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The comparative biology of latent herpes simplex virus type 1 and type 2 infections: latency-associated transcript promoter activity and expression *in vitro* and in infected mice

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HSV-1 and HSV-2 express abundant latency-associated transcripts (LATs) without which these viruses reactivate in animals inefficiently. To further characterize the importance of LATs to the comparative biology of latent HSV-1 and -2 infections, we assessed the relative activities of the viral LAT promoters *in vitro* using transient transfection assays, and the accumulation of LATs *in vivo* using a mouse ocular infection model. *In vitro*, the HSV-2 LAT promoter proved to be six to tenfold more potent than the HSV-1 promoter in driving reporter gene expression. In mice HSV-1 and -2 achieved comparable levels of virus replication in the eye, but HSV-2 grew to higher titers than HSV-1 in trigeminal ganglia and brain. Quantitative-competitive DNA and RNA (RT) PCR and *in situ* hybridization showed that ganglia latently infected with HSV-2 contained sixfold more copies of DNA (P=0.003), eightfold more LATS (P=0.01), and ninefold more LAT *in situ*-positive neurons. However, the numbers of LATs per latent genome were equivalent for both viruses. Although the HSV-2 LAT promoter is more potent than the HSV-1 promoter in transient expression assays, the accumulation of HSV-1 and 2 LATs in mouse trigeminal ganglia is comparable.

Keywords: animal models; herpes simplex virus; latency-associated transcripts; virus latency; viral pathogenesis

Herpes simplex virus types 1 and 2 (HSV-1 and 2) are closely related—structurally, molecularly, antigenically, and biologically (Plummer *et al*, 1970; Roizman and Sears, 1996). Both cause acute infections of human and animal skin and mucous membranes and lifelong latent infections of sensory neurons. Latent virus reactivates episodically in these neurons and spreads centrifugally to mucocutaneous surfaces, where it is shed either asymptomatically or in association with lesions (Cook and Stevens, 1973; Hirsch *et al*, 1995). Neuronal latency is characterized by the abundant expression of transcripts from only one diploid gene of these viruses (Croen *et al*, 1987, 1991; Deatly *et al*, 1987; Mitchell *et al*, 1990; Puga *et al*, 1978; Spivack and Fraser, 1988; Stevens *et al*, 1987, 1988; Suzuki and Martin, 1989). These RNAs are called the latency-associated transcripts, or LATs.

For both HSV-1 and -2, LAT expression is driven by complex upstream promoter elements, the dominant of which is known as latency-associated promoter 1, or LAP1, and includes classical TATA and CAAT boxes and binding sites for nuclear factors such as Sp1, Ap2, and ATF/CREB, among others (Batchelor and O'Hare, 1990, 1992; Dobson *et al*, 1989; Frazier *et al*, 1996a, b; Kenny *et al*, 1994; Lieb *et al*, 1991; Soares *et al*, 1996; Wagner *et al*, 1988; Wang *et al*, 1995; Zwaagstra *et al*, 1989, 1990, 1991). A weaker, second downstream sequence array lacking classical promoter elements is called LAP2 (Chen *et al*, 1995; Goins *et al*, 1994; Yoshikawa *et al*, 1996). The LAT family includes the primary, or low-abundance, 'minor' polyadenylated transcripts that extend from just downstream of the LAP1 TATA element to the 3′ end of the ICP4 transcript, a distance of over 8 kb. Smaller, colinear, nonpolyadenylated transcripts accumulate in neurons to high levels. These 'major' LATs are stable

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introns spliced from the minor LATs starting just downstream of LAP2 (Farrell *et al*, 1991; Mitchell *et al*, 1990; Wagner *et al*, 1988; Zwaagstra *et al*, 1990). HSV-1-infected neurons harbor a 2.0 kb major LAT and a 1.5 kb major LAT spliced again from it (Devi-Rao *et al*, 1991; Krause *et al*, 1988; Wagner *et al*, 1988). The abundant HSV-2 LAT species is 2.2 kb in length (Croen *et al*, 1991; Krause *et al*, 1991; Suzuki and Martin, 1989). While the precise function of the minor and major LATs is not known, there is considerable evidence that their expression modulates virus reactivation. Mutant HSV-1 and -2 strains that are impaired in LAT expression spontaneously reactive or can be induced to reactivate from experimentally infected animal sensory nerve ganglia, but in most studies they do so with a reduced efficiency (Bloom *et al*, 1994, 1996; Devi-Rao *et al*, 1994; Dobson *et al*, 1995; Krause *et al*, 1995; Leib *et al*, 1989; Sawtell *et al*, 1992; Steiner *et al*, 1989; Trousdale *et al*, 1991).

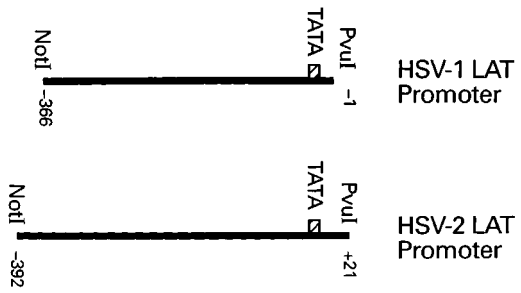
The DNA sequence across several kilobases of the LAT regions of HSV-1 and -2 is well conserved, largely due to the preservation of critical open reading frames within the important regulatory and virulence genes that are expressed anti-sense to the LATs: ICP4, IPC34.5, and ICP0 (McGeoch *et al*, 1991). There are differences in the organization of the LAPs, the dominant promoters, and there is considerable divergence in the sequences of the major LATs themselves (Krause *et al*, 1991; Wang *et al*, 1995). These differences in the HSV-1 and -2 LATs and their promoters could confer meaningful differences in rates of LAT expression and their accumulation in latently infected neurons. If substantive differences in LAT expression and accumulation are found, they could relate to differences in viral phenotypes such as the greater rate at which HSV-2 infections recur in humans (Lafferty *et al*, 1987). Evidence that they influence site-specific rates of virus reactivation was obtained in recent studies with recombinant strains in which LAT sequences of each virus type were swapped (Yoshikawa *et al*, 1996). To further investigate the implications of sequence divergence in the LAT region, we analyzed the relative LAT promoter activities of each virus *in vitro* by transient expression assays and quantitated latent viral load and LAT accumulation in experimentally infected mouse neurons.

Results

The HSV-2 LAT promoter is more potent in vitro

Based on the existing data regarding the structure and organization of the HSV-1 and HSV-2 LAT promoters, we directly compared their activity by transient expression assay using both non-neuronal (HeLa) and neuronal (PC12) cell lines. Similar fragments released from each genome by digestion with *Pvu*I and *Not*I were cloned into the pCAT. Basic plasmid (Figure 1). The HSV-1 LAT promoter examined spans 365 base pairs extending from 1 base upstream of the LAT start site. The corresponding HSV-2 LAT promoter element is 413 bases in length extending upstream of the *Pvu*I site at position +21 relative to the LAT start site.

Following three independent transfections into HeLa and PC12 cells, the mean percent acetylation of chloramphenicol was over sixfold greater for the HSV-2 promoter than for the HSV-1 promoter in PC12 cells and over 10 times greater in HeLa cells ($P<0.01$ by analysis of variance; Figure 1). We confirmed these results with an additional four independent transfections in PC12 cells and 2 transfections in HeLa cells using a second pair of promoter CAT constructs. An HSV-2 promoter construct extending from position +34 to -392 relative to the LAT start site proved to be 6.1 ± 0.8 (mean $\pm$ s.e.m.) times more active in PC12 cells and 10.4 ± 0.9 times more active in HeLa cells than an HSV-1 promoter element extending from bases -1 to -280, respectively (data not shown). That these latter constructs yielded nearly identical



Mean Percent Acetylation

LAT Promoter	HeLa Cells	PC12 Cells
HSV-1	2.1± 0.4*	3.5 ± 1.7
HSV-2	20.2 ± 1.1	18.7 ± 7.7
HSV-2/HSV-1 Ratio	10.4 ± 1.7	6.6 ± 1.3

*Mean ± S.E.M.

Figure 1 *In vitro* comparison of the promoter activity of the HSV-1 and HSV-2 LAT promoters in human HeLa and rat neuronal PC12 cells. The schematic structure of the DNA fragments used for each promoter is shown at the top with base pair numbering relative to the start of the primary (minor) LAT. The promoter activity was quantitiated by CAT assay. For each experiment, the amount of the transfected cell extract used in the assay was adjusted so that the percent acetylation always peaked within the linear range of the assay. The mean ($\pm$s.e.m.) percent acetylation and the ratios of the HSV-2 : HSV-1 promoter activities for each cell line are tabulated. The data presented are results of three independent transfections each in HeLa and PC12 cells.

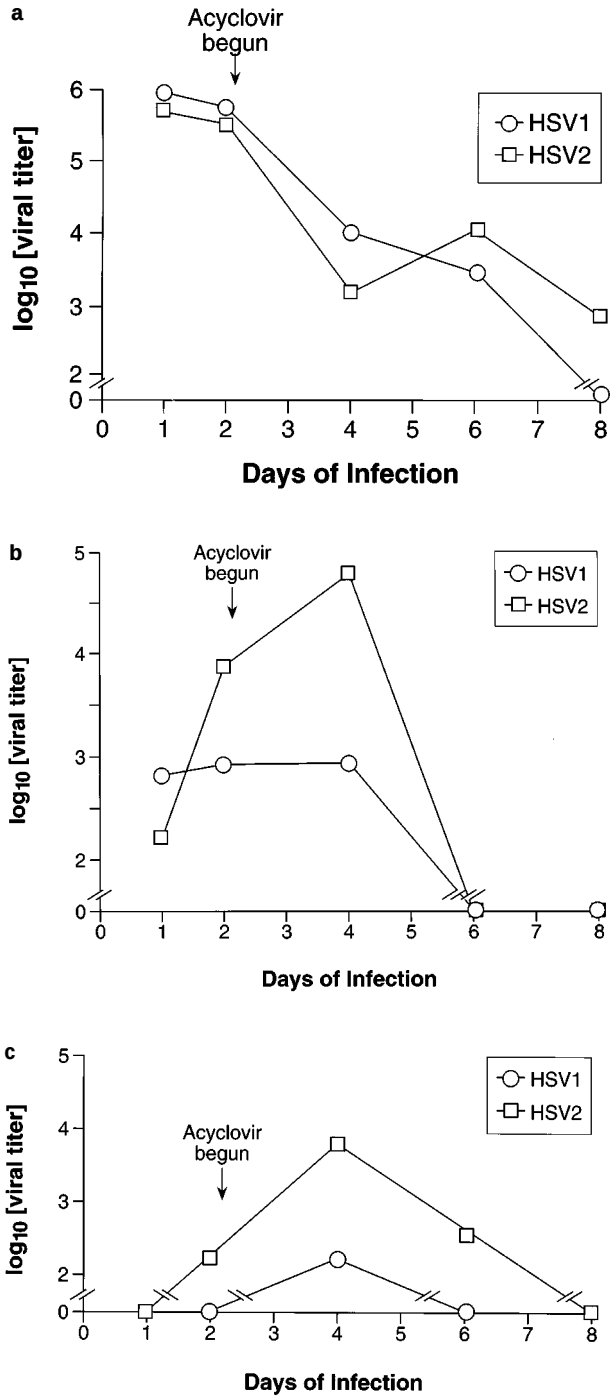


Figure 2 Replication of HSV-1 and HSV-2 in eyes and spread to trigeminal ganglia and brain. Four-week-old C3H female mice were inoculated with 10^5 p.f.u. of either HSV-1 strain 17 syn+ or HSV-2 strain 333 via corneal scarification. Three mice from each group were sacrificed on days 1, 2, 4, 6 and 8 and tissues recovered for viral titers. Acyclovir (Burroughs Wellcome) was added to drinking water following the sacrifice of the day 2 animals. Titers were performed in duplicate on Vero cell monolayers shown as an average of 3 mice for each time point. (**a**) HSV-1 and HSV-2 grew equally well in the eyes. (**b**) HSV-2 achieves titers in trigeminal ganglia that are two logs higher than those achieved by HSV-1, despite the use of acyclovir. (**c**) HSV-2 spreads to the brain and achieves higher titers than HSV-1, despite the use of acyclovir. These results are in keeping with the known greater neurovirulence of HSV-2.

ratios of HSV-2 : HSV-1 promoter activity to those shown in Figure 1 argues that the major determinants of their activity *in vitro* reside well within the bounds of the sequences analyzed. Thus, any differences in construction of the plasmids studied do not explain the greater activity of the HSV-2 LAT promoter *in vitro* than of the HSV-1 LAT promoter.

HSV-2 achieves higher titers in trigeminal ganglia and brain after corneal inoculation

Having shown that the HSV-2 LAT promoter is more potent than the HSV-1 promoter *in vitro*, we wished to determine whether there are corresponding differences in LAT expression *in vivo*. Lacking an ability to define directly the promoter activity *in vivo*, we analyzed LAT accumulation in latently infected mouse trigeminal neurons. We inoculated mouse corneas with equivalent doses of strains of each virus, each sufficiently virulent to cause acute ocular disease and fatal encephalitis. Acyclovir treatment was begun on day 2 so that the animals, particularly those infected with HSV-2, could survive these acute infections. Beginning treatment on day 2 allowed abundant virus to reach the ganglia while limiting subsequent spread to and replication within the brain. The courses of the acute HSV-1 and HSV-2 infections in mouse eyes and spread of the viruses proximally to ganglia and brain were assessed by titration of tissue specimens. Four-week-old female C3H mice were inoculated bilaterally with 5×10^4 p.f.u. of HSV-1 or HSV-2 (total of 10^5 p.f.u. per mouse) by corneal scarification. Mice from each group were sacrificed on days 1, 2, 4, 6 and 8. Eyes, trigeminal ganglia, and brains were removed with sterile instruments, homogenized, and dilutions titered on duplicate Vero cell monolayers. Figure 2a shows that HSV-1 and HSV-2 replicate efficiently in mouse eyes; however, titers of HSV-2 exceeded those of HSV-1 in the trigeminal ganglia and brains between days 2 and 6 (Figure 2b and c).

Quantitative competitive PCRs performed on DNA extracted from acutely infected trigeminal ganglia yielded results that were concordant with those of the more insensitive virus titration assays (Figure 3), thereby validating the quantitative PCR assay. Specifically, levels of HSV-2 DNA were 2 log units higher in acutely infected trigeminal ganglia on days 2 and 4 than HSV-1. Thus, even in the presence of acyclovir, HSV-2 remains demonstrably more neuroinvasive in the mouse model than HSV-1, with greater spread to the trigeminal ganglia and brain.

Reactivation of HSV-1 and HSV-2 from trigeminal ganglia ex vivo *is equivalent*

As seen in Figure 4, latent HSV-1 and HSV-2 can be reactivated from mouse trigeminal ganglia with virtually identical kinetics and efficiency. The

fact that both viruses reactivated efficiently confirms that both established latency in trigeminal ganglia. These data do not indicate whether the viruses possessed comparable potentials to reactivate *in vivo*. In fact, HSV-1 and -2 do not cause recurrent disease in mice. Furthermore, given the potent physical and biochemical stimulus to virus reactivation inherent to the explant cocultivation assay, however, the efficient reactivation of both HSV-1 and HSV-2 in this assay does not imply that there are comparable levels of HSV-1 and HSV-2 genome held latent in the ganglia. It was, then, important to address this issue.

HSV-2 DNA persists in higher copy numbers in latently infected trigeminal ganglia

The comparative abilities of HSV-1 and HSV-2 to establish latency were examined by quantitative competitive PCR using two independent sets of PCR primers and competitor plasmids representing sequences in the regions of the major LATs and the gC genes of each virus (Figure 5). In preliminary studies, we found that the most reproducible and internally consistent results were obtained with PCR assays involving direct incorporation of ^{35}S-dATP for quantitation of HSV-1 gC and HSV-2 LAT region sequences, Southern hybridization with ^{32}P-dATP end-labeled internal oligonucleotides for quantitation of HSV-1 LAT region sequences, and direct incorporation of ^{32}P-dCTP for quantitation of HSV-2 gC DNA. Each DNA sample was amplified in both target regions in duplicate, and the number of genomic copies detected varied consistently by less than one-half log (a threefold difference) from experiment to experiment.

Examples of such quantitative competitive assays are shown in Figure 6. In the experiment shown, the upper bands represent the competitors, created by the addition of a random 50-base-pair insert into the wild-type sequences. The lower bands represent the wild-type sequences from the amplified, latently infected trigeminal DNA. Equivalence of band intensity reflects the presence of equimolar amounts of competitor and wild-type HSV DNA in the sample. Copy numbers of HSV genome were calculated based on the known amount of competitor DNA and its molecular weight.

As shown in Table 1, the quantity of latent HSV-1 DNA ranged from 6.8×10^3 to 2.1×10^4 genome copies per mouse trigeminal ganglion. The geometric mean copy number of latent HSV-2

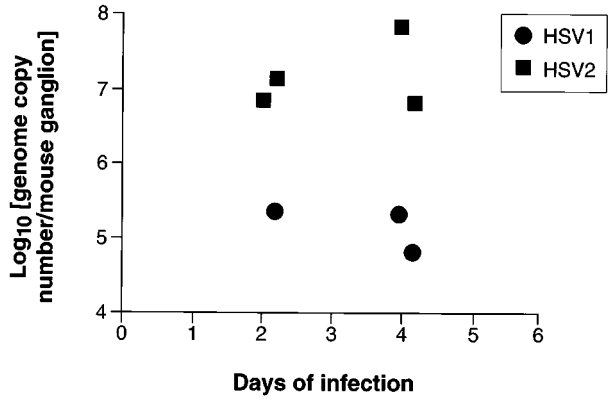


Figure 3 Viral DNA content in acutely infected ganglia. Quantitative competitive PCR on DNA extracts from acutely infected mouse trigeminal ganglia indicate higher genome copy numbers in mice infected with HSV-2 than with HSV-1 both on day 2 prior to start of acyclovir therapy and on day 4. These results corroborate those seen by titering acutely infected mouse trigeminal ganglia (Figure 2b) and validate the PCR assay.

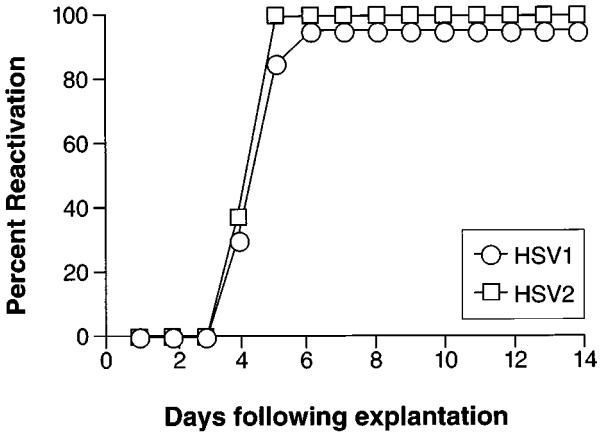


Figure 4 Explant reactivation of latent virus. Trigeminal ganglia were harvested 28 days after infection with either HSV-1 or HSV-2. The ganglion explants were cocultivated on Vero cell monolayers using N,N′-hexamethylene-bis-acetamide in Eagle's Modified Essential Media supplemented with 2% fetal bovine serum. Results indicate that HSV-1 and HSV-2 reactivate equally well from mouse ganglia *in vitro*.

Table 1 The geometric mean numbers of latent HSV-1 and HSV-2 DNA copies and LAT copies in trigeminal ganglia*.

	HSV-1 (n=*6*)	*HSV-2* (n=*5*)	P *value*$^+$
DNA copy no.	1.2×10^4 ($6.8 \times 10^3 - 2.1 \times 10^4$)**	6.8×10^4 ($2.1 \times 10^4 - 2.1 \times 10^5$)	0.003
LAT copy no.	2.6×10^7 ($9.1 \times 10^6 - 2.7 \times 10^8$)	2.2×10^8 ($1.7 \times 10^8 - 5.3 \times 10^8$)	0.01
LAT copies per latent genome	2.2×10^3	2.5×10^3	>0.20

*Copies per 200 ng of ganglion DNA or RNA. **Range of experimental values. $^+$By analysis of variance.

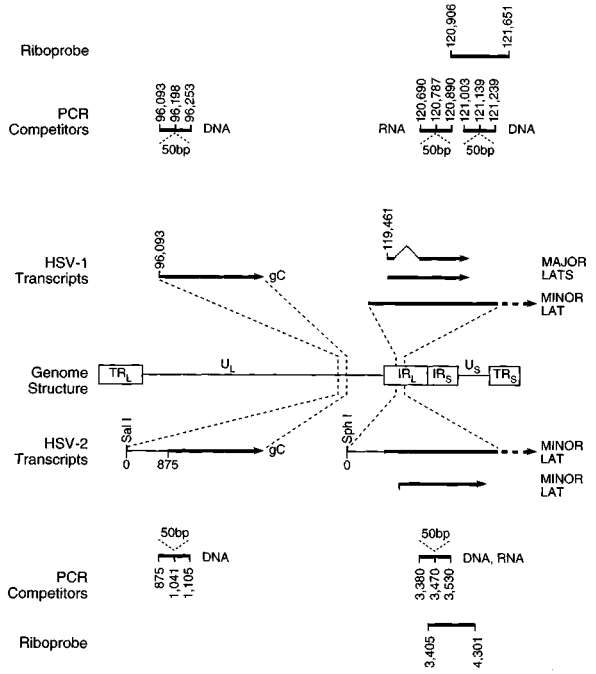


Figure 5 Diagram of the HSV genome depicting the long and short terminal (TR_L, TR_S) and internal (IR_L, IR_S) repeats and unique long (U_L) and short (U_S) regions with schematic drawings of structures pertinent to the present studies of HSV-1 (above) and HSV-2 (below). Line 1: Riboprobe used for *in situ* hybridization of tissues latently infected with HSV-1. Line 2: PCR competitors indicating the 50-base-pair insert for DNA PCR (glycoprotein C[gC] and major LAT) and RNA PCR (major LAT). Line 3: HSV-1 transcripts of gC and major and minor LAT species. Line 4: Genome structure. Line 5: HSV-2 transcripts of gC and major and minor LAT with restriction enzyme cutting sites indicated. Numbers given are relative to the restriction enzyme sites. Line 6: PCR competitors showing the 50-base-pair insert for DNA and RNA PCR. Numbers given are relative to the restriction enzyme cutting sites. Line 7: Riboprobe used for *in situ* hybridization of tissues latently infected with HSV-2.

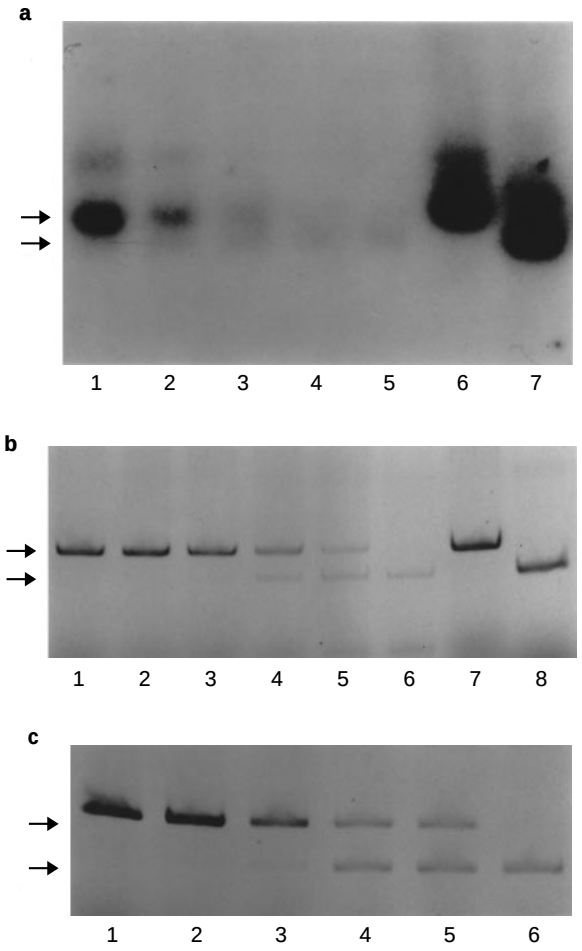


Figure 6 PCR quantitation of latent viral DNA. Quantitative competitive PCR was performed on DNA extracts of mouse trigeminal ganglia latently infected with HSV-1 or HSV-2. (**a**) Southern blot of QC-PCR reactions using the competitor and primers in the region of the HSV-1 major LAT and probed with a ^{32}P-dATP end-labeled internal oligonucleotide. Lanes 1–5 are samples from PCR reactions each containing 100 ng of mouse genomic DNA and dilutions of competitor plasmid (30 fg, 10 fg, 3 fg, 1 fg, 300 ag). In the experiment shown, competition occurs at 3 fg of competitor (lane 3) or a calculated 2.1×10^4 copies per pair of trigeminal ganglia. Lanes 6 and 7 are competitor and wild-type sequences only, respectively. (**b**) QC-PCR results using the competitor and primers in the region of HSV-1 gC detected by direct incorporation of ^{35}S-dATP. Lanes 1–6 are samples from PCR reactions each containing 100 ng genomic DNA and dilutions of competitor plasmid (300 fg, 100 fg, 30 fg, 10 fg, 3 fg, 1 fg). Competition here occurs at 3 fg of competitor (lane 5) or a calculated 2.1×10^4 copies per pair of trigeminal ganglia. Lanes 7 and 8 contain competitor and wild-type sequences only, respectively. (**c**) QC-PCR results using the competitor and primers in the region of HSV-2 major LAT detected by direct incorporation of ^{35}S-dATP. Lanes 1–6 are samples from PCR reactions each containing 100 ng genomic DNA and dilutions of competitor plasmid (300 fg, 100 fg, 30 fg, 10 fg, 3 fg, 1 fg). Competition in this example occurs at 10 fg of competitor (lane 4) or an estimated 6.9×10^4 copies per pair of trigeminal ganglia.

DNA in similarly infected trigeminal ganglia averaged nearly sixfold greater, ranging from 2.1×10^4 to 2.1×10^5 genome copies per ganglion (P=0.003). These results, again, indicate that both HSV-1 and HSV-2 efficiently establish latency in mouse trigeminal ganglia following corneal inoculation; however, significantly greater numbers of HSV-2 genomes persist as compared with HSV-1.

HSV-2 LATs accumulate to higher levels in latently infected trigeminal ganglia

Levels of expression and accumulation of HSV-1 and HSV-2 LAT RNAs in the trigeminal ganglia were quantitated by competitive RT–PCR. Known amounts of *in vitro*-transcribed competitor RNAs were added to trigeminal ganglion RNA, reverse transcribed, and amplified by PCR. An example of these assays is seen in Figure 7, in which the upper bands represent the competitor created by the addition of a random 50-base-pair insert into the wild-type major LAT sequence. The lower bands represent the wild-type sequence of major LAT

amplified from RNA extracts of latently infected trigeminal ganglia. Again, equivalence of band intensity reflects the presence of equimolar amounts of competitor and wild-type HSV LAT RNAs in the sample. Copy numbers of RNA LATs were calculated based on the known amount of competitor RNA and its molecular weight. As shown in Figure 5, the number of copies of major LATs per ganglion latently infected with HSV-1 ranged from 9.1×10^6 to 2.7×10^8. The geometric mean number of major LATs in ganglia latently infected with HSV-2 averaged over eightfold higher, ranging from 1.7×10^8 to 5.3×10^8 copies (P=0.01). If, however, we adjusted the numbers of LATs for differences in the quantities of latent genomes, HSV-2 and HSV-1 expressed equivalent numbers of LATs per genome.

In situ *hybridization reveals more neurons expressing HSV-2 LATs*

Latently infected mice (8 weeks post infection) were perfused with buffered 4% formaldehyde peri-mortem, and 5-micron sections of trigeminal ganglia were hybridized to 1 kb ^{35}S-dUTP-labeled riboprobes generated from the regions of the major LAT for each virus. Neurons expressing LAT were detected by *in situ* hybridization of ganglia from two of three mice latently infected with HSV-1 and three of three mice latently infected with HSV-2. No signals were detected in sections hybridized to antisense probes or in sections treated prior to hybridization with RNase A and T1 (not shown). There was no loss of signal seen on sections treated with RNase-free DNase1 prior to hybridization (also not shown). Figure 8 shows more LAT-positive neurons in HSV-2-infected than in HSV-1-infected ganglia. Segments of the ganglia contained most of the *in situ*-positive signals, indicating a clustering of neurons serving the ophthalmic distribution.

To estimate the proportion of LAT-expressing neurons, eight noncontinuous ganglion sections from each of six mice (three HSV-1-infected, three HSV-2-infected) were hybridized, and all *in situ*-positive and -negative neurons were counted and averages calculated. The frequency of LAT-expressing neurons in HSV-2 latently infected ganglia was one in 34 neurons, while the frequency of LAT-expressing neurons in HSV-1 latently infected ganglia was about 1/9th that for HSV-2, or one in 288 neurons.

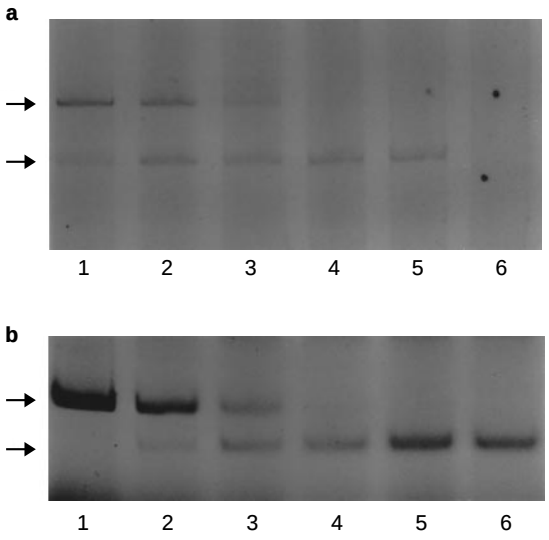


Figure 7 RT–PCR quantitation of LATs produced in latently infected trigeminal ganglia. Quantitative competitive RT–PCR was performed on RNA extracts of mouse trigeminal ganglia latently infected with equivalent doses of HSV-1 or HSV-2. (**a**) QC-RT–PCR results using *in vitro*-generated competitor and primers in the region of the HSV-1 major LAT detected by direct incorporation of ^{35}S-dATP. Lanes 1–5 are samples from RT–PCR reactions containing 100 ng of genomic RNA and dilutions of competitor transcripts (1 pg, 300 fg, 100 fg, 30 fg, 10 fg). Competition in the experiment shown occurs at 100 fg of competitor (lane 3) or a calculated 9.1×10^7 copies per trigeminal ganglion. Lane 6 shows the RT–PCR products of genomic RNA without added reverse transcriptase. (**b**) QC-RT–PCR results using *in vitro*-generated competitor and primers in the region of HSV-2 major LAT detected by direct incorporation of ^{35}S-dATP. Lanes 1–6 are samples from RT–PCR reactions each containing 100 ng of genomic RNA and dilutions of competitor transcripts (1 ng, 300 pg, 100 pg, 30 pg, 10 pg, 3 pg). Competition occurs at 100 pg of competitor (lane 3) or calculated 5.7×10^{10} copies per trigeminal ganglion.

Discussion

Using a transient expression assay, we determined the HSV-2 LAT promoter to be 6–10 times more potent than the HSV-1 LAT promoter *in vitro*, depending on whether epithelial (HeLa) or neural (PC12) cell lines were transfected (Figure 1). In latently infected mice, by both quantitative-competitive RT–PCR (Table 1) and *in situ* hybridization (Figure 8) HSV-2 LATs accumulated to higher numbers than HSV-1 LATs, seeming to suggest that the HSV-2 LAT promoter is also more potent *in vivo*. However, careful quantitation of latent viral DNA load by PCR showed that more copies of the HSV-2 genome persisted than HSV-1, but the quantity of LATs per latent genome and per *in situ*-positive neuron were comparable.

It was necessary in these experiments to briefly treat the mice with acyclovir. In the absence of acyclovir, HSV-2 – which is more pathogenic than HSV-1 – quickly achieves higher titers in neural tissues, kills a far greater proportion of the mice, and thus would leave few animals for which the numbers of latent viral sequences, the relative potency of the LAT promoters and LAT accumulation could be determined. Acyclovir muted the progression of virus replication so that the courses of the acute infections in the eyes were equivalent

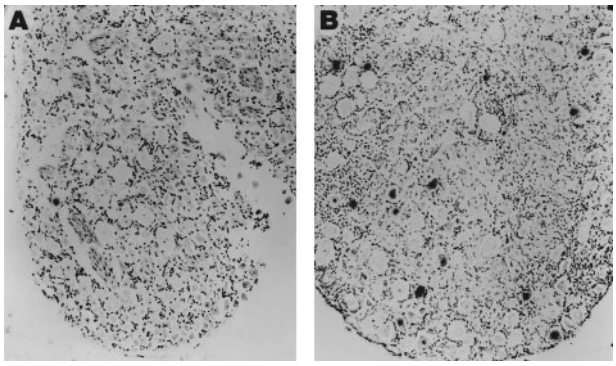


Figure 8 Sections of latently infected trigeminal ganglia probed for HSV-1 or HSV-2 LATs by *in situ* hybridization. (**a**) Low power (100×) photomicrograph of a section from HSV-1 latently infected trigeminal ganglia showing one intensely labeled LAT-positive cell. (**b**) Low power (100×) photomicrograph of a section of HSV-2 latently infected trigeminal ganglia showing 12 LAT-positive cells.

and the rates of mortality, and ultimately, the levels of latent viral genomes were more comparable for HSV-1 and -2. Although the use of acyclovir prevents our drawing fine distinctions on the relative biology of the acute infections it does not influence our ultimate conclusions regarding later accumulation of LATs *in vivo*.

What might be the implications of these observations? First, from a clinical standpoint, HSV-2 infections are known to recur more frequently in humans than HSV-1 infections and it is desirable to understand whether LAT expression and accumulation might influence that behavior (Hanna *et al*, 1976; Lafferty *et al*, 1987; Wald *et al*, 1995). Since LAT expression appears to permit efficient reactivation of latent virus, if HSV-2 were to possess a more potent LAT promoter, or at least its LATs were more stable and accumulated to higher levels than HSV-1 LATs, they could contribute to the higher rate of HSV-2 recurrences in humans.

Although the transient expression assays suggested that the HSV-2 LAT promoter is more potent (Figure 1), the *in vivo* studies did not confirm greater HSV-2 LAT content per latently infected neuron (Table 1). Clearly, these viral promoters evolved to respond to the regulatory milieu of the sensory neuron in a manner that is not well emulated by cultured cells, even ones of neural lineage like PC12 cells. It is possible that terminally differentiated neurons would have given different results than we obtained with these PC12 cells. Nonetheless, that the LAT content of latently infected neurons proved comparable suggest that any differences in rates at which HSV-1 and HSV-2 reactivate do not arise because of differences in LAT accumulation. Rather, the greater rate of HSV-2 recurrence might reflect its capacity to persist in more neurons and at higher genome copy levels than HSV-1. Thus, reactivation would depend largely on the quantity of latent virus and not the relative expression and accumulation of LATs. Although this conclusion is not supported by the recent report by Thompson and Sawtell (1997) describing impaired rates of latency and reactivation in HSV-1 LAT-negative mutants, it is consistent with work involving mice by Maggioncalda *et al* (1996). It also agrees with our recent analyses of LAT-impaired and LAT-negative mutants of HSV-2 (Wang *et al*, 1997). Even more recent experiments in which we directly correlated latent viral load with the frequency of spontaneous virus reactivation in the guinea pig further support this conclusion (Lekstrom-Himes *et al*, manuscript submitted).

Materials and methods

Cells and viruses

Vero cells, HeLa cells, and rat PC12 cells were grown in Dulbecco's Modified Eagle medium (Quality Biological, Inc., Gaithersburg, MD) supplemented with 10% fetal calf serum (Sigma Chemical, St. Louis, MO) and 1% L-glutamine/aureomycin/streptomycin/penicillin (Quality Biological) in a 5% CO_2 humidified chamber at 37°C. PC12 cells are commonly used neural cells. They are adrenal chromaffin tumor cells of neural crest origin that differentiate into neuronal cells when treated with nerve growth factor. Stocks of HSV-1 strain 17 syn+ and HSV-2 strain 333 were prepared in Vero cells, divided into cell-free aliquots, titered, and stored at −80°C until use.

Constructs

The construct containing the HSV-2 LAT promoter is called p-392 (Wang *et al*, 1995). It was created by inserting the HSV-2 strain 333 DNA fragment from nucleotides −392 to +38 relative to the transcription start site into the plasmid pCAT.Basic. The construct representing the HSV-1 LAT promoter is called pH280 and contains the HSV-1 strain 17 promoter from nucleotides −280 to −1 relative to the LAT start site inserted into pCAT.Basic at its *Hind*III site (Figure 1).

Transfection and CAT assay

Cells used for transient transfection assays were plated onto 60 mm tissue culture dishes 2 days before transfection and maintained at 37°C, 5% CO_2. Five μg of the appropriate DNAs were transfected by the calcium phosphate coprecipitation method of Graham and Van der Eb. Two days later, the cells were harvested and extracted in 0.25 M pH 7.4 Tris-HCl, with three cycles of freezing on dry ice and thawing at 37°C.

CAT assays were performed as described previously (Krause *et al*, 1991; Wang *et al*, 1995). Briefly, cell extracts were mixed with 2 μl (50 nCi) of [^{14}C]chloramphenicol (Amersham, Arlington

Heights, IL), 20 μl of 4 mM acetyl-CoA and 0.25 M pH 7.4 Tris-HCl to make a final volume of 150 ml. The mixtures were incubated at 37°C for 1 h. Reactions were stopped by extraction with 1 ml cold ethyl acetate, and the organic phases were recovered and lyophilized. The pellets were resuspended in 30 μl of ethyl acetate and subjected to thin layer chromatography (TLC). The radioactivity of the TLC sheet was quantitated using a Phosphor Imager 445 SI (Molecular Dynamics, Inc., Sunnydale, CA). To ensure that the CAT assays were conducted within their linear ranges, the amounts of cell extracts in each assay were adjusted (about 15 μg of HeLa cell extract and 4 μg of PC12 cell extract) so that the percent acetylation of the p-392 construct was about 50%. The percent acetylation in each experiment was then converted into percent CAT activity relative to that of the construct p-392 in the same experiment.

Mice
Four- to 6-week-old inbred C3H female mice were housed in AALAC-approved facilities and studied according to approved protocols. Mice were anesthetized with ketamine and xylazine and inoculated by corneal scarification with 10^5 p.f.u. of virus per mouse in a 2.5 to 5 μl volume. Acyclovir 1.2 mg/ml (Burroughs Wellcome, Research Triangle Park, NC) was added to the drinking water on day 2 and continued for 14 days in all experiments because of the high rates of animal mortality, particularly following infection with HSV-2. In the absence of acyclovir we could not identify an equivalent infectious inoculum for both HSV-1 and -2 that would result in detectable latency in most HSV-1 infected animals and the survival of most HSV-2 infected animals.

Virus titration
At desired times after infection animals were sacrificed for quantitation of virus in particular anatomical sites. Eyes and trigeminal ganglia were plated into 1 ml of Dulbecco's Modified Eagle medium on ice. Brains were placed into Dulbecco's Modified Eagle medium on ice at approximately 10% wt/vol concentration. Tissues were homogenized using a tissumizer (Tekmar, Cincinnati, OH), and dilutions were plated onto Vero cell monolayers in duplicate. Following 1 h adherence, cells were washed and overlaid with medium containing 0.5% human immunoglobulin. Plaques were counted 2 days later.

Explant cocultivation
Trigeminal ganglia were placed whole onto Vero cell monolayers in 6-well dishes and overlaid with Dulbecco's Modified Eagle medium containing 2% fetal calf serum, 1% L-glutamine/aureomycin/streptomycin/penicillin (Quality Biological, Inc.), amphotericin (3 μg/ml), and N,N′-hexamethylene-bis-acetamide (HMBA; Sigma Chemical) (1 mg/ml). Monolayers were observed daily for signs of virus-induced cytopathic effects.

Extraction of nucleic acids from ganglia
Mice were sacrificed by cervical dislocation and their tigeminal ganglia removed with sterile instruments. Ganglia were placed into 300 μl of cell lysis solution (0.001% SDS, 0.0001% Triton X-100 in buffer containing Tris-HCl 10 mM and EDTA 1 mM) containing 0.6 mg/ml proteinase K (Sigma Chemical) and incubated overnight at 56°C. DNA was extracted using a Puregene kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. RNA was extracted using the Purescript kit (Gentra Systems) according to the manufacturer's instructions following mechanical dispersion of the ganglia using 16- to 25-gauge needles and syringes in succession. DNA was stored in buffer containing Tris-HCl 10 mM and EDTA 1 mM at 4°C. Ganglion RNA was stored in DEPC-treated water at −80°C. The yields of genomic RNA and DNA were calculated based on UV spectrographic absorbance at 260 nm.

Quantitative competitive DNA PCR
Competitor plasmid constructs were made by PCR extension of the wild-type sequence internal to the primers, as shown in Figure 5. Known copy numbers of the competitor plasmids were added to each reaction tube containing 5 μl of 10 × PCR buffer (Life Technologies, Gibco, Gaithersburg, MD), 0.25 μM of each primer, 0.15 mM of each triphosphorylated deoxynucleotide, 1.5 mM $MgCl_2$, 5% glycerol, 27 μl of sterile water and 100 ng of genomic DNA. Taq polymerase (Life Technologies, Gibco), 1.5 units per reaction tube, was added during the first annealing cycle.

HSV-1 glycoprotein C (gC) and HSV-2 latency-associated transcript (LAT)-region DNA fragments were detected by the addition of 2.5 μCu of ^{35}S-dATP (Amersham International, Birmingham, UK) to each reaction tube for labeling by direct incorporation. DNA fragments in the regions of HSV-2 gC were detected by the addition of 2.5 μCu of ^{32}P-dCTP (Amersham) to each reaction tube for labeling by direct incorporation. HSV-1 LAT region sequences were detected by Southern hybridization using ^{35}S-dATP end-labeled internal oligonucleotides. Primer sequences and cycling times are given in Table 2. Samples were amplified in a Perkin Elmer T 1000 thermal cycler (Perkin-Elmer Corp., Norwalk, CT). DNA fragments from amplified wild-type and mutant sequences were separated electrophoretically in 10% nondenaturing polyacrylamide gels and exposed to Kodak XAR-2 film for 5 days. Quantitation was determined by a phosphorimager and related software (Molecular Dynamics, Inc.). All quantitative competitive DNA PCR studies were accompanied by simultaneous studies of positive

Table 2 Specifications for DNA PCR of HSV-1 and HSV-2.

Virus type	*Amplified region*	*Method of detection*	*Primer sequence*	*Cycling parameters*
HSV-1	Major LAT	Southern blot hybridization	5′-gccttacgtgaacaagacta	94°C, 3 min; 55°C; 72°C, 3 min
			3′-tcatccagaggctgttccac	94°C, 1 min; 55°C, 1 min; 72°C, 1 min × 20 cycles
			Internal-atcctggacatggagacta	94°C, 30 s; 55°C, 30 s; 72°C, 30 s × 40 cycles
	gC	Direct incorporation	5′-gtgtgtgatgatttcgccataacac	94°C, 3 min; 60°C, 5 min; 72°C, 3 min
			3′-actgaaaaacgacctccacacgga	94°C, 1 min; 60°C, 1 min; 72°C, 1 min × 10 cycles
				94°C, 30 s; 60°C, 30 s; 72°C, 30 s × 40 cycles
HSV-2	Major LAT	Direct incorporation	5′-gccagacgtgcgtgctctgc	94°C, 3 min; 55°C, 5 min; 72°C, 3 min
			3′-tgttggtctttatcatagaacag	94°C, 1 min; 55°C, 1 min; 72°C, 1 min × 20 cycles
				94°C, 30 s; 55°C, 30 s; 72°C, 30 s × 40 cycles
	gC	Direct incorporation	5′-tgtggtcgtggtgctggccaatgcct	94°C, 3 min; 60°C, 5 min; 72°C, 3 min
			3′-catcgcacgggctccgaggatgtct	94°C, 1 min 60°C, 1 min; 72°C, 1 min × 10 cycles
				94°C, 30 s; 60°C, 30 s; 72°C, 30 s × 40 cycles

and negative controls and a control panel containing known amounts of wild-type genomic sequences and known dilutions of the competitors.

Quantitative competitive reverse transcriptase (RT) PCR

Competitor RNA transcripts were generated according to the manufacturer's instructions by *in vitro* transcription (Promega, Madison, WI) of plasmid constructs made by PCR extension of the wild-type sequences internal to primers. Known copy numbers of competitor transcripts were added to each reaction tube containing 100 ng sample RNA and was reverse transcribed using the 3′ primer. HSV-1 and HSV-2 RNA PCR were performed by first generating cDNA in a reaction containing 6 μl of 5 × RT buffer (Life Technologies Gibco), 40 units of RNasin (Boehringer Mannheim, Gaithersburg, MD), 1 mM DTT, 2.5 μM 3′ primer, 0.5 mM of each triphosphorylated deoxynucleotide, 9.5 μl of DEPC-treated sterile water and 600 units of MMLV RT (Life Technologies, Gibco) for 1 h at 39°C followed by 5 min at 94°C. Products of both the HSV-1 and HSV-2 reverse transcription reactions were amplified as described and electrophoretically separated on 10% nondenaturing polyacrylamide gels. All RNA samples were treated with RNase-free DNase1 (Boehringer Mannheim) for 40 min at 37°C, then inactivated for 20 min at 65°C in buffer containing 3 mM $MgCl_2$ prior to transcription. All quantitative competitive RT–PCR studies were accompanied by simultaneous assay of positive and negative controls, RT-negative controls, and a panel of control specimens containing known amounts of wild-type, *in vitro*-generated RNA and known dilutions of competitor RNAs.

In situ *hybridization*

Mice used for *in situ* hybridization were perfused peri-mortem with 4% buffered formaldehyde. Five-micron sections of trigeminal ganglia were prepared on silanized slides with RNase precautions. Sections were probed with ^{35}S-dUTP-labeled riboprobes using 10^6 c.p.m./section as described (Croen *et al*, 1987, 1991). Slides were developed after a 4-day exposure. The specificity of the results was supported by the maintenance or loss of signal on control sections treated with RNase-free DNase1 or RNaseA and RNase T1, respectively. Additional sections were also hybridized to riboprobes generated from the antisense DNA strand.

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