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Review

Molecular circuitry regulating herpes simplex virus type 1 latency in neurons

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Herpes simplex virus (HSV) type 1 (HSV-1) enters nerve endings during a primary infection, is transported to sensory ganglia, and establishes latency within nuclei of a subpopulation of neurons. The latent state is characterized by absence of detectable HSV-1 antigen, minimal transcription of productive cycle genes, and high expression of 1.5- and 2-kb viral transcripts, termed the major latency-associated transcripts (LATs), within nuclei of a subpopulation of infected neurons. Transcription within the HSV-1 LAT genomic locus has been reported to both facilitate the establishment of latency in additional neurons and to increase the frequency of spontaneous and induced viral reactivation in animal model systems. More recent evidence suggests some possible mechanisms that may explain the relationship between LATs and both reactivation and establishment of latency. This review summarizes general aspects of latency, but focuses on the structure, expression, and function of LATs, and the interaction between host transcriptional regulators and viral gene expression that may impact latency and reactivation. A model that incorporates evidence from a number of experimental studies is proposed that summarizes the involvement of the LAT locus in the biology of HSV-1 latency. *Journal of NeuroVirology* (2000) **6,** 6–24.

Keywords: herpes simplex virus; latency; LAT; nervous system; transcription

Introduction

HSV recurrent disease is typically a self-limited infection lasting about seven days, and is characterized by the appearance of blistered lesions on the surface of mucous membranes (Spruance *et al*, 1997; Bader *et al*, 1978). In a normal host, these infections are usually only a nuisance, although they can be much more severe in the immunocompromised host (Pass *et al*, 1979). In addition, infection of the eye is the number one infectious cause of blindness in industrialized nations (Nesburn, 1983), and infection of the central nervous system (CNS) can lead to encephalitis (Linnemann *et al*, 1976). HSV recurrent disease is attributed to the establishment of latency within neurons of the corresponding peripheral sensory ganglia (Cook *et al*, 1974). As long as viral DNA remains intact within nuclei of latently infected sensory neurons, the virus can utilize cellular proteins to initiate viral gene transcription, which can culminate in the synthesis of viral proteins and assembly and release of infectious viral particles. During the latent state, the viral genome exists in an episomal form within nuclei of latently infected sensory neurons (Efstathiou *et al*, 1986; Mellerick and Fraser, 1987; Slobedman *et al*, 1994), and viral replication is either extremely low or non-existent as determined by absence of infectious particles and undetectable lytic phase gene expression as assayed by *in situ* hybridization (Croen *et al*, 1987; Deatly *et al*, 1987; Stevens *et al*, 1987). This situation apparently renders host defense mechanisms ineffective with respect to eradicating the infection.

One of the major discoveries relevant to the molecular aspects of latency was the observation that the viral genome was not entirely quiescent during latency (Stevens *et al*, 1987). A family of viral transcripts termed latency-associated tran-

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scripts (LATs) were detected during the latent phase by *in situ* hybridization of latently infected ganglia using subgenomic and single-stranded probes and by Northern blot hybridization analyses (Stevens *et al*, 1987). Although more sensitive PCR techniques have since shown low levels of expression of other viral genes including ICP4 and thymidine kinase (Kramer and Coen, 1995), the unusually high levels of LAT expression were striking and stimulated a period of intense research involving the role of LATs in the processes of latency and reactivation. Although far from being understood, a substantial amount of evidence suggests that transcription within the LAT locus facilitates establishment and/or reactivation of latent virus infection.

Lytic replication and viral latency

HSV-1 replication

Pioneering studies concerning the regulation of HSV-1 gene expression have been performed by Roizman and coworkers (Batterson and Roizman, 1983; Mackem and Roizman, 1982a,b; McKnight *et al*, 1987; Michael *et al*, 1988; Honess and Roizman, 1974; Roizman *et al*, 1965). These studies have continued for more than three decades and have included detailed investigations of the HSV-1 life cycle by a multitude of investigators. HSV-1 transcription is sequentially ordered and coordinately regulated. Therefore, the replication strategy is typically described according to the kinetics of transcription of different sets of viral genes characterized during viral replication in permissive cells *in vitro*. In its simplest form, this is based on the expression of three sets of genes. The first set of genes transcribed are the immediate-early or α genes, a group that encodes proteins involved in regulating viral gene expression. Expression of α genes is critical to the activation of genes expressed later in the replication cycle. Early or β genes are synthesized next and encode proteins involved in nucleotide metabolism. Synthesized last are the late or γ genes, which encode structural proteins.

The α genes were initially described as the set of genes that can be expressed in the absence of protein synthesis (Honess and Roizman, 1974). This result was consistent with the observation that α gene transcription is activated by the U_L48 gene product, virion-associated tegument protein, VP16 (Campbell *et al*, 1984). Between 500–1000 copies of VP16 enter the cell with each virion (Heine *et al*, 1974; Spear and Roizman, 1972) and are translocated to the nucleus after the membrane fusion event. VP16 does not bind DNA directly, and must form a complex with a host cellular factor designated C1 or HCF, and the cellular transcription factor Oct-1 of the octamer family (McKnight *et al*, 1987). This VP16-containing complex binds to VP16-responsive sequence elements (5′-GyATGnTAATGArATT-3′) in α gene promoters (Mackem and Roizman, 1982c). This multiprotein-DNA interaction facilitates the recruitment of cellular RNA polymerase II transcription machinery to α gene promoters and culminates in high expression of α proteins 2–4 h post-infection (PI). The α gene products include infected cell polypeptide 0 (ICP0), ICP4, ICP22, ICP27 and ICP47 (Roizman and Sears, 1996).

The activation and inhibition of α gene transcription has been suggested to play an important role in determining the outcome of virus infection. In neurons, HSV-1 does not replicate well, and reduced expression of α genes in these cells has been implicated as the principle reason for the impairment of replication (Wheatley *et al*, 1990). A model developed from *in vitro* studies in cultured neurons suggests that neurons possess octamer proteins not found in permissive cells that inhibit the formation of the Oct-1/HCF/VP16 complex on TAATGARAT sequence elements in α gene promoters (Kemp *et al*, 1990; Lillycrop *et al*, 1991).

Although the TAATGARAT element may play a crucial role in activating α genes in permissive cells, the α promoters also contain binding sites for cellular transcription factors including binding sites for Sp1 and CAAT-enhancer binding protein (C/EBP). Sp1 has been shown to upregulate α gene expression (Jones *et al*, 1986), and C/EBP in combination with a factor termed F2, binds a site in the ICP0 promoter and activates ICP0 expression (O'Rourke and O'Hare, 1993). In permissive cells, the function of these cellular factors is mainly constitutive, and their transcriptional activity is expected to augment the strong activation associated with VP16. In addition to Sp1 and C/EBP sites, the ICP0 promoter also possesses an element that shows homology to a cAMP response element (CRE, Wheatley *et al*, 1992). However, the cAMP inducibility of the ICP0 promoter is controversial (Davido and Leib, 1998; Wheatley *et al*, 1992). Due to inhibition of VP16 activity in neurons, activation of HSV-1 genes by cellular transcription factors may be more crucial than in permissive cells. In addition, VP16 mRNA was not detected until after several other viral genes were expressed in reactivating explants of mouse trigeminal ganglia (Tal-Singer *et al*, 1997), and expression of VP16 does not induce reactivation from latency (Sears *et al*, 1991). Therefore, in reactivating neurons, VP16 is not likely to play its well-known role in permissive cells where its presence is critical to efficiently initiate the first steps of the viral replication cascade.

The transactivating function of ICP0 has been shown to play a significant role in the *de novo* synthesis of virus particles produced by transfecting viral DNA in cells (Cai and Schaffer, 1989). This is believed to occur through ICP0 function in activating α, β and γ gene classes (Cai and Schaffer, 1992). ICP0 has been shown to increase replication

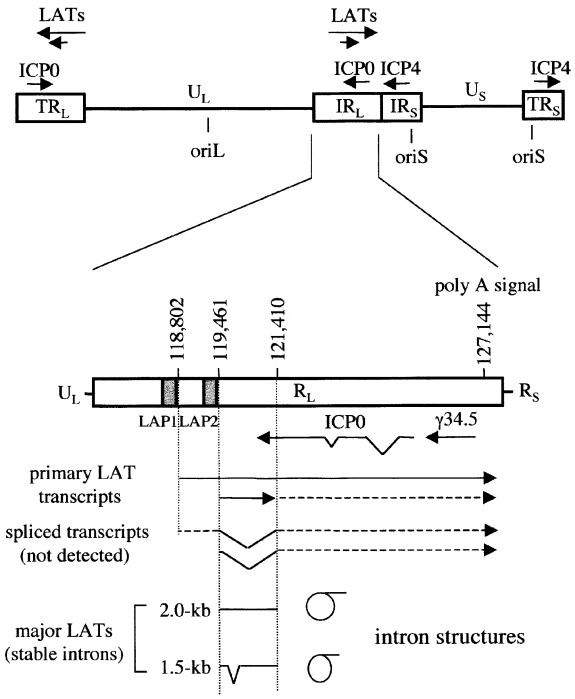

Figure 1 HSV-1 genome architecture and physical characterization of latency-associated transcripts. The positions of the terminal repeat long (TR_L), unique long (U_L), internal repeat long (IR_L), internal repeat short (IR_S), unique short (U_S), and terminal repeat short (TR_S) are shown. The location and orientations of LAT, ICP0, and ICP4 coding sequences are shown as arrows above the genome. The locations of viral origins of replication (ORI_L and ORI_S) are also shown. A close-up view of the R_L region of the genome that codes for LATs is represented at bottom with relevant nucleotide positions marked (Perry and McGeoch, 1988). The approximate locations of the major LAT promoters (LAP1 and LAP2) are shown as gray boxes. The poly A signal used in production of the 8.3 kg primary LAT transcript is also shown. The positions and direction of transcription of the latency-associated transcripts and the ICP0 transcript are depicted by arrows. Dotted lines indicate putative RNAs that have not been detected. The lariat-shaped intron structures of the 2 kb and 1.5 kb LATs are shown.

during acute infection and during reactivation from latency *in vivo* (Cai *et al*, 1993). These properties of ICP0 are consistent with the demonstration that ICP0 facilitates reactivation from latency (Cai *et al*, 1993; Leib *et al*, 1989b).

In addition to expression of viral genes, production of virus particles also requires replication of viral DNA. Viral DNA replication is concurrent with expression of early genes and involves the use of three viral origins of replication, oriL and two identical copies of oriS (Figure 1). These elements bind viral-encoded origin binding proteins (OBP and OBPC) and cellular factors (Baradaran *et al*, 1994; Dabrowski *et al*, 1994; Dabrowski and Schaffer, 1991; Hardwicke and Schaffer, 1995; Nguyen-Huynh and Schaffer, 1998). Although oriL and oriS possess substantial sequence similarity, they are structurally different and are believed to play different roles *in vivo*. For instance, it has been demonstrated that nerve growth factor (NGF) differentiation of PC12 cells inhibits oriS-dependent replication, but has relatively no effect on oriL-dependent replication, suggesting oriL may play a unique role in differentiated neurons (Hardwicke and Schaffer, 1997). In addition, it has been demonstrated that the synthetic glucocorticoid dexamethasone (DEX) can inhibit oriS function and enhance oriL function in NGF-differentiated PC12 cells by a mechanism that requires the integrity of a glucocorticoid response element (GRE) in oriL (Hardwicke and Schaffer, 1997). Because glucocorticoids are mediators of immune suppression and HSV reactivation *in vivo* (Blondeau *et al*, 1993; Padgett *et al*, 1998; McEwen *et al*, 1997), it may be beneficial for the virus to utilize this signaling pathway to boost viral DNA replication in the ganglia at a time that coincides with periods of compromised immune function (Hardwicke and Schaffer, 1997).

Establishment of latency

Although extraneural sites such as the cornea have been suggested to harbor latent HSV-1, most studies suggest that sensory neurons of the trigeminal ganglia constitute the major reservoir of latent HSV-1 that causes recurrent disease (Cook *et al*, 1974; Stevens *et al*, 1972). In order to establish latency in the trigeminal ganglion, HSV-1 enters neurons at nerve endings that innervate the area of primary infection. Viral glycoproteins and a host cell molecule similar to heparin sulfate are believed to mediate virion entry (WuDunn and Spear, 1989). After entry, HSV-1 is shuttled to the cell body by intra-axonal retrograde transport along microtubules (Cook *et al*, 1974; Kristensson *et al*, 1986; Lycke *et al*, 1988; Openshaw *et al*, 1978; Topp *et al*, 1994). Restricted viral replication can occur within the ganglia during the first few days after infection but within 7–10 days viral titers drop significantly (Leib *et al*, 1989a; Sawtell and Thompson, 1992a). Interestingly, during acute ganglionic infection, experimental evidence suggests that two different populations of neurons may become infected by HSV-1: one population in which the LAT promoter was active in the absence of HSV-1 antigenic expression, and the other in which HSV-1 antigenic expression was readily detectable in the absence of detectable LAT promoter activity (Margolis *et al*, 1992). These relatively distinct neuronal populations were further differentiated from each other by expression of the cellular antigen SSEA-3 (Margolis *et al*, 1992). These studies suggested that at any given point in time the LAT promoter was active in only a subset of neurons. It was suggested that the neuron plays a major role in determining the outcome of infection.

During the establishment and maintenance of latency, neurons are not casual bystanders to the infection. Following infection with HSV-2 in the mouse footpad, neurons of the dorsal root ganglia have been shown to undergo metabolic and structural changes indicative of neurite regeneration starting at 2 weeks and sustained at 1 month after infection (Henken *et al*, 1995). Similarly, expression of GAP-43, a neuronal protein involved in axon regeneration has been demonstrated to be induced in the trigeminal ganglion following corneal inoculation with HSV-1 (Martin *et al*, 1996). Neuronal transcription patterns are therefore likely to change significantly following acute infection with either HSV-1 or HSV-2. In another study, mice were infected by corneal scarification, and the trigeminal ganglia were examined for HSV-1 protein expression and inflammatory infiltration at various times after infection (Liu *et al*, 1996). From day 3 PI to day 7 PI, HSV-1 protein expression was reduced to undetectable levels. Also during this time, there was an increase in immune cells expressing γ interferon and interleukin-4, suggesting the possible involvement of these cytokines in the establishment of viral latency. Cytokines and/or other signaling molecules may ultimately guide or regulate the establishment of latency within the infected neuron by altering the course of HSV-1 gene expression and replication.

Maintenance of latency

Once latency is established, viral gene expression becomes severely restricted and viral replication ceases. The only substantial transcription occurs within the two LAT domains of the long repeat regions. The genome exists predominantly in an episomal form within the nuclei of neurons (Mellerick and Fraser, 1987), and the ends of the virion genomic DNA are covalently joined (Efstathiou *et al*, 1986; Rock and Fraser, 1985). A study utilizing the rabbit ocular model has shown that the amount of HSV-1 DNA as well as the number of LAT-expressing cells in the ganglia do not significantly change between 20 and 360 days after infection (Hill *et al*, 1996a). Spontaneous reactivation was also observed during this period, and these events did not alter either the quantity of DNA or number of LAT-expressing cells. The trigeminal ganglion apparently maintains a highly stable reservoir of HSV-1 DNA. Although LAT expression assayed by *in situ* hybridization is often used as a measure of the establishment of latency, *in situ* polymerase chain reaction (PCR) techniques have since shown that the number of neurons containing HSV-1 DNA is 2–3-fold greater then the number of LAT-expressing cells quantified by *in situ* hybridization (Mehta *et al*, 1995).

Quantitative analysis of viral nucleic acid during latency has more recently been examined at the single cell level using a method referred to as contextual analysis (CXA; Sawtell, 1997). This technique involves fixation of latently infected ganglia, dissociation and purification of single cells, and PCR-based quantification of viral DNA in individual cells. Although HSV-1 DNA was observed in nonneuronal murine cells prior to day 10 PI by this technique, the vast majority of DNA was restricted to neurons after this point (Sawtell, 1997). Surprisingly, the viral genome copy number within individual neurons ranged over three orders of magnitude from <10 to >1000. The ability of HSV-1 to establish large reservoirs of viral DNA in a high percentage of neurons is an important characteristic that can determine the frequency of reactivation (Sawtell, 1998; Sawtell *et al*, 1998).

Reactivation from latency

In humans, physical and psychological stress are frequently associated with reactivation of latent virus and recurrent herpetic disease (Schmidt *et al*, 1985; Young *et al*, 1976). In animal models of latency, physical stress such as neurectomy (Walz *et al*, 1974), application of chemical stimuli to the skin (Harbour *et al*, 1983), UV irradiation (Spurney and Rosenthal, 1972), mild trauma (Hill *et al*, 1978), ocular iontophoresis of epinephrine (Hill *et al*, 1983), and transient hyperthermia (Sawtell and Thompson, 1992b) have been shown to reactivate latent HSV. Psychological stress induced by disruption of the social hierarchy within colonies of mice has also been demonstrated to cause reactivation of latent HSV infection (Padgett *et al*, 1998). In rabbits, reactivation often occurs spontaneously (Kocisova *et al*, 1985). Although some of the above stressors have been studied at the physiologic and/or molecular level, it is uncertain which of the characterized responses are directly involved in signaling pathways leading to reactivation of latent HSV-1 infection.

In the rabbit ocular model, virus particles have been observed by electron microscopy in unmyelinated axons following epinephrine iontophoresis-induced reactivation of latent virus infection (Rivera *et al*, 1988). The transported particles were nonenveloped and were closely associated with microtubules. In an *in vitro* model utilizing cultured dorsal root ganglia (DRG) cells in a dual-chamber tissue culture system, nucleocapsids have also been observed to be transported by axonal transport mechanisms at 2–3 mm/h and viral glycoproteins were observed to be transported separately to the termini of the axons (Penfold *et al*, 1994). This evidence is consistent with the model that nucleocapsids are assembled within the neuron nucleus prior to anterograde axonal transport, and that final envelopment occurs near the axon termini.

Viral replication during reactivation from latency is likely to be different than replication initiated by direct inoculation of permissive cells with infectious particles, since initiation of replication in

permissive cells *in vitro* involves the virion-associated protein VP16. VP16 is not present in latently infected cells, nor would it be expected to be synthesized until late in the replication cascade since it is a member of the late gene family. Initiation of HSV-1 DNA replication (Nichol *et al*, 1996) and expression of HSV-1 early genes (Tal-Singer *et al*, 1997) have been demonstrated to constitute the first events leading to viral replication in reactivating neurons. This evidence suggests that inducible neuronal transcription factors may substitute for VP16 and/or HSV-1α proteins such as ICP0 at the onset of reactivation. Cellular immediate-early transcripts for *oct-1*, c-*jun*, c-*fos*, and c-*myc* have been shown to be induced in explanted trigeminal ganglia by 1–4 h post-explant (Tal-Singer *et al*, 1997). Since the first viral transcripts were not detected until 4–8 h post-explant, the kinetics of expression of these inducible cellular transcriptional regulators is consistent with a role for them in stimulating viral gene expression in explanted latently infected ganglia.

Latency-associated transcripts

Discovery of LATs

One of the first major discoveries relevant to the molecular aspects of latency was the observation that the viral genome was not entirely quiescent during latency. A family of viral transcripts termed latency-associated transcripts (LATs) were detected in a subpopulation of sensory neurons during the latency phase by *in situ* hybridization of latently infected murine ganglia using subgenomic and single-stranded probes and by Northern blot hybridization analyses (Stevens *et al*, 1987). It has also been demonstrated that LATs are expressed at levels similar to that found in mice in sensory neurons of rabbits and humans latently infected with HSV-1 (Croen *et al*, 1987; Rock *et al*, 1987; Stevens *et al*, 1987). By *in situ* hybridization, LATs have been observed in approximately 3% of the neurons in the ganglion (Croen *et al*, 1987; Rock *et al*, 1987). Although more sensitive PCR techniques have since shown low levels of expression of other viral genes including ICP4 and thymidine kinase (Kramer and Coen, 1995), the unusually high levels of LAT expression were striking and stimulated a period of intense research involving the role of LATs in the processes of latency and reactivation.

Physical properties of LATs

Since the reported discovery of LATs in 1987, a significant body of literature concerning the physical characteristics of the transcripts as well as their patterns of expression has been accumulated (Figure 1). The LATs are a family of at least three co-linear transcripts of sizes 8.3, 2.0, and 1.5 kb (Rock *et al*, 1987; Spivack and Fraser, 1987; Zwaagstra *et al*, 1990). Of the three LAT RNA species, only the 8.3 kb transcript appears to be polyadenylated (Dobson *et al*, 1989; Zwaagstra *et al*, 1990). The 8.3 kb species is usually referred to as the minor LAT because it is detected at very low levels compared to the 2.0 and 1.5 kb LATs.

It has long been suggested that the smaller LATs are stable introns formed during splicing reactions (Devi-Rao *et al*, 1991; Farrell *et al*, 1991; Krause *et al*, 1990; Wagner *et al*, 1988): the 2.0 kb LAT from the 8.3 kb primary transcript and the 1.5 kb LAT from the 2.0 kb LAT (Figure 1). Recent evidence reported by several groups has quite clearly established the validity of this hypothesis, and the structures of the small LATs are the expected lariat shape of group II spliced introns (Wu *et al*, 1996; Zabolotny *et al*, 1997). However, the LATs are highly unusual introns because a typical intron is degraded rapidly, and the LATs are very stable. Their stability is thought to be a result of an unusual splice junction that is not recognized by cellular degradation factors (Zabolotny *et al*, 1997).

Another unusual characteristic of LAT synthesis is the absence of a detectable ~6.3 kb spliced exon-containing transcript (LATex) that should be formed during the splicing reaction generating the 2.0 kb LAT intron (Figure 1). In an *in vitro* model used to study the splicing of LATs, a truncated LATex has been detected following transfection of CV-1 cells with a LAT minigene-containing eukaryotic expression vector (Zabolotny *et al*, 1997). In this study, the LATex was a cytoplasmic 1.4 kb transcript produced by excision of a 2.0 kb LAT intron from a 3.4 kb primary transcript. It is entirely possible that this truncated LATex is more stable than the 6.3 kb LATex.

Expression of LATs

The 2 kb LAT can be expressed in both latently infected cells and acutely infected cells including nonneuronal cell types (Spivack and Fraser, 1987; Wagner *et al*, 1988). However, the 1.5 kb LAT is only detected in cell populations that support latency. LAT synthesis increases in the infected sensory neurons during the establishment of latency when other HSV-1 genes are silenced and continues throughout latency (Spivack and Fraser, 1988). This characteristic differentiates the regulation of these transcripts from the major HSV-1 gene classes. Expression of the major LATs is primarily nuclear, although a fraction of molecules may be present within the cytoplasm (Goldenberg *et al*, 1997; 1998; Nicosia *et al*, 1994; Zabolotny *et al*, 1997).

Surprisingly, many HSV-1 genome-containing neurons do not express LATs at appreciable levels during latency. Although the reason for this observation remains uncertain, since the number of HSV-1 genomes occupying a particular cell is highly variable (Sawtell, 1997), an attractive hypothesis is that the genome copy number per cell

may impact the accumulation of LATs. Another reasonable hypothesis is based on studies by Margolis and coworkers (1992) that suggested the pathway of neuronal infection characterized by transcription of abundant latency-associated transcripts and minimal viral protein synthesis was much more likely to occur in DRG neurons expressing the cellular antigen SSEA-3. Therefore, expression of LATs may be regulated differently in specific neuronal subtypes. In addition, in an *in vitro* model of latency, it has been demonstrated that the kinetics of LAT expression in HSV-1-infected sensory neuronal cultures is highly sensitive to culture conditions that drive the infection down a latent or lytic pathway (Smith *et al*, 1994). Under culture conditions that support lytic infection, LATs and HSV-1 antigens were observed in the majority of neurons by 24 h PI, whereas under conditions that drive the infection toward latency, the percentage of LAT-positive neurons increased from ~10% to nearly 80% between days 7 and 21 PI (Smith *et al*, 1994). Therefore, it is possible that differences in cellular physiology may impact expression of LATs in neurons.

LAT expression and/or stability may also be modulated during viral reactivation. In an *in vitro* model of latency, reactivation induced by heat shock was associated with increased expression of minor LAT between 12 and 38 h after heat shock (Danaher *et al*, 1999). Although this result could be due to increased stability of the minor LAT, a more likely explanation is that the LAT promoter was activated with kinetics similar to cellular delayed-response genes. It remains uncertain if this observation is a general phenomenon of reactivation or if it is specific to *in vitro* reactivation induced by heat shock. Expression of major LAT has been reported not to change for up to 96 h during reactivation induced by explant coculture of murine trigeminal ganglia (Tal-Singer *et al*, 1997). However, this lack of LAT response may be attributed to the high stability of the major LAT compared to the minor LAT or differences between the two reactivation models. Interestingly, the kinetics of minor LAT expression during reactivation as reported by Danaher and coworkers (1999) is similar to the kinetics of LAT expression observed during initiation of lytic infection in neurons reported by Smith and coworkers (1994). This evidence suggests that activation of the LAT promoter during reactivation may be very similar to LAT promoter activation that occurs in neurons supporting lytic replication early in the primary infection of sensory ganglia.

LAT promoters

The promoter that initiates LAT transcription was mapped to a region 660 bp upstream of the major 2.0 kb LAT (Figure 2; Zwaagstra *et al*, 1989, 1990). This promoter, now designated latency-active promoter 1 (LAP1), contains numerous

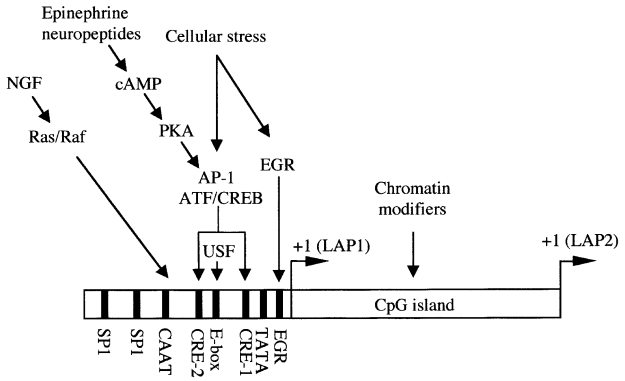

Figure 2 Promoter structures of LAP1 and LAP2 and potential signaling pathways that may regulated promoter activity. Nerve growth factor (NGF) signaling through the Ras/Raf pathway has been shown to operate through LAP1 by unknown factors that interact with LAP1 (Frazier *et al*, 1996a, b). The cAMP/protein kinase A (PKA)/CREB pathway has been reported to operate through the CRE-1 element (Ackland-Berglund *et al*, 1995; Leib *et al*, 1991). A cellular stress pathway is predicted to operate through an EGR binding site (Tatarowicz *et al*, 1997), as well as the CRE-1 and CRE-2 elements (Millhouse *et al*, 1998). Transcriptional regulation by the CpG island region has been suggested to occur through chromatin modifying mechanisms (Bloom *et al*, 1996). Regulation of LAP2 by cellular signaling pathways is unclear.

binding sites for RNAP II transcriptional regulators, including a TATA box, Sp1 sites, and a CAAT element (Figure 2; Wechsler *et al*, 1989). Primer extension analyses demonstrated that transcription initiation mapped to a location close to 28 nucleotides downstream of the first T of the TATA box (Zwaagstra *et al*, 1990). The distance between this position and a putative polyadenylation signal was consistent with the observed size of the ~8.3 kb LAT transcript (Zwaagstra *et al*, 1990). The region of LAP1 located in close proximity to the TATA box has been demonstrated to be critical for expression of LATs during latent infection (Nicosia *et al*, 1993), and mutation of the TATA box strongly reduces LAT expression in neurons *in vivo* (Rader *et al*, 1993).

Numerous studies have suggested that LAP1 contains *cis*-acting elements necessary for efficient transcription in cells of neuronal origin. When mouse neuronal cells (C1300) were compared to mouse nonneuronal cells (L929), neuron specificity in transient transfection was mapped to nucleotides −161 to −613 (Zwaagstra *et al*, 1990, 1991), −212 to at least −348 (Kenny *et al*, 1994), and −75 to −83 (Kenny *et al*, 1994). In human neuroblastoma cells (IMR-32), nucleotides −143 to −158 and −177 to −251 have been implicated in neuronal specificity (Batchelor and O'Hare, 1992). *In vivo* deletion analysis also implicated promoter sequences upstream of −250 as playing an important role in stimulating transcription within HSV-1-infected ganglia 4 days PI (Dobson *et al*, 1995).

Studies have also centered on the sequences between LAP1 and the 2 kb splice junction, a region 660 bp in length. In 1993, it was reported that a LAP1 deleted virus was capable of expressing the 2 kb LAT during lytic infection in culture and during reactivation, but not during latency (Nicosia *et al*, 1993). This evidence implied the presence of a second promoter that is sufficient to drive transcription through the 2 kb region under certain conditions. Goins and coworkers (1994) have mapped this promoter sequence to between positions −257 and −58 relative to the 5′ end of the 2 kb LAT sequence and designated it latency active promoter 2 (LAP2; Figures 1 and 2). Although no TATA element was found, initiation of transcription was determined to be close to the 5′ end of the 2 kb LAT and it was suggested that this promoter may function in a manner similar to TATA-less housekeeping gene promoters. In contrast to the report by Nicosia and coworkers (1993; 1994), additional studies reported LAP2 activity in neurons during latency, although LAP2 activity was much lower than LAP1 activity (Goins *et al*, 1994). However, more recent evidence suggests that LAP1 is the principle promoter during latency (Chen *et al*, 1995). LAP2, on the other hand, is primarily active during acute infection (Chen *et al*, 1995). LAP2 may function under conditions when LAP1 activity is inhibited. Possible inhibitors of LAP1 activity during acute infection are ICP4 (Farrell *et al*, 1994) and cellular transcription factors of the early growth response (EGR) family (Tatarowicz *et al*, 1997). Since LAP2 function has only been assayed in the absence of LAP1, it may be important to note that it remains to be demonstrated whether LAP2 can function in the presence of LAP1.

Another group of investigators have examined the LAP2 region from a different perspective. Lokensgard *et al* (1994), have presented evidence that LAP1 (without LAP2), though capable of driving transient transcription in ganglionic neurons, was not sufficient to drive long-term expression of a reporter gene in ganglionic neurons. They suggested that a long-term expression element (LTE) must exist somewhere else in the genome. The LTE was shortly thereafter shown to reside somewhere within the LAP2 region (Lokensgard *et al*, 1997). Therefore, although both promoters may be able to function independently, it is possible that in the context of the viral genome, sequences within, or in close proximity to, each promoter may affect the activity of the other promoter.

Transcription factors interfacing with LP1

The first discrete *cis*-acting element characterized in LAP1 was the LAT promoter binding factor (LPBF) element (Zwaagstra *et al*, 1991), an element that is more generally referred to now as an E-box motif (Kenny *et al*, 1997). This element is located ~64 nucleotides upstream of the transcription start site (Figure 2). Mutation of this element was shown to strongly reduce LAT promoter activity *in vitro*, and *DNase*I protection assays demonstrated that this element was likely to be bound by a cellular factor. Evidence indicates that this factor in neuronal and non-neuronal cells includes members of the upstream stimulatory factor (USF) family (Kenny *et al*, 1997).

The USF proteins have been shown to antagonize cell proliferation (Luo and Sawadogo, 1996) and participate in the inducible expression of genes (Kahn, 1997). Although USF factors are ubiquitous (Sirito *et al*, 1994), it has recently been reported that a non-ubiquitous coactivator of USF is required for USF function in specific cell types (Qyang *et al*, 1999). USF has been implicated in regulating cdc2 expression in avian nerve cells during neuroretinal development (North *et al*, 1999). Also, USF-deficient mice are growth impaired and some mice exhibited spontaneous epileptic seizures, suggesting that USF is important in development and in normal brain function (Sirito *et al*, 1998). It has been suggested that USF factors may play an important role in stimulating LAT expression throughout latent infection (Kenny *et al*, 1997).

In addition to the E-box element, the LAT promoter contains two CRE-like elements termed CRE-1 and CRE-2 that are located 38 (CRE-1; Leib *et al* 1991) and 66 (CRE-2; Kenny *et al*, 1994) nucleotides upstream of the transcription initiation site (Figure 2). Similar elements in other promoters frequently interact with the b-Zip family of transcription factors, which includes the two subfamilies AP-1 (Jun/Fos) and ATF/CREB, distinguished mostly by the mechanism of their regulation as well as by small but significant differences in DNA binding sequence specificity. Family members CREB-1, ATF-1 and CREM comprise a distinct ATF/CREB subfamily because they contain consensus cAMP-dependent protein kinase (PKA) phosphorylation sites and are regulated by cAMP *in vivo* (Montminy, 1997). The AP-1 family, on the other hand, is more involved in transmitting a variety of cellular stress signals in the cell nucleus. One of the LAP1 CRE elements (designated here as CRE-1) was characterized based on its ability to confer cAMP inducibility to LAP1 (Leib *et al*, 1991), and consistent with this observation, DNA binding studies have shown that this element recruits the cAMP inducible transcription factor CREB-1 (Leib *et al*, 1991; Millhouse *et al*, 1998). Interestingly, this element also binds AP-1 family members, suggesting that the regulatory properties of this element may involve signaling pathways other than cAMP (Millhouse *et al*, 1998). The CRE-2 element was discovered based on its functional involvement in stimulating LAP1 activity in C1300 murine neuroblastoma cells, and this element has been shown to bind members of both ATF/CREB and AP-1 factors

(Kenny *et al*, 1994; Millhouse *et al*, 1998). Evidence in transiently transfected murine neuroblastoma cells suggests that endogenous AP-1 factors contribute to LAP1 activity (Millhouse and Wigdahl, 1999, unpublished results).

In addition to ATF/CREB, AP-1, and USF, some additional cellular factors have been implicated in regulating LAP1 activity. One of these is the EGR family of transcription factors. These are inducible cellular transcription factors that have been shown to inhibit LAP1 activity *in vitro* by binding to an element immediately adjacent to and downstream of the LAP1 TATA box (Figure 2; Tatarowicz *et al*, 1997). A variety of stimuli, including cellular stress, induce EGR mRNA expression in the absence of *de novo* protein synthesis, making these genes members of the cellular immediate-early gene family (Kendall *et al*, 1994). DNA binding studies conducted with purified TBP and EGR-1 suggest that EGR proteins may inhibit the interaction between TBP and the LAP1 TATA box (Tatarowicz *et al*, 1997). Studies have not yet been reported that demonstrate the expression of EGR factors during the course of HSV infection.

With respect to neuron-specific promoter activity, a DNA-binding factor designated IC-1 was shown to be selectively present in neuroblastoma but not HeLa cells and bind to a region in LAP1 associated with neuron-specific promoter activity (Batchelor and O'Hare, 1992). However, further characterization of this factor has not been reported. Several other cellular factors have been suggested to regulate LAT transcription based on sequence analyses, including Sp1 (Hill *et al*, 1996b; Wechsler *et al*, 1989), AP2 (Hill *et al*, 1996b), and C/EBP (Kriesel *et al*, 1997b).

Signaling pathways

Although recurrence of herpetic disease may involve mechanisms that facilitate replication at the peripheral site, a prerequisite to this event is that the latent viral genome must become transcriptionally active. Therefore, in order to understand HSV-1 reactivation, we must learn how the various environmental reactivation signals are transduced to the latent HSV genome. Some of the signaling molecules implicated thus far include epinephrine, nerve growth factor (NGF), and interleukin-6 (IL-6). Introduction of anti-NGF antibody into eyes of latently infected rabbits has been shown to induce HSV-1 reactivation, suggesting that withdrawal of this neuroprotective factor induces a reactivation signal (Hill *et al*, 1997). In contrast, application of anti-IL-6 antibodies to the eyes of latently infected mice inhibited reactivation induced by UV light and hyperthermia (Kriesel *et al*, 1997a), and hyperthermic stress has been shown to elicit a transient rise in IL-6 mRNA in the trigeminal ganglia. Thus, IL-6 may be involved in stimulating reactivation induced by these mechanisms. Epinephrine is of interest because it is a hormone released by the adrenal gland following a variety of psychological stressors. Systemic treatment with propranolol, a receptor antagonist, significantly decreased hyperthermic stress-induced reactivation in mice (Kaufman *et al*, 1996), and administration of epinephrine into the eyes of latently infected animals by iontophoresis induces reactivation (Hill *et al*, 1983). Therefore, epinephrine may play an important role in the HSV life-cycle by inducing reactivation following a variety of stressful events that result in epinephrine release.

It is possible that viral reactivation measured in previous studies is due to direct interaction of ligands with receptors located on the surface of latently infected neurons. Receptors for these molecules have been shown to send a variety of signals to the cell nucleus. Epinephrine binds β-adrenergic receptors on the surface of cells, and this interaction results in elevation of intracellular cAMP and activation of the transcription factor CREB (for a review, see Montminy, 1997). IL-6 can also stimulate transcription through activation of the transcription factor NF-IL6 (also designated C/EBP-β; for a review see Akira *et al*, 1990). NGF can activate CREB (Ginty *et al*, 1994; Riccio *et al*, 1997), but it can also activate other transcription factors including those of the EGR family (Kendall *et al*, 1994). It is logical that these and other inducible cellular transcription factors may play a pivotal role in transducing reactivation signals to the latent HSV-1 genomic regulatory elements. Within the HSV-1 genome, several DNA binding sites have been proposed and characterized for transcription factors such as these (Deb *et al*, 1994; Kenny *et al*, 1994; Kriesel *et al*, 1997b; Leib *et al*, 1991; Tatarowicz *et al*, 1997).

In 1990, observations were reported that demonstrated the presence of a functional cAMP response element (CRE) located $\sim$11 nt upstream of the TATA box in LAP1 (Figure 2; Leib *et al*, 1991). In these studies, treatment of PC12 cells with 1 mM dibutyryl cAMP (a membrane permeable cAMP analog), forskolin (an activator of adenylate cyclase), NGF, or phorbol myristic acetate (PMA) induced a transiently transfected LAP1 construct 1.5–2.9-fold depending on the agent used. Later studies demonstrated that LAT promoter induction by dibutyryl cAMP could be blocked by coexpression of a PKA inhibitor designated PKI (Ackland-Berglund *et al*, 1995), suggesting that elevating intracellular cAMP levels in PC12 cells leads to activation of protein kinase A. This event is expected to cause the subsequent activation of CREB, which could be responsible for the induction of LAT promoter activity observed in *in vitro* experiments. These experimental results were deemed relevant to the viral cycle since a virus genome carrying a mutated LAP1 CRE-1 element exhibited reduced reactivation kinetics in ex-

planted ganglia (Rader *et al*, 1993). Also, more recently, a virus carrying a mutated LAP1 CRE-1 element was significantly restricted in its ability to reactivate from latently infected rabbits, either spontaneously or following ocular iontophoresis of epinephrine (Bloom *et al*, 1997). These observations were exciting because they linked LAT expression with signal transduction pathways that are associated with virus reactivation.

The potential ramificiations of AP-1 interaction with LAP1 CREs are numerous. Most importantly, it expands the potential repertoire of signals that LAP1 may respond to during virus reactivation from latency. AP-1 activation occurs in response to a wide variety of stress-related stimuli, many of which correlate with HSV-1 reactivation. Activation of AP-1 factors occurs in a variety of cell types including neurons following several different types of stress signals, and involves the upregulation of Jun and Fos mRNA and protein, as well as phosphorylation of proteins by c-Jun N-terminal kinase (JNK; Herdegen *et al*, 1997; Morgan and Curran, 1989). Activation of AP-1 is believed to play a pivotal role in regulating neuronal responses to injury or insult. Environmental signals associated with neuronal AP-1 activation include ionizing radiation (Ferrer *et al*, 1995) and nerve transection (Hull and Bahr, 1994; Jenkins *et al*, 1993; Leah *et al*, 1993; Tetzlaff *et al*, 1994). Also, HSV-1 lytic infection has been demonstrated to activate AP-1 in nonneuronal cells (Jang *et al*, 1991; Zachos *et al*, 1999). Expression of GAP-43, a protein involved in axon regeneration, is regulated in parallel with c-Jun following neuronal damage (Bisby *et al*, 1995; Schreyer and Skene, 1993; Tetzlaff *et al*, 1994), and is induced in neurons during HSV-1 and HSV-2 infection of ganglia (Henken *et al*, 1995; Martin *et al*, 1996). Therefore, activation of AP-1 is likely to occur within infected ganglia due to stress of infection and following insults that are associated with HSV-1 reactivation. AP-1 factors may therefore play a modulatory role in directing both establishment and reactivation of HSV latent infection.

Another signal transduction pathway investigated with respect to LAP1 is that which is activated by treating PC12 cells with NGF and/or sodium butyrate (NaB; Frazier *et al*, 1996a). In these studies, 8–12-fold induction of LAP1 activity was observed with NGF or NaB alone, and 40–60-fold when the two agents were used in combination. Induction of α, β and γ promoters was also observed with NGF treatment although the level of activation was only 2–4-fold. LAP1 sequences involved in NGF induction have been mapped to the region between -159 and -81, although no known transcription factors were identified in mediating the NGF signal to this region of the promoter (Frazier *et al*, 1996b). The signaling pathway involved in LAP1 induction by NGF was demonstrated to follow a characterized receptor tyrosine kinase pathway (for a review, see Marshall, 1995). This pathway involves the GTP-binding protein, Ras, and the serine/threonine kinase, Raf, which activate downstream kinases to regulate gene transcription (Frazier *et al*, 1996a). Overall, this evidence suggests that LAP1 is responsive to Ras/Raf-mediated signals, and that extracellular molecules, such as NGF, that utilize this pathway may play a role in modulating LAP1 activity.

In vivo models and examination of LAT function

Overview of experimental approach

A multitude of HSV-1 mutants have been generated for the purpose of characterizing the involvement of LATs in the processes of establishment, maintenance and reactivation of latent infection in animal model systems. In these experiments, HSV-1 mutants that contain genetic alterations within the LAT locus are compared to unmodified or marker rescued viruses for their ability to establish latency and/or reactivate from latency. Establishment of latency is frequently characterized by assaying the percentage of neurons harboring HSV-1 DNA and/or the total amount of HSV-1 DNA contained within a ganglion at a point in time when latency should be established. Methods for assaying viral reactivation are more numerous and more complex. This can be accomplished by examining the kinetics and frequency of reactivation *ex vivo*, by explanting latently infected ganglia onto permissive cells, and assaying for infectious virus production (explant cocultivation assay). Alternatively, *in vivo* reactivation can be assessed utilizing ocular models. In this experimental approach, infectious particles can be assayed from tear film of animals infected through the ocular route.

Typically, in addition to assaying for specific phenotypes by one of the methods described above, HSV-1 mutants are tested for their ability to synthesize the major LATs (2.0- and 1.5-kb species) in trigeminal neurons during latency. As a result, many HSV-1 LAT mutants are designated as being either LAT positive (major LATs are abundantly expressed during latency) or LAT negative (major LATs are not detected during latency). Unfortunately, the significance of the LAT-expressing status is not yet clear since it is uncertain whether neurons that support viral reactivation are those that express LATs during latency. As previously stated, LAT expressing neurons comprise only a subset of the total pool of latently infected neurons in sensory ganglia.

Initial studies have clearly demonstrated that LAT expression is not necessary for HSV-1 to either establish latency or reactivate from latency (Ho and Mocarski, 1989; Javier *et al*, 1988; Steiner *et al*, 1989). However, more detailed studies revealed that LAT negative viral constructs are impaired in their

ability to reactivate, but are normal with respect to replication properties associated with acute infection. Nevertheless, it has been difficult to generate a cohesive model of LAT function from these experiments for one very good reason: the effector molecule(s) has not been clearly defined. This molecule(s) may be one or both of the major LATs, or a corresponding protein product, or it may involve synthesis of another less abundant transcript coded within this region not yet characterized. Because of this fact, when HSV-1 LAT domain mutants are examined for changes in phenotype, it is not known for certain if the mutation is altering the amino acid sequence of a protein coded within the domain, or if a functional RNA is modified, or if a transcriptional or translational regulatory mechanism is being modified, or some combination of the three possibilities. Another difficulty involves evidence that viral strains display varying sensitivities to genetic alterations within the LAT domain (Loutsch *et al*, 1999). The following discussion is aimed at highlighting selected *in vivo* studies that represent several aspects of current discussion concerning LATs and their involvement in viral latency.

LATs involvement in viral reactivation

In terms of the LAT promoter, deletion of a ~200 nt region of LAP1 (strain 17ΔPst; Figure 3) containing the TATA box, CREs, USF, and transcription start site, resulted in a strong reduction in reactivation frequency in both mouse (Devi-Rao *et al*, 1994) and rabbit (Bloom *et al*, 1994) model systems. This virus did not express LATs during latency and was impaired in explant coculture reactivation in mice as well as ocular recovery in the rabbit model. This evidence strongly suggested that transcription initiation at LAP1, and the expression of the major LATs, was involved in regulating HSV-1 reactivation.

In vivo analysis of LAP1 has also been conducted at a more precise level. Mutation or deletion of the LAP1 TATA box reduces LAT accumulation during latency (Rader *et al*, 1993). This observation could be expected, given that LAP1 has been shown to be the critical promoter during this period of time. Of interest with respect to potential signaling pathways involved in regulating LAT expression is a virus that was constructed containing a mutated CRE-1 element (Bloom *et al*, 1997). This virus was shown to be significantly impaired in its ability to reactivate either spontaneously or by induction using epinephrine iontophoresis in a rabbit ocular model. Interestingly, ganglia latently infected with this virus exhibited wild-type levels of LAT expression during the latent state, making this virus LAT-positive. This evidence suggested that an uncharacterized LAT domain-associated transcriptional process, activated by CRE-1-binding cellular factors, AP-1 and ATF/CREB, may be involved in

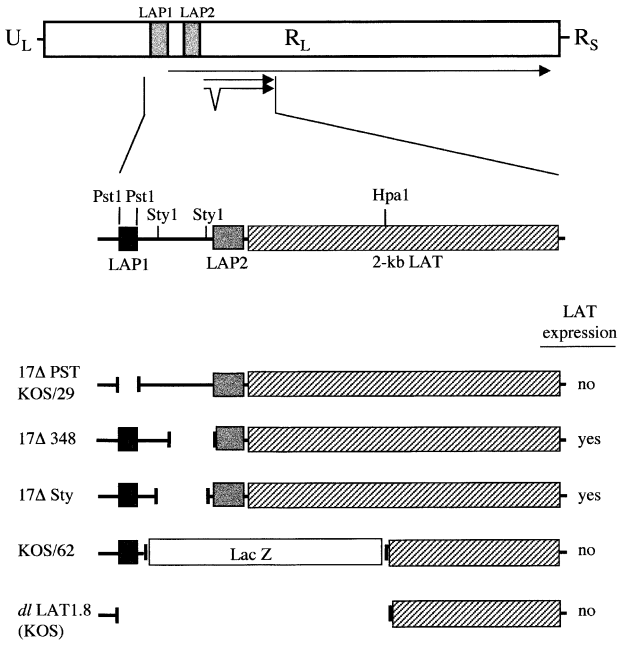

Figure 3 HSV-1 LAT mutants used in animal models of latency. The positions of structural features located within the LAT locus are shown at top. The LAT promoters (LAP1 and LAP2) are indicated by shaded boxes. The 2 kb LAT coding sequence is indicated by the hatched area. At bottom, the features of some genetically modified HSV-1 strains are shown. The name of the construct is indicated to the left and the 2 kb LAT expression phenotype (detected by *in situ* or Northern hybridization techniques during latent infection) is given to the right of the construct. Regions deleted in the virus are bounded by vertical lines, and insertion of a reporter gene (Lac Z) is shown in the one construct. References are cited in the text.

stimulating *in vivo* reactivation in latently infected rabbits. Although additional experimental approaches are required to ascertain the mechanism by which this element supports reactivation in the rabbit ocular model, it is possible that CRE-1 may be involved in relieving transcriptional inhibition caused by chromatin structure since both AP-1 (Bannister *et al*, 1995) and CREB (Chrivia *et al*, 1993) factors can recruit the transcriptional coactivator/histone acetylase, CBP/p300.

There exists additional evidence that an uncharacterized LAT domain-associated transcriptional process may regulate viral reactivation. This is based on data collected using two different recombinant viruses (17Δ348 and 17ΔSty; Figure 3) that contain similar deletions located downstream of LAP1 and in close proximity to LAP2. Both of these viral constructs were impaired in their ability to reactivate in the rabbit ocular model (Bloom *et al*, 1996; Hill *et al*, 1996b), and were LAT-positive. Since both of these deletions were located within the primary transcript coding region for the 8.3 kb minor LAT, it is possible that expression of the minor LAT in some cells may play a role in reactivation. Alternatively, the regions deleted in

17Δ348 and 17ΔSty may modulate expression of an unknown transcript, or possibly the major LATs in a small but critical subpopulation of cells. The DNA sequences within the regions deleted in 17ΔSty and 17Δ348 share homology to a classical CpG island (Bloom *et al*, 1996; Hill *et al*, 1996b). CpG islands are unusually rich in CpG dinucleotides and are believed to regulate the transcription of genes located in close proximity to these islands by facilitating nucleosomal positioning; a process that may be regulated by cytosine methylation (Patel *et al*, 1997). This may be highly relevant to HSV-1 latency, since HSV-1 DNA is associated with nucleosomes in a chromatin structure during latency but not during acute infection (Deshmane and Fraser, 1989). Therefore, it may be hypothesized that the LAT CpG island, in combination with LAP1 CREs, plays a role in regulating transcription within the LAT locus by modifying chromatin structures that form during latency. Additional studies are required to elucidate the role of the LAT CpG island in transcriptional regulation and reactivation from latency.

Although the phenotypes of 17Δ348 and 17ΔSty may suggest the existence of a functional molecule other than the major LATs, it should be pointed out that these viral constructs have not been shown to have reactivation defects in murine models of latency. This characteristic differentiates these HSV-1 mutants from 17ΔPst (LAT-negative), which exhibits reactivation defects in both rabbit and murine models of latency. This observation indicates that the LAT domain may possess two different functions that can modulate HSV-1 reactivation. As will be discussed next, a possible explanation is that one of these functions may participate in the establishment of latency, whereas the other may participate in events that are timed more closely with reactivation of latent virus.

LAT involvement in establishment of latency: lessons learned from the murine model

The frequent association of LAT-negative virus strains with impaired reactivation phenotypes does not rule out a functional role for LATs in the establishment of latent infection. By taking advantage of the very high sensitivity of PCR-based CXA in the mouse model, it has been demonstrated, quite convincingly, that two different LAT-negative strains that have reactivation defects (KOS/62 and KOS/29; Figure 3), also show a ~66% reduction in the establishment of latency as quantified by the percentage of neurons harboring latent genomes (Thompson and Sawtell, 1997). Interestingly, by increasing the input titer of a LAT-negative strain so that latency establishment was equal to a LAT-positive virus, the frequency of reactivation (scored by titering virus in the ganglia 22 h after transient hyperthermia) was equivalent between the LAT-positive and LAT-negative strains, suggesting that in this model, the reactivation defect of these LAT-negative strains is secondary to a LAT defect related to the establishment phase.

Results obtained in the rabbit model have been somewhat different than those obtained in the mouse model, since LAT-negative HSV-1 strains appear normal with respect to establishment, yet these strains still displayed reduced reactivation frequencies. This apparent conflict between the two animal models remains to be resolved, but some explanations appear reasonable: (1) it is possible that more sensitive techniques need to be utilized to assay establishment in the rabbit model, and (2) genetic differences between animals, and in some cases between viral strains, may negate or accentuate functions that occur at either the establishment phase or later during reactivation. As previously proposed, there may exist two LAT domain-encoded functions based on phenotype differences between 17ΔPst (LAT-negative and reactivation impaired in both murine and rabbit models) and 17ΔSty (LAT-positive and reactivation impaired in the rabbit model but not in the murine model). One might speculate that one function may be timed during the establishment of latent infection, and another function may be timed to operate during viral reactivation.

Models of LAT function

Inhibition of productive cycle gene expression

It has been proposed that LATs may function by repressing accumulation of productive cycle gene transcripts in sensory neurons. This model is based on evidence that a LAT-negative virus (*dl*LAT1.8; Figure 3) displayed increased accumulation of productive cycle gene transcripts during acute infection of murine sensory ganglia (Garber *et al*, 1997) and during latency (Chen *et al*, 1997), even though replication of *dl*LAT1.8 in tissue culture was no different than the LAT-positive rescuant virus. Although mechanisms underlying these observations are not yet known, gene silencing may occur through inhibition of specific viral mRNAs by antisense RNA interactions since LATs are antisense to ICP0 transcripts (Farrell *et al*, 1991; Garber *et al*, 1997; Stevens *et al*, 1987). Alternatively, gene silencing may occur at the genomic level by a mechanism similar to X chromosome silencing by XIST RNA (Bloom *et al*, 1996). Taken in context with murine latency establishment studies, this evidence is consistent with the hypothesis that LAT expression facilitates the establishment of latent infection within sensory neurons of murine trigeminal ganglion by restricting expression of viral productive cycle transcripts.

Inhibition of ICP0 translation

As previously discussed, ICP0 has been demonstrated to increase replication during acute infection and during viral reactivation *in vivo* (Cai *et al*, 1993). Therefore, regulated expression of this protein may play an important role in the molecular processes of latency. Although still speculative, one of the most appealing mechanisms that may explain the observed relationship between LATs and productive cycle transcripts is that LATs may hybridize with ICP0 transcripts *in vivo* and subsequently block their processing, transport, and/or translation. This could be advantageous to the establishment of latent infection by restricting viral replication in the ganglion which otherwise may activate host antiviral defenses. The LATs have been shown to reduce ICP0 mRNA levels in a neuronal cell line through a mechanism that does not involve the ICP0 promoter (Mador *et al*, 1998), and the expression of LATs *in vivo* increases in the trigeminal ganglia within the same time frame that viral titers drop. ICP0 has been shown to be an activator of viral replication when the multiplicity of infection is low, so it is very likely to play an important role within the neuronal environment. Although the intracellular environment of the neuron has been shown to inhibit HSV-1 α gene expression and viral replication, which alone may facilitate the establishment of latent infection, the ICP0 promoter may be activated at low but significant levels by cellular factors or through virion-associated proteins such as VP16.

Translation of a LAT-encoded protein

The LATs possess small to moderately sized open reading frames. It has been reported that an antiserum raised against a protein generated by *in vitro* transcription/translation of LAT templates could detect expression of the antigen in latently infected ganglia (Doerig *et al*, 1991). However, this observation has not been confirmed and it remains uncertain if the antiserum reactivity was specific to a protein coded by the LATs. Stronger evidence for translation of LAT proteins comes from studies involving characterization of polyribosomes (Goldenberg *et al*, 1997; 1998). Although circumstantial, this evidence indicated that the major LATs are associated with polyribosomes in trigeminal ganglia of latently infected mice, and to a much lesser degree, in brainstem. Although this evidence suggests that LATs can physically interact with the translational machinery in tissues that support reactivation of latent HSV-1 infection, it does not demonstrate that a protein was generated in the process.

Very recently, it was demonstrated that one of the open reading frames in the HSV-1 2 kb LAT, if expressed constitutively and as a polyadenylated transcript, encodes a $\sim$30 kD protein that enhances HSV-1 growth in the relatively nonpermissive ND7 neuronal cell line (Thomas *et al*, 1999). In addition, expression of the LAT ORF completely compensated for replication deficiencies of an ICP0 deletion mutant of HSV-1 (*dl*1403) in both ND7 cells and the highly permissive BHK fibroblast cell line, and also compensated for replication deficiencies of a VP16 mutant of HSV-1 (*in*1814) in BHK cells (Thomas *et al*, 1999). It has been suggested that expression of this ORF would have to be tightly regulated, possibly through a translational mechanism, and should coincide with reactivation of latent virus infection (Thomas *et al*, 1999). The critical missing observation is the demonstration that a LAT-encoded protein is produced *in vivo* from an RNA transcribed off of a natural genomic template. This is no small consideration given the current understanding that the ORF is positioned within a nonpolyandenylated, nonlinear molecule. The characteristics of the stable LATs suggest the possibility that an uncharacterized but related transcript could be involved in providing an RNA template for a LAT protein. The possibility that an uncharacterized LAT-related transcript has eluded

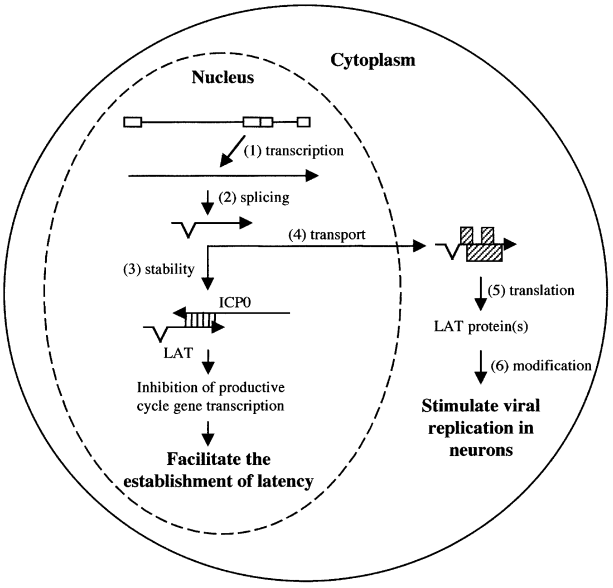

Figure 4 A model of LAT function. The functional involvement of LATs in latency and reactivation may involve two distinct molecular pathways. One of these pathways may increase establishment of latency by inhibiting productive cycle gene expression. In this pathway LATs may bind ICP0 transcripts and inhibit their translation, resulting in reduced levels of ICP0 transactivator protein. In the second pathway, LATs may be transported to the cytoplasm, where they associate with the host translational machinery to produce LAT-encoded proteins that stimulate viral replication. Regulation of this complex scheme could occur at multiple steps including: (1) activation and inhibition of promoter activity as well as selection of transcription initiation sites, (2) alternative splicing, (3) regulation of RNA stability, (4) RNA transport between the nucleus and cytoplasm, (5) regulation of translation, and (6) post-translational modification of LAT-encoded proteins.

detection seems reasonable if one accepts a model in which expression of the transcript is transient, and if only a small fraction of neurons are involved in *in vivo* viral reactivation.

Putting it all together

Presently, there does not exist a consensus among investigators on an overall model of LAT function in HSV-1 neurobiology and latency. However, we and others have proposed two reasonable mechanisms of LAT function that seem consistent with most experimental observations (Figure 4): (1) if present within the nucleus, stable LATs may inhibit ICP0 translation, resulting in reduced lytic gene expression, repression of viral replication, and efficient establishment of latent infection (Chen *et al*, 1997; Garber *et al*, 1997; Thompson and Sawtell, 1997), and (2) if a cytoplasmic LAT mRNA is produced, then translation of this mRNA(s) may produce a protein(s) that stimulates replication in the neuronal environment where α gene expression is either not yet activated or otherwise deficient (Thomas *et al*, 1999).

These potential mechanisms are not necessarily mutually exclusive. The choice of a particular mechanism could be regulated by altering the structure and/or subcellular localization of the transcript. This could occur through differential usage of transcription initiation sites, as well as regulation of RNA transport, translation, or splicing. Regulation of the LAT promoter(s) by cellular factors could be important in determining the efficiency of establishment and the timing of viral reactivation. The concept that one genetic locus could provide for two seemingly opposite functions is not entirely unique. One example of this type of mechanism involves the CREM gene. This gene encodes several isoforms of CREM that function as transcriptional activators or repressors depending on alternative splicing and the use of an internal intronic promoter (for a review see Sassone-Corsi, 1998).

If the LAT promoter(s) has an inducible function within sensory neurons that stimulates viral reactivation, then it seems likely that at least some of the stimuli that induce reactivation may impact the LAT promoter(s). This could occur in response to the release of chemical signals by the host neuroendocrine system, and/or chemical signals induced in the host by noxious environmental stimuli. In the context of LAT promoter transcriptional regulation, this question comes down to determining which cellular transcription factors are activated (or de-activated) in response to reactivation signals and which ones are involved in promoter function. If more than one promoter is involved, then promoter switching must be analyzed.

Although no specific transcription factor family has been shown to induce LAP1 during reactivation, the CRE-1 element in LAP1 has been shown to be necessary for frequent spontaneous reactivation and epinephrine iontophoresis-induced HSV-1 reactivation in the rabbit ocular model. This observation, and evidence that the CRE-1 mutation does not affect basal LAT expression in sensory neurons during latency, suggests that transcription factors that bind to this element may activate LAP1 or some other local cryptic promoter in response to a signal that initiates viral reactivation. The types of factors that bind to CRE-1 and CRE-2 (ATF/CREB and AP-1) are highly regulated by cellular signal transduction pathways and would be strong candidates to perform this type of function.

Conclusions

To decipher the role that LATs play in the biology of HSV-1 latency has proven to be a difficult task. In part, this may be a reflection of the unusual characteristics of the LATs that have surfaced since their discovery. However, it is apparent that the LAT locus is involved in HSV-1 neurobiology. The locus is conserved among HSV-1 variants, and the promoter proximal region of LAP1 that contains the TATA box, USF site and CRE-1 site is almost identical between HSV-1 and HSV-2 (McGeoch *et al*, 1991). Such high conservation of a promoter that is active within cells latently infected with HSV-1 and HSV-2 almost necessitates one to ascribe a beneficial function of LAT production to HSV-1 neurobiology. Furthermore, experimental evidence has clearly shown that this rather large region of the HSV-1 genome is involved in regulating HSV-1 biology within sensory neurons of the peripheral ganglia.

With the increasing sensitivity of modern molecular techniques, many of the unanswered questions surrounding HSV-1 latency and LAT are likely to be answered. These questions relate to: (1) characterizing LAT-encoded effector molecules and their function, (2) characterizing the population of latently infected cells that supports viral reactivation *in vivo*, (3) characterizing the cellular signaling pathways and transcriptional regulators that direct HSV-1 gene expression and viral reactivation in neurons *in vivo*, and (4) characterizing the kinetics of viral gene expression and replication in neurons *in vivo*.

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