Chromatin control of herpes simplex virus lytic and latent infection

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Abstract | Herpes simplex viruses (HSV) can undergo a lytic infection in epithelial cells and a latent infection in sensory neurons. During latency the virus persists until reactivation, which leads to recurrent productive infection and transmission to a new host. How does HSV undergo such different types of infection in different cell types? Recent research indicates that regulation of the assembly of chromatin on HSV DNA underlies the lytic versus latent decision of HSV. We propose a model for the decision to undergo a lytic or a latent infection in which HSV encodes gene products that modulate chromatin structure towards either euchromatin or heterochromatin, and we discuss the implications of this model for the development of therapeutics for HSV infections.

Many viruses, such as influenza viruses and noroviruses, undergo an acute infection in human hosts and are then cleared by the immune system. Other viruses remain in the host for long periods of time, causing either chronic or latent persistent infections. In chronic infections, infectious virus is produced continuously. For example, in the chronic infection caused by hepatitis B virus, infectious virus is produced continuously in liver hepatocytes[1]. In latent infections, such as those caused by herpesviruses, the virus is quiescent and no infectious virus can be detected. Periodic reactivation results in recurrent infections, recrudescent disease and transmission to new hosts[2]. The molecular and cellular biology of herpes simplex virus (HSV) has been reviewed recently[3]. In this Review, we examine the mechanisms by which HSV can undergo a lytic or latent infection in different cell types.

HSV-1 causes oral cold sores, whereas HSV-2 infects the genitals, but both viruses can establish a latent infection in innervating ganglia and persist for the lifetime of the host. The burden of HSV infection is high because, although primary and recurrent oral and genital infections are generally self-limiting in immunocompetent individuals, HSV causes significant morbidity and mortality in immunocompromised individuals, in whom there is an increased risk of serious herpetic disease[3,4]. Neonates infected during delivery or *in utero* can develop a life-threatening disseminated infection. Approximately 1,500 newborns are infected with HSV each year in the United States[5], and despite the availability of antiviral drugs there are still significant levels of morbidity and mortality in infected babies. Patients with AIDS are at risk for disseminated HSV infections, and much of the drug-resistant HSV arises in these patients. Immunocompetent individuals can also suffer serious herpetic disease[3]. Approximately 300,000 cases of ocular herpes are diagnosed each year in the United States. Recurrent herpes keratitis (corneal infection) can lead to corneal scarring and blindness. More serious are central nervous system infections with HSV or herpes encephalitis. There are an estimated 1,500 cases of herpes encephalitis per year in the United States, and despite the availability of antiviral drugs there is considerable mortality. For the survivors, there are severe neurological sequelae. Finally, genital herpes infection increases the likelihood of HIV infection and transmission by 2–4 fold[6,7]. Current antiviral strategies for HSV only affect the lytic infection, so tackling the HSV reservoir that is present through latent infection of neurons with new therapeutic strategies is a public health priority.

The co-evolution of HSV with its human host has resulted in a complex relationship in which the host does not completely eradicate the virus. Latent infection is not a passive process in which a virus infects a non-permissive cell. Rather, a successful latent infection by a virus involves several processes. In the case of HSV:

- Lytic gene expression must be silenced to prevent the cytopathic effects of lytic infection.
- Host cell responses, such as apoptosis and innate immunity, must be blocked.
- The acquired immune response must be evaded or blocked so that the infected cell is not cleared.

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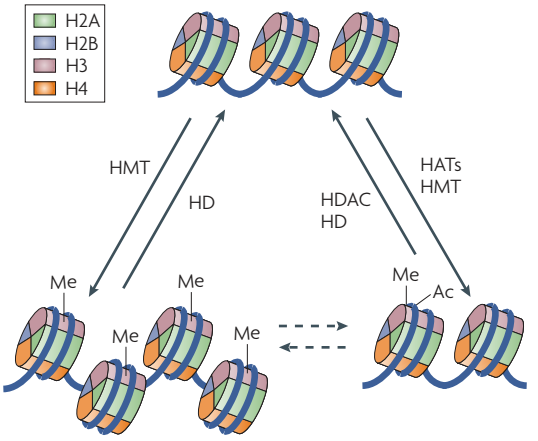
doi:10.1038/nrmicro1794
Published online
11 February 2008

Box 1 | **Chromatin structure and the regulation of gene expression**

In eukaryotic cells, DNA is tightly associated with histones and other proteins to form chromatin. The structure of chromatin has increasingly been recognized as highly important in gene regulation, genome propagation, differentiation, ageing and oncogenesis. The fundamental unit of chromatin is the nucleosome, which is composed of ~145 base pairs of DNA wrapped around a tetramer of the core histone proteins (H2A, H2B, H3 and H4; see the figure). The DNA duplex that links the nucleosomes is associated with histone H1, which serves as a linker histone. Higher-order folding of the nucleosomal DNA can give rise to either the less condensed, active euchromatin or to the highly condensed, silent heterochromatin. Post-translational modifications of the core histone tails that stick out from the nucleosomes have been directly linked to the regulation of chromatin structure, a concept known as the histone code. Modifications of the core histones include acetylation, methylation, ubiquitylation and phosphorylation, and function to alter the interactions of histones with DNA and the recruitment of chromatin associated proteins. The best characterized histone modifications are acetylation and methylation. Acetylation of histone tails, carried out by histone acetyltransferases, is primarily associated with active gene expression. Histone acetylation results in the relaxation of the basic chromatin structure through increased charge repulsion and by serving as binding sites for protein complexes of chromatin-modifying and transcriptional activators. Histone methylation can be found in both heterochromatin and euchromatin. Trimethylation of histone H3 on lysine residue 9, H3K9me3, is bound by heterochromatin protein 1 (HP1), which results in chromatin compaction and heterochromatin formation. These patterns of histone modifications, which cause gene silencing or activation, can be inherited in daughter cells, a phenomenon called epigenetics.



Examples of histone modifications

Heterochromatin
- H3K9me2,3
- H3K27me3
- H4K20me3

Euchromatin
- H3K4me2,3
- H3K9ac, H3K14ac
- H4K5ac, H4K8ac
- H2AK5ac, H2BK12ac
- H2BK15ac

One of the main experimental techniques that has allowed the elucidation of chromatin structure and function is chromatin immunoprecipitation (ChIP). The first step in ChIP analysis is to cross-link the chromatin associated protein to DNA in live cells. The cells are then lysed and DNA complexes are sheared into small fragments, and the protein of interest is immunoprecipitated. Consequently, DNA sequences that interact with the protein of interest are enriched in this immunoprecipitation stage. Protein–DNA cross-links are subsequently reversed and the amount of DNA precipitated is quantified, usually by real-time PCR or microarray analysis. Thus, ChIP analysis, using antibodies to specific chromatin-associated proteins, together with an analysis of individual histone modifications, enables the characterization of endogenous chromatin structure at individual genomic regions.
HATs, histone acetyltransferases; HD, histone demethylase; HDAC, histone deacetylase; HMT, histone methyltransferase.

In this Review we focus on the role of histone modifications — which have increasingly been recognized in the regulation of eukaryotic genes[8] (BOX 1) — in the expression of HSV genes during lytic and latent infections. We propose a new model in which chromatin modification provides an epigenetic switch that determines whether a lytic or a latent infection occurs. We also specify those viral gene products that can 'flip' this switch towards either type of infection.

Lytic and latent infection by HSV

The life cycle of HSV involves both lytic (productive) and latent (non-productive) infection (FIG. 1). Upon entry at a mucosal surface, or at a break in the skin, HSV infects epithelial cells and undergoes a productive infection (FIGS 1,2; see REF. 3). Entry involves binding of the virion to the cell surface, which is followed by fusion of the virion envelope and the cell plasma membrane. The viral nucleocapsid is transported along microtubules and then docks with the nuclear pores to release the viral genome into the nucleus. The linear viral DNA circularizes rapidly and is transcribed to sequentially express immediate–early (IE), early (E) and late (L) viral gene products. The nucleus is reorganized to form replication compartments in which viral DNA is replicated and transcribed and progeny nucleocapsids are assembled. The nucleocapsids acquire tegument proteins and an envelope during budding through the inner nuclear membrane. According to the most generally accepted model, extracellular virions are produced by de-envelopment of the nucleocapsids at the outer nuclear membrane, which is followed by budding into the Golgi apparatus and secretion to the outside of the cell. Progeny viruses can infect surrounding cells and cause either primary herpetic disease or an asymptomatic infection.

As HSV spreads from the primary site of infection, the virus also infects sensory neurons by fusion with the neuronal membrane at the axonal termini, and the nucleocapsid is carried by retrograde axonal transport to the nucleus in the cell body of the neuron in a ganglion (FIG. 1). Viral DNA is released into the nucleus, presumably in a similar mechanism to that used in lytic infection, where it circularizes. HSV DNA persists in the nucleus in a circular episomal form that is associated with nucleosomes. Lytic gene expression is repressed, but the latency-associated transcript (LAT) is expressed at high abundance, which helps to silence lytic gene expression. Latent infection was classically defined as an absence of infectious virus in ganglionic tissue accompanied by the appearance of infectious virus upon co-cultivation of the ganglionic tissue with susceptible cells[3]. Although nearly all of the infected ganglionic neurons have severely restricted lytic gene expression, recent studies have shown that lytic gene expression and reactivation occur in a few neurons[9]. Similarly, genital shedding of HSV-2 can occur frequently in some individuals[10]. Thus, in a sensory ganglion, the bulk of the infected neurons

Epigenetic
Factors that affect gene action without changing nucleotide sequence. Epigenetic modifications function by changing the structure of chromatin, and are facilitated by DNA methylation and histone modification.

Nucleosome
A subunit of chromatin that is composed of DNA wrapped around a tetramer of histone proteins.

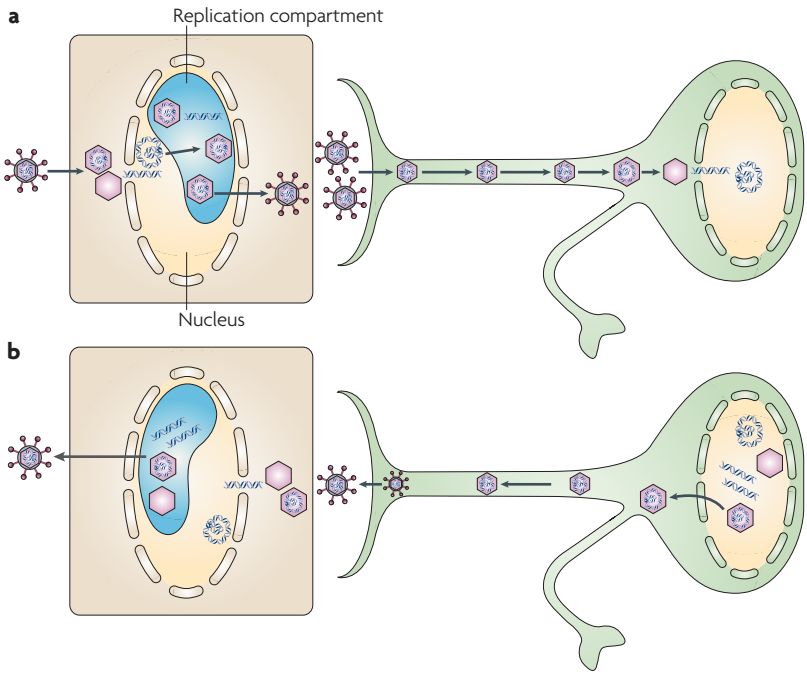


Figure 1 | **Stages of herpes simplex virus infection. a** | Infection of epithelial cells in the mucosal surface gives rise to productive replication, resulting in the production of progeny virions, which can spread to infect additional epithelial cells. Virus enters innervating sensory neurons, and nucleocapsids are transported to the neuronal cell body. The viral DNA is released into the neuronal nucleus and circularizes. Circular viral DNA persists in the neuronal cell nucleus, and the latency-associated transcript is expressed. **b** | Upon reactivation, viral lytic gene expression is initiated, and newly formed capsids are transported to the axonal termini. Infectious virus is released from the axon and infects epithelial cells, resulting in recurrent infection and virus shedding.

have a latent infection, whereas a minority undergo reactivation. Reactivation likely involves expression of early and late proteins and viral DNA replication in amounts that are sufficient to produce progeny virions. New components of the virion move by anterograde transport to both peripheral and central branches of the neuron. Envelope proteins might be transported independently of capsid protein components, and axonal localization of certain glycoproteins and capsid and teguments could be linked. Virion assembly and release along the axon shaft and at the axon tip release viruses into the periphery, where recurrent infection, a recrudescent lesion and transmission can occur.

Chromatin and lytic versus latent infection

We propose that an important factor in the lytic or latent infection decision by HSV is how the virus deals with the host cell response that assembles chromatin on naked DNA upon entry into cells (FIG. 3). HSV DNA is not associated with histones inside the virion so, upon HSV entry into the host cell nucleus, it is likely that host cell mechanisms attempt to assemble chromatin on the viral DNA to silence the viral genes. We propose two alternative pathways for the regulation of chromatin that could result in lytic infection of epithelial cells or latent infection of sensory neurons (FIG. 4). In epithelial and other non-neuronal cells, viral proteins function to minimize histone association with viral lytic gene promoters and to promote euchromatin histone modifications on those histones that are associated with viral DNA (FIG. 4, left-hand panel). In infected neurons, however, one or more viral functions promote heterochromatin assembly on lytic gene promoters so that viral lytic genes are silenced and a latent infection can ensue (FIG. 4, right-hand panel). Below we consider how viral functions might regulate chromatin assembly on the HSV genome and effect an epigenetic switch.

Chromatin and lytic infection

During lytic infection, more than 80 viral genes[3] are expressed in a cascade pattern[11]. IE gene products are expressed from 2–4 hours post-infection. These include infected cell protein 0 (ICP0), ICP4, ICP22, ICP27 and ICP47. The genes that encode these proteins are transcribed in the absence of *de novo* viral gene expression. IE gene promoters have several binding sites for cellular transcription factors, and their transcription is also activated by the virion protein VP16. VP16 is a well characterized transcriptional activator protein that has an acidic activator domain[12]. VP16 forms a complex with host cell factor (HCF) and localizes in the cell nucleus, where the VP16–HCF complex binds to the host transcription factor octamer-binding protein 1 (OCT1). OCT1 binds to specific sites in the upstream regulatory sequences of IE genes, tethering the VP16–HCF complex to IE gene promoters and enabling the activator domain of VP16 to recruit transcription factors that stimulate IE gene transcription. IE gene products activate expression of the E gene products. ICP4 is required for all subsequent viral gene expression, most likely through its association with transcription factors and their adaptor proteins[13,14]. The E gene products are involved in viral DNA replication, after which the L genes are expressed. Activation of L gene expression requires DNA synthesis and at least 3 viral proteins: ICP4, ICP27 and ICP8. ICP27 reportedly stimulates transcription of L genes, cytoplasmic transport of viral mRNAs and translation of L mRNAs, but the mechanism that underlies these functions has not been fully defined[3]. ICP8 probably has a role in viral chromatin modulation.

HSV DNA in virions is not associated with histones[15], nor are there histones present in the virion[16,17]. Upon entry of the viral DNA into the cell nucleus, host functions will assemble chromatin on the naked viral DNA to silence incoming genes, as is observed for transfected DNA[18]. Nuclease-digestion studies have shown that there are few, if any, nucleosomes on viral DNA[17,19,20]. Some completely protected viral genomes were observed, but they are likely to be present in nucleocapsids. Furthermore, viral DNA is replicated, and accumulates, in replication compartments that exclude histones[21,22]. Nevertheless, chromatin immunoprecipitation (ChIP) studies have revealed that histones are associated with lytic genes during lytic infection[23,24], although it was concluded that "histones are underrepresented at the promoters of actively transcribed genes."[23] Our own studies comparing viral gene promoters with the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene promoter have shown reduced levels of

Anterograde transport
The direction of anterograde axonal transport is from the cell body to the synapses. By contrast, retrograde axonal transport is from the synapses to the cell body.

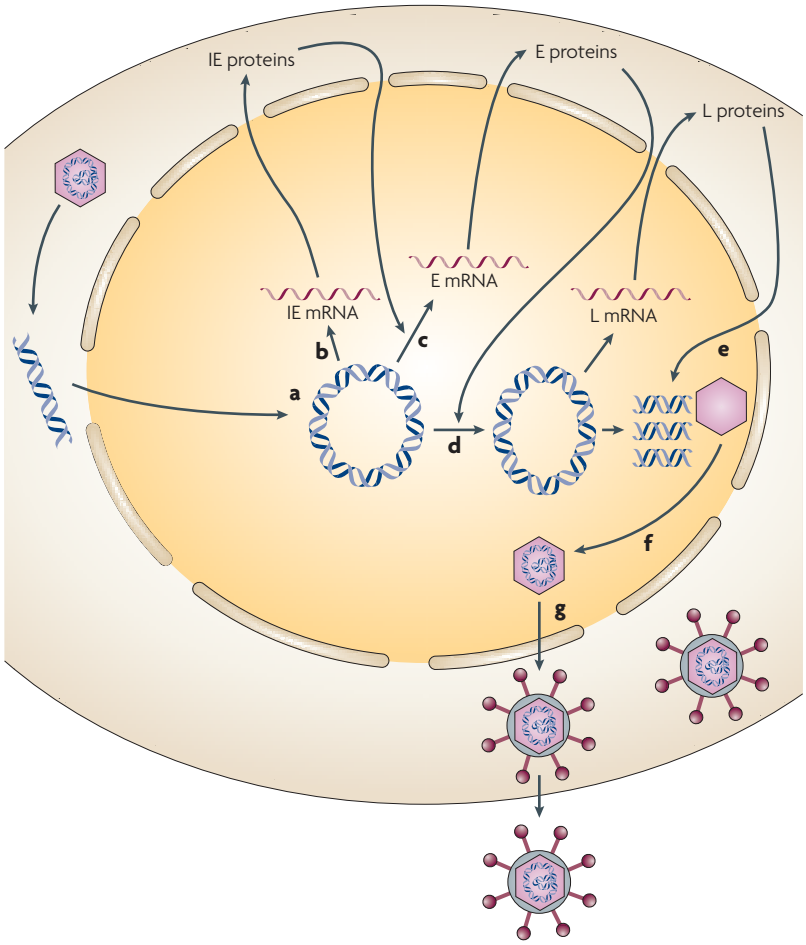


Figure 2 | **Overview of the herpes simplex virus lytic infection cycle. a** | Parental viral DNA enters the host cell nucleus and rapidly circularizes. **b** | The first genes to be expressed are the immediate–early (IE) genes, the transcription of which, by host RNA polymerase II, is stimulated by the viral tegument protein VP16. **c** | IE proteins are transported into the nucleus and transactivate early (E) gene expression. The products of E genes include proteins that are required for viral DNA replication. **d** | DNA replication stimulates the expression of the late (L) genes, many of which encode viral structural proteins. **e**,**f** | Viral capsid assembly and progeny DNA encapsidation take place in the nucleus. **g** | Virions egress from the nucleus and the cell.

histones associated with the viral genes by 6–8 hours post-infection, as compared with the GAPDH promoter (A.C. and D.M.K, unpublished observations). The specific form in which histones are organized on HSV DNA remains to be defined — are they nucleosomal or present in a different form? Nonetheless, the histones that are associated with lytic genes bear modifications that are characteristic of euchromatin: methylation of lysine 4 of histone H3 (H3K4me) and acetylation of lysines 9 and 14 of histone H3 (REF. 24). These euchromatin-style modifications may stimulate viral gene transcription because an inhibitor of protein methylation, which would reduce methylation of H3K4, reduced viral gene expression, although specific knockdown of H3K4 methyltransferases reduced mRNA expression of *ICP0* (an IE gene) and *VP16* (an L gene) by only twofold and had no effect on mRNA expression of thymidine kinase (an E gene)[25].

Several viral gene products have been implicated in the modulation of chromatin structure on HSV lytic genes during lytic infection, including the *VP16*, *ICP0* and U_S3 (unique S component open reading frame 3) gene products.

VP16. Herrera and Triezenberg[23] provided the first direct evidence that a viral gene product can have an active role in reducing chromatin and heterochromatin on viral lytic gene promoters[23]. ChIP assays showed that the VP16 virion transactivator protein, in addition to recruiting transcription factors to IE gene promoters, recruited the chromatin-modifying co-activators CBP (cAMP response element binding (CREB)-binding protein) and p300, as well as components of the human orthologues of the yeast SWI/SNF ATP-dependent chromatin-remodelling complex (BRG1 and BRM) to viral IE gene promoters. In the absence of VP16, increased levels of histone H3 are associated with IE gene promoters and decreased levels of acetylated histones are associated with E gene promoters. Therefore, VP16 protein has a role in reducing total chromatin levels on IE genes during lytic infection and in promoting euchromatin modifications on the histones that are associated with HSV lytic genes.

ICP0. ICP0 is an IE protein that increases the expression of HSV and non-HSV genes that are transfected into mammalian cells[26–30]. In addition, ICP0 increases viral gene expression in cells that are infected at low multiplicities of infection[31–35]. ICP0 defects can be complemented, at least in part, by inhibitors of histone deacetylases (HDACs)[36,37]. Thus, it seems that part of the function of ICP0 is to inhibit HDACs and prevent silencing of the viral genome.

Two general models have been proposed to explain how ICP0 relieves host cell silencing mechanisms. First, ICP0 may cause the degradation of a host protein that is involved in the silencing of viral genes[38]. One host protein that contributes to repression of HSV gene expression is the promyelocytic leukaemia (PML) protein[39], at least certain forms of which are degraded by *ICP0* (REF. 40). One study showed that knockdown of *PML* expression in human cells partially restored the ability of *ICP0* mutant viruses to express lytic genes[39]. However, another study of $PML^{-/-}$ mouse embryonic fibroblasts showed no increased growth or gene expression of $ICP0^{-}$ viruses[41]. It is likely that there are differences in the functional role (or roles) and the relative contributions of these functions of ICP0 in different cell types.

Second, ICP0 could inhibit histone deacetylation by associating with and inhibiting the activity of HDACs. ICP0 that is expressed in transfected cells forms a complex with HDAC5, HDAC6 and HDAC7 and thereby reduces their activity[42], although this function has not been demonstrated in infected cells. ICP0 also forms a complex with the RE1 silencing transcription factor–co-repressor to REST (REST/CoREST)–HDAC repressor complex that leads to the dissociation of HDAC1 from the complex[43,44], which could inactivate its repression activity. ICP0 expression in cultured cells that are quiescently infected with

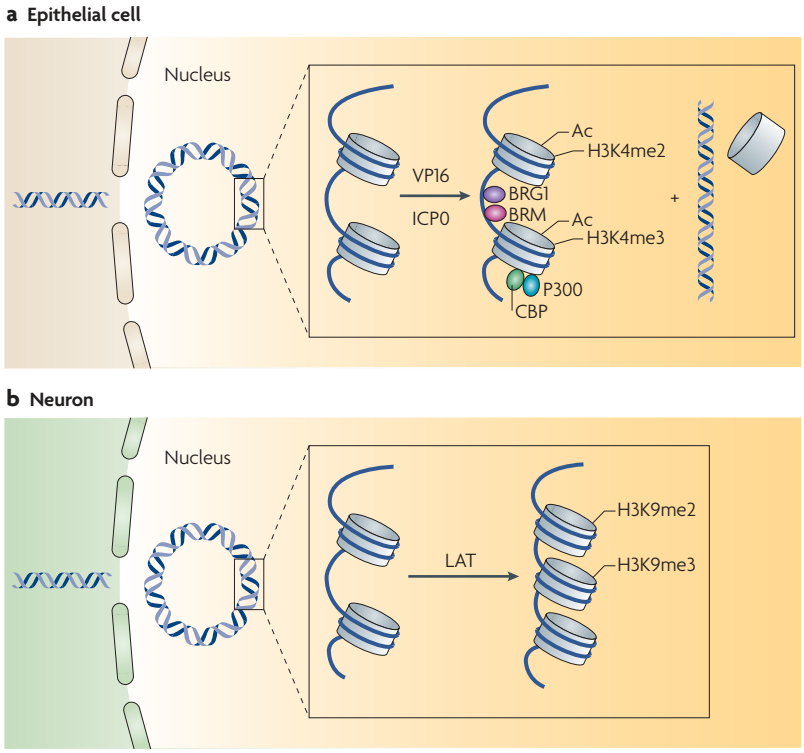


Figure 3 | **The fate of viral DNA. a** | The majority of the virion-encapsidated double-stranded DNA genomes are linear, although a small portion may be circular. The genome is not associated with histones and is wrapped as a toroid or spool. Following infection of epithelial cells, the viral genome circularizes. Early in infection, at least a proportion of the viral genome associates with histones. The presence of the virion protein VP16 in the nucleus results in the recruitment of histone-remodelling factors, such as BRG1 and BRM, and the histone acetyltransferases CBP and p300 to promoters of immediate–early genes. The histones that are associated with the viral genome bear markers of active euchromatin, such as acetylation of histone H3 on residues K9 and K14 (H3K9, H3K14), H3K4me2 and H3K4me3. Viral proteins that are expressed following infection, such as infected cell protein 0 (ICP0) and ICP8, allow further remodelling of the associated histones, which are removed by the recruitment of host chromatin-remodelling factors. **b** | Upon neuronal infection, the genome also circularizes. VP16 remains in the cytoplasm and a high proportion of the DNA associates with nucleosomes. The levels of acetylated histones on lytic promoters are low. Expression of latency-associated transcript (LAT) promotes the assembly of heterochromatin in the form of histone H3K9me2, H3K9me3 and H3K27me3 on lytic gene promoters.

HSV-1 leads to histone H3 acetylation at viral promoters[45]. Consistent with this, recent ChIP studies showed that during lytic infection *ICP0* expression results in an increase in euchromatin formation at HSV lytic genes and in a decrease in total histone association with IE and E gene promoters (A.C. and D.M.K., unpublished observations). Thus, ICP0 contributes to the under-representation of chromatin at HSV lytic genes and to active modifications on the limited amount of histones that are assembled on viral DNA during lytic infection.

ICP0 might act directly to decrease chromatin on viral genes by recruiting chromatin-remodelling complexes or other enzymes to the viral DNA. Alternatively, ICP0 might act indirectly to decrease chromatin content on viral DNA by stimulating histone acetylation, which activates transcription and reduces chromatin loading on viral lytic genes. The former model is supported by a study that demonstrated that removal of a nucleosome on the long terminal repeat (LTR) of HIV occurs before transcription[46].

U_S3. The U_S3 protein kinase reportedly blocks histone deacetylation and enables baculoviral gene expression[47]. U_S3 can phosphorylate HDAC1 and HDAC2 (REFS 24,26,27), which could lead to inactivation of the HDACs. It is not yet clear whether U_S3 has such a role in HSV-infected cells.

ICP8. ICP8 recruits chromatin-remodelling complexes into replication compartments and onto progeny viral DNA[48]. ICP8 can stimulate L gene expression independently of its role in stimulation of viral DNA replication[49], and this may be due to a reduction of chromatin on viral progeny DNA in replication compartments (V. Leautaud, A.C. and D.M.K., unpublished observations).

Chromatin and latent infection

Viral gene expression (of IE, E and L genes, and of LAT) is observed in trigeminal ganglia of infected mice during the first 24–72 hours post-infection[50–52]. There is a transient accumulation of viral IE and E gene transcripts[53], but LAT continues to be expressed and accumulate[52], so that by the time a latent infection is established, LAT is the only abundant viral transcript that is expressed in the infected neurons[54]. Although the order of events during establishment of latency has not been defined, latent infection can be established without lytic infection[55,56]. Furthermore, studies have indicated that distinct populations of cells express LAT and lytic gene transcripts[57,58]. This suggests, but does not prove, that the neurons expressing lytic genes die and are cleared, whereas neurons that express LAT go on to establish latent infection.

LAT was first detected in latently infected murine ganglia[54] and subsequently in latently infected human[59,60] and rabbit[61] ganglia. The LAT primary transcript (FIG. 5) is spliced into several RNA species that are collectively referred to as LATs. The full-length transcript accumulates at low levels in latently infected neurons, whereas the 2.0- and 1.5-kb introns that are processed from the primary transcript are abundant and accumulate in the nucleus[62]. The stability of these introns has been attributed to their unusual lariat structures[63]. The LAT transcriptional unit contains upstream regulatory sequences from approximately 800-base pairs (bp) upstream from the transcriptional start site and an enhancer that confers long-term expression that maps downstream of the transcriptional start site (FIG. 5; REFS 64–66). The LAT gene promoter shows neuronal specificity[67,68], and although the promoter elements that mediate neuron-specific expression have not been completely mapped, activating transcription factor (ATF)/ CREB sites might be involved[69]. A 2-kb LAT-related transcript can be detected in productively infected cells at late times post-infection[70], but this is likely due to splicing from read-through transcripts that are common at this stage of infection[71] rather than to transcription from the LAT promoter. Despite isolated reports of detection of proteins that are encoded by LATs[72,73], most researchers have found no

Trigeminal ganglia
The trigeminal ganglion is a sensory ganglion of the trigeminal nerve that occupies a cavity in the *dura mater* that covers the trigeminal impression near the apex of the petrous part of the temporal bone.

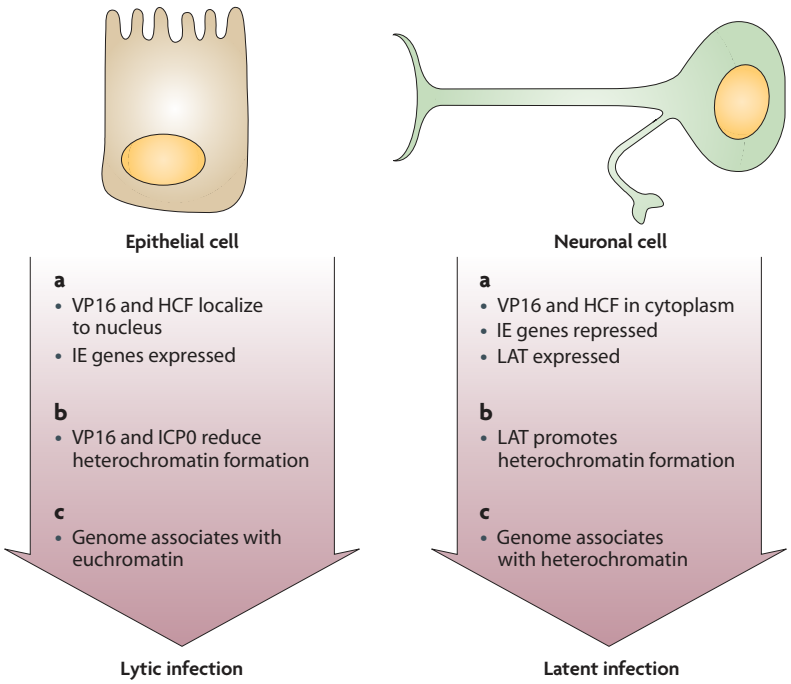


Figure 4 | **Summary of potential mechanisms that might determine the outcome of viral infection of epithelial cells and neurons.** The figure shows the chromatin switch model for the mechanism of the decision by herpes simplex virus (HSV) to undergo lytic versus latent infection pathways. HCF, host cell factor; ICP0, infected cell protein 0; IE, intermediate–early; LAT, latency-associated transcript.

evidence for LAT-directed protein expression (see REF. 74). Therefore, LAT function is likely to be effected by the transcript itself.

Functions of LAT. Some studies reported that recombinant viruses that lack various LAT domains establish latency at normal levels[75–77], whereas others reported that the number of neurons harbouring LAT$^-$ viruses was decreased by 3–5 fold in the absence of LAT[78,79]. Further studies reported reduced explant reactivation of virus from ganglia that are latently infected with LAT$^-$ mutant viruses[76], although in some cases LAT$^-$ mutant viruses had reduced replicative ability[80]. The region of the LATs that is associated with decreased reactivation has been mapped to a 348-bp sequence in the 5′ region[81,82].

Several studies have reported that LAT affects viral gene expression or replication. First, LAT$^-$ virus mutants show elevated productive viral gene expression in sensory neurons during acute infection[53] and during latent infection[83]. Consistent with this, expression of LAT in cultured cells has been shown to reduce lytic viral gene expression and replication in those cells[84]. Second, LAT$^-$ mutant viruses produce more severe pathology in trigeminal tissue[76], cause more neuronal death[85] and cause higher mortality in infected mice[85,86] than wild-type virus. Finally, LAT is associated with the prevention of apoptosis[87]. Thus, a common theme is that LAT protects infected neurons from cell death[88] by: reducing viral gene expression; protecting against apoptosis; or by other mechanisms. In summary, LAT has been reported to have several different potential functions. These might be independent or related functions. This Review focuses on viral gene regulation, so it will address only the effects of LAT on gene regulation.

Chromatin and latent infection. During latent infection, HSV DNA is circular[89–92] and is assembled into nucleosomes[93]. However, the form of the chromatin on the viral DNA during latent infection has only recently been revealed. Recent studies with eukaryotic cells have defined the importance of histone modification in chromatin structure and function (BOX 1). Application of molecular techniques, including ChIP, to the study of viral chromatin in neurons has provided the basis for an epigenetic model of HSV gene regulation in different cell types (FIG. 4). Kubat *et al.*[94] showed that there are increased levels of acetylated H3 histone associated with the LAT promoter and enhancer compared with the *ICP0* gene, which indicates that active chromatin was associated with the LAT gene only. Furthermore, Wang *et al.*[86] showed that as latent infection is established the HSV lytic genes are progressively associated with chromatin that contains histones with modifications that are indicative of heterochromatin — specifically dimethylation of H3K9me2. Given that methylation of the viral DNA cannot be detected during latent infection, HSV lytic genes are likely silenced by heterochromatin rather than by DNA methylation[95,96]. Therefore, during latent infection the LAT gene is associated with euchromatin, whereas the lytic genes are associated with heterochromatin. How are these chromatin domains maintained separately on the latent viral genome? Amelio *et al.*[97] identified candidate insulator elements that contain CCCTC sites that are bound by the CCCTC-binding factor (CTCF) upstream of the LAT promoter boundary (bp 120,503–120,635) and in the LAT intron (bp 117,158–117,342) (FIG. 5). Insulators are DNA sequences that bind protein factors that maintain chromatin boundaries, and Amelio and co-workers proposed that insulators keep the LAT euchromatin activity within a boundary and heterochromatin outside of the same boundary.

Studies on reactivation have yielded results that are consistent with chromatin control of lytic gene expression during latent infection. After induction of reactivation by *in vitro* explant, LAT transcript levels decrease[98] and the histones associated with the LAT gene become deacetylated[99]. Similarly, lytic genes, such as the *ICP0* gene, become associated with acetylated histones[99], and lytic gene transcripts accumulate[100,101]. Also, treatment of *in vitro* latent infections with the HDAC inhibitor trichostatin A increases expression from the *ICP0* gene promoter[102]. Furthermore, treatment of latently infected mice with butyrate, another inhibitor of HDACs, causes acetylation of histones on lytic genes and reactivation of virus[103].

Silencing of lytic genes in neurons. There are several mechanisms that activate HSV IE gene expression during lytic infection. So, the crucial question is: how is viral lytic gene expression silenced in sensory neurons? Several mechanisms, either individually or in

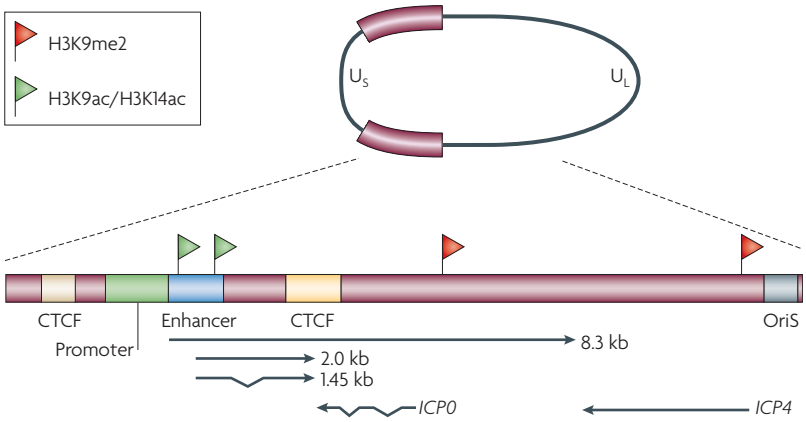


Figure 5 | **Schematic representation of the latency-associated transcriptional unit of the herpes simplex virus genome.** Transcription of the 8.3-kb primary latency-associated transcript (LAT) initiates from the latency-associated promoter. The primary transcript is spliced to form a 2-kb stable intron. Alternative splicing can give rise to a 1.5-kb species. A long-term expression element that is located in the 5′ exon of LAT is the only region of the viral DNA that has been found to be associated with acetylated histone H3 during latent infection. The infected cell protein 0 (*ICP0*) and *ICP4* genes are associated with histone that bears markers of heterochromatin (H3K9me2, H3K27me3). Spreading of euchromatin and heterochromatin into neighbouring regions of the genome is presumably prevented by the presence of insulator to which the host protein CCCTC-binding factor (CTCF) binds. U_S, unique short sequence; U_L, unique long sequence; OriS, origin of replication.

combination, could underlie silencing of lytic gene expression. First, transcription factors that bind to sites in IE gene promoters might be missing from sensory neurons[51]. Second, it has been proposed that transcriptional repressors bind to HSV IE genes in sensory neurons. It has also been postulated that the host protein OCT2 represses IE gene expression through its interaction with IE promoters that block OCT1 binding and activation[104,105]. However, other investigators failed to detect OCT2 expression in sensory neurons, and even when OCT2 was overexpressed in cells, it did not inhibit expression from a reporter gene that contained the complete IE promoter and enhancer[106], reducing the potential for IE gene repression by OCT2.

HSV IE gene expression is activated by VP16 during lytic infection, and there has been extensive investigation of VP16 function in neuronal cells. First, Roizman and colleagues hypothesized that VP16 might not be capable of translocation to the cell body of a sensory neuron[107]. Ectopic expression of VP16 in neurons under the control of the metallothionein promoter from the viral genome or in transgenic mice did not affect the ability of HSV to establish latent infection[107]. Therefore, absence of VP16 could not account for restricted IE gene expression.

Other studies have proposed that VP16-associated proteins might not be expressed or function in neurons. Using *in situ* hybridization it was shown that *OCT1* mRNA is not expressed in sensory ganglia cells[51,108]. More sensitive band shift assays showed that OCT1 is present, but at low levels, in sensory neurons[106]. Another study showed that HCF or C1, a host cell factor that mediates VP16 stimulation of IE gene transcription, is localized to the cytoplasm of uninfected sensory neurons, but moves to the nucleus when ganglia are explanted into culture[109]. Thus, HCF might not be able to localize to the nucleus during HSV infection and therefore might not transactivate IE gene transcription. In addition, HCF has been reported to transport VP16 into the cell nucleus[110]. Therefore, even if the VP16 in the incoming virion is translocated to the neuronal cell body in sensory neurons, it would not be localized into the neuronal nucleus and it could not participate in the transactivation of IE genes.

Finally, data on the mechanism of lytic gene silencing in neuronal cells points to a role for the LAT gene, or its transcript, in the repression of lytic gene expression during acute infection[53] and latent infection[83] in murine trigeminal ganglia. The LAT gene or transcript promotes the formation of heterochromatin and reduces euchromatin on HSV lytic gene promoters[86]. A LAT^- mutant virus showed reduced levels of a heterochromatin marker, the dimethyl form of H3K9me2 and elevated levels of a euchromatin marker, H3K4me2 (REF. 86). Further studies have shown that expression of LAT correlates with increases of other heterochromatin markers, H3K9me3 and H3K27me3 (A.C. and D.M.K., unpublished observations).

How do the LATs mediate gene silencing and heterochromatin formation on viral lytic genes? There is no good evidence that any of the LATs encode a protein product, so it is tempting to speculate that this effect is mediated through the transcript itself. In recent years a role for non-coding RNAs in the assembly and maintenance of heterochromatin has been defined. For instance, in the fission yeast, *Schizosaccharomyces pombe*, components of the RNA interference machinery are required for heterochromatin formation and spreading through the induction of H3K9 methylation[111]. Given that high levels of H3K9 methylation can be found in HSV chromatin during latent infection, and given the role of a viral RNA in promotion of heterochromatin formation, it is tempting to speculate that a similar mechanism to that operating in fission yeast also occurs during heterochromatin assembly on the latent genome. However, there is little evidence that this process occurs in vertebrates. One recent study showed that small interfering RNAs can cause transcriptional silencing in a mammalian cell in a process that involves Argonaute-1 (REF. 112). We propose that this mechanism could be operating during latent infection, in which case HSV latent infection might provide a useful mammalian system in which to study RNA-induced transcriptional silencing.

Alternatively, LAT could enhance heterochromatin formation through a process that is similar to the mammalian X-inactivation process, in which a non-coding RNA, known as the X-inactivation short transcript (Xist), binds to one X chromosome and triggers chromosomal silencing. Stable silencing of the X chromosome is maintained with Polycomb proteins[113]. Long non-coding RNAs have recently been shown to induce transcriptional silencing in human cells through Polycomb proteins and heterochromatin[114]. To our knowledge, no one has looked to see whether Polycomb proteins are associated with HSV lytic genes during latent infection.

Box 2 | **Latent infection and herpes simplex virus therapy**

Herpes antiviral drugs suppress lytic replication, but do not affect the latent reservoir of herpes simplex virus (HSV). Many HSV antivirals are nucleoside analogues that are activated by the viral thymidine kinase and inhibit the HSV DNA polymerase[3]. Prevention of latent infection might be possible if drugs are administered prophylactically, but this may not be desirable or feasible. Antiviral drugs can be applied topically to reduce HSV infection and indirectly reduce the establishment of latent infection[3]. Similarly, HSV vaccines can be used prophylactically to prevent acute and latent infection and therapeutically to reduce recurrent infection and recrudescent disease[3,128]. However, immunization does not reduce the viral load in HSV latent infection (M. Kramer and D.M.K., unpublished observations). Thus, new approaches are needed to target HSV latent infection directly.

It might be possible to target latent viruses with agents that reduce heterochromatin. HIV is maintained as a latent provirus in resting $CD4^+$ T cells owing to the formation of heterochromatin[46]. One proposed mechanism for eliminating the latent reservoir of resting $CD4^+$ T cells is to use histone deacetylase (HDAC) inhibitors to disrupt HIV latent infection and then to use antiretroviral therapy to kill the infected cells and prevent viral spread[126,129]. Treatment of 4 individuals with a new HIV antiviral and valproic acid (VPA), an HDAC inhibitor, led to a decline in the number of infected resting $CD4^+$ T cells[130].

Similarly, Epstein–Barr virus (EBV) tumour cells are infected with a latent form of EBV, and treatment with activators of lytic infection, including VPA, has been used to activate lytic EBV infection *in vitro*[131,132]. VPA and chemotherapy were more effective in inhibiting EBV-positive tumour formation in severe combined immune deficient (SCID) mice than was chemotherapy alone[132]. Based on these approaches, HSV latent infection could be induced to lytic infection through reactivation, as observed in one animal model[133], and then treatment with antiviral drugs or immunization to induce an immune response could be used to clear virus from reactivated cells. Reactivation could be induced by: treatment with HDAC inhibitors or other drugs that induce active chromatin; or by reducing latency-associated transcript (LAT) expression using RNA interference. Two possible risks are the potential toxicity of HDAC inhibitors and the potential for an infection of the central nervous system following reactivation.

Alternatively, the effect of LAT could be indirect. For example, the LAT transcripts might exert micro-RNA (miRNA) or antisense effects on *ICP0* expression. As discussed above, ICP0 increases active chromatin on viral lytic gene promoters, so reducing *ICP0* expression could lead to increased heterochromatin. Small amounts of lytic transcripts can be detected during latent infection[115], including *ICP0* spliced and unspliced transcripts[116]. Studies with a LAT^- virus showed no appreciable increase in *ICP0* transcripts[116]. Thus, there is no evidence that LAT reduces *ICP0* expression by reducing *ICP0* mRNA levels. miRNAs encoded in LAT might reduce translation of *ICP0* mRNA. Two predicted miRNAs that are encoded within the LAT transcriptional unit are miRNA #1 of Pfeffer *et al.*[117] and miRNA #10 of Cui *et al.*[118]. The miRNA #1 of Pfeffer *et al.*[117] is complementary to an intron of the *ICP0* transcript and thus is not likely to exert effects on mature mRNA function. By contrast, miRNA #10 of Cui *et al.*[118] is complementary to sequences near the 3′ end of the *ICP0* transcript and could conceivably affect the 3′ untranslated region and translation. However, no HSV-encoded miRNA has as yet been shown to be expressed during latency[118,119] or to have any role in latent infection, despite claims to the contrary[119,120].

Basis for a chromatin switch

The model in FIG. 4 predicts that, in epithelial cells, HSV VP16 enters the cell in the virion, reduces the total chromatin and promotes euchromatin formation on HSV IE genes. This allows expression of the IE gene *ICP0*, and then of the E gene *ICP8* and the proteins encoded by each in turn reduce total chromatin and promote euchromatin formation on the HSV lytic genes, thereby promoting lytic gene expression. By contrast, in neuronal cells LAT is expressed and this promotes heterochromatin formation on the HSV genes. In short, the chromatin 'switch' is thrown to euchromatin in non-neuronal cells, but to heterochromatin in neuronal cells. Thus, in common with many cellular development pathways, specific cascades are triggered by transcriptional control mechanisms or by the availability of host cell factors in different cell types. For HSV, the transcription controls do not just turn specific genes on or off — which would in turn upregulate or downregulate other specific genes — but instead affect the formation of active chromatin or inactive chromatin across the genome, which allows the coordinate regulation of the entire viral chromosome. The use of chromatin for global regulation of the HSV genome might be necessary owing to the large number of HSV transcriptional units[3].

Why would HSV use an RNA-mediated mechanism to silence viral lytic genes in neurons? The use of RNA might avoid the expression of proteins that could be recognized by the host immune system. However, viral RNA might be recognized by cytoplasmic sensors, such as RIG-I (retinoic-acid-inducible gene I), as part of the innate immune response. Perhaps the stable portion of LAT is hidden inside the nucleus to avoid these cytoplasmic sensors.

Chromatin and other viral infections

HSV is not unique in having to control the chromatin 'response' of the host cell. The DNA genomes of all viruses that replicate in the host cell nucleus face the challenge of subverting the cell's attempt to assemble chromatin on foreign or naked DNA as it enters the cell, just like transfected DNA. Therefore, DNA viruses must regulate chromatin structure to express their genes[121]. The polyomaviruses, such as SV40, have nucleosomes that are assembled on the virion DNA

Nucleoside analogues
Nucleoside analogues are chemically similar enough to nucleosides to be incorporated into growing DNA strands, but different enough to ensure that the resultant DNA is non-functional.

molecule, but have a nucleosome-free region over the origin and enhancer region to keep the early promoter open for transcription[122]. Adenoviral DNA is associated with core proteins in the virion, but the virus does not prevent chromatin assembly on the DNA genome in infected cells[123]. Instead, the E1A gene product recruits host proteins with histone acetyltransferase activities to keep the chromatin in a euchromatic or active form. The herpesviruses, in general, show an under-representation of chromatin on their viral genomes during lytic infection, at least during early and late times post-infection. However, by contrast, they show nucleosomal organization on their genomes during latent infection. Human cytomegalovirus encodes several gene products that also promote assembly of active chromatin on viral genomes during lytic infection. The virion protein pp71 causes the degradation of the Daxx protein and relieves its gene-silencing effects[124]. The IE1 72-kDa and IE2 86-kDa proteins inhibit HDACs to stimulate viral gene expression[125]. In addition to the nuclear DNA viruses, HIV latent infection of resting $CD4^+$ T lymphocytes is established by repression of transcription from the integrated proviral genome. This is accomplished by recruitment of HDACs to the viral promoter[46]. Treatment of HIV-infected, resting CD4 cells with valproic acid, an HDAC inhibitor, leads to the activation of HIV transcription[126,127], which is consistent with the proposed role of HDACs in HIV latent infection.

So, heterochromatin formation drives latent infection for a number of viruses. So far, HSV is unique in encoding a gene product that promotes heterochromatin formation, lytic gene silencing and latent infection.

Perspectives

The available evidence strongly supports the differential regulation of HSV gene expression during lytic and latent infection of HSV by chromatin structure. Furthermore, there is genetic evidence that during lytic infection HSV encodes several gene products that reduce chromatin levels and keep the remaining chromatin in an active form, but that during latent infection HSV encodes at least one gene product that increases the level of heterochromatin on the viral genome. HSV is the only virus that is known, so far, to encode a gene product that promotes heterochromatin assembly on lytic gene promoters, gene silencing and latent infection. It is crucial to determine whether other viruses also use this mechanism for the regulation of lytic versus latent infection. Other exciting questions for the future are the mechanisms by which these viral gene products affect chromatin structure on the viral genome in such different ways. These studies might answer basic scientific questions, including how chromatin is removed from eukaryotic cell DNA and how transcriptional silencing occurs in mammalian cells. Furthermore, these studies have the potential to yield therapies (BOX 2) that could interrupt, control or possibly even cure latent HSV infections and reduce the huge burden of HSV infection.

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Acknowledgments
Studies on HSV latent infection in the authors' laboratory are supported by NIH grant P01 NS35138.

DATABASES

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