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Mutations in the 5′ End of the Herpes Simplex Virus Type 2 Latency-Associated Transcript (LAT) Promoter Affect LAT Expression In Vivo but Not the Rate of Spontaneous Reactivation of Genital Herpes

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The primary herpes simplex virus type 2 (HSV-2) latency-associated transcript (LAT) promoter influences LAT expression and rates of virus reactivation. We explored the biological importance of particular neuronally responsive regions within the promoter by creating new recombinant viruses bearing a targeted deletion (246 bp [strain 524]) or a point mutation (2 bp [strain 167]) in this region. These recombinant viruses grew efficiently in vitro and in vivo, caused acute genital disease in guinea pigs, and, as measured by quantitative-competitive (QC) DNA PCR, established latency, all as well as did the wild-type parental HSV-2 strain 333, the rescuant strain 524R, and the previously described 624-bp LAT$^-$ promoter deletion mutant. By QC-reverse transcriptase PCR of RNA from latently infected ganglia, mutant 167 expressed wild-type levels of LAT and the deletion mutant 524 expressed 9- to 15-fold less LAT than normal, while the LAT expression of the LAT$^-$ mutant was undetectable or at least 5 log units less than that of the wild type. The rates of recurrence of genital lesions were normal for recombinant viruses 524 and 167 but reduced (as expected) for the LAT$^-$ mutant. Alteration of a subset of LAT promoter elements reduced LAT expression by 1 log unit but did not influence the rate of spontaneous disease reactivation in vivo. Far greater reductions in LAT expression are necessary before reactivation rates are noticeably changed.

Herpes simplex virus type 2 (HSV-2) causes acute genital herpes and establishes latent infection in dorsal root ganglia. The latent virus reactivates episodically, resulting in recurrent lesions that serve as reservoirs for transmission of the virus to susceptible individuals. The mechanism underlying the transition between lytic and latent infection and virus reactivation is poorly understood.

During latency, the viral genome is transcriptionally silent, with the single prominent exception of the gene encoding the latency-associated transcripts (LATs). The LAT gene is located in the long repeat regions (TR_L and IR_L) of the genome flanking the U_L region. It codes for a primary transcript, the minor LAT, and a stable intron derived from it, the major LAT (Fig. 1). LATs accumulate to high levels in latently infected sensory neurons (4–6, 25, 26). The transcriptional regulation of this gene is complex. Two promoter regions have been identified, and each may play a different role in various stages of infection (3, 8, 10, 13, 28, 29). A classical promoter domain is very potent, especially in neuronal cell lines (28), and appears to be primarily responsible for the expression and accumulation of LATs during latency (8, 14, 29). Our studies have identified within this classical HSV-2 LAT promoter a TATA box, an ATF/CREB element which is similar to one in the HSV-1 LAT promoter (16), a novel nuclear factor binding element we termed LAT-3, and a distal neuronally responsive sequence (*Not*I-*Not*I fragment [Fig. 1]) among others (28). A second, weaker promoter element lies downstream of the first. This promoter lacks a TATA box and other features of classical promoters. Its role in regulating LAT expression in neurons is uncertain, but it does not appear to be as strong as the primary promoter during latency (3, 29).

Despite researchers' increasing knowledge about the structure and regulation of LAT expression, its actual role in HSV-1 and HSV-2 infection remains uncertain. The function of the 5′ end of the HSV-1 LAT gene has been most extensively studied. Various deletions, insertions, and targeted mutations have been made in its sequence in a number of HSV-1 strains, extending from the primary promoter region and well into the major LAT, and tested in several animal models. The results differ somewhat according to the HSV-1 strain and the animal model used and the methods employed to assess reactivation and quantitate the latent virus burden (1, 7, 9, 12, 15, 18–20, 24). Most data but not all suggest that the LAT region is required for the efficient spontaneous or induced reactivation of latent virus in animal models. Nevertheless, recent studies suggest alternative possibilities for LAT function. Thompson and Sawtell (27) reported that HSV-1 LAT region mutants reactivate less efficiently because they establish latency in fewer neurons.

Little work has been done with HSV-2 despite the fact that the only animal model system in which disease reactivates spontaneously to produce lesions is the guinea pig infected genitally with HSV-2. Deletion of a 624-bp DNA fragment encompassing the primary LAT promoter and part of its downstream region (a fragment bounded by *Not*I sites [see Fig. 1, *Not*I-*Not*I fragment]) yielded a virus with a reduced rate of recurrence of guinea pig genital herpes (14).

A number of mechanisms could explain the above observations with HSV-1 and HSV-2 mutants. For example, deletion of the LAT upstream regulatory region could eliminate the expression of LAT, which in some unknown way modulates

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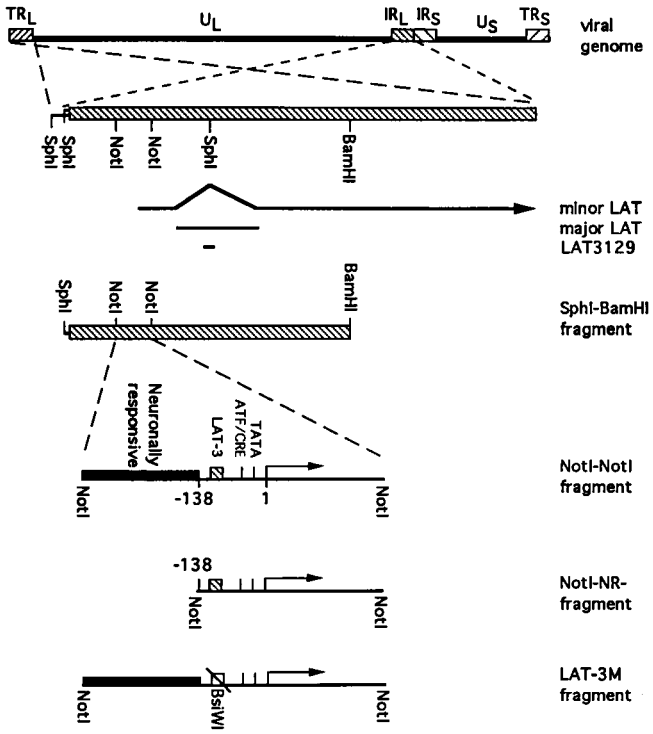

FIG. 1. Schematic representation of the HSV-2 genome structure and DNA fragments from the LAT region. At the top are the structure of HSV-2 genome, the more detailed structure of the repeat regions flanking U_L, the genomic location of the minor and major LATs, and a probe termed LAT3129 that was used for Northern blotting assay. The *Sph*I-*Bam*HI fragment of the IR_L sequences was cloned and used to rescue the mutant 524 to yield recombinant virus strain 524R. The *Not*I-*Not*I fragment encompassing the LAT promoter is shown with some of its best-characterized *cis*-elements (24). In a plasmid bearing this fragment, the neuronally responsive region was deleted to make the *Not*I-NR$^-$ fragment shown or 2 bp in the LAT-3 element was mutated to create a new *Bsi*WI site and generate the LAT-3M fragment. These deleted or mutated fragments were ligated in place of the native *Not*I-*Not*I sequences in the *Sph*I-*Bam*HI fragment, and the resulting plasmids were used to generate mutant viruses 524 and 167, respectively.

reactivation; deletion of the upstream region might affect expression not only of LAT but also of some other gene products which could influence viral latency, and LAT expression might influence the quantity of virus that persists and is amenable to subsequent reactivation.

To further examine these and related competing hypotheses, we introduced a deletion or a point mutation into the LAT promoter domain of HSV-2, specifically within the neuronally responsive region and the LAT-3 element, and tested their effects on levels of latent viral DNA, LAT expression, and rates of spontaneous reactivation in the guinea pig genital herpes model.

MATERIALS AND METHODS

Plasmid constructs. Plasmids pSphI-BamHI and pSB-N$^-$ containing portions of the HSV-2 LAT gene were described previously (14). In pSphI-BamHI, the CAT gene and its flanking sequences between the *Sph*I and *Bam*HI cleavage sites of pCAT.Basic (Promega, Madison, Wis.) were replaced with the 6.2-kb DNA fragment bounded by *Sph*I and *Bam*HI sites in the HSV-2 strain 333 IR_L (Fig. 1). Plasmid pSB-N$^-$ was created by deletion of the 624-bp *Not*I-*Not*I fragment encompassing the LAT promoter. Both plasmids were generous gifts from Philip Krause. To generate pSB-NR$^-$, which has a 246-bp deletion of the neuronally responsive region (bases −384 to −139 relative to the LAT start site) of the promoter, DNA fragment *Not*I-NR$^-$ (Fig. 1) was inserted into the *Not*I site of pSB-N$^-$. To construct plasmid pSB-LAT3M, which has a 2-bp mutation in the LAT-3 element, the LAT-3M DNA fragment (Fig. 1) was inserted into the *Not*I site of pSB-N$^-$.

Viruses. HSV-2 strain 333 was used as the wild-type virus from which all other viruses studied here were derived. LAT$^-$ (a generous gift from Philip Krause) was engineered from strain 333 by the deletion of the entire 624-bp *Not*I-*Not*I fragment (14). All of the viruses used in this study were propagated in Vero cells, and stocks of cell-free viruses made by using three cycles of freezing and thawing were stored at −80°C. The virus titers of the stocks were determined by plaque assay on Vero cells.

Construction of mutant viruses. The neuronally responsive region deletion mutant virus, termed strain 524, and the 2-bp LAT-3 mutant, termed strain 167, were generated by cotransfection of Vero cells as previously described (11, 14) with purified LAT$^-$ virion DNA and plasmids pSB-NR$^-$ and pSB-LAT3M, respectively.

The progeny viruses were plaque purified and screened by slot blot hybridization. The ^{32}P-labeled *Not*I-*Not*I fragment was used as a probe to screen mutants 524 and 167. Minicultures of positive clones were subjected to two further cycles of plaque purification and selected by DNA slot blot hybridization. The virion DNAs of the final selected positive clones were prepared and further characterized by restriction endonuclease (*Bam*HI, *Not*I, *Sph*I, and *Bsi*WI) mapping and Southern blot hybridization with the ^{32}P-labeled *Sph*I-*Bam*HI fragment (Fig. 1) as the probe.

The rescuant virus 524R was generated in the same general way as the other recombinants, except that cotransfection was performed with DNA purified from mutant 524 virus and the wild-type plasmid pSphI-BamHI DNA, so that the deletion in 524 was repaired. The DNA fragment encompassing the neuronally responsive region (*Not*I-*Not*I fragment [Fig. 1]) was used to screen rescuant 524R.

One-step virus growth curve. The efficiency of replication of strains 524, 524R, 167, and LAT$^-$ and the parental strain 333 was measured by means of a one-step growth assay in Vero cells as described previously (14) with a multiplicity of infection of 0.01. Infected cells were harvested at 0, 3, 9, and 20 h postinfection, and the virus titers were then determined by plaque assay on Vero cell monolayers.

Guinea pig studies. (i) Acute infection. Female Hartley strain guinea pigs weighing 450 to 500 g (Charles River Laboratories, Southbridge, Mass.) were inoculated intravaginally with 2×10^5 PFU of virus in 50 μl of solution. Herpetic lesions appearing in the perineal region were scored daily with the scale described by Stanberry et al. (23). At predetermined times postinfection, samples of vaginal secretions were taken with Dacron-tipped swabs and stored at −80°C. The virus titers of these samples were determined by plaque assay. To quantitate infectious virus in lumbosacral ganglia, guinea pigs were sacrificed by CO_2 narcosis on predetermined days postinfection, and lumbosacral ganglia were recovered aseptically. Pooled ganglia from each animal were homogenized in 1 ml of complete minimal essential medium 199 with a Tissumizer (Tekmar Co., Cincinnati, Ohio) and clarified by centrifugation. A 0.5-ml portion of the supernatant was promptly titrated by plaque assay on monolayers of primary rabbit kidney cells (BioWhittaker Inc., Walkersville, Md.).

(ii) Latent infection. Inoculation was the same as in the study on acute infection. Two experiments were conducted to verify the results. In the first experiment, no acyclovir was used, and about 50% of the animals died of the acute infection. In experiment 2, in order to reduce mortality, animals were treated once daily for 7 days with 25 mg of acyclovir administered intraperitoneally starting 24 h postinfection.

From day 12 or 15 postinfection, animals were examined daily, and all recurrent lesions were recorded.

Quantitative-competitive (QC) DNA PCR and reverse transcriptase (RT) PCR. To quantify latent HSV-2 DNA, lumbosacral ganglia were recovered on day 66 postinfection in experiment 1 and on day 116 postinfection in experiment 2. DNA was isolated from pooled ganglia from one side of the spine of each animal, and RNA was isolated from the ganglia on the other side of the spine. For isolating DNA, ganglia were soaked in 300 μl of a proteinase K mixture (10 mM Tris-Cl [pH 8.0], 0.001% Triton X-100, 0.0001% sodium dodecyl sulfate, 1 mM EDTA, 0.6 mg of proteinase K per ml) and incubated at 56°C overnight. Proteins were precipitated with the protein precipitation solution from the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, Minn.) at room temperature for 5 min and pelleted at 12,000 × *g* for 5 min. Then, DNA was precipitated with a 0.75× volume of isopropanol at 18°C for 10 min and pelleted at 12,000 × *g* for 5 min. The DNA pellet was washed with 75% ethanol, dried, and resuspended in 50 to 100 μl of H_2O.

QC PCR was used to estimate the viral DNA copy numbers in latently infected ganglia. Oligonucleotide primers termed LATMA4 (5′GCCAGACGTGCGT GCTCTGCACGAT3′) and LATMA5 (5′TGTTGGTCTTTATCATAGAACA GAG3′) were used to amplify DNA in the major LAT region. A competitor plasmid termed pHSV2e was used as well. It contains a 150-bp viral DNA insert spanning the region of the major LAT bounded by oligonucleotides LATMA4 and LATMA5. This region was cloned into pSP18 (Boehringer Mannheim, Indianapolis, Ind.). A 71-bp nonspecific stuffer DNA sequence was then inserted in the middle of the cloned viral DNA fragment so that the amplified products of this plasmid would be 71 bp longer than the amplified latent viral DNA. An internal oligonucleotide termed LATMA4.5 (5′TCGTTCTGGCTCCCT GTCTTGGT3′) was used as a probe to monitor the specificity of the PCRs. In

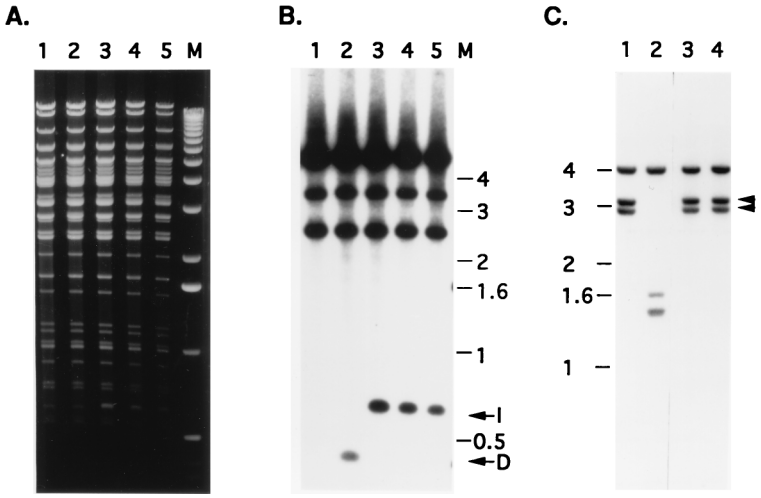

FIG. 2. Restriction enzyme mapping of virion DNA from mutant virus. (A) Purified virion DNA digested with the *Not*I restriction enzyme was resolved on a 0.8% agarose gel and stained with ethidium bromide. Lanes 1 to 5, DNAs of the LAT$^-$, 524, 524R, 167, and 333 viruses, respectively; lane M, a 1-kb DNA marker ladder. (B) The DNA from the gel shown in panel A was transferred to a nylon membrane and hybridized with ^{32}P-labeled *Sph*I-*Bam*HI fragment DNA (Fig. 1) to confirm the deletions in the 624-bp *Not*I-*Not*I fragment. Arrow I indicates the position of the intact 624-bp *Not*I-*Not*I fragment; arrow D indicates the expected band of the *Not*I-*Not*I fragment from which the 246-bp neuronally responsive region had been deleted in mutant 524. The positions corresponding to DNA markers are shown to the right in kilobases. (C) DNA from virus 333 (lanes 1 and 3) and mutant 167 (lanes 2 and 4) was digested with *Sph*I plus *Bsi*WI (lanes 1 and 2) or *Sph*I only (lanes 3 and 4). Arrowheads indicate the two expected *Sph*I DNA fragments released from across the TR_L-U_L and U_L-IR_L junctions (Fig. 1) encompassing the LAT promoter. The newly created *Bsi*WI site within those fragments of mutant 167 is shown in lane 2. The positions of DNA markers are shown to the left in kilobases.

each PCR experiment, four HotStart50 tubes (Molecular Bio-products, Inc., San Diego, Calif.) were used for quantitating the viral DNA content in each sample. In each tube, 8 μl of a 50-ng/μl ganglion DNA solution was mixed with PCR reagents (2.5 μl of 10× PCR buffer, 2.5 μl of 4 mM each deoxynucleoside triphosphate) 2.5 μl each of 5 μM LATMA4 and LATMA5 primers, 2 μl of H_2O, and 5 μl of pHSV2e competitor DNA at fourfold-increasing molar concentrations. Tubes were incubated first at 70°C for 3 min to melt the wax in the HotStart50 tubes and then at room temperature for 5 min so that the wax would harden and seal the reaction mixture. Then, 25 μl of a mixture of 0.65 μl (2.3 U) of polymerases of the Expand High Fidelity PCR system (Boehringer Mannheim), 2.5 μl of 10× PCR buffer, and 22 μl of H_2O was layered into each tube over the wax. In every set of experiments, both water and DNA from uninfected guinea pigs were included as negative controls. The PCR mixtures were incubated in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, Conn.) with the following programs: (i) 3 min each at 97, 57, and 72°C; (ii) 1 min at each of these three temperatures for 20 cycles; and (iii) 30 s at each of these three temperatures for a final 40 cycles. To visualize the PCR products, 16 μl of each reaction mixture was resolved on a 1.25% agarose gel and stained with ethidium bromide. The amplified viral DNA products migrated as 150 bp, while the amplified competitor DNA migrated as 221 bp (see Fig. 5A).

QC-RT PCR was used to estimate the number of LAT copies in latently infected guinea pig ganglia. Guinea pig ganglia were harvested, as described above, and homogenized in 1 ml of RNA Stat-60 with a Tekmar Tissumizer. RNA was purified by the RNA Stat-60 method according to the manufacturer's instructions, resuspended in diethyl pyrocarbonate-treated water, and stored at −80°C. Competitor RNA in each reaction represented a 271-base in vitro transcript, derived by T7 RNA polymerase from plasmid pHSV2e, which had been linearized at an *Xba*I site. Five QC-RT PCRs were performed to quantitate the LAT in each sample. About 1 ng of sample RNA in 5 μl was added to each tube. From tubes 1 to 4, 5 μl of competitor RNA was added in fourfold-increasing concentrations. Five microliters of water was added to tube 5. Then, 7 μl of the following reagent mixture was added to each tube: 6 μl of 5× RT buffer, 1 U of Tuffin RNase inhibitor (Trevigen Inc., Gaithersburg, Md.), and 10 U of RNase-free DNase I (Boehringer Mannheim). Samples were incubated at 37°C for 20 min, and then another 10 U of DNase I was added to each tube and the tubes were incubated 30 min more at 37°C. DNase was inactivated by heating to 65°C for 15 min, and then the samples were chilled on ice. For cDNA synthesis, 12 μl of the following reagent mixture was added to each tube: 3 μl of 10 mM each deoxynucleoside triphosphate, 2 μl of 100 mM dithiothreitol, 5 μl of a 5 μM solution of the LATMA5 primer, and 2 μl of Moloney murine leukemia virus RT (Life Technologies, Gaithersburg, Md.). No RT was added to tube 5. All tubes were incubated at 37°C for 60 min and at 95°C for 5 min. Then, 5 μl (one-sixth of each RT reaction product) of cDNA was subjected to DNA PCR as described earlier for QC PCR, but no DNA competitor was added. Sixteen microliters of each reaction product was resolved on a 1.25% agarose gel to visualize the PCR products. The amplified viral LAT products migrated as 150 bp, while the amplified competitor RNA migrated as 221 bp (see Fig. 5B).

Statistical analysis. Since the numbers of latent HSV-2 genomes and LATs were found to follow a normal log distribution, analyses were performed on the logs of the data points. Geometric means ± standard errors are reported. Comparisons among the results of experiments with each virus strain were made with Tukey's Studentized Range (HSD) Test, with *P* values adjusted for multiple comparisons, and by analysis of variance, with multiple comparisons.

RESULTS

Construction of mutant viruses 524 and 167. By specifically truncating, deleting, and mutating sequences of the primary HSV-2 LAT promoter and studying its residual activity in transient expression assays, we showed previously that the 5′ portion of this promoter is particularly active in neuronal cell lines. Promoter constructs lacking this region possess 90% less activity. A novel nuclear protein binding element we called LAT-3 was also identified and shown to be neuronally responsive, with 80% less promoter activity of constructs in which this element was mutated (28). Based upon these findings, we modified the LAT promoter of HSV-2 strain 333 and studied the effects of these changes on LAT expression in vivo and on virus latency and reactivation.

We deleted 246 bp of the neuronally responsive region or introduced a 2-bp point mutation at the critical bases of the LAT-3 element and recombined DNA fragments containing these alterations into HSV-2 strain 333. The neuronally responsive region deletion mutant was designated strain 524, and the LAT-3 mutant was designated strain 167. A rescuant virus termed 524R was generated from deletion mutant 524 by using similar procedures.

The genomic structure and mutations in these three new recombinant viruses were confirmed with restriction endonuclease mapping and Southern blot hybridization. Shown in Fig. 2A are the restriction profiles of virion DNA from HSV-2 strains LAT$^-$, 524, 524R, 167, and 333, following *Not*I digestion. The digestion pattern of each virus is similar, with the exception of bands containing the desired changes. The deletion in recombinant 524 and its repair in strain 524R are best visualized in the Southern blot shown in Fig. 2B. Here, a

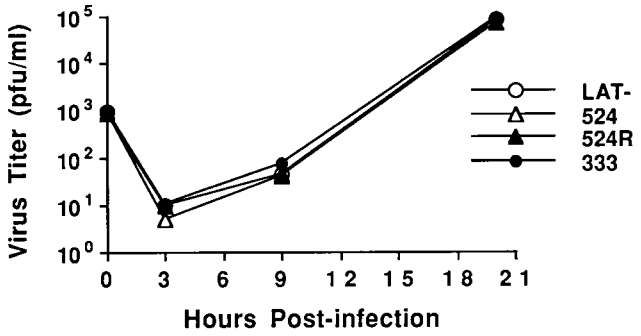

FIG. 3. Virus replication in Vero cells. One-step growth curves of viruses 524 and 524R are compared with those of the LAT$^-$ mutant and wild-type virus strain 333. Vero cell cultures were infected with each of these viruses at a multiplicity of infection of 0.01 and incubated for 0, 3, 9, or 20 h at 37°C. Titers of the cell-free viruses recovered at different time points for each culture were determined by plaque assay.

^{32}P-labeled *Sph*I-*Bam*HI fragment (Fig. 1) was used as a probe. As expected, *Not*I digestion of the parental, wild-type strain 333 (lane 5) yielded the intact 624-bp fragment encompassing the LAT promoter region, and this fragment was absent from the LAT$^-$ mutant genome (lane 1). Only 246 bp of this fragment had been deleted in strain 524, so that *Not*I digestion yielded the appropriate 378-bp residual segment of DNA (lane 2). In lane 3, DNA from 524R showed restoration of the intact *Not*I band. Recombinant 167 (lane 4) harbors only a 2-bp point mutation, so the length of the *Not*I fragment was the same as in the wild-type virus.

The base changes in mutant 167, however, introduced a new *Bsi*WI site, as documented by the *Sph*I and *Bsi*WI mapping shown in Fig. 2C. Since the *Sph*I site lying upstream of the LAT promoter falls within the U_L region, and the nearest downstream *Sph*I site is in the long repeat elements, two different *Sph*I fragments encompass the LAT promoter (lanes 1, 3, and 4). Codigestion of these two fragments with *Bsi*WI released three fragments from mutant 167, two of which migrated together (lane 2), but did not affect the fragments from wild-type DNA.

HSV-2 neuronally responsive region mutants replicate at wild-type levels in vitro. The above data confirmed the desired structures of five viruses. The ability of all of these viruses to replicate efficiently and comparably in vitro was then verified by one-step growth studies in Vero cells. As shown in Fig. 3, the new recombinant strains, 524 and 524R, replicated as well as the LAT$^-$ mutant and the wild-type strain 333. The mutant 167 also replicated as well as the wild-type strain 333 (data not shown).

HSV-2 LAT mutants replicate and induce lesions in guinea pigs, similar to wild-type virus. Before latent infection and reactivation of the mutants could be addressed in the guinea pig model of genital herpes, we first verified that each of the recombinants replicates in the periphery, causes lesions, and spreads efficiently to the lumbosacral ganglia. After intravaginal inoculation, guinea pig genital lesions were scored daily, up to 14 days postinfection. As shown in Fig. 4A, all five viruses caused comparable acute genital disease with respect to lesion severity scores and duration. In the experiment shown, the LAT$^-$ mutant yielded higher lesion scores on days 6 and 7, but this was not seen in our two preliminary experiments terminated at day 9 postinfection (data not shown) or in a prior publication about this virus (14). Thus, the higher lesion scores here most likely indicate experimental variation.

Virus replication in the vaginal epithelium was monitored by collecting secretions on swabs on days 1 to 3, 5, and 7, and the titers of recovered virus were determined by plaque assay. As shown in Fig. 4B, all five viruses replicated efficiently in the vaginal epithelium.

To investigate the ability of the mutants to reach and replicate in sensory ganglia, lumbosacral ganglia were harvested at days 5 and 6 postinfection. Here, virus titers of the ganglionic homogenates were determined by plaque assay on primary rabbit kidney cell monolayers. As shown in Fig. 4C, all five viruses were recoverable from the ganglia. For four of them (viruses 524, 524R, 167, and 333) the titers were comparably low. Interestingly, the titers for mutant LAT$^-$ were, if anything, higher than those achieved with the 524R and 333 viruses. In previous studies of the LAT$^-$ mutant, no such differences in ganglion virus titers were noted (14).

HSV-2 LAT mutants are not impaired in their ability to establish latency. To investigate the effects of each of the mutations on the establishment of virus latency, LAT expression, and disease reactivation, we conducted two experiments. In the first experiment, 10 guinea pigs were inoculated intravaginally with 2×10^5 PFU of each virus. Animals that survived the acute infection (about five per group) were then scored daily for recurrent lesions from days 12 to 60 postinfection. On day 66 postinfection, all animals were sacrificed, and their lumbosacral ganglia were recovered for further studies. In a second experiment, 17 guinea pigs were infected in each group. The LAT$^-$ virus was not included in this experiment. To reduce the considerable mortality associated with acute infection, these animals were treated with acyclovir for 7 days, starting at 24 h postinfection. Recurrent skin lesions were scored daily from days 15 to 90 postinfection. Animals were then sacrificed on day 116, and their lumbsacral ganglia were harvested.

The quantity of latent viral DNA in all animals was determined by QC PCR. An example of the resulting data is shown in Fig. 5A. In the set of reactions labeled "A" (lanes 1 to 4), the competitor DNA and latent viral DNA yielded equivalent bands in lane 1 (0.078 fg of competitor). Equivalence was achieved in sample B in lane 7 (1.25 fg of competitor). Since the DNA in sample C was from uninfected ganglia, no competition occurred.

The results of QC PCR determinations for two independent animal experiments are plotted in Fig. 6. The geometric mean number of copies of latent HSV-2 strain 333 DNA was 1.4×10^3 (95% confidence interval [CI] of 4.0×10^2 to 5.1×10^3) for the first experiment and 2.5×10^3 (95% CI of 6.3×10^2 to 1.0×10^4) for the second experiment. Although the quantity of latent DNA varied somewhat from these geometric means in each experiment, and the numbers of latent LAT$^-$ virus DNA copies were highest, none of these differences reached statistical significance ($P > 0.2$ by analysis of variance). Thus, the abilities of the mutants to establish latency are not impaired.

Deletion of the neuronally responsive region reduced LAT expression during latency. Total RNA was isolated from ganglia dissected from latently infected animals on day 66 (experiment 1) or day 116 (experiment 2) postinfection. To determine the total LAT copy number in the extracts, each RNA sample was subjected to QC-RT PCR. An example of the method used and the data obtained by it are shown in Fig. 5B. Here, in vitro-synthesized competitor RNA in fourfold-increasing concentrations was mixed with 1.25 ng of sample RNA. The amount of LAT was estimated according to how much competitor RNA yielded an equivalent band after reverse transcription and PCR amplification. In the examples shown in Fig. 5B, equivalence of amplified products is seen

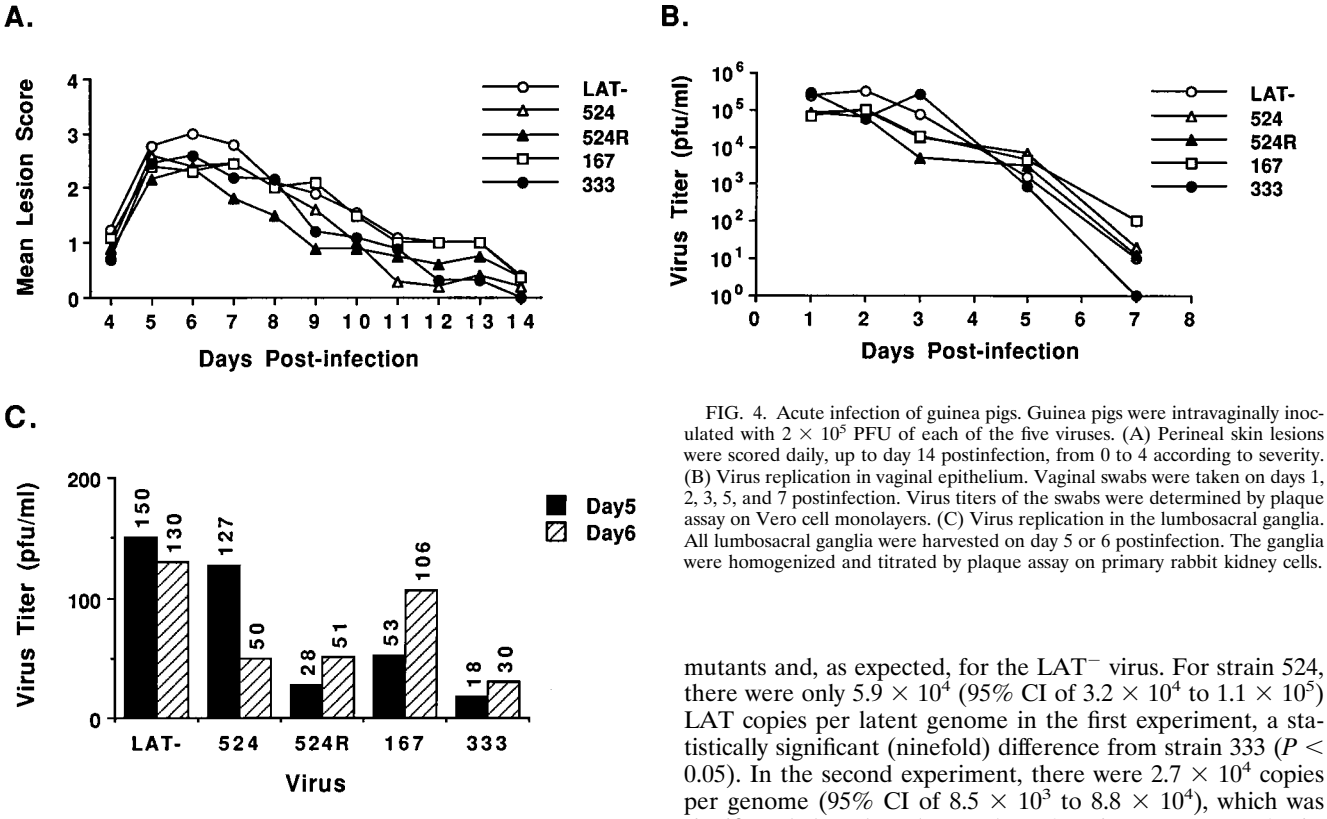

FIG. 4. Acute infection of guinea pigs. Guinea pigs were intravaginally inoculated with 2×10^5 PFU of each of the five viruses. (A) Perineal skin lesions were scored daily, up to day 14 postinfection, from 0 to 4 according to severity. (B) Virus replication in vaginal epithelium. Vaginal swabs were taken on days 1, 2, 3, 5, and 7 postinfection. Virus titers of the swabs were determined by plaque assay on Vero cell monolayers. (C) Virus replication in the lumbosacral ganglia. All lumbosacral ganglia were harvested on day 5 or 6 postinfection. The ganglia were homogenized and titrated by plaque assay on primary rabbit kidney cells.

between lanes 1 and 2 and also in lane 7, corresponding to estimated LAT concentrations of 0.98 and 1.95 fg of competitor RNA, respectively. RNA from an uninfected guinea pig (sample C [lanes 11 to 14]) showed no LAT signal.

Using these data and the already determined quantities of latent HSV-2 DNA in each guinea pig, we calculated the ratio of LAT copies per latent viral genome (Fig. 7). The geometric mean number of HSV-2 strain 333 LATs per latent genome was 5.2×10^5 (95% CI of 3.0×10^5 to 9.2×10^5) for the first experiment (Fig. 7A) and 4.0×10^5 (95% CI of 1.1×10^5 to 1.5×10^6) for the second experiment (Fig. 7B). The geometric mean numbers for recombinant strains 167 and 524R were similar to the levels for the wild type in both experiments, but the numbers of LATs per latent genome were lower for the 524 mutants and, as expected, for the LAT$^-$ virus. For strain 524, there were only 5.9×10^4 (95% CI of 3.2×10^4 to 1.1×10^5) LAT copies per latent genome in the first experiment, a statistically significant (ninefold) difference from strain 333 ($P < 0.05$). In the second experiment, there were 2.7×10^4 copies per genome (95% CI of 8.5×10^3 to 8.8×10^4), which was significantly less than the number of copies per genome for its rescuant, 524R ($P < 0.05$). The previously described LAT$^-$ virus was included in the first experiment as a negative control. LAT signal was undetectable by QC-RT PCR, being at least 5 logs less than that of strain 333 ($P < 0.001$), or less than 2.1 LATs per latent genome.

Virus reactivation is not impaired in HSV-2 deleted for the neuronally responsive region of LAT. To assess the effects of the targeted promoter mutations in strains 524 and 167 on rates of spontaneous disease reactivation, guinea pigs that survived acute infection were scored daily for recurrent genital skin lesions, up to day 60 in the first experiment and day 90 in the second experiment. The mean cumulative recurrences for each animal group are shown in Fig. 8. As previously reported, the rate of reactivation of the LAT$^-$ virus was reduced compared with that of the wild-type virus 333 (examined only in the

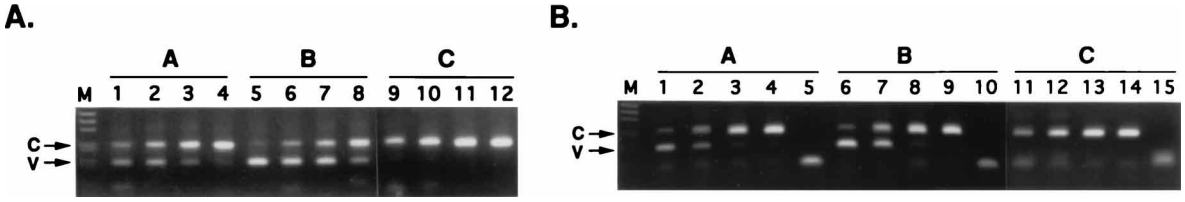

FIG. 5. Examples of QC PCR and QC-RT PCR analyses. A pair of PCR primers termed LATMA4 and LATMA5 was used to amplify DNA in the major LAT region in the presence or absence of the competitor plasmid pHSV2e DNA. Four PCRs were run for each sample by adding fourfold-increasing amounts of the competitor (0.078, 0.31, 1.25, and 5 fg) from tubes 1 to 4 to 400 ng of ganglion DNA and the PCR reagents. (A) A typical ethidium bromide-stained agarose gel of the QC PCR products from three different DNA samples. Samples A and B were from mutant 524- and LAT$^-$-infected animals, respectively, while sample C was from an uninfected animal. Arrow V indicates the 150-bp PCR products from amplified viral DNA, and arrow C indicates the 221-bp products from the amplified competitor DNA. No viral sequence was detected in sample C. Lane M, a 1-kb DNA ladder. (B) A stained agarose gel of QC-RT PCR products. Five reactions were performed to quantitate the LATs in each sample. cDNAs were synthesized by adding 1.25 ng of ganglion RNA to each tube, fourfold-increasing amounts of competitor RNA (0.48, 1.95, 7.81, 31.25 fg), primer LATMA5, and reagents for reverse transcription. Tube 5 served as a competitor and RT-negative control. Five microliters of cDNA was then used for DNA PCR with both the LATMA4 and LATMA5 primers. Samples A and B were RNAs from animals infected with strains 167 and 333, respectively, while sample C was from an uninfected animal. Arrow V indicates the 150-bp PCR products from LAT, and arrow C indicates the 221-bp PCR products from competitor RNA. No LAT signal was detected from sample C. There were no PCR products in RT-negative reactions (lanes 5, 10, and 15).

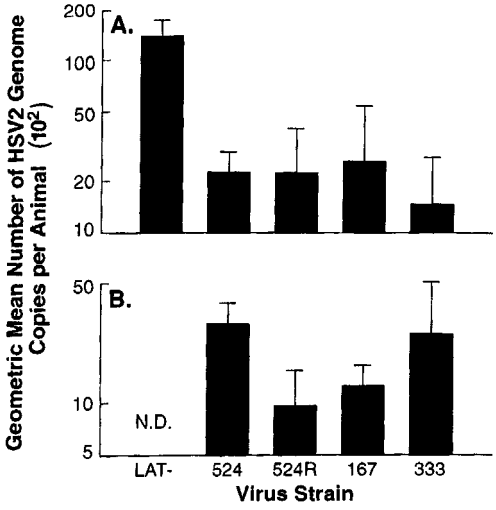

FIG. 6. Quantitation of viral DNA in latently infected ganglia. The geometric means (± standard error) of HSV-2 DNA copies in latently infected ganglia from two independent experiments were determined by QC PCR. In the first experiment (A), guinea pigs were intravaginally inoculated with 2×10^5 PFU of each of the five viruses, and ganglia were harvested 66 days postinfection. Data are for four animals per group infected with virus strains 524, 167, and 333, three animals infected with the LAT$^-$ mutant, and five infected with rescuant 524R. In the second experiment (B), to reduce mortality, animals were treated with acyclovir intraperitoneally (25 mg/animal) once a day for 7 days starting 24 h after infection. Ganglia were harvested on day 116 postinfection. The data are for three animals per group infected with strains 524 and 167, four infected with strain 524R, and two infected with the parental strain, 333. In both experiments, the DNA content of ganglia infected with the mutant viruses was not significantly different from that of ganglia from animals infected with strain 333. N.D., not done.

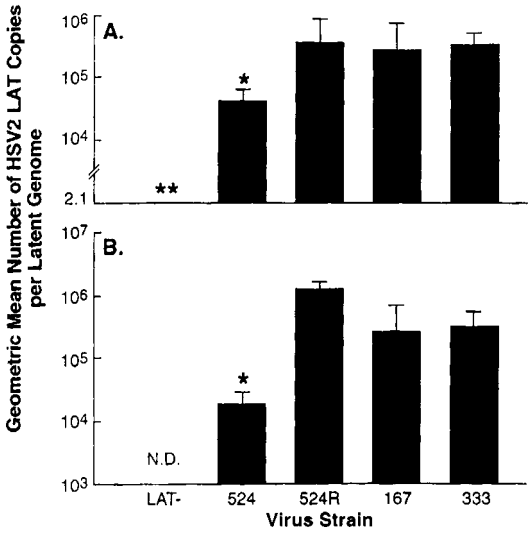

FIG. 7. LAT content in latently infected ganglia. The numbers of LAT copies in latently infected ganglia were determined by QC-RT PCR in two independent experiments, as explained in the legend to Fig. 6. The data shown represent the geometric means (± standard errors) of LATs per latent viral genome. The numbers of animals used to generate these data were the same as those described in the legend to Fig. 6. No LATs were detected in animals infected with the LAT$^-$ mutant. The limit of the assay was 2.1 LATs per latent genome. Asterisks indicate statistically significant differences relative to values for strain 333: *, $P < 0.05$; **, $P < 0.001$. N.D., not done.

first experiment). Infection with mutant strain 524 appeared to reactivate slightly less frequently than strain 333 infection in the first experiment (Fig. 8, panel A), but this result was not confirmed in the second experiment (Fig. 8, panel B). The rescuant, 524R, and the point mutant 167 appeared to reactivate at a normal rate.

DISCUSSION

The promoter of the HSV-2 LAT gene is a complex linear array of *cis*-acting elements whose complete deletion leads to a profound reduction in LAT accumulation in latently infected neurons (not detectable by Northern blot analysis or RT PCR) and a substantial decrease in the rate at which the virus reactivates (Fig. 8) (14). In the present study, we sought to delineate promoter elements most essential to LAT expression in neurons and to estimate the capacity of LATs to modulate disease reactivation. We compared wild-type (333) and rescuant (524R) strains of HSV-2 with mutants that express normal levels of LATs in vivo (strain 167), 9- to 15-fold-reduced levels of LATs (strain 524), and no detectable LATs (LAT$^-$ strain). By quantitative PCR assay, all of the mutants established latency efficiently, but the ability of the LAT$^-$ strain to cause recurrent genital herpes was impaired. The mutant that expressed moderately reduced levels of LATs recurred normally.

All evidence indicated that mutant 524, in which the 246-bp neuronally responsive promoter region had been deleted, its rescuant, 524R, and the mutant 167, in which the LAT-3 element had been specifically altered to abrogate nuclear factor binding, were constructed as desired (Fig. 2). All these strains replicated in vitro (Fig. 3), infected guinea pig genitalia, spread to the lumbosacral ganglia (Fig. 4), and established latency (Fig. 6) as well as did the wild-type HSV-2 strain 333 from which they were derived. As evidenced by the results of QC PCR, all five of the viruses studied were shown to persist in comparable levels in latently infected ganglia.

The fact that the present HSV-2 mutants established latency efficiently is in agreement with many published reports involving HSV-1 mutants in mice (15, 17, 22) and rabbits (12, 18). These data are not readily reconciled, though, with the earlier data and more recent data of Sawtell and Thompson (21, 27) indicating that LAT-negative (KOS/62) and LAT-impaired (KOS/29) mutants of HSV-1 established latency in about one-third of the number of neurons that the parental virus strain did. It is possible, of course, that pertinent methodological differences exist between their studies and ours. In this regard, it is particularly noteworthy that reduced establishment of latency was seen by Sawtell and Thompson (21) in trigeminal but not in lumbosacral ganglia, the site of HSV-2 persistence in the genital herpes model we employed.

Of further relevance to these issues are the recent data of Garber et al. (9a) indicating that LAT-deleted HSV-1 expressed lytic genes in a greater number of neurons in ganglia during acute infection, implying a defect in the switch from active to latent infection. This would, perhaps, leave a smaller reservoir of neurons from which virus could subsequently reactivate. Some of the present data are, in fact, concordant with that observation. The HSV-2 LAT$^-$ mutants, and to a lesser extent the 524 and 167 mutants, achieved relatively higher titers in ganglia on day 5 and/or 6 than did the parental and rescuant viruses (Fig. 4C). Nevertheless, they yielded wild-type or, in the case of the LAT$^-$ mutant, higher rather than reduced levels of latent DNA (Fig. 6A). We suspect that the higher levels of LAT$^-$ virus in acutely infected ganglia reflect experimental variation. Similar results were not obtained in its original characterization studies (14).

By altering or deleting *cis*-acting promoter regions, each of which markedly influences LAT promoter expression in vitro,

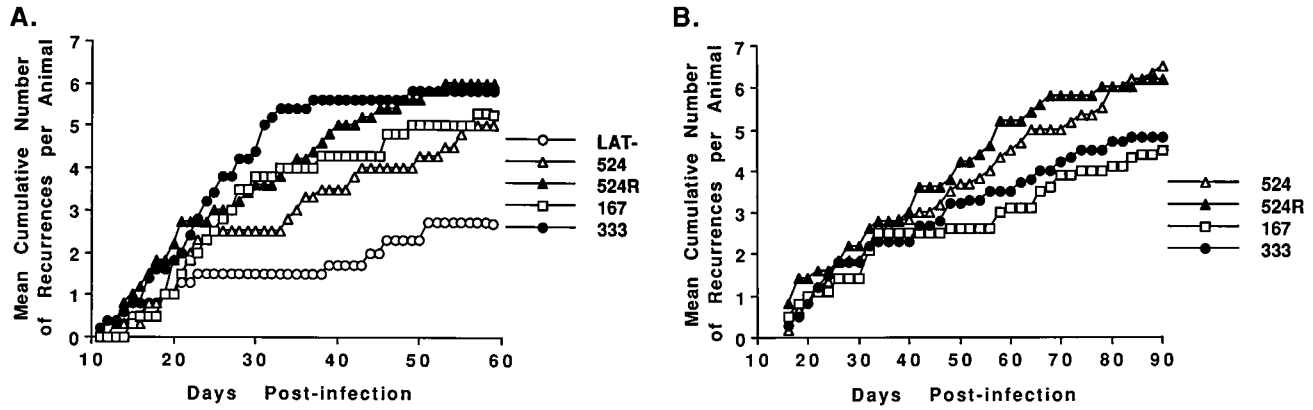

FIG. 8. Cumulative recurrences of genital herpes. In the two independent experiments described in the legend for Fig. 6, recurrent guinea pig perineal skin lesions were scored daily from day 12 to day 60 or 90 postinfection. Animals were infected with five viruses (A) or with four (B).

we created new recombinant viruses that expressed in vivo essentially normal (mutant 167) or moderately reduced (mutant 524 [9- to 15-fold]) quantities of LAT relative to those expressed by parental strain 333 and the rescuant 524R. For mutant 524, the reduction in LAT expression in vivo closely paralleled that predicted by the in vitro transient expression studies (90% less promoter activity in PC12 or IMR32 cells) (28). Thus, *cis*-acting elements within the neuronally responsive region of the LAT promoter are functional and available to neuronal transcription factors during latent infection in ganglia. This finding indicates that this portion of the promoter may not be extensively modified and remains active in the latent viral genome. The fact that mutant 167 did not yield reduced quantities of LATs in vivo implies either that the LAT-3 *cis*-element is blocked in vivo or that the transcription factor is not present in latently infected neurons.

The finding that guinea pigs infected with mutant 524 accumulated about 1 log (9- to 15-fold) fewer copies of LATs in their ganglia than did animals infected with wild-type virus but still had over 4 logs more copies (95% CI of 3.5 to 5.4 logs) than seen with the LAT$^-$ virus afforded us the unique opportunity to determine the efficiency with which LATs modulate spontaneous disease reactivation. Animals infected with mutant 524 developed wild-type or near-wild-type rates of genital recurrences (Fig. 8). The LAT$^-$ mutant reactivated at a rate that was about one-fourth to one-third of the wild-type rate in the present experiment and even lower than that in previous experiments (14). The reactivation rates of LAT$^-$ virus in this study, which are relatively higher than those that have been previously reported, may reflect the levels of virus achieved in acutely infected ganglia (Fig. 4C) and the levels of latent LAT$^-$ genome copies (Fig. 6A), which were somewhat higher and higher, respectively, than those in the prior study (14).

In prior studies, researchers discerned that mutants which are severely impaired for LAT expression reactivate poorly (14, 15, 18, 19), but they could not estimate the potency of the LATs in mediating this effect. The present data indicate that moderate (9- to 15-fold) reductions in HSV-2 LATs are not sufficient to impact perceptibly the rates of spontaneous disease recurrence in the guinea pig genital model; far more profound reductions in LAT accumulation are required to appreciably alter recurrence rates. This finding indicates that the LATs, at least as reflected by the quantity of the major LATs in ganglia, are inefficient and weak determinants of disease reactivation. Studies of a pair of epidemiologically unrelated HSV-2 isolates with widely differing rates of clinical reactivation and LAT expression yielded similar conclusions (2). The present experiment confirms that earlier observation by careful creation and quantitative analyses of targeted mutations on an otherwise isogenic background.

If the accumulation of LATs in latently infected ganglia has at best an inconsistent effect on establishment of latency and is a poor predictor of virus reactivation, then what role does this gene play? It remains possible that the arbiter of reactivation is not LAT accumulation as much as the continued presence and integrity of LAT region sequences. It is obvious now that the LAT promoter region far upstream affects LAT accumulation, but it does not determine reactivation rates, because its partial deletion in strain 524 had no obvious impact on disease recurrence. Regions downstream of the LAT start site might be important, however. It has been hypothesized that the CpG-rich sequences downstream of the primary LAT promoter influence HSV latency and gene expression (1). The fact that the LAT$^-$ mutant, which lacks these sequences, reactivates poorly and that mutant 524, which retains these sequences, reactivates well may be relevant to this hypothesis. This notion can be directly addressed by specifically mutating the CpG-rich elements in HSV-2 and analyzing the effects of those alterations on spontaneous reactivation of the virus in the guinea pig.

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