map of the internal repeat and joint region of the HSV-1 genome. (A) Schematic of the repeat and joint regions of the HSV-1 genome with the map locations of the repeat ends, the ends of LAT, and the joint indicated. Transcripts expressed only during productive infection are shown at the top, and LAT and the stable introns processed from it during the latent phase of infection are shown at the bottom. Finally, a schematic representation of the in situ hybridization signal intensity seen when probes are hybridized to latently infected neurons is shown. The details of the mapping of transcripts and the properties of proteins encoded have been recently reviewed (301). The functions of the proteins encoded by the major transcripts (α0, α4, and α22) and the mRNA encoding ICP34.5 are well documented; proteins encoded by less abundant transcripts [Trscp-Y, Trscp-X, LAT(x), α-X, β-X, and Ori(s)] have yet to be carefully characterized. (B) Higher-resolution map of the HSV-1 LAT, with potential protein translational reading frames encoded by the 17*syn*$^+$ strain shown in parentheses. The translational frames which have been specifically mutagenized in this strain and shown to play no role in the efficient reactivation phenotypes in rabbits are shown as heavy bars in parentheses (17, 74). Other translational frames in the 5′ and other portions of LAT can be eliminated as playing a role based on data obtained by using inserted transcription termination/polyadenylation signals and by constructing deletions with other strains of virus (17, 72, 207). Finally, translation frames B, O, and P have been excluded from playing a major role in viral pathogenesis by specific mutagenesis (146).

The evidence that these two RNA species are introns is overwhelming. Most convincingly, Farrell et al. have expressed the primary LAT unit in transfected cells and have demonstrated the processing of this unit to produce a poly(A)$^-$ LAT (73). Precise mapping of the 5′ and 3′ ends of the poly(A)$^-$ LAT species from latently infected tissue has been carried out by both S1 nuclease and RNase protection analysis, and the 5′ and 3′ ends map to canonical splice signals (299, 300). Furthermore, the 2-kb poly(A)$^-$ LAT is uncapped (57) and can be isolated as a lariat form (321). The work of Farrell et al. demonstrates that this intron is stable in transfected cells and accumulates as it does in neurons, although we have found that the intron is not stable in all cells in which it is expressed—for example, mouse neuroblastoma cells (73). More recently, several laboratories have demonstrated that the two stable RNA species can be isolated as lariats (233, 321).

The smaller (1.4-kb) species is related to the larger by the removal of another 600-base intron (300). This "respliced" LAT intron is seen only in latently infected tissue, and the relative amounts seen depend upon the strain of virus used to establish latent infection. While the fact that secondary splicing of introns is not a well-understood or described phenomenon has led some to speculate that an independently expressed small species is generated from a "second" LAT promoter, the recent demonstration that it can be isolated in lariat form is clearly consistent with a resplicing mechanism for its formation. Finally, while some laboratories have reported that the lower-abundance smaller species is actually made up of two partially contiguous transcripts differing by 50 bases (52, 179, 304, 305), the report by Wu et al. demonstrates that these two species are actually the same RNA differing in their physical configuration (321).

Primary LAT. Northern blot analysis of poly(A)$^+$ RNA from productively infected cultured cells reveals the presence of an 8.5-kb transcript expressed from the same DNA strand as that encoding the LAT strand beginning just 3′ of the LAT promoter and extending to a polyadenylation signal in the short repeat region (57, 60). The presence of such a primary transcript can be inferred from in situ hybridization data for latently infected neurons (reviewed in reference 224), and one laboratory has reported the detection of the primary transcript by using Northern blots of RNA from latently infected neural tissue (332). Furthermore, transcripts in latently infected tissue initiating at the expected cap site and extending to near the putative polyadenylation signal have been detected by RNase protection analysis (57, 60) and confirmed by PCR analysis (16, 56).

RNase protection assays demonstrate that the 5′ end of this poly(A)$^+$ transcript is located approximately 25 bases 3′ of the TATA box element of the LAT promoter (see below) and that RNA from latently infected murine sensory ganglia protects the same fragment as that seen with productive-cycle poly(A)$^+$ RNA. Although the 3′ end of LAT is not as precisely located as the 5′ end of the primary transcript, it is clear that the polyadenylation signal situated in the short repeat region just 3′ and on the opposite strand of DNA from that controlling the polyadenylation of α4 is used in lytic infection. RNase protection probes from that region are protected by poly(A)$^+$ RNA isolated from productively infected cells, and PCR products from oligo(dT)-primed cDNA from latently infected rabbit

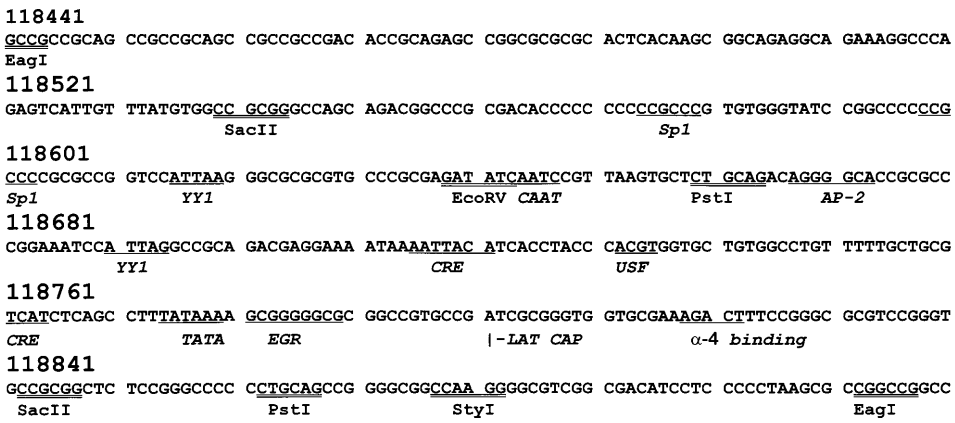

FIG. 2. Promoter for the HSV-1 LAT. The sequence of the 17*syn*$^+$ strain is shown, and specific transcription factor binding and/or transcription-regulatory sites are shown. As discussed in the text, some of these sites have only been suggested to play a role in latent phase transcription, but the CRE has been shown to have a mediating effect on the reactivation phenotype (8, 9, 19, 60, 132, 225, 267, 305, 306, 331). The region between the two *Pst*I sites at 118659 and 118866 were deleted to generate the 17ΔPst LAT-negative mutant.

and murine sensory nerve ganglia have been generated with primers located upstream, but not downstream, of this polyadenylation signal. This strongly suggests that a major portion of latent phase transcription terminates here. It should be noted, however, that weak in situ hybridization signals have been found with HSV-2 probes extending into the C-terminal portion of the α4 protein (179); therefore, there may be more than one 3′ end to the latent transcript.

Processed forms of the primary LAT. Since the 2- and 1.4-kb stable, poly(A)$^-$ latent phase transcripts are introns, it is clear that processed forms of the exons in the primary poly(A)$^+$ transcript must occur. To date, these have not been extensively characterized, but in their in vitro system, Farrell et al. were able to detect processed RNA from which the major LAT intron had been spliced out, demonstrating the stability of the LAT intron (73). Evidence for such a processed RNA was also seen in PCR analysis of trigeminal neuron-derived RNA following induced reactivation of HSV in latently infected rabbits (16), but we have not been able to isolate such a poly(A)$^+$ RNA from productively infected or uninduced latently infected tissue.

Other and/or independent species of LAT. A 2-kb poly(A)$^+$ RNA extending from the LAT cap site to a position near the 3′ end of α0 mRNA can be readily isolated from productively infected cells (57); however, it is not clear whether its contiguous 3′ end communicates with the LAT polyadenylation signal some 7 kb downstream via a splice or whether polyadenylation occurs at a noncanonical termination/polyadenylation signal. No role is known for this transcript.

As discussed below, there are some data which support the idea of an independent promoter or, at least, transcription start site near the 5′ end of the stable 2-kb intron. Although it has been posited that this promoter could give rise to an RNA species partially colinear with the 2- and 1.4-kb LAT introns, no convincing demonstration of this mechanism has been made.

Other processed forms such as one using the splice signals seen in the 1.4-kb LAT intron, along with those seen in the primary transcript, can also be posited, but there is no direct evidence for their occurrence in either lytic or latent infections. It should be noted, however, that during infections of cultured cells the expression of the translational reading frame present in the region of the viral genome encompassed by the 2-kb intron should involve the expression of some poly(A)$^+$ transcript mapping in this region (61, 224). While none has been clearly identified, Nicosia and colleagues have suggested that it is colinear with the 5′ portion of the LAT unit (193).

One of the problems with attempting to work with such low-abundance transcripts is that no biological function related to the expression of LAT maps to the regions identified. Another major problem is that there are a number of productive-phase, low-abundance transcripts mapping within the contiguous limits of the LAT unit whose kinetics of expression and regulation are unusual in that they are inhibited by immediate-early transcription (21, 322). Such transcripts can greatly complicate the analysis of low-abundance transcripts thought to be associated with latent infection, since much of the analysis must be done in cultured cells. Finally, transcription patterns from LAT and other transcripts colinear with its 5′ portions are also expressed during productive infection and may or may not be under the control of novel promoters.

PROMOTER CONTROLLING HSV LATENT PHASE TRANSCRIPTION

With HSV-1, readily detectable transcription is abolished in latent infections with a recombinant virus (17Δ*Pst*) containing a deletion of approximately 200 bases containing RNA polymerase II promoter elements located at −1,700 bases 3′ of the α0 transcript terminus (16, 56, 60). Transcription is also eliminated during productive and latent infection, with more extensive deletions encompassing this region (60, 119, 180, 266). Full confirmation that this region indeed contains the HSV-1 LAT promoter was achieved by showing that high levels of a reporter gene mRNA could be expressed when recombined in the appropriate position just downstream and by using RNase protection experiments which establish the initiation site of the LAT primary transcript just 3′ of a canonical TATA box within the 202-base region deleted in the recombinant (138). The location of this latent phase promoter coincides exactly with a canonical RNA polymerase II promoter element suggested by sequence analysis, and its sequence (17*syn*$^+$) is shown in Fig. 2. There is a high degree of sequence homology between HSV-1 and HSV-2 in the immediate region of this promoter, but not immediately upstream or downstream of it (138).

Analysis of Functional Elements of the HSV-1 Latent Phase Promoter by the Transient-Expression Assay

The sequence of the HSV-1 latent phase promoter contains a number of potential control elements which could play a role in transcription during latent infections. These include canonical cyclic AMP response elements (CRE), CAT box, Sp1, USF, YY1, and AP-2 sites, and an early growth response element, as well as a sequence element just at the cap site which is quite similar to the strong α4 DNA binding site involved in the autoregulation of that regulatory protein (8, 9, 132, 225, 267, 305, 306, 331). Although determination of the actual importance of such sequences in the latent phase activity of this promoter requires animal studies, a number of studies have been carried out in cell culture which suggest that some of these and other elements have a potential function. Thus, promoter activity is responsive to cAMP levels in cell culture, and the α4 binding site represses the ability of LAT expression to be induced by α4 in transient-expression assays (9, 82, 83, 152, 225).

The latent phase promoter behaves unusually in transient-expression assays with cultured cells; it has a rather high basal activity and is not highly activated by HSV superinfection compared with productive-cycle promoters (7, 57, 330). There is a marked enhancement of basal promoter activity in cells of neuronal origin, and the region of the LAT promoter responsible for such enhanced neuronal activity has been shown to bind to a potential transcription factor found in neuron-derived IMR-32 cells (332).

The following approach described by Devi-Rao et al., with the β-galactosidase gene as a reporter, serves as a rough guide to transient-expression assays designed to detect neuron-specific elements and to examine possible neuronal specificity of the LAT promoter in vitro (57). A construct containing 360 bases upstream of the LAT cap expressed somewhat more β-galactosidase activity than did a productive-cycle promoter in uninfected rabbit skin cells. Upon transcriptional activation by viral superinfection, however, levels of indicator enzyme activity controlled by the latency promoter increased only approximately 3-fold compared to a better-than-50-fold increase with the productive transcript promoter. This result is due, in part, to the presence of the α4 autoregulatory site at the cap site.

Transient-expression assays with murine neuroblastomas displayed a significantly different pattern of expression. Here, efficient expression of the LAT promoter required sequences 180 bases and more upstream of the cap site, and the elements in question seem to reduce LAT promoter activity in rabbit skin cells, since increased activity was observed with the deletions in such cells.

It is important to note that despite the evidence that sequence elements influence neuronal expression in the latent phase promoter, this promoter is not profoundly specific for neural cells, since its activity is readily detectable in productive infection of cultured cells and in animals (57, 162, 168, 270, 299, 300, 304). Furthermore, the analysis of the LAT promoter in cultured cells and during productive infection leaves at least one rather obvious conundrum. Since LAT promoter activity appears to be repressible by α4 protein and the LAT promoter has rather high basal levels of activity, it would be predicted that LAT should act somewhat like a weak immediate-early transcript during lytic infection. Despite this, there is no evidence for its expression under conditions of infection where only such immediate-early transcription is occurring. This suggests that the actual state of the transcription template, as well as the LAT promoter itself, is important in LAT expression in latent infection.

Analysis of Latent Phase Promoter Elements In Vivo

The study of functional elements of the latent phase promoter is complicated by the fact that the precise level of activity of the promoter during latent infection is not known. As discussed above, viral genomes in many latently infected neurons are transcriptionally silent, and the high stability of the introns processed from the primary latent phase transcript in transcriptionally active neurons makes a ready estimate of promoter activity impossible. Furthermore, Margolis et al. have shown that reporter gene expression from the latent phase promoter declines with time after establishment of latency (166). It is important to note, however, that because the virus used had a deletion downstream of the LAT cap site, it is possible that this deletion affected the long-term expression characteristics of this virus. Adding to this argument is the observation that the total number of reporter-positive neurons dropped dramatically with time (166), a pattern which does not mirror the expression of LAT. Still, taken together, the best interpretation of all the data is that the very small amount of RNA corresponding to the primary transcript recoverable suggests that extremely low promoter activity was present during the latent phase of infection in neurons.

Despite the technical difficulties in carrying out precise studies, it is clear that the functional elements of the latent phase promoter seen in neurons in vivo do not fully correspond to those identified in vitro. Lokensgard et al. suggested that DNA sequence elements downstream of the latent phase transcript cap should play an important role in latent phase transcription, since they found that even extensive portions of upstream promoter elements controlling a reporter gene did not maintain neuronal expression when recombined into the unique long region of the viral genome (158). Although such an analysis could not preclude a role for the template structure as being important instead of the presence of an actual promoter element(s), more recent studies by Soares et al. (267) provide very strong evidence for the presence of a neuronal "enhancer" element within 60 bases or so immediately downstream of the transcript start site. Whether this element plays a role in expression in vivo is unclear, however. Lastly, analysis of mutant virus in which upstream promoter elements have been altered demonstrates the functionality of the CRE most proximal to the latent transcript start site; furthermore, the importance of the TATA box in maximal expression was established (152, 218).

Clearly, further analysis is necessary, but it is also clear that the relatively laborious approach of generating viruses bearing defined mutations in putative control elements will provide the only reliable means of identifying elements in the latent phase promoter which demonstrate neuron and latent phase specificity. The fact that viruses which are lacking or impaired in latent phase transcription are inefficient reactivators in vivo makes surveys of potentially important sites a somewhat more tractable task. For example, recent work by Bloom et al. has demonstrated a partial role of the proximal CRE in epinephrine-induced reactivation in rabbits (19).

Is There a Second Latent Phase Promoter?

Although deletions of the latent phase promoter abolish readily measurable transcription during latent infection as assayed by in situ hybridization and PCR analysis (16, 30, 55–57, 60), some workers continue to speculate about the existence of a second latent phase promoter just upstream of the splice

acceptor region of the stable 2-kb intron. This speculation is based on the following observations. (i) Insertion of a β-galactosidase reporter gene upstream of the intron resulted in low levels of reporter protein expression in latently infected mice, even though a transcript initiating at the latent phase transcript start site would have a very long leader (115). (ii) A β-galactosidase reporter gene construct controlled by the putative TATA-less promoter from this region, when recombined into the long unique region of the viral genome, expressed a small amount of enzyme activity for a very long period following neuronal infection (94). (iii) A 2-kb transcript which is partially colinear with the 2-kb intron is expressed during productive infection by mutant viruses lacking a functional latent phase promoter (30, 81). (iv) Goins et al. have found that a putative TATA-less promoter is active in transient-expression assays, although some 10-fold less so than is the weak latent phase promoter itself (94).

While the high stability of the functional β-galactosidase enzyme makes it unsuitable as a reporter to measure promoter activity at any given time (166), the presence of the enzyme in neuronal tissue and the transient-expression data do suggest that there is a low-activity promoter functioning upstream of the stable 2-kb intron. However, the great preponderance of the published data indicate that this promoter is active in productive, not latent, infection. It is therefore invalid to define it as a second latent phase promoter. Despite their nomenclature (latency-associated promoter 2 [LAP-2]), Soares et al. appear to agree with this assessment (267), and thus the terminology is unfortunate and potentially misleading.

ROLE OF LATENT PHASE TRANSCRIPTION IN LATENCY AND REACTIVATION

A (if not the) major question regarding latent phase gene expression by HSV is whether it plays a significant biological role in some aspect of latency. Since a latent infection can be established in the absence of measurable viral gene expression in all animal models tested, it is clear that latent phase transcription cannot be a requirement. Furthermore, careful quantitative analysis of the levels of viral genomes within neurons latently infected with virus mutants not expressing such transcripts argues against LATs playing any required role in the maintenance of viral genomes.

By elimination, then, it would be expected that any role for such latent phase transcription would be in the reactivation process. This has been found to be the case in rabbit, guinea pig, and some murine models. Despite this, the actual details of the role of such gene expression may differ depending on the model system used, and the specific regions of the LAT unit critical for the effects seen do not precisely co-map between murine and in vivo models.

Latent Phase Transcription Is Required for Efficient Reactivation in In Vivo Models

The fortuitous finding of a latent transcription-negative mutant of strain 17*syn*$^+$ of HSV-1 (X10-13) with a 1,200-base deletion of the latency-associated promoter and 5′ region of the primary transcript which was derived from stocks of virus generated in the formation of HSV-1 × HSV-2 recombinants (125, 300) allowed Hill et al. to show that lack of such gene expression was correlated with a low frequency of induced reactivation of virus in vivo in the rabbit eye model (113). A second independent 17*syn*$^+$ mutant with a similar extensive deletion was also shown be a poor spontaneous reactivator (294).

The extensive nature of the deletions in these viruses required comprehensive confirmation of the role of latent phase transcription per se with a mutant of strain 17*syn*$^+$ containing a defined, engineered deletion of the latent phase promoter itself (16). These studies demonstrated a measurable effect on both spontaneous and induced virus reactivation as measured by recovery of infectious virus in the tear film. PCR analysis was also used to demonstrate the lack of any observable transcription extending to the polyadenylation signal of the primary transcript and that the levels of viral DNA as measured by ratios to cellular DNA were essentially the same in latent infections with both mutant, *wt*, and latent phase transcription-restored rescue virus. The reduced spontaneous reactivation in a rabbit model was confirmed with an independent mutant virus by Perng et al. (205).

HSV-2 is a much more efficient spontaneous reactivator than HSV-1 in the guinea pig vaginal model, and an HSV-2 mutant with a similar deletion of the LAT promoter was tested by Krause et al. (139) with essentially identical results—latent phase transcription plays a significant role in efficient in vivo reactivation. Again, no effect was observed in the amount of viral DNA seen in latently infected tissue.

The Region of the Latent Phase Transcript Important in the Efficient Reactivation Phenotype in Rabbits Is Confined to Region of 480 bp or Less within Its Extreme 5′ End

Bloom et al. described a series of mutants of HSV-1 in which a polyadenylation signal was inserted between the cap site of LAT and its 3′ end. Termination of transcripts even 1,500 bases downstream of the cap had no effect on induced reactivation in the rabbit eye model (17). In this same communication, it was shown that the specific deletion of a 348-bp DNA element situated between −205 and −554 bp 3′ of the cap site, containing three potential translation initiator codons, did result in a virus which expressed both normal amounts of LAT and the stable introns but which could not efficiently reactivate upon epinephrine induction. In agreement with this observation, Hill et al. have shown that a strain 17*syn*$^+$ mutant containing a 370-bp deletion encompassed by two *Sty*I restriction sites which partially overlaps the critical 348-bp region did not efficiently reactivate upon epinephrine induction (111). Despite this agreement, however, a similar mutant of the McKrae strain of HSV-1 did not show reduced spontaneous reactivation (208); however, the low rate of spontaneous reactivation could statistically mask an effect.

The sequence of the 17*syn*$^+$ strain of HSV-1 from the start of LAT to the 3′ terminus of the critical 348-bp deletion is shown in Fig. 3. Finer mapping of the region important in high-frequency induced reactivation was attempted by Bloom et al., but three smaller subdeletions spanning the 348-bp critical region are themselves dispensable for the reactivation function (17). More recently, substitutional mutagenesis has fully confirmed the conclusions derived from the deletional analysis (20). Taken together, the smallest contiguous DNA element required for reactivation could be situated within the 138-bp region from −77 to −216 bp downstream of the cap site; alternatively, multiple partially redundant sites could exist within the full 478-bp region spanned in the two mutants. Current data obtained with mutants in which the sequences have been mutagenized instead of deleted confirm the observation that no element further than 544 bases downstream of the start of LAT is involved in efficient induced reactivation. Other deletion and substitution mutants are currently being examined to further characterize this region.

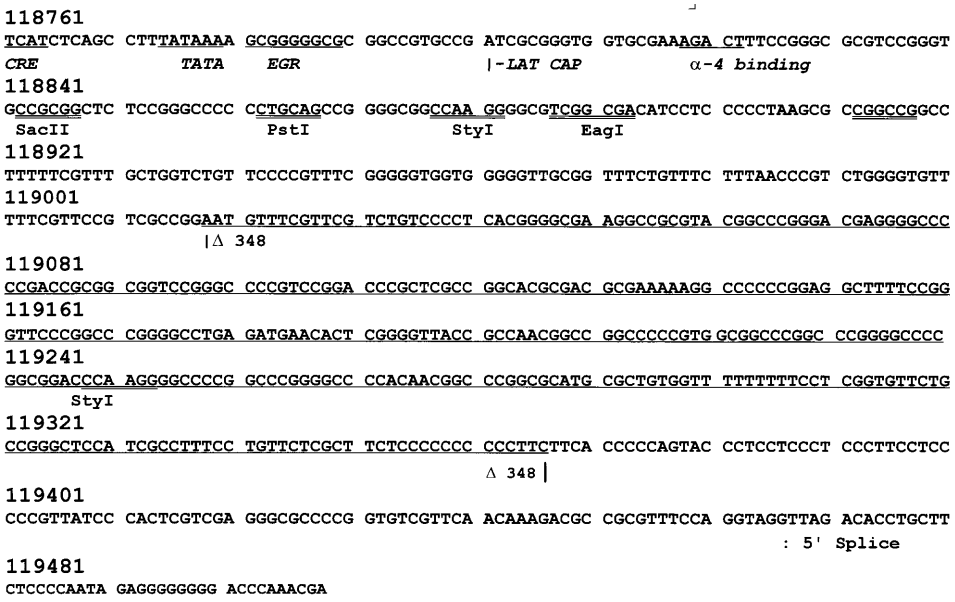

FIG. 3. The region of the 5′ end of the HSV-1 LAT mediating efficient reactivation in rabbits. As discussed in the text, deletion and poly(A) signal insertion mutagenesis have demonstrated that the region within the deletion boundaries of the Δ348 mutant is all that is required for efficient reactivation; an overlapping deletion between the two indicated *Sty*I sites has also been shown to be reactivation impaired (17, 111).

Evidence that Modulation of Expression of LAT during the Latent Phase or at the Initiation of Reactivation Plays a Role in the Efficient Induction of Virus in the Rabbit Eye Model

The fact that a new class of reactivation-impaired mutants now exists which express normal amounts of LAT during the latent infection (17) reinforces the observation that the amount of LAT produced by naturally occurring HSV strains does not easily correlate with the relative reactivation potentials of these strains (23, 68). This suggests that the influence of LAT on reactivation may be exerted as a transient change in its rate of transcription following the reactivation stimulus.

The finding of altered reactivation rates following latent infection with a mutant virus in which one of the CREs within the LAT promoter was mutagenized supports the idea that increased expression of LAT occurs during reactivation (19). While a similar mutant (17CRE) examined in the mouse showed only slight reduction in reactivation efficiencies by cocultivation (218), it showed a more dramatic reduction in reactivation in the rabbit, particularly with respect to spontaneous reactivation frequencies. Since CREs are critical in some stress response pathways, the data suggest that levels of cyclic AMP modulate the induction of LAT transcription and hence modulate the levels of stress-induced reactivation. The studies also highlight the apparent differences among the various animal models used for these studies.

Latent Phase Transcription Facilitates but Is Not Required for the Efficient Recovery of Infectious Virus from Explanted Latently Infected Murine Ganglia

The murine explant model which is not an in vivo reactivation model exhibits a more varied pattern of dependence on LAT expression. Thus, latent phase transcription with at least some strains of HSV-1 has an effect on the rate and level of virus production in explanted latently infected murine ganglia. This was first reported by Leib et al. (150), who showed that fewer trigeminal ganglia explanted following latent infection with a mutant of the KOS strain of HSV-1 in which the latent phase promoter was specifically deleted were able to produce virus than were control ganglia latently infected with *wt* virus. Mutants of the 17*syn*$^+$ strain of HSV-1 showed similar reduced and/or delayed recovery levels from latently infected murine dorsal root and trigeminal ganglia (15, 56, 275). Despite this, Izumi et al. (119) and Devi-Rao et al. (56) did not see such a delay or reduction in virus recovery from spinal ganglia latently infected with several KOS(M) derived LAT-negative mutants. The reason for such a difference may be related to the fact that the KOS(M) strain of virus has patterns of neuroinvasiveness and neurovirulence different from those of the 17*syn*$^+$ strain (291).

The HSV-1 Latent Phase Transcript May Play a Role in the Efficiency of Establishment of Latent Infection in Murine Trigeminal Ganglia

Sawtell and Thompson (244, 245), using their hyperthermic reactivation model to assay productive phase gene expression in the neuron itself, reported a reduction in reactivation frequency with the LAT-negative KOS(62) mutant of the KOS (M) strain of HSV-1. They also reported that the number of latently infected neurons seen in trigeminal ganglia in mice infected in the eye with the latent phase transcription-negative mutant was measurably smaller than the number in mice infected with the *wt* virus, and they suggested that the reduced reactivation frequency as well as delayed kinetics of reactivation observed in explant models could be explained by this difference.

Two other laboratories have also reported a slightly reduced efficiency of establishment of latent infections by LAT-negative viruses in murine eye models. Devi-Rao et al. assayed viral DNA levels by PCR and found a slight but measurably significant reduction in viral DNA recovered from murine trigeminal ganglia latently infected with LAT-negative mutants of strain 17*syn*$^+$ and KOS(M) but (as noted above) observed no difference with KOS(M) derived LAT-negative virus in spinal ganglia (56). A measurable reduction in the number of latently

infected neurons in trigeminal ganglia following infection of mice with a LAT-negative mutant of strain 17*syn*$^+$ compared to infections with *wt* virus was also reported by Maggioncalda et al. by using PCR-amplified in situ hybridization (164).

Such data suggest that there is a significant difference in the phenomenology of establishment of latency in mice compared to rabbits. This follows from the fact that following explant cocultivation, there is no evidence for delayed recovery of latent phase promoter-defective virus from latently infected rabbit trigeminal ganglia (16), and, as noted above, no quantitative difference in viral DNA recovery was found in rabbits latently infected with this or *wt* virus. As discussed below, however, such a difference(s) does not necessarily require the role of LAT in critical cells during the reactivation process to differ markedly in the different systems.

Murine Explant Models Do Not Reveal a Region Critical for Virus Recovery Equivalent to That Characterized for Reactivation In Vivo in Rabbits

It is striking that despite the excellent agreement for different mutants and different strains of HSV-1 in localizing the reactivation function to a specific region of the viral genome in rabbit models, this region does not appear to be critical for the recovery of virus from explanted latently infected murine trigeminal or dorsal root ganglia. Thus, Maggioncalda et al. showed that the deletion of the 370 bp between the *Sty*I sites in strain 17*syn*$^+$, which so profoundly affects induced reactivation in rabbits, has no effect at all on the recovery of virus from explanted latently infected trigeminal ganglia (163). Bloom et al. (17) reported the same for their 348-bp deletion mutant. The possible interpretation of such results in light of the comparison of the mode of action of latent phase transcription in rabbits and mice is briefly considered in the following section.

MECHANISM OF ACTION OF HSV LAT IN REACTIVATION

Since the expression the 5′ portion of the latent phase transcription unit is involved in efficient reactivation in several in vivo models, it would seem no great trick to be able to determine both the cell types in which it acts and the molecular mechanism of this action. Unfortunately, this trick has not been successfully performed to date. There is a large and accumulating body of evidence that the mode of action is not a simple one. LAT mediates reactivation in highly differentiated tissue and clearly in only a very limited subpopulation of cells—possibly a single neuron. In addition, the basal level of latent phase LAT expression varies among neurons. These factors combine to make the study of what is really going on in HSV reactivation very difficult.

There Is No Evidence for a Major Antisense-Mediated Repressive Action in Animal Models

The fact that the LAT unit and its stable intron are antisense to the α0 transcript, which is critical in the mediation of low-multiplicity virus replication, suggested that LAT might work as an antisense modulator of immediate-early gene expression initiating the productive cascade (282). Such modulation might protect certain critical neurons or peripheral cells from productive virus infection. While such a role would seem to run counter to the positive effect of latent phase transcription on reactivation, one could posit that certain critical cells must have been protected at the very initiation of the latent phase of infection or that the modulation protects the nerve ganglia by limiting vegetative replication in the ganglion during the process of reactivation itself.

This model is attractive in its simplicity, and it might explain the significant differences between the course of reactivation seen with HSV and that of the "naturally" LAT-negative alphaherpesvirus, VZV (see above). Unfortunately, none of the model systems that were used to examine reactivation support such a role. Thus, while several groups have reported that high levels of LAT intron in productively infected cells have a small but measurable negative effect on the course of productive infection (73, 243), levels normally seen in productive infection appear to have no effect (57). Most critical, of course, is the fact that mutant viruses which express the stable LAT intron but do not reactivate efficiently in the rabbit model can be constructed, and viruses which do not express the latent phase transcript intron but reactivate normally can also be constructed (16, 207, 208).

There Is No Evidence for a Latent Phase-Expressed Viral Protein Involved in Reactivation

The prominent open translational reading frame within the HSV-1 LAT intron prompted speculation and experimental study of the possibility that it or another protein expressed during the latent phase of infection could mediate the reactivation phenotype (300). The expression of this open reading frame during productive infection has been detected (160, 224), but its elimination by mutation has no effect upon efficient spontaneous or induced reactivation in rabbits (16, 74), nor does such mutation have an effect on explant-induced recovery of virus from latently infected mouse neurons (72).

Possible roles for the expression of proteins encoded by other translational reading frames during latent infection can also be eliminated. Bloom et al. specifically set out to eliminate the three potential translation initiators found in the LAT-induced reactivation-critical 348-bp region in the 5′ portion of the latent phase transcription unit to assess the possible expression of a protein. They found that all such mutations had no effect on the efficient recovery of virus from rabbits with epinephrine-induced latent infection (17). Furthermore, reactivation rates were determined to be normal for a mutant virus (RHA6) in which latent phase transcripts are terminated 1,500 bases 3′ of the LAT start by insertion of an efficient polyadenylation signal. This effectively eliminates appreciable expression of any translational reading frames downstream of this (17).

Of course, only directed mutation of each open translational reading frame found within the whole latent phase transcription unit will completely rule out the expression of a minute amount of some protein playing a role, and such analysis will also be required to fully eliminate expression of one of these translational reading frames during the early events following successful reactivation in a specific cell. This is a laborious task, and somewhat difficult to justify as a high-priority one with the data at hand. Still, sufficient work has been done to rule out the expression of the ICP34.5 protein by a transcript from the same DNA strand encoding the α0 transcript, which plays a role in neurovirulence, as having an influence on HSV recovery from rabbits in vivo or mice in vitro (22, 26, 32, 204, 206, 297). Similarly, the ORF-P protein expressed from one or another low-abundance transcripts which are partially colinear but independently controlled by productive-phase promoters (Fig. 1) does not seem to play any measurable role in the reactivation process (17, 21, 145, 189).

Possible *cis*-Acting Mechanisms for the Influence of LAT on Reactivation

Perhaps the best interpretation of the evidence discussed above is that HSV latent phase transcription mediates reactivation via a *cis*-acting mechanism. Two possible models for such activity are discussed below.

LAT could play a role in methylation and transcriptional availability. It is possible that the reactivation-critical region at the 5′ end of LAT acts as a transcriptional enhancer. As such, it could be required during the latent phase of infection to maintain an appropriate structure of the viral genome which allows the LAT promoter to be accessible to transcription. This critical region contains a cluster of CpG dinucleotides which extend into the LAT promoter (17), and such CpG islands have long been known to be implicated in transcriptional regulation of eukaryotic gene expression through methylation. In addition to directly interfering with the binding of specific transcription factors, methylation of CpG dinucleotides can change the twist of the DNA, affecting the nucleosomal structure. It is possible, then, that the deletion of critical CpG islands alters the DNA structure so that the LAT promoter is inaccessible at a critical point in reactivation.

The idea that methylation might play a role in reactivation is not a new one: 5-azacytidine has long been known to modulate transcription of thymidine kinase and to induce reactivation in in vivo systems (35, 326). The actual significance of these in vivo studies was difficult to interpret, however, due to the potential global and indirect effects of 5-azacytidine. In addition, initial studies of latent HSV DNA demonstrated that the latent genome was not extensively methylated (63). However, the identification of the CpG dinucleotide islands in this reactivation-critical region of the LAT makes investigation of the methylation status of this specific region of the latent HSV genome during latent and productive infection particularly important.

Could the critical region of the latent phase transcription unit act as a cell-specific origin of genome replication? The potential methylation patterns as defined by CpG islands in the critical *cis*-acting region of the latent phase transcript are similar to those seen near the origins of replication of the virus. This, and the requirement for transcription through the region, could be interpreted to imply that the critical region acts as a tissue-specific origin of replication operating just in the cell(s) immediately involved in productive reactivation. The only way to really test such a possibility is to identify a tissue or cell type in which it can be demonstrated that this critical region is required for genomic amplification. Certainly, there is no evidence based on the recovery of plasmids from neuronal cells transfected with this region of the latent phase transcription unit and then superinfected with virus that this region acts as a readily measurable origin of replication in cultured cells (57).

Does Latent Phase Transcription Supply an Essential Function in Neurons or Peripheral Cells Initiating Reactivation?

One of the major difficulties in developing a full understanding of the role of HSV latent phase transcription in the reactivation process is that we do not yet have a definitive picture of where it works. Although it has been often tacitly assumed that it must play its role in a subset of neurons, it is just as possible that the critical site for LAT to act is in a peripheral cell or tissue either just prior to establishment of latency or during the early stages of reactivation (or both). In this scenario, LAT-negative virus would have a small, but significant, replication impairment.

Certainly, the evidence that LAT-negative mutants are less efficient in establishing latent infections in murine trigeminal ganglia (discussed above) is fully consistent with a peripheral site of action. Further, a good way of rationalizing the differences seen between the lack of any role for latent phase transcription on the efficiency of establishment of latent infections in rabbits, guinea pigs, and murine dorsal root ganglia compared to that seen in the mouse eye model is that this restriction would generally be seen in the critical first step following the appearance of virus at the periphery of the latently infected neuron in in vivo reactivation but could also operate at some level in primary infection in the mouse eye. Here, the reduced yield of virus would result in a lower inoculation of latent genomes into the neurons themselves.

One approach to the study of potential peripheral sites is to carry out a precise analysis of the earliest events in the reactivation process. It has long been known that small amounts of HSV and/or viral transcripts and proteins can be detected in some neuronal ganglia in the absence of evidence of reactivation in the periphery (i.e., at the site of infection). Recently, this phenomenon has been carefully described by using PCR analysis in murine ganglia (98, 99, 136, 280). These sporadic events could be the result of abortive, atypical, or dead-end virus production with no clear role in the actual process of reactivation itself. Such a conclusion is consistent with the fact that careful PCR analysis of RNA from ganglia latently infected with LAT-expressing and LAT-negative viruses in the few hours following either induction of reactivation in rabbits with epinephrine or insult of an explant in mice showed essentially no difference in the levels or timing of the appearance of productive-cycle transcripts (16, 56). Alternatively, however, these results could imply that LAT expression plays no role in the appearance of infectious virus as a result of limited replication in one or several neurons. Rather, the differences in the level of virus replication seen in the periphery would be due to a limited impairment of replication of LAT-negative virus at the onset of peripheral virus replication.

Clearly, the investigation of growth impairment due to the lack of LAT function would be most readily studied in cell culture. Unfortunately, the expression of LAT has yet to be shown to play a measurable role in virus replication in cultured cells. Although there is a report in the literature of such a role based upon plaque size reduction of a deletion mutant of HSV which did not express LAT (14), the interpretation is complicated by the fact that the mutant virus used eliminates transcription upstream of LAT. From the work of Rosen-Wolff et al. (238), this area is known to mediate some aspects of neuropathology and virus replication in CV-1 cells.

A careful analysis of the process of transcription and viral genome replication in both trigeminal neurons and the cornea during the early stages of induced reactivation in rabbits is currently being investigated in our laboratories (20). Preliminary data demonstrate that there is no restriction of replication of LAT-negative virus at moderate and high multiplicities of infection. Hopefully, continued analysis will clarify more of the issues involved.

FUTURE DIRECTIONS

Does HSV Latency Provide a Complete Model for Alphaherpesvirus Latent Infections and Reactivation?

While HSV is the prototype for the alphaherpesviruses, it is important to recognize that there are important areas of divergence between HSV and other members of this group with respect to latency and reactivation. Unlike VZV (discussed

above), other members of this group examined (bovine herpesvirus type 1 [BHV-1] equine herpesvirus type 1, and pseudorabies virus) have been shown to express LATs (10, 31, 159, 214, 215, 227, 230, 231). While the LATs expressed by these viruses are located in equivalent regions of their respective genomes, the size of the transcripts (and intron species) can vary significantly among them. Thus, while the pseudorabies virus LAT is a large transcript like that of HSV, the BHV-1 transcript does not extend antisense through the cognate immediate-early transcript at its 3′ end. Sequence comparison of the DNAs encoding these LATs reveals only distant similarity, although the CpG island frequency in the promoter region is conserved.

Functional analyses of LAT in BHV-1 have shown that, like the situation with reactivation of HSV in vivo, LAT null mutants are restricted in reactivation in rabbits (226). From this, it may be that the LAT-expressing members of the alphaherpesvirus group share similar general mechanisms of LAT enhancement of reactivation. The fact that the sizes of the transcripts and the DNA sequences encoding them differ so greatly is consistent with their having a *cis*-acting mode of action.

Other possibilities exist, however. For example, the BHV-1 LAT encodes a protein related to cyclin that may act to inhibit cell cycle advance (116, 247). If this function is confirmed, BHV-1 would stand in distinct contrast to the situation with HSV. While it is not at all clear that such a protein is actually involved in reactivation, the data may indicate that the common features of latent phase transcription in the alphaherpesviruses manifest different sorts of mechanistic approaches toward modulating latent infection and/or reactivation. Such possibilities form one of the best arguments in favor of carrying out comparative studies between related herpesviruses—the sum total of the studies will provide much more than simple variations on a single mechanistic theme!

Course of Future Experiments

All the work reviewed here suggests that the role of LAT in reactivation is subtle and may be manifested differently in the different cellular environments seen in various model systems. Future work will have to be directed at the level of individual neurons due to the differences in how the virus interacts with the diverse neuronal cell types within a sensory ganglion.

It is also important to consider that the transcriptionally complex LAT region of HSV may well encode other functions relevant to reactivation. Even though only the 5′ end of LAT has been shown to play a role in induced reactivation, it is possible that downstream portions influence aspects of establishment or maintenance of the latent infection in certain systems or cells. Further genetic dissection of the LAT region is required for the elucidation of the mechanism(s) influencing efficient reactivation. This information will set the stage for ultimately determining those other factors which affect clinical reactivation, including the role of immunity in limiting the recurrent disease and the physicochemical mediators of reactivation.

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