

Expression of the Herpes Simplex Virus Type 2 Latency-associated Transcript Enhances Spontaneous Reactivation of Genital Herpes in Latently Infected Guinea Pigs

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Summary

The latency-associated transcript (LAT) is the only herpes simplex virus (HSV) gene product detectable in latently infected humans and animals. In this report, we show that a 624-bp deletion in the promoter of the HSV-2 LAT had no discernable effect on viral growth in tissue culture or in acute genital infection of guinea pigs, but impaired LAT accumulation and led to a marked decrease in spontaneous genital recurrences when compared with the behavior of wild-type and rescuant strains. Differences in the ability of the mutant to replicate, or in how readily it established or maintained latency did not account for this finding. Thus, HSV LAT expression facilitates the spontaneous reactivation of latent virus.

After replication at sites of initial inoculation, herpes simplex virus types 1 and 2 (HSV-1 and -2)¹ establish lifelong latent infections of the sensory neurons of the ganglia serving those sites. Periodically, the virus reactivates, and travels centripetally along the neuronal axon to cause symptomatic or asymptomatic recurrent mucocutaneous infections (1, 2). While many factors may influence the likelihood of a reactivation giving rise to a recurrent lesion, considerable interest has been focused recently on the molecular mechanisms of reactivation.

Of the more than 75 HSV genes, only the latency-associated transcripts (LATs) are known to be expressed during latency (3, 4). The LATs are transcribed from within the long repeats of the viral genome, and consist of both an 8–9-kb “minor LAT” species (5, 6), and the abundant “major LATs” (of about 1.9 and 1.5 kb in HSV-1 [7–10] or of 2.2 kb in HSV-2 [11–13]; see Fig. 1) which appear to be processed from the minor species (14). The major LATs overlap the 3' terminus of the viral immediate-early transactivator ICP0 in an antisense direction. The minor LATs extend even further, overlapping not only the ICP0 gene but also the gene encoding the neurovirulence

protein ICP34.5 and possibly the 3' end of the gene for ICP4, which encodes another immediate-early regulatory protein. Promoter sequences required for LAT transcription during latency reside upstream of the minor LAT (15–17). Studies of latently infected animals and humans have revealed no evidence for the translation of the LAT into a protein product. While HSV-1 and HSV-2 are genetically related viruses, and both encode LATs, the sequences of the HSV-1 and HSV-2 major LATs share essentially random homology throughout the major LAT sequences (12, 18, 19).

In humans, spontaneous reactivation of latent virus results in recurrent disease. Whereas several animal models have been used to study HSV-1 latency and the role of the LATs in the pathogenesis of infection, there is no model of latent HSV-1 infection in which spontaneous reactivation produces recurrent lesions. Hence, studies to define the effect of LAT expression on HSV-1 reactivation have used nonphysiological methods including models that use various induction stimuli in order to provoke recurrences, or the use of explant cocultivation of latently infected ganglia in tissue culture to produce *ex vivo* reactivation. Studies using a rabbit ocular model of virus reactivation have shown that LAT-deleted HSV-1 mutants reactivate with reduced frequency compared to LAT-positive viruses when reactivation is induced by ocular iontophoresis of epinephrine, but that in this model, LAT deletion does not affect the number of spontaneous culture-positive

¹ Abbreviations used in this paper: HSV-1 and -2, herpes simplex virus types 1 and 2; LAT, latency-associated transcripts; MOI, multiplicity of infection; U_L, unique long.

reactivations (20, 21). In other experimental systems, some HSV-1 mutants with deletions or interruptions in the LAT sequences or its promoter showed lower rates of reactivation (22–25), whereas others showed no effect (26–29). Because of the limitations of the HSV-1 animal models, these studies could not address the role of LAT in controlling spontaneous reactivation of latent virus. While the molecular biology of HSV-2 has been less extensively studied than that of HSV-1, there is an animal model of HSV-2 infection in which spontaneous reactivation of latent virus occurs. The guinea pig model of genital HSV-2 disease shares many of the features of genital herpes in humans, including a natural route of inoculation, generally self-limited primary infection, establishment of latency, and development of spontaneous recurrent genital lesions (30–32). In this report, we describe the construction of two HSV-2 mutants, one in which sequences from the LAT promoter were deleted and a second in which the deletion was repaired. To examine the role of LAT in the control of spontaneous reactivation, we have characterized the primary, latent, and spontaneous recurrent infections produced by these mutants in the guinea pig model of genital herpes.

Materials and Methods

Cells and Viruses. Vero and SK-N-SH cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD), and maintained in 1:1 minimum essential medium/medium 199, with 10% heat-inactivated FCS, and 1% L-glutamine/aureomycin/streptomycin/penicillin (all from Quality Biologicals, Gaithersburg, MD). Primary rabbit kidney cells were prepared from New Zealand white rabbits (HRP, Denver, PA), maintained in basal medium Eagles supplemented with 10% heat-inactivated FCS, amphotericin B, penicillin, streptomycin, and L-glutamine. HSV-2, strain 333, was obtained from Gary Hayward (Johns Hopkins University, Baltimore, MD). To determine one step growth characteristics of wild-type and mutant viruses, $\sim 10^6$ Vero cells were inoculated in duplicate at time 0 with a multiplicity of 0.1 plaque forming units/cell of each virus. For the 3 and 20 h time points, virus was allowed to adsorb for 90 min before addition of medium. Cells were scraped, freeze-thawed three times, and plaque-titered in duplicate at the specified time points.

Clones. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA), Boehringer Mannheim Corp. (Indianapolis, IN), or Life Technologies (Gaithersburg, MD), and used in accordance with the instructions of the manufacturers. The SphI–BamHI clone of HSV-2 strain 333 was constructed by inserting a Sall–BamHI fragment from the plasmid pgr90 (containing the HSV-2 HindIII IK fragment, obtained from Gary Hayward) into the Sall and BamHI sites of the previously described SphI–Sall CAT construct (this insertion replaces the CAT gene in this construct with the HSV-2 Sall–BamHI fragment). To delete the NotI–NotI fragment, the SphI–BamHI clone was digested with NotI, and religated.

Construction of Mutant Viruses. For the LAT promoter deletion mutant, the 624 bp NotI–NotI fragment was deleted from a clone spanning the SphI–BamHI fragment shown in Fig. 1. This DNA was cotransfected into Vero cells with purified HSV-2 strain 333 viral DNA at a molar ratio of approximately 100:1. The resultant virus was plated in serial dilutions on 6-well plates, and individual plaques were selected and grown. DNA was purified from these

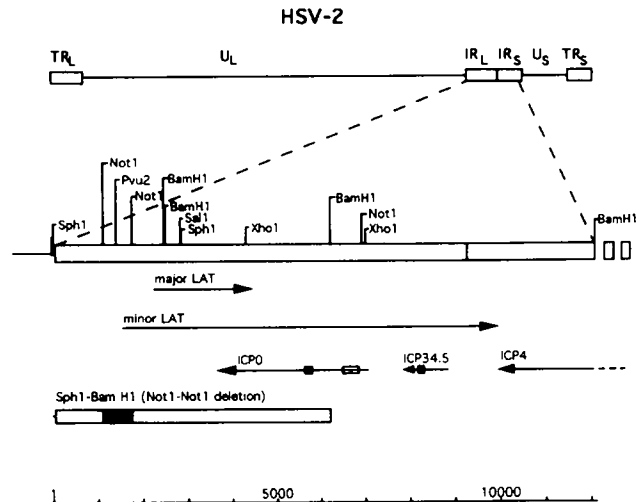


Figure 1. HSV-2 genome. Restriction endonuclease cleavage sites in the region of the LAT are shown relative to the HSV genome and the major LAT, the minor LAT, ICP0, ICP34.5, and ICP4. Introns in ICP0 and ICP34.5 are shown with boxes. The SphI–BamHI insert of the plasmid used to construct the mutant viruses is also shown; the deleted LAT promoter sequences from NotI–NotI are shaded. Distance in base pairs are shown relative to the first SphI site. Abbreviations: TR_L, terminal repeat long; IR_L, internal repeat, long; IR_S, internal repeat short; U_S, unique short; TR_S, terminal repeat short.

stocks by freeze-thawing three times, resuspending 70 μ l of stock in 330 μ l of lysis buffer (30 mM Tris, pH 7.5, 3.6 mM CaCl₂, 5 mM MgAc, 125 mM KCl, 0.5 mM EDTA, 0.5% NP-40, 4 μ g/ml RNase A) adding proteinase K to 100 μ g/ml and incubating at 56°C for 60 min, extracting with phenol/chloroform and precipitating in 2.5 volumes of ethanol. The resultant crude DNA was resuspended in 20 μ l of TE (10 mM Tris, 1 mM EDTA) buffer, digested with the restriction endonuclease PvuII or BamHI (New England Biolabs) in the supplied buffer, subjected to electrophoresis, and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Southern hybridizations using probes spanning this region were performed according to the instructions of the membrane manufacturer. The frequency of plaques positive for recombinant virus ranged from ~ 0.5 –5% of the total screened. 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 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 333pLAT⁻. This procedure was repeated, using intact SphI–BamHI DNA and 333pLAT⁻ DNA to produce the rescuant, 333pLAT^R. Additional Southern hybridizations were carried out on purified viral DNA digested with the enzymes NotI, BamHI, XhoI, and SphI. Blots were probed with ³²P-radiolabeled SphI–BamHI plasmid, as well as with ³²P-radiolabeled intact virion DNA. Other than the NotI–NotI deletion in 333pLAT⁻, no differences among the viruses were identified.

Guinea Pig Studies. Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, MA) weighing 400–525 g were inoculated with approximately 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 to 4) until resolution of the

acute infection. Acyclovir-treated guinea pigs received 25 mg/dose intraperitoneally twice a day for 7 d, starting 12 h after inoculation. These guinea pigs were observed daily for recurrence frequency from days 15 to 100. (Two guinea pigs infected with HSV-2 333 had mild, unresolved lesions persisting until day 17. These lesions were not counted as recurrences.) All observations of the guinea pigs were performed by investigators blinded as to virus inoculum. Guinea pigs that were not evaluable for the entire observation period (either acute or latent infection) were excluded from analysis. Animals were housed in American Association for Accreditation of Laboratory Animal Care approved facilities and cared for in accordance with institutional guidelines. To determine viral titers during acute infection, vaginal swab cultures were obtained on days 1, 3, and 5 from guinea pigs inoculated in the absence of acyclovir and virus titers were obtained by plaque titration on primary rabbit kidney cells. To determine nervous system titers of acutely infected guinea pigs, animals were killed 4 d after inoculation. Their dorsal root ganglia and spinal cords were removed aseptically by dissection, snap frozen, homogenized, and plaque titered on primary rabbit kidney cells.

Northern Hybridizations. Vero cells were infected with each virus at a multiplicity of 1.0 pfu/cell. 20 h after infection, RNA was purified (33) from the infected cells. RNA was also extracted from latently infected guinea pig ganglia. 5 μ g of each RNA was subjected to electrophoresis on 1.5% agarose/formaldehyde denaturing gels, and transferred to nitrocellulose membranes (Schleicher and Schuell) and hybridized according to the instructions of the manufacturer. The blot was probed with the SphI-BamHI clone, radiolabeled with α -[32 P]dCTP using a nick translation kit (Amersham Corp., Arlington Heights, IL).

Ganglion Cocultivations. Ganglia were removed after enumeration of recurrences was completed from the guinea pigs untreated with acyclovir, chopped with a razor blade, and placed in tissue culture with rabbit kidney cells. Cultures were observed daily for evidence of cytopathic effect, and reported as positive on the first day that HSV cytopathic effect was evident.

Semiquantitative Polymerase Chain Reactions. 100 ng of viral DNA (quantified spectrophotometrically) extracted from pooled S1-S3 ganglia of each guinea pig killed 27 d after inoculation was subjected to polymerase chain reaction (94°C for 1.5 min, 52°C for 3 min, 72°C for 3 min) for 35 cycles, simultaneously using the primers CACCTGCCAGTTCGAACGACCTCAT and AGCCGC-CACCCCCTCCCCTGCGT (which are specific for a 500-bp product in the HSV-2 major transforming region [mtr-2], located in the HSV-2 BglII N fragment), and AGTCCATTCTTGTC-TGCTCTCT and CTGGGGAACAAAGTAAGAGTCAAC (which are specific for a 500 bp product in guinea pig lactalbumin). The products were electrophoresed and subjected to Southern hybridization using 32 P-radiolabeled gel-pure internal amplified fragments from a plasmid containing the HSV-2 BglII N fragment, and strain 2 fetal guinea pig DNA as probes. Membranes were exposed to XAR film (Eastman Kodak, Rochester, NY) for 6 h, and bands were quantified by densitometry on a digital imaging system (IS-1000; Alpha Innotech Corp., San Leandro, CA). For each sample, the ratio of the HSV-2/gplac signals was calculated.

Results

Mutant Virus Design and Construction. Based on sequence comparisons with HSV-1 the HSV-2 LAT promoter was assumed to reside in the 624-bp NotI-NotI fragment. This fragment contains TATA, CREB, and other homologies, with a high percentage identity with previously identified HSV-1

LAT promoter sequences. In preliminary experiments (not shown), deletion of this fragment from the SphI-SalI CAT construct abolished detectable promoter activity in transient expression assays. DNA fragments deleted for the 624-bp NotI-NotI fragment, flanked by native HSV-2 long repeat sequences, were cotransfected with wild-type HSV-2 strain 333 DNA to produce by homologous recombination the LAT promoter deletion mutant, designated 333pLAT⁻. After plaque purification, this deletion was restored with wild-type sequences to produce a rescuant virus, denoted 333pLAT^R.

The deletion and its restoration were verified by Southern hybridization. DNA from HSV-2 strains 333, 333pLAT⁻, and 333pLAT^R was analyzed in Southern hybridizations with a 32 P-radiolabeled SphI-BamHI plasmid probe (Fig. 2 A). In wild-type HSV-2 333 (Fig. 2 A, lane 1), digestion with BamHI yielded the 3.9-kb BamHI P fragment, and \sim 7 and \sim 10 kb fragments representing segments from either end of the unique long (U_L) portion of the genome that span its junction with the repeats. These fragments were diminished in size by \sim 600 bp in 333pLAT⁻ (Fig. 2 A, lane 2), and were restored in the rescuant (Fig. 2 A, lane 3). Digestion with NotI gave identical restriction patterns (Fig. 2 A, lanes 4–6) among the three viruses, with the exception of the loss of the deleted 624-bp fragment in 333pLAT⁻ (Fig. 2 A, lane 5). In 333pLAT⁻, deletion of the NotI-NotI fragment eliminated the sole PvuII site in the long or short repeats of HSV-2 (Fig. 2 A, lane 8), causing changes in the sizes of all four bands seen in HSV-2 333 (Fig. 2 A, lane 7) and 333pLAT^R (Fig. 2 A, lane 9). Digestion with XhoI (Fig. 2 A, lanes 10–12) also produced restriction fragment changes in 333pLAT⁻ (Fig. 2 A, lane 11) compatible with the loss of the 624-bp deletion from the two XhoI fragments that span the U_L-repeat junctions. Using 32 P-radiolabeled HSV-2 333 DNA as a probe (Fig. 2 B), hybridization of whole virus DNA digested with BamHI (Fig. 2 B, lanes 1–3) and PvuII (Fig. 2 B, lanes 4–6) also revealed no differences among the viruses other than those associated with the deletion of the 624-bp NotI-NotI fragment in 333pLAT⁻. In other experiments (data not shown), a probe spanning the NotI-NotI fragment was shown not to hybridize with DNA extracted from 333pLAT⁻ virus.

Characterization of Acute Infections with Wild-type and Mutant Viruses. To determine whether the LAT promoter deletion has any effect on acute viral replication, we compared the ability of each virus to grow in culture and in guinea pigs.

To examine growth of virus in tissue culture, Vero cells were inoculated with each virus at a multiplicity of infection (MOI) of 0.1 pfu/cell and were harvested at 3 h and at 20 h after inoculation. Virus yield at each time point was determined by plaque titration. One-step growth curves for each virus were comparable, with similarly reduced titers of virus at 3 h (corresponding to virus penetration of infected cells), and increased titers at 20 h post inoculation (corresponding to one-step growth of virus), as seen in Fig. 3 A. Similar one-step growth experiments were performed in SK-N-SH human neuroblastoma cells and in primary human fetal sensory ganglion cultures, and also showed no differences among the viruses (data not shown).

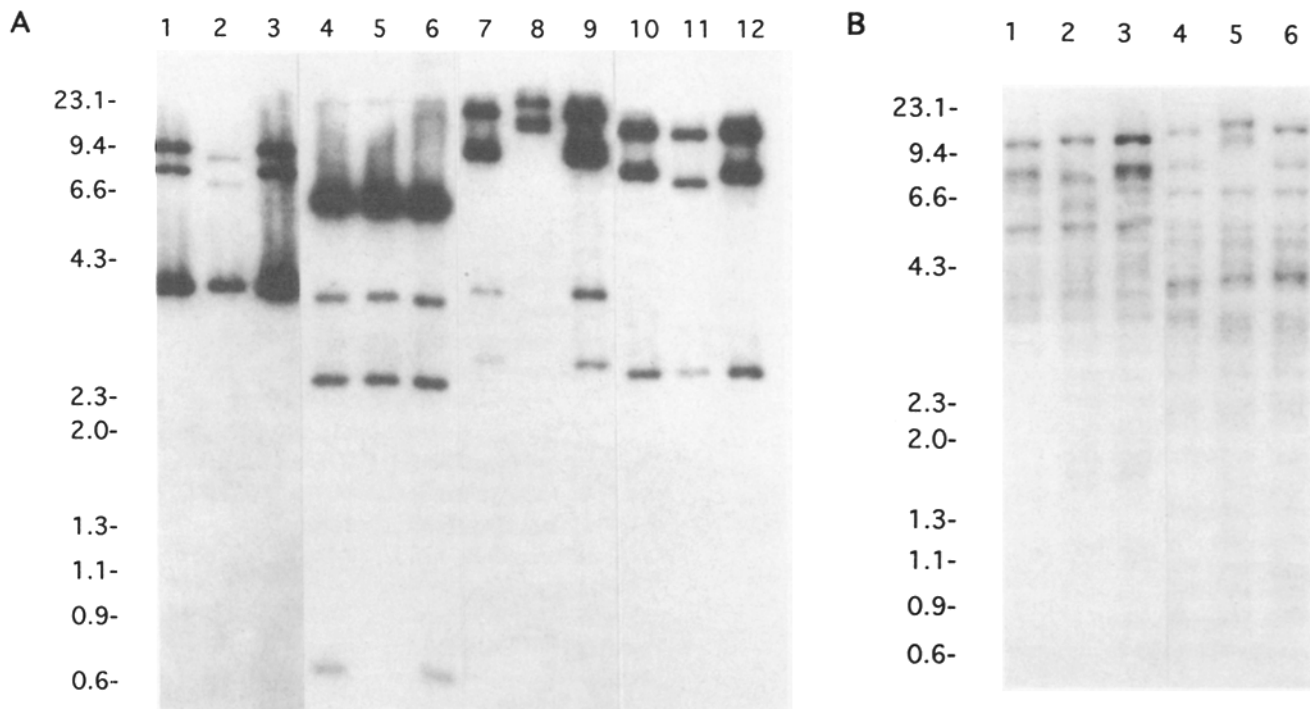


Figure 2. Characterization of wild-type, mutant, and rescuant viruses by Southern hybridization. (A) DNA from HSV-2 333 (lanes 1, 4, 7, and 10), 333pLAT⁻ (lanes 2, 5, 8, and 11), and 333pLAT^R (lanes 3, 6, 9, and 12) was digested with BamHI (lanes 1–3), NotI (lanes 4–6), PvuII (lanes 7–9), and XhoI (lanes 10–12) and subjected to Southern hybridization with a ³²P-radiolabeled SphI–BamHI probe. Marker locations in kilobases are shown to the left of the autoradiogram. (B) DNA from HSV-2 333 (lanes 1 and 4), 333pLAT⁻ (lanes 2 and 5), and 333pLAT^R (lanes 4 and 6) was digested with BamHI (lanes 1–3) and PvuII (lanes 4–6), and subjected to Southern hybridization with a ³²P-radiolabeled whole virus HSV-2 333 DNA probe.

The severity and course of primary genital infection was studied in two independent experiments. Hartley guinea pigs were intravaginally inoculated with 10^{5.7} pfu of each virus and scored daily either in the absence (Fig. 3 B) or the presence (Fig. 3 C) of intraperitoneal acyclovir treatment. To compare primary infections, the area under the lesion score-day curves (AUC) shown in Fig. 3, B and C were calculated for each virus, and used as a measure of the severity of acute infection. In the experiment involving untreated animals, the mutant 333pLAT⁻ caused acute infections comparable to those of the rescuant 333pLAT^R (AUC of 9.1 and 7.8, respectively), while the parental HSV-2 333 caused somewhat higher scores (AUC 14.3). The severity of the acute infections was appropriately lower in the acyclovir-treated guinea pigs (AUC of 5.7 for 333pLAT⁻, 6.1 for 333pLAT^R, and 9.7 for HSV-2 333). In each experiment, strain 333 yielded slightly higher scores than the other viruses, but no difference in lesion scores were observed between the promoter deletion mutant and its rescuant.

To determine whether viral replication in the guinea pigs differed, virus titers were obtained from vaginal swab specimens and neuronal tissues of the untreated guinea pigs. Virus titers from vaginal swabs collected at days 1, 3, and 5 post inoculation were compared among animals infected with each virus (Fig. 3 D). No differences in vaginal titers were observed. Moreover, virus titers obtained from homogenized sacral ganglia and spinal cord from guinea pigs killed 4 d after the ini-

tial infection with HSV-2 333pLAT⁻ and 333pLAT^R were also comparable (Fig. 3 E). Thus, as measured by lesion scores and virus titers in genital and neural tissues, there were no significant differences in the course or severity of primary infections attributable to the LAT promoter deletion, or in spread of virus to or replication in the nervous system.

Effect of LAT Promoter Deletion on Recurrent Disease. Following recovery from acute infection, animals were examined daily from days 15–100 post inoculation for recurrent disease. Fig. 4, A and B show cumulative recurrences over time for each experiment. In animals not treated with acyclovir (Fig. 4 A), strain 333 caused slightly more recurrences (mean 11.9, SE 2.0) than the rescued virus 333pLAT^R (mean 8.2, SE 1.9), but markedly more than the LAT promoter deletion mutant 333pLAT⁻ (mean 0.8, SE 0.5). In animals treated with acyclovir for the first 7 d of their primary infection (Fig. 4 B), 333pLAT⁻ lesions appeared spontaneously only an average of 1.4 times (SE 0.5) per animal, as compared with 6.3 (SE 1.8) and 11.4 (SE 2.2) recurrences for strains 333 and 333pLAT^R, respectively. In each experiment, differences between 333pLAT⁻ and its parent or rescuant strains were statistically significant. Using the two-tailed Wilcoxon test, median recurrence frequencies and *p* values for pairwise comparisons of recurrence frequencies associated with each virus were as follows. Untreated animals: 333pLAT⁻ (*n* = 5, median 0 recurrences) vs. HSV-2 333 (*n* = 7, median 11), *p* = 0.006; 333pLAT⁻ vs. 333pLAT^R (*n* = 6, median 8),

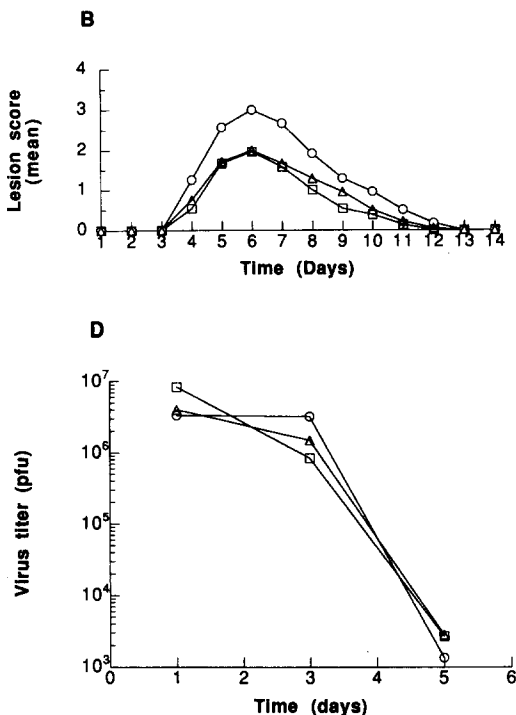
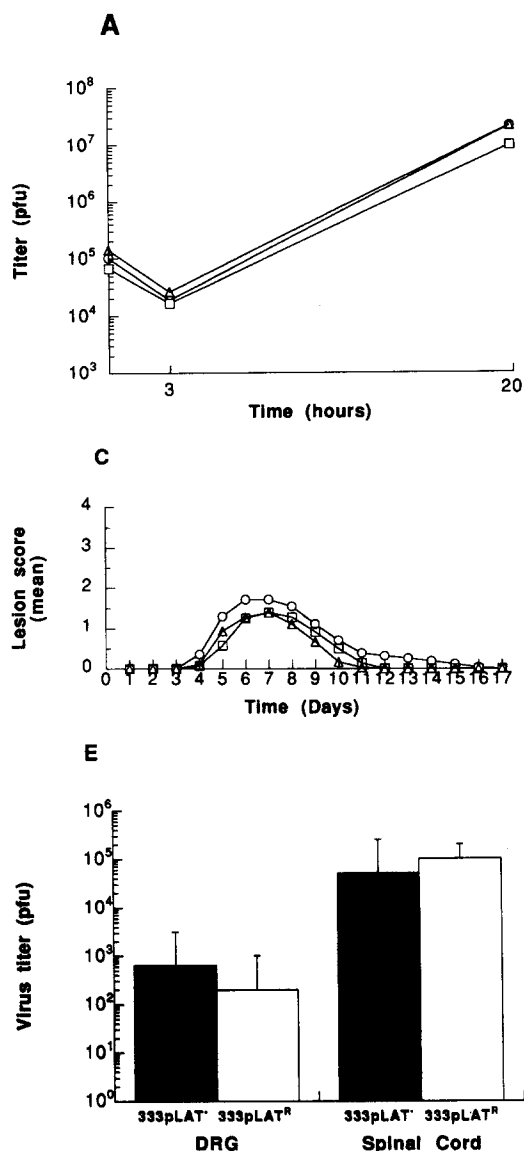


Figure 3. Characterization of acute infections with each virus (333 [O], 333pLAT⁻ [Δ], and 333pLAT^R [□]). (A) 10⁶ Vero cells were inoculated with each virus at a MOI of ~0.1 at time 0. Cultures were plaque-titered at 0, 3, and 20 h after inoculation. (B) Severity of primary genital infection in guinea pigs inoculated on day 0 with 10^{5.7} pfu of HSV-2 333 (*n* = 12), 333pLAT⁻ (*n* = 12), and 333pLAT^R (*n* = 12) were scored daily on a scale from 0 to 4. (C) Lesions of guinea pigs infected on day 0 with 10^{5.7} pfu of HSV-2 333 (*n* = 14), 333pLAT⁻ (*n* = 18), and 333pLAT^R (*n* = 18) were scored daily. These guinea pigs received intraperitoneal acyclovir (25 mg/kg) twice daily for 7 d, starting 12 h after inoculation. (D) Vaginal swab cultures of guinea pigs were plaque titered 1, 3, and 5 d post inoculation. (E) Spinal cords and pooled lumbosacral dorsal root ganglia of guinea pigs infected with 333pLAT⁻ (*n* = 5) and 333pLAT^R (*n* = 4) were removed 4 d after infection, homogenized, and plaque titered. Error bars show standard errors.

$p = 0.004$; HSV-2 333 vs. 333pLAT^R, $p = \text{NS}$. Acyclovir-treated animals: 333pLAT⁻ (*n* = 11, median 1) vs. HSV-2 333 (*n* = 12, median 4.5), $p = 0.016$; 333pLAT⁻ vs. 333pLAT^R (*n* = 12, median 12), $p < 0.001$; HSV-2 333 vs. 333pLAT^R, $p = \text{NS}$. Thus, in both experiments, guinea pigs infected with the LAT promoter deletion mutant 333pLAT⁻ experienced at least an 80% reduction in recurrences as compared with those infected with strain 333 or the rescued virus 333pLAT^R.

Effect of the LAT Promoter Deletion on LAT Expression during Latency and Acute Infection. To determine whether 333pLAT⁻ produced LAT during latency, and whether there was any evidence of viral replication in the ganglia, we performed Northern hybridizations of RNA extracted from guinea pig sacral ganglia removed 27 d after inoculation. The 2.2-kb HSV-2 LAT was not detected from 333pLAT⁻ infected or uninfected guinea pigs, but was readily detected in tissues from animals harboring the 333pLAT^R strain (Fig. 5 A).

Thus, the LAT promoter deletion abolished detectable LAT transcription during latency. ICPO mRNA was not detected in any latently infected guinea pigs, indicating no active virus replication.

In contrast, during productive infection of Vero cells, the 2.2-kb LAT was readily identified in RNA extracted 20 h after infection with HSV-2 333 and 333pLAT^R. Cells infected with 333pLAT⁻ also produced a 2.2-kb LAT, but the signal was clearly diminished (Fig. 5 B). At immediate-early time points, the LAT promoter deletion had no effect on ICPO transcription (data not shown).

Effect of the LAT Promoter Deletion on Establishment and Maintenance of Latency. To determine whether the 80% reduction in rate of spontaneous genital recurrences of the 333pLAT⁻ virus could be explained by differences in establishment or maintenance of latency, we performed both ganglia explant-cocultivation experiments and semi-quantitative PCR studies to assess the relative viral DNA burden in guinea pig ganglia

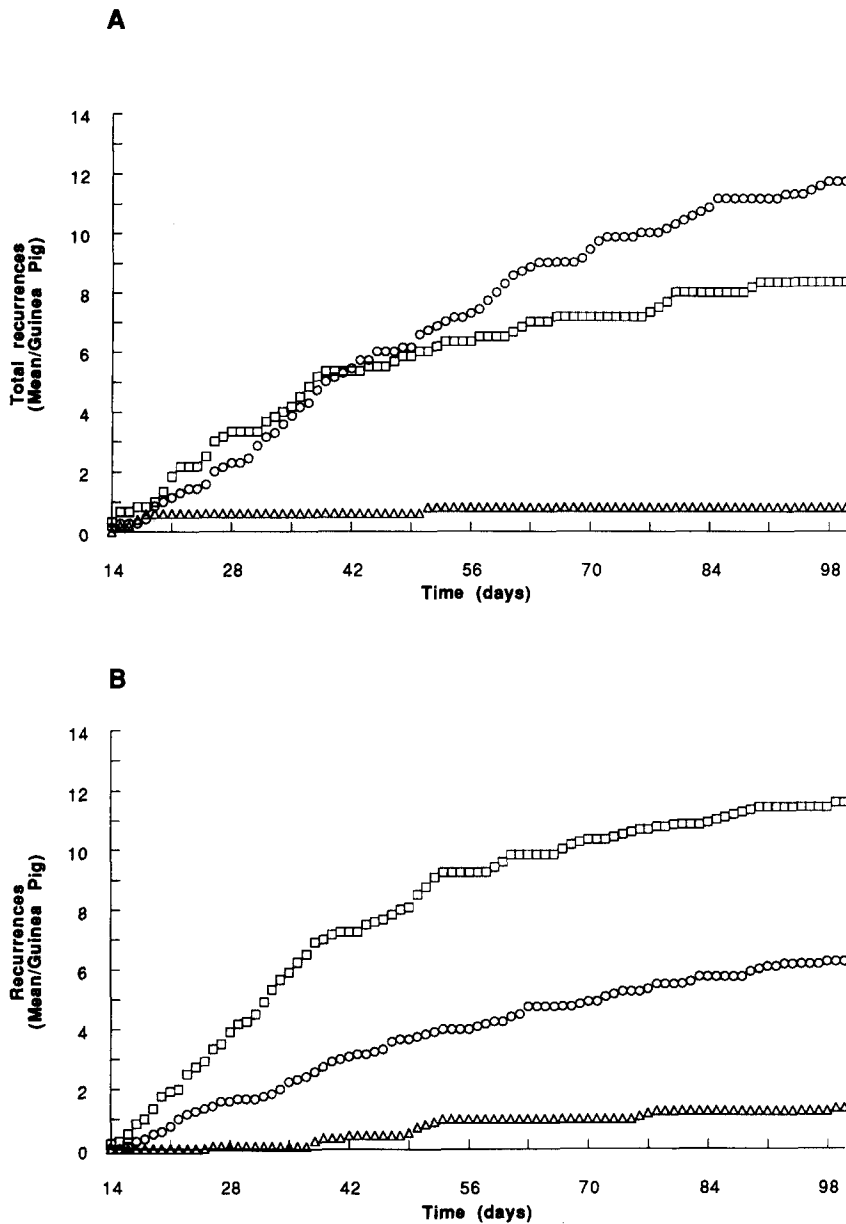


Figure 4. Spontaneous recurrences in latently infected guinea pigs. Guinea pigs infected with each virus (333 [O], 333pLAT⁻ [Δ], 333pLAT^R [□]) were observed for recurrences starting 14 d post infection. (a) Cumulative recurrences are shown for guinea pigs infected with HSV-2 333 (*n* = 7), 333pLAT⁻ (*n* = 5), and 333pLAT^R (*n* = 6), whose acute infection is depicted in Fig. 3, B and D, untreated with acyclovir. (b) Cumulative recurrences in guinea pigs inoculated with HSV-2 333 (*n* = 12), 333pLAT⁻ (*n* = 11), and 333pLAT^R (*n* = 12) and treated with acyclovir during the acute infection.

latently infected with the three virus strains. After enumeration of the recurrences reported in Fig. 4 A, ganglia from guinea pigs (untreated with acyclovir) latently infected with strain 333, 333pLAT⁻, or 333pLAT^R were harvested and cocultivated with primary rabbit kidney cells (Table 1). Virus was recovered from all ganglia tested and in approximately the same period of time (15–21 d). Southern hybridizations confirmed the appropriate restriction endonuclease digestion patterns for each virus recovered by cocultivation (not shown).

Similar conclusions were reached regarding viral DNA content in the PCR experiments. Ganglia were harvested 27 d post inoculation and subjected to PCR simultaneously with primers specific for HSV-2 DNA, and with primers specific

for the guinea pig lactalbumin gene. Southern hybridization of the PCR products was carried out using radiolabeled gel-purified specific internal amplified fragments as probes (Fig. 6). The ratio of signal intensities for HSV DNA to lactalbumin DNA for each animal was determined by densitometry, and used as an estimate of latent virus burden. All of the signal ratios fell between the ratios obtained in reconstruction experiments using 100 and 1,000 copies of the HSV-2 viral genomes. The ratio of signal intensities found with each virus overlapped (ratios for 333pLAT⁻ ranged from 1.62 to 3.07; for 333pLAT^R they ranged from 0.88 to 3.26), indicating no difference in the amount of viral DNA persisting in the sacral ganglia of each animal. These cumulative data

Table 1. *Explant Cocultivation of Latently Infected Guinea Pig Ganglia*

Virus	No. positive/no. tested	Time to
		positive culture
		<i>days</i>
333	3/3	15, 15, 15
333pLAT ⁻	2/2	15, 21
333pLAT ^R	3/3	15, 15, 20

imply that the 333pLAT⁻ mutant was able to establish and maintain latency with a frequency comparable to that of the parental and rescued virus strains.

Discussion

In the present series of experiments, it was determined that deletion of sequences directing latent transcription of the HSV-2 LAT had no apparent effect on productive infections in tissue culture or in guinea pigs, or on the establishment or maintenance of latency, yet there was a marked decrease in the frequency of spontaneous herpetic recurrences. Because the deletion in the LAT promoter did not impair viral growth in peripheral or neuronal tissues, recurrences can be correlated with the rate of ganglionic reactivation. This indicates that the LAT is required for efficient spontaneous reactivation of latent virus from latently infected neurons. Since it is the only gene whose transcription is detectable in these cells during latency, these data argue that LAT may be involved in the mechanism by which reactivation is triggered.

We found the LAT promoter deletion mutant to be capable of transcribing small amounts of LAT during productive infection of Vero cells, but not during latency. This may be due to additional promoter elements not contained within the deleted NotI–NotI fragment, or to run on transcription from upstream genes transcribed during acute infection. In HSV-1, there is also evidence of previously unidentified LAT regulatory sequences (34, 35) which may play a role in acute phase LAT transcription, but not in latent phase transcription.

Our cocultivation studies revealed that an approximately equivalent amount of reactivatable virus was present in each ganglion, regardless of the infecting virus strain. While it is possible that there were some differences in DNA quantities that we were unable to discern by PCR, it is reasonable

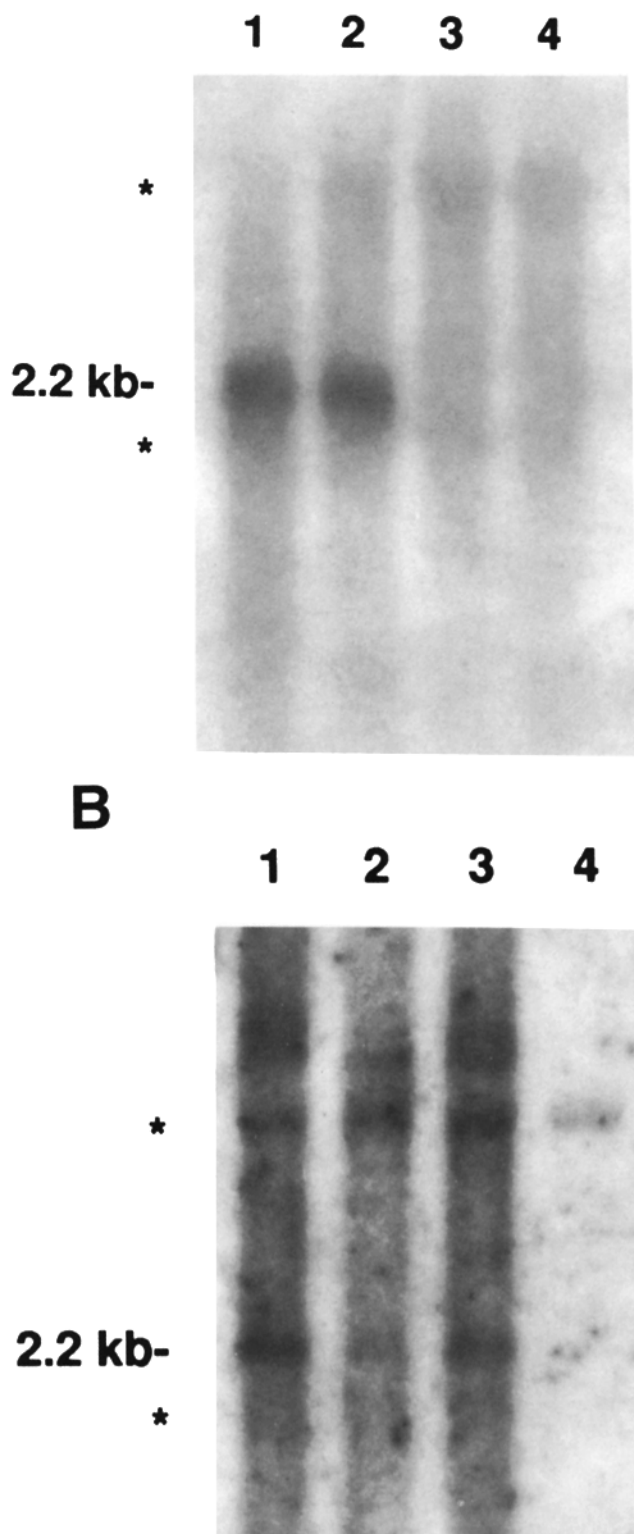


Figure 5. LAT production during infection with each virus strain. (A) LAT production during latent infection of guinea pigs by Northern hybridization. RNA was extracted from guinea pigs latently infected with

333pLAT^R (lanes 1 and 2), 333pLAT⁻ (lane 3), or uninfected guinea pigs (lane 4), subjected to electrophoresis, and probed with a radiolabeled SphI–BamHI probe. (B) RNA was extracted 20 h after infection from Vero cells infected at a MOI of 1 pfu/cell with HSV-2 333 (lane 1), 333pLAT⁻ (lane 2), 333pLAT^R (lane 3) and uninfected Vero cells (lane 4). The blot was probed with a radiolabeled SphI–BamHI plasmid probe. The size of the 2.2-kb LAT is marked. Asterisks denote the positions of 18S and 28S rRNAs.

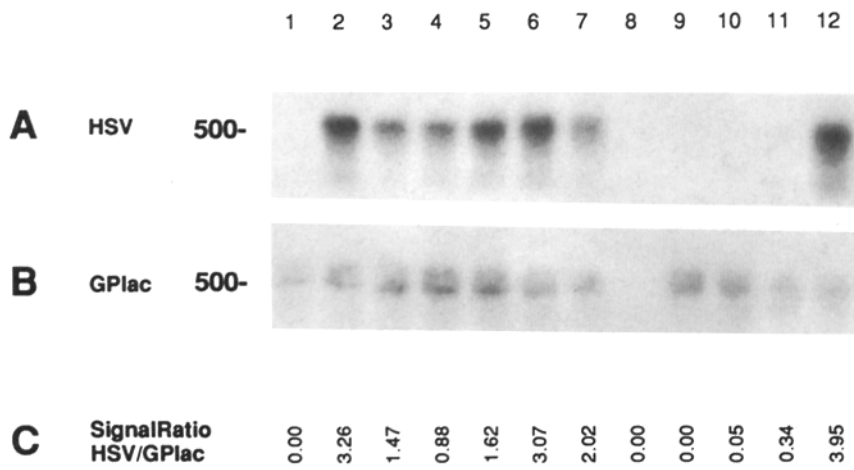


Figure 6. Coamplification of guinea pig lactalbumin and HSV-2 DNA in latently infected ganglia by PCR. Samples included DNA from uninfected guinea pig (lane 1), guinea pigs latently infected with 333pLAT^R (lanes 2–4) and 333pLAT⁻ (lanes 5–7), sterile water (lane 8), and strain 2 fetal guinea pig cellular DNA spiked with 0, 10, 100, and 1,000 copies of HSV-2 strain MS genomes, respectively (lanes 9–12). (A) primers specific for 500 bp HSV-2 product, probed with amplified 250 bp internal probe. (B) primers specific for 500 bp guinea pig lactalbumin product, probed with amplified 250 bp internal probe.

to exclude differences in rates of the establishment or maintenance of latency as a sufficient explanation for the observed differences in recurrence frequency.

Several studies used the recovery of virus by cocultivation of ganglia as an endpoint for reactivation. We were able to recover virus from all latently infected ganglia by this method, and the likelihood of doing so and time required to do so did not correlate with frequency of spontaneous recurrences. Thus, our data suggest that cocultivation is not truly representative of the biological process of reactivation. This conclusion is consistent with reported comparisons of cocultivations and induced reactivations of an HSV-1 × HSV-2 intertypic recombinant in latently infected rabbits (20), but differs from conclusions drawn from other experiments using HSV-1 in rabbits (23) and in mice (22, 24), in which mutations in the LAT region either led to reduced frequency or delayed kinetics of reactivation by explant cocultivation. In all published comparisons, rates of virus recovery using explant cocultivation exceeded those of virus recovery by induced reactivation. Explant cocultivation appears to be useful in studying some aspects of LAT function in some animal models, but clearly represents a different situation from spontaneous reactivation of HSV-2 in latently infected guinea pigs. It could be that HSV-1 and HSV-2 differ in this regard, or that different animal models have differing thresholds for reactivation by cocultivation. It is also possible that an ability to recover latent virus by cocultivation correlates with the quantity of latent virus DNA (as was reported in mice [22]), or that the very strong stimulus to reactivation (explantation presumably followed by cell death) generated with this model is not relevant to “clinically” meaningful outcomes like spontaneous disease recurrences.

While the acute infections with HSV-2 333 scored as being slightly more severe than those with the other two viruses (Fig. 3, B and C), this did not correlate with a difference in recurrence frequency (in the first experiment, HSV-2 333 recurred more frequently than the rescuant, in the second, it recurred less frequently). Conceivably, the difference in acute

infection severity could be attributable to some unrecognized genotypic difference between the parent and the other two viruses in a region distant from the LAT. There was, however, no evidence for any genotypic differences (besides the LAT promoter deletion) between the mutant 333pLAT⁻ and strain 333pLAT^R, derived from it, and no phenotypic differences between these two strains except in rates of spontaneous recurrence.

These data show that HSV-2 LAT expression influences spontaneous recurrence rates of latent virus. The LATs also appear to play a role in HSV-1 recurrence. While the primary infections of HSV-1 and HSV-2 in humans are indistinguishable, HSV-1 recurs most frequently from primary infections involving the trigeminal dermatome and latent HSV-2 recurs most frequently from sacral ganglia (2). This observation suggests that these viruses differ in their ability to either establish or reactivate from latent infections in a site-specific manner. Because the major LAT sequences of these viruses share essentially random homology, it is possible that the LATs of these viruses have evolved to facilitate reactivation in specific cellular environments.

The development of a ganglionic reactivation into a mucocutaneous recurrence involves many factors including viral replication in neurons, peripheral replication, and the immune system. While deletion or mutation of other viral genes (e.g., thymidine kinase, ICP0) reduces recurrence frequency in some models, identifiable effects of those mutations on the growth and spread of virus has precluded making firm conclusions about their effect on reactivation. In contrast, deletion of sequences from the HSV-2 LAT promoter did not affect neuronal or nonneuronal replication either in vitro or in vivo in immunocompetent guinea pigs. Thus, our findings assign a role to the LAT in reactivation of latent HSV-2. Further study of interactions between the LAT and other viral and cellular genes will be required to precisely establish the mechanisms of HSV reactivation, and how LAT modulates disease recurrence.

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