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# Downstream Regulatory Elements Increase Acute and Latent Herpes Simplex Virus Type 2 Latency-Associated Transcript Expression but Do Not Influence Recurrence Phenotype or Establishment of Latency

TETSUSHI YOSHIKAWA,[1] LAWRENCE R. STANBERRY,[2] NIGEL BOURNE,[2] AND PHILIP R. KRAUSE[1]*

*Division of Viral Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland,[1] and Division of Infectious Diseases, Children's Hospital Research Foundation, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio[2]*

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**The role of putative promoter or activator sequences downstream of the herpes simplex virus type 2 latency-associated transcript (LAT) promoter and upstream of the LAT intron was investigated in vivo by constructing and evaluating mutant viruses with deletions in this region. The deletion of LAT promoter sequences upstream of the primary LAT transcript reduced levels of LAT expression during productive infections, compared with the LAT expression level of wild-type virus, and abolished LAT expression during latency. The deletion of the putative downstream regulatory elements reduced but did not eliminate LAT expression during productive and latent infections. The deletion of both regions almost completely eliminated acute LAT transcription, although additional acute LAT-region transcription directed by sequences upstream of either region was detected by reverse transcriptase PCR. The deletion of the downstream elements did not influence the ability of the virus to reactivate from latently infected guinea pigs relative to the ability of the wild-type virus to reactivate; thus, decreased LAT expression did not affect the frequency of recurrence. The deletion of both regions did not affect the ability of the virus to establish latency. We conclude that downstream regulatory elements are necessary for maximal acute LAT expression but do not constitute an independent promoter during latency and do not play an obvious role in the establishment of or reactivation from latency.**

After replication at a site of inoculation, herpes simplex virus (HSV) establishes latency in the sensory ganglia innervating that site. Periodically, the virus may reactivate to cause symptomatic or asymptomatic recurrences (4, 20). Of the 80 or so described genes of HSV, only the latency-associated transcript (LAT) is uniformly transcribed during latency (6, 8, 35). Mutations in sequences directing HSV type 1 (HSV-1) (2, 14, 21, 28, 34, 36) and HSV-2 (17) LAT transcription impair virus reactivation but do not influence the establishment of latency in rabbits (15) or guinea pigs (17). The role of these sequences in the establishment of virus latency in mice is unclear (28, 34).

During HSV-1 latency, abundant LAT introns of 2.0 and 1.4 to 1.5 kb (18, 30, 38, 40) are spliced (12) from a less abundant primary LAT transcript (37, 41) (approximately 8 to 9 kb in size). During productive infections, only the 2.0-kb LAT intron and the primary LAT have been detected. While transcription during HSV-2 latency has not been as thoroughly investigated, a single 2.2-kb LAT intron is also readily detected both during acute and latent infection (5, 19, 24).

Promoter sequences which appear to direct transcription of the HSV-1 primary LAT are present approximately 600 to 700 bp upstream of the LAT intron (1, 9, 10, 42). This promoter contains a TATA box, a cyclic AMP-responsive-element binding (CREB) site (27), Sp1 sites, and a binding site for a protein denoted the LAT promoter binding factor (43). The deletion of these promoter sequences abolishes latent HSV-1 LAT transcription but permits continued detection of the HSV-1 LAT intron during acute infections in tissue culture (26). This observation, together with the identification of promoter activity in transient transfection and infection assays in sequences downstream of the primary LAT 5′ end but upstream of the LAT intron (13), led to the hypothesis that acute HSV-1 LAT transcription may be regulated by two independent promoters. The traditional promoter upstream of the primary LAT has been referred to as LAP1, and the putative downstream regulatory sequences between LAP1 and the LAT intron have been denoted LAP2. The LAP2 sequences contain potential AP2 and Sp1 binding sites in a high-GC-content region, together with a CT-rich element which is characteristic of several TATA-less constitutive eukaryotic promoters (16). In a recently published experiment, a *Sty*I-*Sty*I fragment upstream of the CT-rich element contained within the HSV-1 LAP2 region was removed from wild-type HSV-1, and this deletion did not affect acute or latent HSV-1 LAT expression or the HSV-1 reactivation phenotype in explanted mouse trigeminal ganglia (23).

HSV-2 LAT promoter sequences homologous to the HSV-1 LAP1 region containing TATA, CREB, and Sp1-AP2 homologies have also been described (19, 25). More-detailed functional analyses of this region revealed multiple AP2 binding sites, an AP1 binding site, the CREB site, and four additional unidentified DNA binding footprints (39). The deletion of sequences homologous to HSV-1 LAP1 from HSV-2 abolishes latent LAT transcription and dramatically reduces spontaneous recurrences from latently infected guinea pigs but permits acute HSV-2 LAT transcription (17). HSV-2 also contains sequence elements homologous to HSV-1 LAP2 between the 5′ ends of the primary LAT and the LAT intron, including Sp1-AP2 homologies and CT-rich elements (Fig. 1B). To explain acute LAT transcription in LAP1$^-$ mutants, as in HSV-1, we hypothesized that HSV-2 LAP2 may contain important

* Corresponding author. Mailing address: HFM-457, Bldg. 29A, Rm. 1C16, MSC4555, 29 Lincoln Dr., Bethesda, MD 20892-4555.

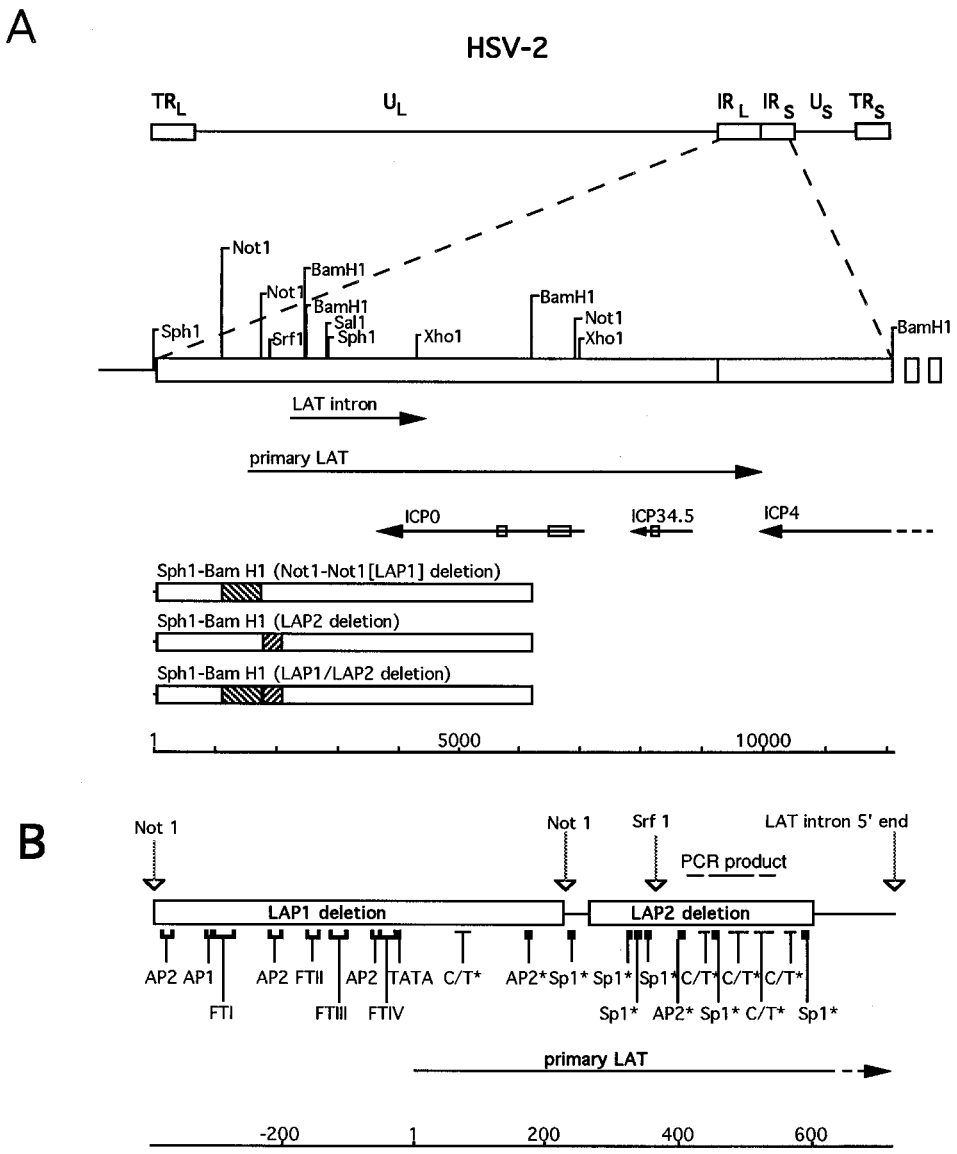


FIG. 1. Map of HSV-2 LAT and constructs used to generate mutations. (A) Restriction endonuclease sites are shown relative to the positions of the HSV-2 major and primary LATs, ICP0, ICP34.5, and ICP4. Introns are denoted within each transcript by a box. Regions corresponding to the LAP1 and LAP2 deletions are shaded within boxes corresponding to the *Sph*I-*Bam*HI fragment, which was the insert in the plasmid vectors used to construct the mutant viruses. Distance in base pairs is shown relative to the first *Sph*I site, within the unique long region. Abbreviations: $U_L$, unique long; $TR_L$, terminal repeat, long; $IR_L$, internal repeat, long; $IR_S$, internal repeat, short; $U_S$, unique short; $TR_S$, terminal repeat, short. (B) Transcription factor binding sites within LAP1 and LAP2 regions are shown. Upstream of the primary LAT 5′ end, these sites were mapped by functional assays (39). In addition, a CREB site overlies FTIV. Downstream of the primary LAT 5′ end, the sites marked with an asterisk are based only on sequence analyses. Consensus sequences used were Sp1 (GGGCGG), AP2 (CCGCCCGCG), and CT (more than 20 bases exceeding 90% CT content). The expected product of the reverse transcriptase PCR experiments is denoted with a dashed line. Distance in base pairs is shown relative to the 5′ end of the primary LAT, as mapped in vitro (39).

regulatory elements, possibly including an additional promoter. The roles of these sequences in LAT transcription and in latency establishment and reactivation phenotypes have not yet been investigated.

To delineate the role of downstream sequences in HSV-2 LAT transcription and latency, we deleted putative HSV-2 LAP2 sequences from a mutant virus already lacking the HSV-2 LAP1 promoter and from a virus with an HSV-2 333 wild-type genotype. We then characterized productive and latent infections of wild-type HSV-2 and of strains with LAP1, LAP2, or LAP1 and LAP2 deletions. Latent infections and reactivation patterns were observed in guinea pigs, which provide the only animal model of HSV latency in which the virus spontaneously reactivates to cause recurrent lesions, as it does in humans (31–33).

## MATERIALS AND METHODS

**Cells and tissue culture experiments.** Vero and SK-N-SH (human neuroblastoma) cells were obtained from the American Type Culture Collection (Rockville, Md.), and maintained in 1:1 minimum essential medium-medium 199, with 1% GASP (a mixture of glutamine, aureomycin, streptomycin, and penicillin) and 10% heat-inactivated fetal bovine serum (all from Quality Biologicals, Gaithersburg, Md.). To determine one-step growth characteristics of parental virus and mutant viruses, confluent Vero cells in 6-well dishes were inoculated in duplicate at time zero with $10^5$ PFU (at a multiplicity of infection [MOI] of approximately 0.1 PFU per cell) of each virus. For the 3- and 16-h time points, virus was allowed to adsorb for 60 min before the addition of medium. Cells were

scraped and freeze-thawed three times, and the plaque titers of the virus were determined in duplicate at the specified time points.

**Clones.** Restriction endonucleases and modifying enzymes were purchased from New England Biolabs (Beverly, Mass.), Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or Life Technologies (Gaithersburg, Md.) and used in accordance with the instructions of the manufacturers. To make LAP2 deletions, most of the sequences between the 5′ end of the primary LAT and the 5′ end of the LAT intron (positions 4081 to 4382) were deleted by *Bal* 31 digestion around an *Srf*I site from a clone spanning the *Sph*I-*Bam*HI fragment (17) shown in Fig. 1, blunting with T4 DNA polymerase, and religation. A clone containing the appropriate deletion was identified by restriction endonuclease digestion and sequencing. A clone with deletions of LAP1 and LAP2 was obtained by removing a *Not*I-*Not*I fragment (containing LAP1) from this plasmid.

**Construction of mutant viruses.** The parent virus for all of the viruses used in this study was 333pLAT$^-$, in which a *Not*I-*Not*I fragment spanning the HSV-2 LAP1 had been deleted. The rescue of this virus into 333pLAT$^R$ (renamed 333WT for convenience), which has a wild-type genotype and phenotype, has been described elsewhere (17). LAP2 sequences were deleted (via homologous recombination of deleted plasmids) from 333pLAT$^-$ (renamed 333LAP1$^-$ for convenience) and 333pLAT$^R$ to construct 333LAP1&2$^-$ and 333LAP2$^-$, respectively.

In order to make the LAP2 deletion mutant and the LAP1-LAP2 double promoter deletion mutant, appropriate plasmid DNA was cotransfected into Vero cells with purified 333pLAT$^R$ viral DNA and 333pLAT$^-$ viral DNA, respectively, with lipofectin (Life Technologies). The resultant viruses were plated in serial dilutions on 6-well plates, and individual plaques were selected, grown, and screened by Southern hybridization as previously described (17). After identification of mutant viruses, plaque purifications were performed until Southern hybridization identified no evidence of contamination with the parents. At this point, two additional plaque purifications were performed to yield stocks of each mutant. Additional Southern hybridizations were carried out on purified viral DNA digested with the enzyme *Sph*I, *Bam*HI, *Srf*I, *Not*I, or *Not*I plus *Xho*I. Blots were probed with *Sph*I-*Bam*HI plasmid or intact virion DNA that was radiolabeled with $^{32}$P with a random primer labeling kit (Amersham, Arlington Heights, Ill.).

**Guinea pig studies.** Animal studies were approved by the Children's Hospital Research Foundation Institutional Animal Care and Use Committee. Female Hartley guinea pigs (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 400 to 500 g were inoculated with approximately $10^{5.7}$ PFU of 333WT, 333LAP1$^-$, 333LAP2$^-$, or 333LAP1&2$^-$ on day 0 by rupture of the vaginal closure membrane with a moistened calcium alginate-tipped swab and instillation of 0.1 ml of virus. Lesion severity was scored daily (on a scale from 0 to 4) until resolution of the acute infection (32). All observations of the guinea pigs were performed by investigators who were unaware of the virus inoculum. Guinea pigs that were not evaluable for the entire observation period were excluded from analysis. Animals were housed in American Association for the Accreditation of Laboratory Animal Care-approved facilities and cared for in accordance with institutional guidelines.

**Northern (RNA) hybridization.** Vero cells and SK-N-SH cells were infected with virus at an MOI of 1.0 PFU per cell. Sixteen hours after infection, RNA was extracted from the infected cells. RNA was also extracted from latently infected guinea pig ganglia. Five micrograms of each RNA was subjected to electrophoresis on 1.5% agarose-formaldehyde denaturing gels, transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.), and hybridized according to the instructions of the manufacturer. The blots were probed with $^{32}$P-radiolabeled gel-pure *Sal*I-*Xho*I double-stranded DNA restriction fragments. A radiolabeled human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe (Clontech, Palo Alto, Calif.) was used to control for RNA quantity in some experiments.

**PCR amplification of viral RNA.** Vero cells were infected at an MOI of 5.0 PFU per cell. RNA was extracted from the infected cells 10 h after infection. Five micrograms of each extracted total RNA sample was treated with 80 U of RNase-free DNase I (Boehringer Mannheim) for 1 h and then denatured at 94°C for 15 min. Each sample was then incubated at 39°C for 1 h in a 30-μl solution containing Moloney murine leukemia virus reverse transcriptase (Life Technologies) and the downstream primer. Reverse-transcribed samples and RNA samples without reverse transcription were used in standard 100-μl PCR mixtures with both primers (upstream primer, AGAGCGAGACAGACACACGCAG; downstream primer, CGTTCCGTTTCTTCTCCCTCC).

Amplification was carried out in a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Norwalk, Conn.) under the following cycle conditions: 94°C for 1 min; and then 2 cycles at 98°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and 28 cycles at 95°C for 1 min, 55°C for 2 min, and 72°C for 2 min. After completion of amplification, 10 μl of the reaction mixture was loaded on a 1.5% NuSieve GTG agarose gel (FMC BioProducts, Rockland, Maine). The products were electrophoresed and subjected to Southern hybridization with $^{32}$P-radiolabeled gel-pure *Not*I-*Sal*I fragments from a plasmid containing the HSV-2 *Sph*I-*Bam*HI fragment.

**Semiquantitative PCR.** Semiquantitative PCRs to assess the quantity of viral DNA in latently infected ganglia were performed as previously described (17). Briefly, 100 ng of genomic DNA (quantified spectrophotometrically) extracted from pooled S1 to S3 ganglia of guinea pigs killed 27 days after inoculation with 333WT or 333LAP1&2$^-$ was subjected to PCR (94°C for 1.5 min, 52°C for 3 min, 72°C for 3 min) for 35 cycles, simultaneously using the primers CACCTGCCAG TCGAACGACCTCAT and AGCCGCCACCCCCCTCCCCTGCGT (which are specific for a 500-bp product in the HSV-2 morphologic transforming region, located in the HSV-2 *Bgl*II N fragment), and AGTCCATTTCTTGTCTGTCTC TCT and CTGGGGAACAAAGTAAGAGTCAAC (which are specific for a 500-bp product in guinea pig lactalbumin). The products were electrophoresed and subjected to Southern hybridization with $^{32}$P-radiolabeled gel-pure internal amplified fragments from a plasmid containing the HSV-2 *Bgl*II N fragment and with strain 2 fetal guinea pig DNA as a probe. Membranes were exposed to XAR film (Eastman Kodak, Rochester, N.Y.) for 6 h, and bands were quantified by densitometry on a digital image system (model IS-1000; Alpha Innotech Corp., San Leandro, Calif.). For each sample, the ratio of HSV-2 DNA signal to the guinea pig lactalbumin signal was calculated.

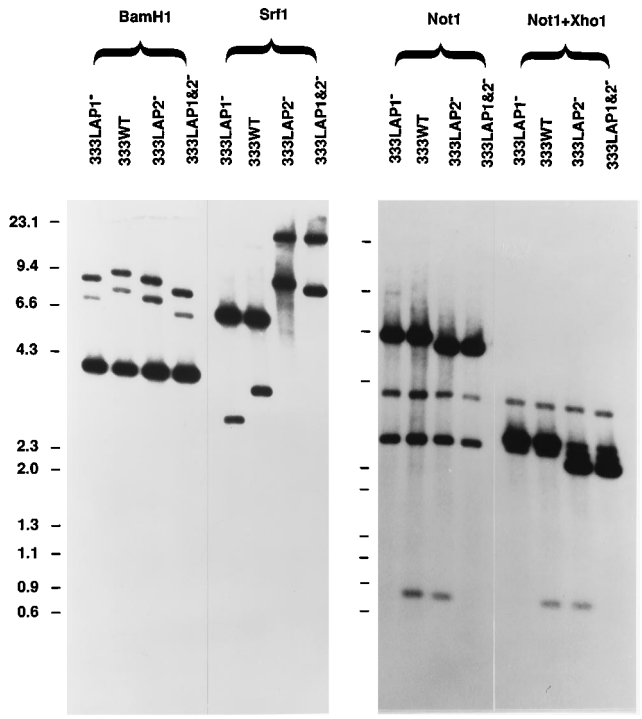


FIG. 2. Southern hybridizations of DNAs extracted from 333WT, 333LAP1$^-$, 333LAP2$^-$, and 333LAP1&2$^-$. Virus DNAs were digested with the denoted enzymes, subjected to electrophoresis, and hybridized with an *Sph*I-*Bam*HI probe. Marker sizes (in kilobase pairs) are shown to the left of each panel.

## RESULTS

**Mutant virus characterization.** To examine the role of LAP2 sequences during acute and latent infection, we constructed and evaluated mutants containing deletions in the LAP1 region (333LAP1$^-$), the LAP2 region (333LAP2$^-$), or both the LAP1 and LAP2 regions (333LAP1&2$^-$). Plasmid inserts used to construct these mutant viruses by homologous recombination with parent virus are shown in Fig. 1A. Binding sites for potential enhancer elements in this region are shown in Fig. 1B, mapped either by DNase footprinting and methylation interference (39) (upstream of the primary LAT start site) or on the basis of sequence analysis.

Appropriate construction of each mutant virus was demonstrated by Southern hybridization of DNA extracted from productively infected Vero cells (Fig. 2). The $^{32}$P-labeled double-stranded DNA probes used in these analyses spanned the *Sph*I-*Bam*HI fragment used in the construction of the mutants.

After digestion with *Bam*HI, each virus DNA yielded the expected 3.9-kb fragment between the two *Bam*HI sites inter-

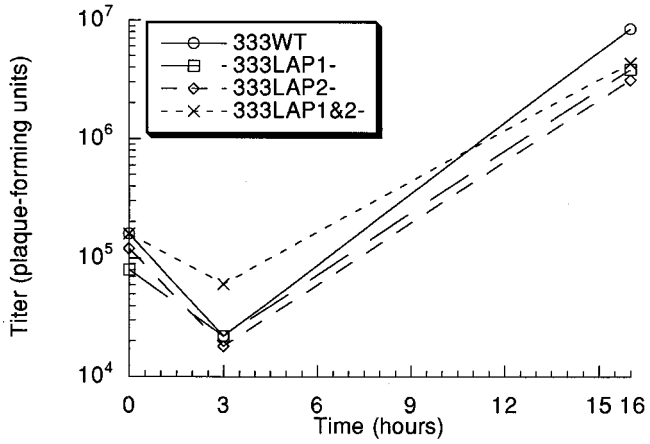


FIG. 3. One-step growth of 333WT, 333LAP1$^-$, 333LAP2$^-$, and 333LAP1&2$^-$ in tissue culture. Vero cells ($10^6$) were inoculated with each virus at an MOI of approximately 0.1 at time zero. The plaque titers of cultures were determined 0, 3, and 16 h after inoculation.

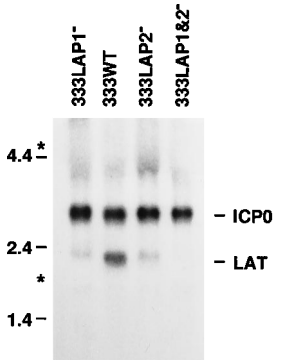


FIG. 4. Expression of LAT by each virus during productive infections. RNA extracted from Vero cells productively infected with each virus was subjected to electrophoresis and hybridized with a double-stranded, gel-pure *Sal*I-*Xho*I probe. Positions of size markers (in kilobases) of 18S and 28S rRNAs (denoted with asterisks) and of the 2.2-kb LAT and 2.6-kb ICP0 transcripts are shown.

nal to the probe. The two other bands represented fragments originating in each unique long segment and ending at the *Bam*HI site within the probe. Relative to the sizes of the band pair obtained with 333WT DNA, each pair of bands from the mutants was appropriately smaller, depending on the size of the deleted segment (624 bp for LAP1, 301 bp for LAP2, 925 bp for both LAP1 and LAP2).

Digestion of 333LAP1$^-$ and 333WT with *Srf*I yielded a pair of ~6-kb bands corresponding to fragments between the *Srf*I site internal to the probe and another site contained within the short repeats close to the junction of the inverted long repeat and inverted short repeat or to the 5′ end of the genome. Another band of similar size, corresponding to the *Srf*I fragment between the unique long segment on one side and the probe, was also superimposed on this band. The reduction in size of one of the three comigrating bands by the 624-bp LAP1 deletion in 333LAP1$^-$ was not resolved on this gel. The ~3.3-kb band (in 333WT), corresponding to a *Srf*I fragment spanning the unique long repeat junction on the other side, was appropriately diminished to about 2.7 kb in 333LAP1$^-$. In 333LAP2$^-$, the *Srf*I site internal to the probe was deleted, so on one side of the unique long repeat the *Srf*I fragment spanned approximately 11 to 12 kb and on the other side it was approximately 8 to 9 kb. These bands appeared to be appropriately smaller in 333LAP1&2$^-$, although resolution of bands of this size is difficult.

The digestion of each LAP1-containing virus with *Not*I yielded the 624-bp *Not*I fragment and 3.4- and 2.3-kb fragments corresponding to sequences originating in the unique long segment. LAP1 deletions in 333LAP1$^-$ and in 333LAP1&2$^-$ were reflected by the loss of the 624-bp *Not*I fragment, and LAP2 deletions were reflected by changes in the size of the largest (~5-kb) *Not*I fragment. These latter changes were more easily identified when *Xho*I was added to the digestion; this band became sufficiently small to readily identify the absence of the LAP2 sequences from the appropriate mutants.

We examined the effect of each deletion on virus infections in one-step growth experiments in tissue culture (Fig. 3). Confluent Vero cells were infected with each virus at an MOI of 0.1 PFU per cell and harvested 3 and 16 h after infection. The virus yield at each time point was determined by plaque assay titration. Each virus displayed similar one-step growth characteristics, with similarly reduced titers of virus at 3 h after inoculation, corresponding to virus entry into cells, and with increased titers after 16 h.

**Characterization of LAT expression in productive infections.** To determine the effect of each mutation on LAT expression during productive infections, Vero cells were infected with each virus at an MOI of 1.0 PFU per cell. Total cellular RNA was extracted from infected cells 16 h after infection and subjected to Northern hybridization with a radiolabeled *Sal*I-*Xho*I double-stranded DNA probe, which is homologous both to the 2.2-kb LAT intron and to ICP0.

In this experiment (Fig. 4), 333WT produced the largest amount of the 2.2-kb LAT, while 333LAP1$^-$ and 333LAP2$^-$ each produced smaller (reduced by 60 to 70%, on the basis of comparison of the signal intensities) but detectable quantities of LAT. No 2.2-kb LAT was detectable by Northern hybridization from cells infected with 333LAP1&2$^-$. Each virus produced comparable quantities of the 2.6-kb ICP0 transcript. Equivalency of the amounts of RNA loaded onto each lane was demonstrated by subsequent hybridization of these RNAs with a radiolabeled G3PDH probe (data not shown). In similar experiments, in which SK-N-SH human neuroblastoma cells were infected with each virus, the relative intensities of the LAT bands with each virus were similar to those observed in infected Vero cells (data not shown).

**Identification of acute transcription unrelated to either LAP1 or LAP2.** To determine whether the residual expression of LAT observed during productive infection by the 333LAP1$^-$ virus could be completely attributed to sequences downstream of LAP1, including LAP2, we performed reverse transcriptase PCRs on RNAs extracted from Vero cells infected with each virus. We used a primer pair near the 5′ end of LAP2 in these experiments. If all residual LAT synthesized by 333LAP1$^-$ were attributable to LAP2, one would expect to detect no primary LAT transcription in this region from 333LAP1$^-$.

The results of this experiment are displayed in Fig. 5. The expected 134-bp PCR product is identified in cells productively infected with 333LAP1$^-$ and 333WT. RNA extracted from cells infected with 333LAP2$^-$ did not contain sequences homologous with these primers and was negative, as was RNA from uninfected Vero cells and from a reaction mixture containing no template. In each case in which a band was observed, omission of reverse transcriptase from the reaction gave no product, supporting the conclusion that the PCR products result from the amplification of reverse-transcribed RNA

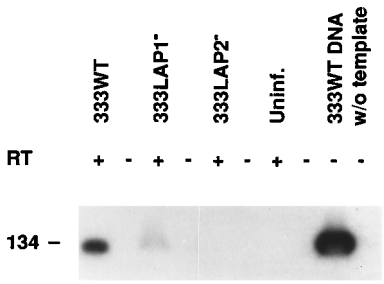


FIG. 5. Identification of RNAs transcribed from promoter sequences outside of LAP1 and LAP2. RNA was extracted from Vero cells productively infected with each virus or with uninfected cells (Uninf.) and subjected either to reverse transcription (RT) (+) or no reverse transcription (−). Additional samples included DNA extracted from cells infected with 333WT (333WT DNA) and PCR reagents without any template (w/o template). PCR was carried out with the primers indicated in Fig. 1B, which give an expected product of 134 bp.

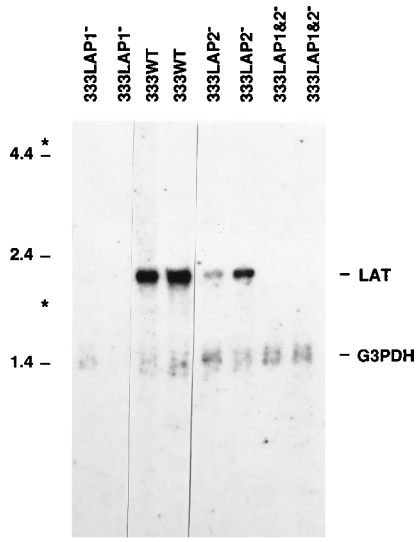


FIG. 6. LAT expression by each virus during latent infections. RNA extracted from guinea pig ganglia latently infected with each virus was subjected to electrophoresis and hybridized with a double-stranded, gel-pure *Sal*I-*Xho*I probe and with a probe to detect cellular G3PDH. Positions of size markers (in kilobases) of 18S and 28S rRNAs (denoted with asterisks) and of the 2.2-kb LAT and 1.5-kb G3PDH transcripts are shown.

rather than from DNA contamination of the samples. Therefore, regulatory sequences upstream of either LAP1 or LAP2 also direct some LAT-region transcription.

**Characterization of LAT expression in latent infections.** The ability of each virus to produce LAT during ganglionic latency was determined by Northern hybridization of RNAs extracted from sacral dorsal root ganglia 27 days after intravaginal inoculation of guinea pigs with each virus (Fig. 6). Figure 6 shows results obtained by hybridization of RNAs extracted from the sacral dorsal root ganglia of two different guinea pigs latently infected with each virus. A $^{32}$P-labeled, double-stranded, gel-pure *Sal*I-*Xho*I DNA probe was also used in this experiment.

As we observed previously, the ganglia of guinea pigs latently infected with 333LAP1$^-$ contained no demonstrable LAT (17). The wild-type virus 333WT synthesized the greatest amount of LAT in the ganglia, 333LAP2$^-$ produced less (but detectable) LAT, and the combined deletion mutant 333LAP1&2$^-$ produced no LAT. Appropriately, ICP0 was not detected during latency. A comparison of the intensities of the LAT bands with those obtained by hybridization with a G3PDH probe showed that differences in levels of LAT expression observed with each virus strain could not be attributed to differences in the amounts of RNA on each blot.

**Characteristics of acute and recurrent infections of guinea pigs.** There were no discernible differences in levels of virulence among the viruses during the productive infection of the guinea pigs (Table 1). The abilities to produce acute disease, as measured by lesion scores, were comparable for each virus. Acute spinal cord and dorsal root ganglion virus titers were comparable for 333WT and 333LAP1&2$^-$, suggesting that LAT did not influence the abilities of these viruses to reach the central nervous system.

While the deletion of LAP2 influenced acute and latent LAT expression, it did not abolish it. To determine whether this reduction in levels of LAT expression influenced virus reactivation, we observed guinea pigs infected with 333WT and 333LAP2$^-$ from days 21 to 63 after intravaginal inoculation with each virus and tabulated spontaneous recurrences.

In this experiment, guinea pigs infected with 333WT experienced spontaneous recurrences a mean ± standard error (SE) of 3.7 ± 0.7 (median = 3) times over the 42-day observation period, compared with a mean ± SE of 4.0 ± 1.3 (median = 4) times for 333LAP2$^-$. Thus, the deletion of LAP2 sequences from HSV-2 333 did not influence recurrence frequency. Appropriately, 333LAP1&2$^-$ recurred much less frequently, i.e., a mean ± SE of 0.5 ± 0.5 (median = 0) time per animal. This experiment demonstrated significant differences between the recurrence frequencies of 333LAP1&2$^-$ and each of the other two viruses ($P < 0.05$ by the Wilcoxon rank sum test [7]) but not between 333WT and 333LAP2$^-$ ($P > 0.5$).

**Establishment of latency.** No studies to date have addressed

TABLE 1. Primary, recurrent, and latent HSV-2 infection in guinea pigs

| Virus | Severity of primary infection[a] | Virus titer[b] in: | | Recurrence[c] | Latent DNA[d] |
|---|---|---|---|---|---|
| | | DRG | SC | | |
| 333WT | 6.2 ± 0.9 (11) | 3.1 ± 0.1 (3) | 4.3 ± 0.4 (3) | 3.7 ± 0.7 (3) | 1.3 ± 0.4 |
| 333LAP2$^-$ | 6.6 ± 1.2 (10) | ND[e] | ND | 4.0 ± 1.3 (5) | ND |
| 333LAP1&2$^-$ | 6.2 ± 0.5 (16) | 3.0 ± 0.2 (3) | 5.1 ± 0.2 (5) | 0.5 ± 0.5 (4) | 1.5 ± 0.3 |

[a] Severity is defined as the area under the lesion score-per-day curve; data are the means of two experiments ± SEs. The numbers of animals tested are shown in parentheses.

[b] Virus titer in spinal cord (SC) or sacral dorsal root ganglia (DRG) 5 days after intravaginal HSV-2 inoculation. Values are the mean $\log_{10}$ PFU per gram of tissue ± SEs. The numbers of animals tested are in parentheses.

[c] Recurrence is defined as the median number of days animals experienced recurrent infections from days 21 to 63 after HSV-2 inoculation. The numbers of animals tested are in parentheses.

[d] Latent virus DNA values were determined by semiquantitative PCR of DNA extracted from four latently infected animals and are reported as means ± SEs.

[e] ND, not done.

the influence that HSV-2 LAT expression might have on the establishment of latency. To determine whether HSV-2 LAT RNA expression influences the establishment of latency, we used a semiquantitative PCR assay to determine the latent virus DNA burdens in guinea pig ganglia 27 days after inoculation with 333WT and 333LAP1&2$^-$ (Table 1). Relative to levels of guinea pig lactalbumin, the mean adjusted relative HSV DNA content for four guinea pigs infected with 333WT was 1.3, while the mean value for four guinea pigs infected with 333LAP1&2$^-$ was 1.5. This difference falls within the expected error of this assay and is indicative of no substantial differences in the abilities of these viruses to establish latency in guinea pigs.

## DISCUSSION

The deletion of both LAP2 and LAP1 eliminated our ability to detect LAT in acute and latent infections. The deletion of LAP2 alone diminished levels of LAT expression in both acute and latent infections. Thus, sequences important for wild-type-level LAT transcription during productive infections and during latency appear to reside within LAP2. LAP2 does not contain promoter elements sufficient to direct latent LAT expression autonomously, as evidenced by the inability of LAP1 deletion mutants to transcribe LAT during latency.

In previously reported experiments, the quantities of LAT expressed in different ganglia latently infected with the same virus varied from animal to animal. Such experimental error could have influenced the LAT quantities we detected in some of the tested guinea pig ganglia. In this study, a consistent effect of the LAP2 deletion on LAT expression was observed in every experiment we performed, including those performed during the productive infection of Vero and human neuroblastoma cells and with the latently infected ganglia. We therefore conclude that LAP2 sequences are necessary for maximum-level LAT expression.

It is unclear whether LAP2 behaves as a completely independent promoter during productive infection. Reverse transcriptase PCR of RNA extracted from cells infected with the LAP1 deletion mutant showed small amounts of RNA transcription in the direction of the LAT intron, between LAP1 and LAP2. This small amount of transcription could represent a modified primary LAT RNA, directed by residual elements of the LAP1 promoter which were not completely eliminated by the *Not*I-*Not*I deletion or by run-on transcription from RNAs further upstream. It is possible that LAP2 sequences merely enhance or activate this residual upstream transcription. Recently, two polyadenylated gamma (late) transcripts have been identified upstream of HSV-1 LAP1 (29). HSV-2 could transcribe similar RNAs during productive infection.

Experiments in which the HSV-1 LAP1 promoter linked to β-galactosidase sequences was inserted into the gC locus did not identify long-term expression of the reporter except when LAT promoter sequences (from LAP1) were juxtaposed with long terminal repeat promoter sequences from Maloney murine leukemia virus (22). It has been suggested that LAP2 sequences may supply such enhancer or activator function to LAP1, contributing to sustained LAT expression in neurons. Our experiments showed that the deleted LAP2 sequences were not required for long-term expression in neurons, suggesting that any required enhancer sequences for long-term expression by LAP1 are not contained in the LAP2 region.

While it is established that LAP1 is required for efficient reactivation of HSV-2 (17), the deletion of LAP2 did not influence the recurrence phenotype of this virus in guinea pigs. This observation is consistent with previous reports that the quantity of HSV-1 or HSV-2 LAT in ganglia or the level of acute HSV-2 LAT transcribed does not influence the frequency of recurrence in viruses that are capable of LAT transcription (3, 11). This finding also makes it less likely that primary LAT sequences upstream of the LAT intron contribute to a virus' reactivation phenotype.

While LAP1 deletion from HSV-2 was shown previously not to influence the establishment of latency (17), these experiments did not fully exclude a role for the LAT in the establishment of latency because the mutation did not totally eliminate acute LAT transcription. Any role which LAT might play in establishing latency is more likely to be related to acute than to latent LAT transcription. In our study of guinea pig infection, we identified no difference in the abilities of the 333WT and 333LAT1&2$^-$ strains to spread to ganglia, as reflected by disease severity and virus titers in neural tissues during acute infections. Moreover, a semiquantitative PCR assay did not discern any differences in the relative concentrations of latent viral DNA attained by wild-type virus and 333LAP1&2$^-$. While a small effect cannot be excluded by an experiment of this size using this technique, these results allow us to conclude that HSV-2 LAT plays no major role in the establishment of latency in guinea pigs, as has previously been concluded for the HSV-1 LAT in rabbits (15).

Although significant similarities with respect to LAT transcription patterns and LAP1 regulatory elements exist between HSV-1 and HSV-2, the present findings may not necessarily extend to HSV-1. It is important to recall that HSV-1 and HSV-2 exhibit distinct reactivation phenotypes (each virus has a preferred site for reactivation [20]) and that the RNA sequences of the HSV-1 and HSV-2 LAT introns are quite different (19). The deletion of HSV-1 LAP1 did not influence acute LAT transcription (26), although deletions in this region affected the ability of this promoter to direct acute transcription when the gene for β-galactosidase was inserted into the virus as a reporter (10). While the LAP1 regions of HSV-1 and HSV-2 are fairly similar, the LAP2 regions exhibit considerably less overall homology, although major elements (AP2, Sp1, and CT-rich sites) are conserved. Recently reported experiments with an HSV-1 mutant from which sequences between two *Sty*I sites within HSV-1 LAP2 were deleted showed no impairment of either acute or latent LAT transcription or of the reactivation phenotype from explanted mouse ganglia (23). Our finding that LAP2 influences acute and latent HSV-2 transcription may be attributable to a more complete deletion of LAP2 sequences, as the related mutant of HSV-1 did not eliminate the putative CT-rich element within LAP2, or to other inherent differences between HSV-1 and HSV-2.

To achieve maximum-level LAT expression in productively and latently infected neurons, HSV-2 requires both the LAP1 and LAP2 regions. Our experiments definitely assign a role to LAP2 in LAT expression, in the context of the whole virus. Despite this role, LAP2 does not appear to be important for virus reactivation or establishment of latency. LAP2 does not constitute a promoter that can function autonomously during latency, and it is not required for latent transcription. Further study of these and other mutant viruses may help to elucidate the molecular mechanisms of HSV-2 reactivation from latently infected ganglia.

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