The Characteristic Site-specific Reactivation Phenotypes of HSV-1 and HSV-2 Depend upon the Latency-associated Transcript Region

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Summary

After replication at sites of initial inoculation, herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) establish lifelong latent infections of the sensory and autonomic neurons of the ganglia serving those sites. Periodically, the virus reactivates from these neurons, and travels centripetally along the neuronal axon to cause recurrent epithelial infection. The major clinically observed difference between infections with herpes simplex virus type 1 and type 2 is the anatomic site specificity of recurrence. HSV-1 reactivates most efficiently and frequently from trigeminal ganglia, causing recurrent ocular and oral-facial lesions, while HSV-2 reactivates primarily from sacral ganglia causing recurrent genital lesions. An intertypic recombinant virus was constructed and evaluated in animal models of recurrent ocular and genital herpes. Substitution of a 2.8-kbp region from the HSV-1 latency-associated transcript (LAT) for native HSV-2 sequences caused HSV-2 to reactivate with an HSV-1 phenotype in both animal models. The HSV-2 phenotype was restored by replacing the mutated sequences with wild-type HSV-2 LAT-region sequences. These sequences or their products must act specifically in the cellular environments of trigeminal and sacral neurons to promote the reactivation patterns characteristic of each virus.

Primary or initial infections with herpes simplex virus type 1 and 2 (HSV-1 and HSV-2)[1] are clinically indistinguishable. While social habits and other factors contribute to varying sites of initial infections, the predilection of virus to reactivate from specific ganglionic sites maintains the well-known associations of HSV-2 with genital herpes and of HSV-1 with ocular and oral-facial herpes (1, 2). The HSV latency-associated transcripts (LATs) are a family of transcripts specified by the genomic long repeat regions. The more abundant nuclear LAT introns (2.2 kbp in HSV-2 [3–5], 2.0 kbp and 1.5 kbp in HSV-1 [6–9]) are processed via a splicing mechanism from less stable ~8.5-kbp primary LAT transcripts (10). The LATs of HSV-1 and HSV-2 (11–13) are required for efficient reactivation from latency in vivo, but have not been shown to influence acute replication of virus or establishment of latency (14–19) in most animal models, although there is some evidence suggesting an effect on establishment of latency in a mouse model of infection with HSV-1 (20). The sequences of the LAT regions differ significantly between HSV-1 and HSV-2 (4, 21, 22), leading to the hypothesis that differences in the LATs could be responsible for site-specificity of virus recurrence. To test this hypothesis, we introduced an alteration into HSV-2 strain 333 that substituted the LAT region from HSV-1 strain 17syn+ for native HSV-2 LAT sequences, and constructed a rescuant of this mutant. We then tested the ability of parent, mutant, and rescuant viruses to replicate and reactivate in a rabbit eye model of recurrent ocular herpes, and a guinea pig model of recurrent genital herpes.

[1] *Abbreviations used in this paper:* HSV-1 and HSV-2, herpes simplex virus type 1 and 2; LAT, latency-associated transcript; PI postinoculation; PRK, primary rabbit kidney.

Materials and Methods

Cells and Viruses. Vero cells were obtained from American Type Culture Collection (ATCC, Rockville, MD), and maintained in 1:1 minimum essential medium/medium 199, (Quality Biologicals, Gaithersburg, MD) with 10% heat-inactivated fetal bovine serum (Quality Biologicals, Gaithersburg, MD) and 1% GASP (a mixture of L-glutamine, aureomycin, streptomycin, and

penicillin, Quality Biologicals, Gaithersburg, MD). Herpes simplex virus type 2, strain 333, was obtained from Gary Hayward (Johns Hopkins University, Baltimore, MD). Herpes simplex virus type 1, strain 17syn+, was obtained from Dr. John Hay (SUNY-Buffalo, Buffalo NY). Virus stocks were grown in Vero cells, and plaque titered in duplicate before inoculation of animals.

Mutant Virus Construction. Sequences homologous to the Xho1 site in the HSV-2 LAT region were introduced via site-directed mutagenesis into an Avr2-Alu1 clone of the HSV-1 LAT region sequences. This mutation was verified by DNA sequencing. For the mutant virus, the 2820 bp HSV-1 strain 17syn+ Not1-Xho1 fragment was inserted between Not1 and Xho1 sites of a clone spanning the HSV-2 strain 333 Sph1-BamH1 fragment shown in Fig. 1. This DNA was used to construct the mutant virus by homologous recombination with HSV-2 strain 333 DNA as described (18). After identification of mutant virus, plaque purification was performed until Southern hybridization identified no evidence of contamination with the parent. At this point, two additional plaque purifications were performed to yield a stock of HSV-2 333/LAT1. This procedure was repeated, using wild-type Sph1-BamH1 DNA and 333/LAT1 DNA to produce the rescuant, HSV-2 333/LAT1R. Additional Southern hybridizations using ^{32}P-radiolabeled probes were performed to validate the correctness of each virus stock.

Animal Studies. All animal experiments were performed in AAALAC-certified facilities. New Zealand white rabbits weighing 2,000–2,500 *g* were infected with each virus in three independent experiments. Scarified (in one experiment) or unscarified (in two experiments) rabbit eyes were inoculated by gentle rubbing of a 25-μl suspension containing 10^5 PFU of HSV-1 17syn+, HSV-2 333, HSV-2 333/LAT1, or HSV-2 333/LAT1R. In each experiment, except for virus inoculated, animals infected with each virus were treated identically. After inoculation, all manipulations and observations were performed by investigators who were masked as to inoculating virus. 5–6 wk after inoculation, transcorneal iontophoresis of 0.01% epinephrine (0.8 mA for 8 min, once a day for three consecutive days) was used to attempt induction of ocular viral shedding from unscarred eyes. No eyes shed virus for any of the 3 d before iontophoresis. Eye swabs were taken daily for 7–9 d after the first iontophoresis. Swabs were cultivated on primary rabbit kidney (PRK) cells, and determined to be positive if cytopathic effect consistent with HSV infection was observed. Two independent experiments were performed in guinea pigs. Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington MA) weighing 400–525 *g* were inoculated with $10^{5.7}$ PFU of each virus on day 0 by rupture of the vaginal closure membrane with a moistened calcium alginate tipped swab and instillation of 0.1 cc virus. Lesion severity was scored daily (on a scale from 0–4) until resolution of the acute infection. Disease severity was calculated as the area under the lesion score-day curve, for the duration of the acute infection. These same guinea pigs were observed daily for recurrence frequency from days 15–63 after inoculation. All observations of the guinea pigs were performed by investigators masked as to inoculating virus. Guinea pigs that were not evaluable for the entire observation period (either primary or recurrence phase) were excluded from analysis.

Cocultivation and Quantitative Polymerase Chain Reaction. Trigeminal ganglia were removed from rabbits after completion of the epinephrine iontophoresis experiments, removed from the outer

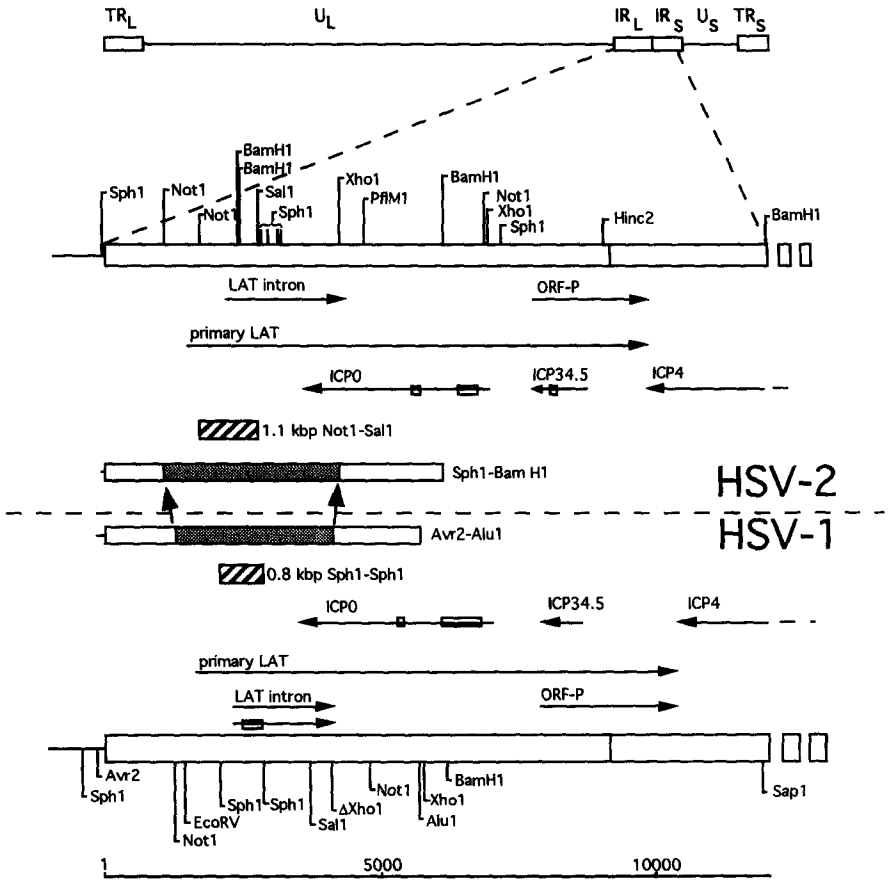

Figure 1. HSV-2 and HSV-1 LAT regions. HSV-2 sequences are shown above the dashed line, HSV-1 sequences are below the dashed line. Transcripts and restriction endonuclease cleavage sites in this region are shown for each virus. Boxes within transcripts represent introns. The 2.8 kbp of HSV-1 LAT sequence that was inserted into the Sph1-BamH1 clone including the HSV-2 LAT region is shaded. The Sph1-BamH1 fragment was also used as a probe in the Southern hybridizations, along with the HSV-1 Sph1-Sph1 and the HSV-2 Not1-Sal1 fragments (*hatchmarked boxes*). *LAT*, latency-associated transcript; *ICP0*, infected cell protein 0, an immediate-early viral transactivator; *ICP4*, infected cell protein 4, an immediate-early viral transactivator; *ICP34.5*, infected cell protein 34.5, implicated in prevention of neuronal apoptosis; *ORF-P*, open reading frame P transcript, function unknown; TR_L, terminal repeat, long; U_L, unique, long; IR_L, internal repeat, long; IR_S, internal repeat, short; U_S, unique, short; TR_S, terminal repeat, short.

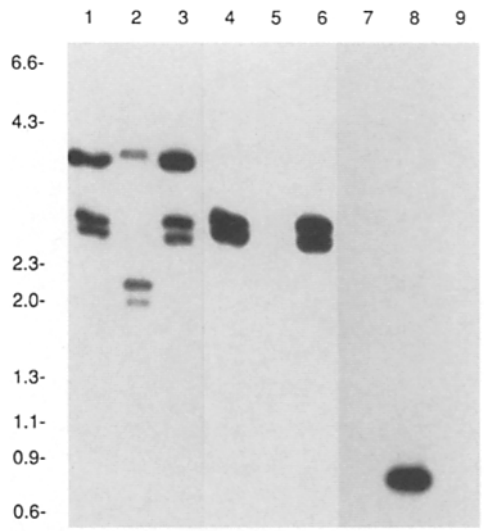

Figure 2. Southern hybridization of DNA extracted from HSV-2 333 (lanes *1*, *4*, and *7*), HSV-2 333/LAT1 (lanes *2*, *5*, and *8*), and HSV-2 333/LAT1R (lanes *3*, *6*, and *9*). DNA was cleaved with Sph1, and subjected to Southern hybridization with an HSV-2 Sph1-BamH1 probe (lanes *1–3*), an HSV-2 sequence-specific Not1-Sal1 probe (lanes *4–6*), or an HSV-1 sequence-specific Sph1-Sph1 probe (lanes *7–9*). Marker sizes (in kbp) are shown to the left of the gel.

sheath, separated into two or three pieces, and placed in tissue culture with PRK cells. Cultures were observed daily for evidence of cytopathic effect, and reported as positive on the first day that HSV cytopathic effect was evident.

Ganglionic DNA was extracted from trigeminal ganglia of three rabbits latently infected with each virus and from three uninfected rabbits. PCR controls consisted of 100 ng of DNA extracted from an uninfected rabbit trigeminal ganglion spiked with 10^1 to 10^6 copies of HSV-2 DNA in 10-fold increments. In each reaction, 100 ng (quantified spectrophotometrically) of DNA was subjected to coamplification with two primer pairs by polymerase chain reaction (94°C × 1 min, 55°C × 1 min, 72°C × 2 min) for 30 cycles. The primers were GAACCACGGGCTGATGTTTG and CAATGCGTAGACGGAGAAAAAGAG (which are specific for a 181-bp product in the HSV-2 glycoprotein C region), and CCATTCATTGACCTCCACTACATGG and TCGCTCCTGGAAGATGGTGATG (which are specific for a 134-bp product in rabbit glyceraldehyde-3-phosphate dehydrogenase). The products were alkaline denatured and subjected to slot blotting on Nytran membranes (Schleicher & Schuell, Keene, NH) using a ^{32}P-radiolabeled probes derived either by end-labeling an oligonucleotide internal to the rabbit G3PDH product (TGTTCCAGTATGATTCCACC) or by random priming of a 1-kbp gel-pure Bgl2-BstE2 fragment from within HSV-2 gC. Signals were quantified using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). For each sample, the ratio of the HSV-2/G3PDH signals was calculated and compared with scores obtained using the spiked controls. The approximate number of copies of HSV DNA in the three ganglia latently infected with each virus was calculated by linear extrapolation between the spiked control scores directly above and below those for each sample. Controls using DNA from the three uninfected rabbits and using no template (oligonucleotide primers only) gave rise to no signal attributable to HSV.

Results

Mutant Virus Construction and Evaluation. To test our hypothesis, we introduced an alteration into HSV-2 strain 333 which substituted the LAT region from HSV-1 strain 17syn+ for native HSV-2 LAT sequences, and designated this virus HSV-2 333/LAT1 (Fig. 1). The substituted sequences start upstream of the LAT promoter and include the LAT promoter, primary LAT 5′ end sequences, and most LAT intron sequences (excluding only 160 bases from the 3′ end of the LAT intron). A rescuant HSV-2 333/LAT1R was constructed by replacing the substituted HSV-1 sequences in HSV-2 333/LAT1 with parent HSV-2 sequences.

The construction of the mutant and rescuant viruses relative to the sequences of the parent virus was verified by Southern hybridization. Purified DNA extracted from HSV-2 333, HSV-2 333/LAT1, and HSV-2 333/LAT1R was cleaved with the restriction endonuclease Sph1 (Fig. 2) and probed with radiolabeled DNA fragments specific for the HSV-2 and HSV-1 LAT regions. In DNA extracted from wild-type and rescuant virus, a probe spanning the HSV-2 Sph1-BamH1 region (Fig. 2, lanes 1–3) detected a predicted 4,083-bp fragment within the long repeat, and a doublet of 3,073 and 2,829 bp representing fragments that span Sph1 sites present within the unique-long segment on either side of the HSV-2 genomic repeats and the first Sph1 site within the repeats. Smaller (<200 bp) Sph1 fragments are not visualized on this gel. Changes in the location of the first Sph1 site within the repeats led to detection of the predicted fragments of 4,169 bp for the larger band, and 2,236 and 1,992 bp for the doublet in the mutant virus. An internal HSV-1 derived 786-bp Sph1 fragment in the mutant lacks homology with the HSV-2 sequences in the probe, and is not detected. A probe spanning the HSV-2 Not1-Sal1 region (lanes 4–6), which is specific for HSV-2 sequences, detected the appropriate doublets in wild-type and rescuant virus, and showed no homology with mutant virus sequences. A probe spanning the HSV-1 786 bp Sph1-Sph1 region (lanes 7–9) detected only the 786-bp Sph1 fragment in the mutant virus, and showed no homology with wild-type or rescuant sequences. Additional Southern hybridizations with BamH1, EcoRV, Not1, Sal1, and Xho1 digests of virus DNA also yielded the predicted results when hybridized with these probes (data not shown). Each virus displayed similar one-step growth characteristics in Vero cells, indicating that the LAT substitution did not influence the ability of the mutant virus to grow in tissue culture (data not shown).

Assessment of LAT Subsitution In Vivo in Rabbit Eyes. The rabbit ocular model of HSV-1 infection mimics natural human infection with HSV-1. The virus causes acute ocular infections, establishes latency in trigeminal ganglia, and can be induced to reactivate by appropriate stimuli (14). Also similar to human infections, HSV-2 does not reactivate well in vivo from rabbit trigeminal ganglia.

After corneal inoculation with HSV-2 333, HSV-2 333/LAT1, HSV-2 333/LAT1R, or HSV-1 17syn+, all rabbits exhibited comparable acute epithelial HSV keratitis during

Table 1. *Induced Reactivation (via Ocular Iontophoresis of Epinephrine) of HSV Mutants from Latently Infected Rabbits*

Virus	No. of rabbits*	Positive eyes/ total eyes‡	Positive swabs/ total swabs§
HSV-1 17 syn+	7	10/14 (71%)	30/101 (30%)
HSV-2 333	6	1/10 (10%)	1/80 (1%)
HSV-2/LAT1	6	5/9 (56%)	14/68 (21%)
HSV-2/LAT1R	10	2/19 (11%)	3/152 (2%)

*Cumulative data from three independent experiments.
‡$P < 0.05$ by Fisher exact test for all pairwise comparisons except between 17syn+ vs. HSV-2 333/LAT1 and HSV-2 333 vs. HSV-2 333/LAT1R.
§$P < 0.001$ by Chi Square analysis for all pairwise comparisons except between 17syn+ vs. HSV-2 333/LAT1 and HSV-2 333 vs. HSV-2 333/LAT1R.

postinoculation (PI) days 3–6. All tested eyes had cleared and showed no epithelial defect by 21 d after inoculation. We enumerated ocular recurrences in three independent experiments of latently infected rabbits subjected to ocular iontophoresis of epinephrine (summarized in Table 1). The procedure efficiently induced reactivation from rabbit eyes inoculated with HSV-1 strain 17 syn+; 71% of eyes and 30% of swabs yielded virus after iontophoresis. As expected, wild-type HSV-2 333 did not reactivate well from the latently infected rabbits (10% of eyes, 1% of swabs). The rescuant 333/LAT1R behaved similarly to wild-type HSV-2 333. However, the mutant 333/LAT1 reactivated almost as efficiently as HSV-1 did (56% of eyes, 21% of swabs). All positive swabs were verified to contain the inoculated virus by Southern hybridization (data not shown). Thus, the ability to reactivate efficiently from rabbit trigeminal ganglia was conferred on HSV-2 by DNA sequences from the HSV-1 LAT region.

In Northern hybridizations of RNA extracted from latently infected rabbit trigeminal ganglia and from Vero cells productively infected with each virus, HSV-2 333/LAT1 appropriately transcribed LATs indistinguishable from those of HSV-1 17syn+, and HSV-2 333 transcribed LATs indistinguishable from those of 333/LAT1R (data not shown). There was also no difference in the quantity of LAT expressed during latent infection by the wild-type, mutant, and rescuant viruses.

Assessment of LAT Substitution In Vivo in Guinea Pig Genitalia. We next examined the effect of the LAT substitution mutation on viral infection in a guinea pig model of genital herpes. As is the case in humans, HSV causes acute infections of guinea pigs, establishes latency in sacral ganglia, and reactivates spontaneously to cause recurrent lesions (23, 24). In the guinea pig genital model, HSV-2 also recurs significantly more frequently than does HSV-1. Two independent experiments were performed in guinea pigs, each yielding similar results. The severity of the primary infections as assessed by the area under the lesion-score curve was similar for guinea pigs infected with HSV-2 333, HSV-2 333/LAT1, HSV-2 333/LAT1R, or HSV-1 17syn+, indicating no differences in the abilities of these viruses to replicate or cause acute disease in guinea pigs (Table 2).

Table 2. *Acute and Spontaneous Recurrent Infections of HSV Mutants in Guinea Pigs*

Virus*	Primary (d1-14) genital skin disease severity mean ± SE (no. of animals)	Days with recurrent genital skin lesions (d15-63) mean ± SE (no. of animals)
HSV-1 17syn+	4.3 ± 0.8 (10)	0.9 ± 0.6 (10)
HSV-2 333	5.3 ± 1.1 (9)	4.1 ± 1.6 (9)
HSV-2/LAT1	5.2 ± 0.8 (13)	1.5 ± 0.5 (11)
HSV-2/LAT1-R	4.8 ± 0.5 (14)	5.9 ± 1.0 (14)

*Cumulative data from two independent experiments.

After recovery from acute infection, guinea pigs were examined for recurrent lesions daily from days 15–63 post inoculation. Infections with HSV-2 strain 333 and HSV-2/LAT1R resulted in frequent recurrences (mean 4.1, SE 1.6, and mean 5.9, SE 1.0, respectively), while the mutant HSV-2 333/LAT1 recurred with a frequency comparable to that of HSV-1 strain 17syn+ (mean 0.9, SE 1.6, and mean 1.5, SE 0.5, respectively). Thus, higher reactivation frequencies from guinea pig sacral ganglia were associated with the presence of sequences from the HSV-2 LAT region.

Assessment of the LAT Region Substitutions on Establishment of Latency. In previous experiments, deletions in the LAT region did not influence establishment or maintenance of viral latency in rabbits (14) and guinea pigs (18), although it did in mice (20). Previous studies in which latent virus was recovered in tissue culture from explanted guinea pig sacral ganglia also showed no difference between recovery of la-

Table 3. *Virus recovery from latently infected rabbit trigeminal ganglia by cocultivation and detection of viral DNA by quantitative PCR*

	Detection by Cocultivation			PCR
Virus	No. positive/ total ganglia	Average days to positive	Range	Mean HSV genomes per 100 ng TG DNA*
			d	
HSV-1 17syn+	1/1	11	11	Not done
HSV-2 333	4/4	14	10–24	1.8×10^3
HSV-2/LAT1	2/2	21	17, 24	1.2×10^2
HSV-2/LAT1-R	5/5	16	12–17	1.0×10^2

*Mean copy number in three ganglia latently infected with each virus.

tent HSV-1 or HSV-2. In our study, virus was recovered from all rabbit ganglia subjected to explant cocultivation, and with approximately the same kinetics (Table 3). Southern hybridizations confirmed the appropriate restriction endonuclease digestion patterns for each virus recovered by cocultivation (data not shown). Similar conclusions were reached regarding latent viral DNA content in the PCR experiments. Although there was variability in the quantity of HSV DNA detected in ganglia, the quantity of viral DNA in rabbit ganglia latently infected with HSV-2 333/LAT1 was comparable to that in animals infected with the wild-type virus. This indicates that potential differences in establishment or maintenance of latency could not explain the differences in reactivation frequency.

Discussion

The present study demonstrates that site-specific virus reactivation depends upon LAT-region sequences. In the context of either HSV-1 or HSV-2, the HSV-1 LAT region is both necessary and sufficient for the HSV-1 site-specific reactivation phenotype from sacral and trigeminal ganglia. The HSV-2 LAT region is necessary for the HSV-2 phenotype of efficient reactivation from sacral ganglia, although a potential requirement for additional, as-yet unidentified HSV-2-specific viral factors is not strictly ruled out by these experiments. The precise mechanism by which the LAT region expresses this phenotype is unclear. It is conceivable, although unlikely, that the site-specific reactivation phenotype could be attributable to the small exchange of 3′ ICP0 sequences between HSV-1 and HSV-2 in the mutant virus. However, the mutation did not shift the ICP0 reading frame, and there are only minor differences in ICP0 3′ amino acid sequences between HSV-1 and HSV-2. We were unable to identify any phenotypic differences between the viruses which would be expected to be associated with alterations in ICP0 function (ICP0 appeared normal on Northern hybridizations of productively infected Vero cells [data not shown]; one-step growth, acute infections, and recovery by cocultivation of the viruses were normal).

The HSV-1 and HSV-2 LAT promoters have very similar sequences, and in this experiment did not differ substantially in their ability to direct latent transcription of LAT. Moreover, previous studies have indicated that differences in the quantities of LATs transcribed by HSV during latency are not associated with differences in recurrence phenotypes (25, 26). Thus, LAT promoter sequences are also unlikely to be directly responsible for site-specific reactivation, although we cannot exclude differences in expression during reactivation which are not evident during latency. Because the reactivation phenotype was independent of the ability to transcribe latent LAT RNA, the LAT-associated phenotype is probably not attributable to an antisense mechanism (e.g., with ICP0). The LAT phenotype also appears unlikely to be attributable to LAT sequences transcribed under the control of the LAT promoter, but downstream of the substituted Not1-Xho1 fragment. Theoretically, HSV reactivation could be controlled by another as yet unidentified transcript under the control of the LAT promoter.

There are no well-conserved potential open reading frames between HSV-1 and HSV-2 in the LAT sequences we substituted. Experiments in which viruses with mutations disrupting potential open reading frames within the HSV-1 LAT intron were evaluated in rabbits and mice, indicated that these theoretical open reading frames do not play a role in virus reactivation (27, 28). Thus, it appears unlikely that a protein encoded by LAT is responsible for its effect on reactivation.

These results imply that the LAT region confers the phenotype of efficient site-specific reactivation, which is the major clinically relevant phenotypic difference between HSV-1 and HSV-2. Site-specificity of reactivation appears to be most dependent on differences in ability to reactivate from the ganglion, rather than differences in access to the ganglion. Consistent with previous reports of LAT mutants, we identified no effect of our mutation on the ability of the virus to replicate acutely or to establish or maintain latency in either animal model. Thus, products of this genomic region (possibly the LAT RNAs themselves) must act in a specific manner within the cellular milieu of either trigeminal or sacral ganglia. This experiment definitively assigns to the LAT region an active and critical role in virus reactivation from latency. Additional studies of chimeric viruses, for example, in which HSV-2 LAT sequences are substituted for wild-type HSV-1 sequences or in which smaller regions are substituted between HSV-1 and HSV-2 will yield further insight into the role of the LAT in site-specific reactivation. Further study of interactions between cellular constituents and the LAT DNA and RNA sequences should yield a fundamental understanding of the mechanisms both of anatomic site-specific reactivation, and of herpes simplex virus reactivation in general.

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