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Two Herpes Simplex Virus Type 1 Latency-Active Promoters Differ in Their Contributions to Latency-Associated Transcript Expression during Lytic and Latent Infections

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Herpes simplex virus type 1 (HSV-1) establishes latency in human sensory ganglia, during which time the viral genome is transcriptionally silent with the exception of the latency-associated transcripts (LATs). The most abundant LAT is a 2-kb RNA whose biosynthesis is poorly characterized. The 2-kb LAT may be a primary transcript, or its synthesis may involve splicing and/or other forms of processing. Two potential RNA polymerase II promoters (LAP1 and LAP2) upstream of the 2-kb LAT 5′ end have been identified. To investigate the role played by LAP1 and LAP2 in the synthesis of the 2-kb LAT under lytic and latent conditions, we analyzed HSV-1 mutants which contain deletions of one or both of these promoters. During lytic infection in cell culture, the *cis* elements critical for the normal accumulation of the 2-kb LAT were mapped to LAP2, while LAP1 sequences were largely dispensable. The 5′ ends of the major 2-kb LATs produced by the wild-type and LAP deletion viruses were examined by primer extension analysis and were all found to be identical (±2 bp). The accumulation of the 2-kb LAT during latent infections of murine trigeminal ganglia was examined by Northern (RNA) blot and by reverse transcription-PCR. In contrast to the results found in lytic infections, the critical *cis* elements needed for 2-kb LAT accumulation during latency were mapped to LAP1. Deletion of LAP1 resulted in a 500-fold reduction in 2-kb LAT accumulation, whereas deletion of LAP2 resulted in only a 2- to 3-fold reduction. Deletion of both LAP1 and LAP2 resulted in undetectable levels of the 2-kb LAT. Our results indicate that both LAP1 and LAP2 are critical for 2-kb LAT expression but under different conditions. LAP1 is essential for LAT expression during latency, while LAP2 is primarily responsible for LAT expression in lytic infections in cell culture. LAP1 and LAP2 may prove to be functionally independent promoter elements that control 2-kb LAT expression during different stages of HSV-1 infections.

Each of the human herpesviruses has developed a novel set of interactions with the host, resulting in lytic infection in some cell types and the establishment of latency in others. Latency is a strategy that these viruses have developed to maintain the presence of their genomes in a nonpathogenic, nonreplicative form. Latent virus serves as a reservoir for transmission to other susceptible hosts following reactivation. Although the molecular mechanism(s) underlying the "decision" to replicate or establish latency is poorly understood, entry into latency is generally accompanied by a change in the viral transcription pattern. Viral gene expression is silenced, with the one exception being the latency-associated genes. For some herpesviruses such as Epstein-Barr virus, expression of the latency-associated genes has been linked to the establishment and maintenance of the latent state (16, 22, 45).

Herpes simplex virus type 1 (HSV-1) is capable of establishing latency in sensory nerve cell nuclei of the peripheral nervous system. During latency, viral gene expression is silent except for the synthesis of the latency-associated transcripts (LATs), which map to repeat regions flanking the unique long (U_L) component of the genome (5, 6, 33, 39, 43). The major LAT species that accumulates during latent infections is a 2-kb RNA that has the unusual features of being largely nonpolyadenylated and intranuclear (43, 47). Current evidence suggests that the 2-kb LAT may be a highly stable intron spliced from an 8.5-kb, polyadenylated RNA species whose 5′ end maps just downstream from a consensus TATA box and extends to a consensus polyadenylation site (8, 10). Smaller LAT species of 1.4 to 1.5 kb are detected solely during latency (39, 47). Sequence analysis of cDNAs generated from these species indicate that they have the same 5′ and 3′ ends as the 2-kb LAT but have been spliced to remove a 560-base intron (41). While these studies have characterized the physical structure of the LATs, no formal precursor-product relationship has been established for any of the LAT RNA species.

The transcriptional control of LAT expression is equally complex, although some progress has been made in the identification of latency-active promoters (LAPs) (13). Sequences associated with an RNA polymerase II promoter, including a TATA box and potential binding sites for a number of transactivators, lie approximately 700 bp upstream of the 5′ end of the 2-kb LAT (Fig. 1A) (48). This LAP was first described by Dobson et al. (10). We have designated this promoter region LAP1 to distinguish it from a second latency-active promoter, LAP2, located just upstream of the 2-kb LAT (14). LAP2 lacks a consensus TATA box and is highly GC rich, properties associated with many eukaryotic "housekeeping" gene promoters (32, 35, 44, 46). Transient-gene-expression assays with transfected-cell cultures have demonstrated promoter activity for both LAP1 and LAP2 (2, 14, 50, 51). Similar analyses have also demonstrated that LAP1 contains upstream sequences that confer higher activity in some neuronal cell lines (2, 3, 52). The most compelling evidence for the role of LAP1 in LAT expression during latency has come from in vivo studies with a

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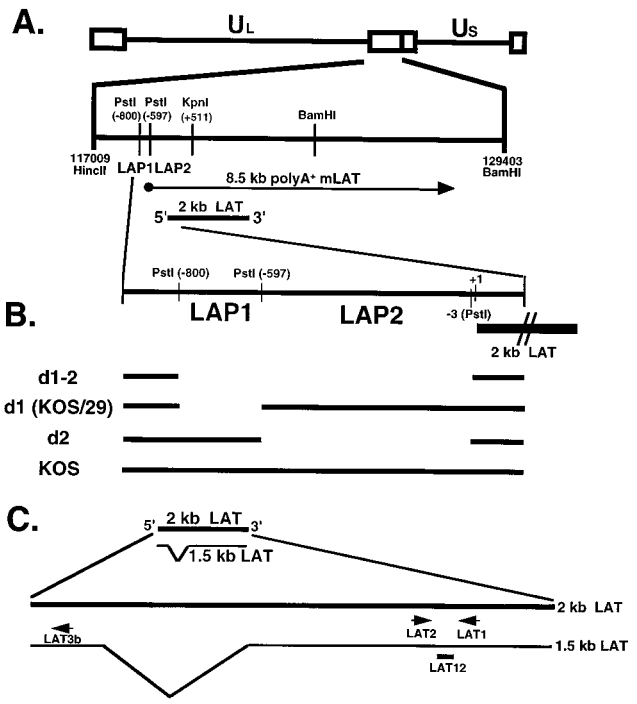

FIG. 1. The LAT region of HSV-1. (A) A schematic representation of the HSV genome is shown, with expanded views of the LATs and the LAT promoter region. The positions of the 8.5- and 2-kb LATs are indicated. (B) The LAP deletion mutants and relevant restriction sites are diagrammed. A *Pst*I site was introduced 3 bp upstream of the 5′ end of the 2-kb LAT in the *Bam*HI B fragment through site-directed mutagenesis. Deletions of LAP2 or of both LAP1 and LAP2 were generated as described in Materials and Methods. Mutant virus *d*1 (KOS/29) bears the deletion of LAP1 alone (10). (C) Oligonucleotide primers used in this study are represented graphically. The polarity of the primers is indicated by arrows. The positions of the primers relative to the putative intron are indicated. The sequences present in the primers are as follows: LAT3b (+66 to +27), LAT1 (+1539 to +1520), LAT2 (+1345 to +1364), LAT12 (+1433 to +1452). Nucleotide numbering is relative to the 5′ end of the 2-kb LAT (nucleotide 119,461 (47) of the HSV genome; GenBank accession number X14112).

viral mutant, KOS/29, in which the sequences for LAP1 and the putative start site of the 8.5-kb LAT have been deleted (10). This mutant establishes latency but does not produce LAT as detected by in situ hybridization (10) or by Northern (RNA) blot (30). This finding is consistent with the hypothesis that the 2-kb LAT is processed from a larger precursor, perhaps the 8.5-kb polyadenylated LAT (8, 10). However, deletion of LAP1 did not affect expression of the 2-kb LAT during lytic infection in cell culture, indicating that LAP1 is not always essential for 2-kb LAT synthesis and that an additional, perhaps cryptic promoter might function during lytic infection (14, 30). This finding raises the possibility that LAP2 is the promoter which drives LAT expression in the absence of LAP1 and possibly in wild-type (wt) virus.

The studies reported here were undertaken to examine the relative roles of LAP1 and LAP2 in LAT expression during lytic infection in cell culture and during latent infections in vivo. Viruses containing precise deletions of LAP2 (*d*2) and of LAP1 and LAP2 (*d*1-2) were constructed and compared with the LAP1 deletion virus (KOS/29; referred to as *d*1 in this paper) and wt KOS virus for their ability to express LAT under different conditions. Our goal was to determine whether both LAP1 and LAP2 were functional, in either an independent or cooperative manner. Our results demonstrate that LAP1 was primarily responsible for LAT expression during latency and that LAP2 was primarily responsible for LAT expression during lytic infection.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells and rat B103 neuroblastoma cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersberg, Md.). The wt HSV-1 KOS strain (19) was used as the viral backbone for all mutants constructed and analyzed in this study. The LAP1 deletion mutant virus, KOS/29 (*d*1), was generously provided by Lawrence T. Feldman, University of California at Los Angeles (10). The *d*1 virus was deleted in both LAT loci for a 203-bp *Pst*I fragment (−800 to −597) containing the LAP1 core promoter (Fig. 1B). The numbering system used in this study defines +1 as the 5′ end of the 2-kb LAT (nucleotide 119461 [47] of the HSV-1 genome; GenBank accession number X14112).

Construction of the LAP deletion mutants. To generate deletions of LAP2 and LAP1-LAP2, plasmids pd2 and pd1-2, respectively, were constructed as follows. The *Pst*I (−597)-*Kpn*I (+511) fragment containing LAP2 (14) was subcloned into the *Pst*I and *Kpn*I sites of pTZ-18U (United States Biochemical, Inc., Cleveland, Ohio) to generate plasmid pTZ18U-PK. The sequence (−8) CGTT TCCAGGTA (+4) was converted to (−8) CGTcTgCAGGTA (+4) by single-stranded in vitro mutagenesis (23). The resulting plasmid, pTZ18U-PPK, now contained a *Pst*I site (the underlined sequence) at position −3 relative to the 5′ end of the 2-kb LAT while preserving the potential splice donor site (5′-AGGT-3′) at the 5′ end of the 2-kb LAT (48). Next, the *Pst*I (−3)-*Kpn*I (+511) fragment from pTZ18U-PPK was inserted in place of the *Pst*I-*Kpn*I (−800 to +511) fragment of plasmid pBB, which contains the *Bam*HI B fragment of the KOS genome. The resulting plasmid, pd1-2, was thus deleted for nucleotides −800 to −3, sequences that comprise both LAP1 and LAP2 in the *Bam*HI B fragment. The junction sequence of the deletion in pd1-2 was confirmed by DNA sequencing. The 203-bp *Pst*I (−800 to −597) fragment containing LAP1 was inserted back into the *Pst*I site of pd1-2 to generate plasmid pd2, deleted for the LAP2 region. The orientation of the inserted LAP1 sequence in pd2 and the junction sequences in pd1-2 and pd2 were confirmed by DNA sequencing.

Deletion mutant viruses *d*1-2 and *d*2 (Fig. 1B) were constructed by cotransfection of linearized pd1-2 or pd2 (5 μg), respectively, with viral DNA (20 μg) from the ICP27 temperature-sensitive (*ts*) mutant, *ts*62 (kindly provided by Priscilla A. Schaffer, Harvard University School of Medicine) (34) by the calcium phosphate procedure (37). Since the *ts*62 mutation could be rescued by the *Bam*HI B fragment, it was possible to select for recombinants at 39°C. Recombinant viruses were purified through three rounds of limiting dilution in 96-well plates. Three isolates for each deletion mutant virus were analyzed by Southern blot hybridization to confirm the deletion and to judge the purity of the virus stock.

Infection of cells and isolation of RNA. Confluent B103 or Vero cell monolayers were mock infected or infected with the LAP deletion mutants or wt virus at a multiplicity of infection of 10. In some experiments, 400 μg of phosphonoacetic acid (PAA) per ml was used to inhibit viral DNA synthesis (27). Total-cell RNA was isolated from Vero or B103 cells productively infected with different viruses at various time points by using RNAzol B (Cinna/Biotex Inc., Houston, Tex.) as recommended by the supplier. Infected cells were lysed in RNAzol B and extracted with 0.1 volume of chloroform. RNA was precipitated from the aqueous phase with an equal volume of isopropanol. The RNA pellets were washed in 75% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated H_2O. The RNA samples were treated with 1 U of DNase I (RNase free; Boehringer-Mannheim, Indianapolis, Ind.) per μl in 100 mM sodium acetate–5 mM $MgSO_4$ for 3 to 4 h at room temperature. RNA was reextracted with phenol-chloroform and precipitated with 2.5 volumes of ethanol. Dried RNA pellets were dissolved in diethyl pyrocarbonate-treated H_2O, and the RNA concentration was determined by measuring the A_{260}.

Northern (RNA) blot analysis. RNA samples (20 μg) were size fractionated by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde and transferred onto Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.) by capillary transfer in 10× SSPE (1.8 M NaCl, 100 mM Na_2HPO_4, 10 mM EDTA). The RNA was covalently linked to the membrane by UV cross-linking. The LAT-specific probe consisted of the *Pst*I-*Kpn*I (−3 to +511) fragment of the 2-kb LAT from plasmid pTZ18U-PPK (Fig. 1B). The glycoprotein C (gC) gene-specific probe used was the 300-bp *Sma*I-*Sma*I fragment corresponding to nucleotides (nt) +772 to +1072 of the gC transcript. These fragments were gel purified and uniformly labeled by random hexamer priming with [α-^{32}P]dCTP (3,000 Ci/mmol; NEN-DuPont, Wilmington, Del.). RNA transfer blots were prehybridized in 50% formamide–5× Denhardt's reagent–5× SSPE–0.1% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml at 42°C for 2 h and subsequently hybridized with 2×10^6 cpm of radiolabeled DNA in the same buffer at 42°C for 12 to 20 h. Blots were washed twice in 6× SSPE–0.1% SDS for 15 min at room temperature, twice in 1× SSPE–0.1% SDS at 37°C, and finally once in 0.1× SSPE–0.1% SDS at 65°C for 45 min and exposed to XAR-5 X-ray film (Kodak, New Haven, Conn.) at −80°C with intensifying screens.

Primer extension and sequencing reactions. The primer LAT3b (5′-CTGGG

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GCGCCCCCTGTCGTTTGGGTCCCCCCCCCTCTATT) is complementary to nucleotides +66 to +27 of the 2-kb LAT (Fig. 1C). A 75-ng portion of primer was 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (6,000 Ci/mmol) and hybridized to 75 μg of whole-cell RNA extracted from infected Vero cells at 12 h postinfection (p.i.). Reverse transcription (RT) was carried out with reverse transcriptase SuperScript II (Gibco BRL). Reaction mixtures containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 1 mM dithiothreitol, 3 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, 50 μg of actinomycin D (Sigma, St. Louis, Mo.) per ml, and 1 U of RNasin (Promega, Madison, Wis.) per ml were incubated at 37°C for 1 h. The reactions were stopped by the addition of 2.75 μl of 0.5 M EDTA, and the mixtures were treated with 2.75 μl of 5-μg/ml RNase A (Sigma) at 37°C for 30 min, phenol-chloroform extracted, ethanol precipitated, and resuspended in 6 μl of TE (10 mM Tris, 1 mM EDTA [pH 7.8]) plus 4 μl of formamide sample buffer. The reaction products were size fractionated on an 8% acrylamide–7 M urea sequencing gel, dried to paper, and exposed to film. Dideoxy sequencing reactions with plasmids pTZ18U-PK, pd2, and pd1-2 and primer LAT3b served as absolute size standards for the primer extension reactions.

Infection of mice and isolation of DNA and RNA. Five-week-old BALB/c mice (Harlan, Indianapolis, Ind.) were used in all experiments. The animals were anesthetized with Metofane (Pitman-Moore, Mundelein, Ill.), and the corneas of both eyes were scarified and inoculated with 2×10^6 PFU of either wt virus or the various LAP deletion mutants or were mock infected. Trigeminal ganglia (TGs) were surgically removed from animals sacrificed at least 4 weeks p.i., and DNA and RNA were extracted with total RNA isolation (TRI) reagent (Molecular Research Center, Inc., Cincinnati, Ohio) as specified by the supplier. TGs from five infected mice were pooled and homogenized in 2 ml of TRI reagent. Following chloroform extraction, RNA was precipitated from the upper, aqueous phase with an equal volume of isopropanol, resuspended in diethyl pyrocarbonate-treated H_2O, and treated with DNase I (RNase free) as described above. DNA was extracted from the lower phase of the initial chloroform extraction of the TG homogenates with an equal volume of TE buffer and precipitated with 2 volumes of absolute ethanol. The resulting DNA precipitates were resuspended in 1 ml of 50 mM Tris-HCl (pH 8.0)–2 mM EDTA–0.5% Tween 20–400 μg of proteinase K (Boehringer-Mannheim) per ml and incubated at 37°C for 12 to 18 h. The lysate was then boiled for 10 min, and the total DNA concentration was determined by measurement of the A_{260}.

Quantitative PCR and RT-PCR. To determine the level of viral DNA harbored in latently infected TGs, quantitative DNA PCR assays were carried out with 10-fold serial dilutions of 400 ng of the DNA samples harvested from the latently infected TGs. The reactions were carried out with 0.5 μg each of either the primer pair ADS1 (5′-AGTGTGCGGGGATGCAGT) and ADS2 (5′-ACGCGAGAGCCCCACGTA) specific for the single-copy mouse adipsin gene (21) or the primer pair gB1 (5′-ATTCTCCTCCGACGCCATATCCACCACC TT) and gB2 (5′-AGAAAGCCCCCATTGGCCAGGTAGT) complementary to the glycoprotein B (gB) coding sequence of HSV-1 (12). The amplifications were carried out with 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min. The PCR products were analyzed by Southern blots with oligonucleotides 5′-end labeled with [γ-^{32}P]ATP (6,000 Ci/mmol; NEN-DuPont) and T4 polynucleotide kinase (Boehringer Mannheim). The oligonucleotide ADS12 (5′-AGTCGAAGGTGTGGTTAC) (21) was used for the detection of the mouse adipsin gene DNA, and the oligonucleotide gB12 (5′-AAGGACGCCCGCGACGCCAT) was used for the detection of the HSV gB gene. The hybridizations were carried out in 6× SSPE–1% SDS at 49°C overnight. The blots were washed twice at room temperature in 6× SSPE–1% SDS for 10 min each and at higher stringency in 1× SSPE–1% SDS at 49°C for 5 min and exposed to X-ray film.

For quantitative RT-PCR analyses of LAT expression during latency, the LAT-specific primer pair LAT1 (5′-ACGAGGGAAAACAATAAGGG) and LAT2 (5′-GACAGCAAAAATCCCCTGAG) (25) (Fig. 1C) were used. Both LAT1 and LAT2 are located downstream of the intron within the 2-kb LAT and will thus amplify all LAT species. Three micrograms of each RNA sample harvested from latently infected mouse TGs was hybridized to 0.5 μg of the reverse primer LAT1. First-strand cDNA synthesis was carried out at 42°C with avian myeloblastosis virus reverse transcriptase (Promega) as specified by the supplier. Tenfold serial dilutions of 1/10 of the RT products were amplified with 0.5 μg each of LAT1 and LAT2 under the same conditions as listed above. RT-PCR amplifications with the gB-specific primer pair were also carried out to find whether each virus had entered latency and was no longer expressing lytic genes or whether the viruses had reactivated from the latent state. In addition, 0.3 μg of the RNA samples was amplified directly with the primer pairs without RT to ensure that the signals detected by RT-PCR were not due to DNA contamination of the RNA samples. The reaction products were subjected to electrophoresis in 3% NuSieve agarose gels and transferred as described above. The blots were hybridized with radiolabeled oligonucleotide gB12 for gB-specific products or LAT12 (5′-TCCCCGACACGGATTGGCTG) (Fig. 1C) for LAT-specific products. Blot hybridization and washing conditions were identical to those described above, except that the hybridization and high-stringency wash temperature was 61°C.

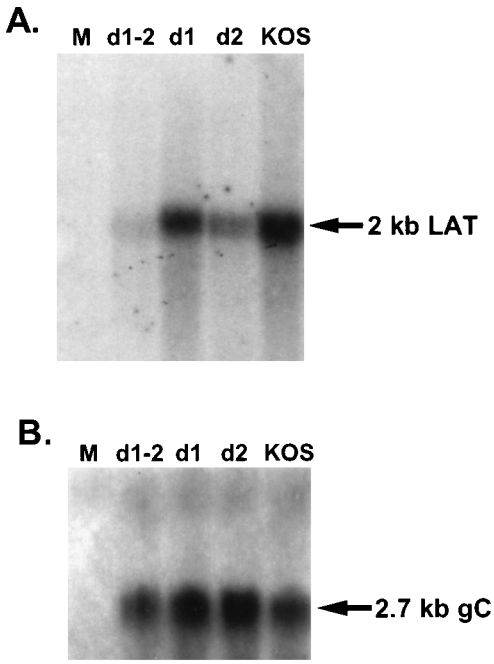

FIG. 2. Northern blot analysis of 2-kb LAT expression during lytic infection. Vero cells were mock infected or infected at a multiplicity of infection of 10 with *d*1-2, *d*1, *d*2, or wt KOS virus, as indicated. Total RNA (20 μg) harvested 12 h p.i. was separated on a denaturing agarose gel, transferred to nylon membrane, and hybridized with a ^{32}P-labeled LAT-specific probe (−3 to +511) (A) or gC-specific probe (B). The mobility of the 2-kb LAT or the 2.7-kb gC RNA was determined by RNA size standards and is indicated by arrows.

RESULTS

Construction of LAP deletion viruses. To determine the respective roles of LAP1 and LAP2 in LAT expression during both lytic and latent infection, we analyzed 2-kb LAT accumulation from viruses that had engineered deletions of either LAP1, LAP2, or both promoters. All viruses were constructed in the HSV-1 KOS background. The LAP1 deletion virus (*d*1) lacks sequences −800 to −597 relative to the 2-kb LAT 5′ end (10). LAP deletion viruses lacking LAP2 (*d*2) or both LAP1 and LAP2 (*d*1-2) from both LAT loci were created by site-directed mutagenesis (Fig. 1B). Plasmids containing the LAP region of HSV-1 were constructed in which either LAP2 (nt −597 to −3) or both LAP1 and LAP2 (nt −800 to −3) were removed. Both deletions preserve the potential splice donor site at the 5′ end of the 2-kb LAT (48) and were confirmed by DNA sequencing (data not shown). The LAP deletions were recombined into the viral genome by cotransfecting the plasmid with HSV-1 DNA from an ICP27 *ts* mutant (34). Recombinant plaques were selected at 39°C, and the recombination of the LAP deletions into both LAT loci was confirmed by Southern blot analysis.

Sequences in LAP2 are important for 2-kb LAT expression during lytic infection. We analyzed the contributions of LAP1 and LAP2 to 2-kb LAT accumulation by Northern blot analysis of total RNA isolated from infected Vero cells at 12 h p.i. by using a LAT-specific probe derived from the *Pst*I-*Kpn*I fragment (−3 to +511) of the 2-kb LAT (Fig. 2A). The results showed that the 2-kb LAT is the predominant transcript detected in RNA isolated during wt HSV-1 infection (Fig. 2A, lane KOS). No RNA hybridizing to this probe was detected from mock-infected cells (lane M). Deletion of both LAP1 and LAP2 resulted in a dramatic reduction in 2-kb LAT accumu-

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lation, demonstrating that the sequences from positions −800 to −3 (LAP1 + LAP2) were essential for 2-kb LAT accumulation. Analysis of the RNA from the LAP1 and LAP2 deletion viruses indicated that the LAP2 sequences (−597 to −3) played a dominant role in 2-kb LAT expression during lytic infections of Vero cells. Deletion of LAP1 sequences (−800 to −597) caused only a slight decrease in 2-kb LAT accumulation, indicating that LAP1 sequences were not critical for 2-kb LAT expression under these conditions. To control for RNA isolation, the same RNA samples were also hybridized to a fragment specific for the viral late-gene gC. Similar levels of the gC transcript were detected in all four infections (Fig. 2B), indicating that the differences in the level of 2-kb LAT expression observed in Fig. 2A were not due to differences in RNA extraction or infection. Similar effects of the LAP deletions on 2-kb LAT expression were also obtained from B103 neuroblastoma cells, indicating that these LAP mutations had equivalent effects of 2-kb LAT expression in cells of neuronal origin (data not shown). The finding that the LAP1-LAP2 double deletion resulted in near elimination of the 2-kb LAT product and that the LAP2 deletion virus produced significantly reduced levels of 2-kb LAT while the LAP1 deletion virus produced near-wt levels of 2-kb LAT suggested that LAP2 played the dominant role in 2-kb LAT expression during lytic infection.

The 5′ end of the 2-kb LAT is independent of promoter usage. The finding that the 2-kb LAT was detected in cells infected with viruses deleted for either or both LAP1 and LAP2 raises important questions concerning its biosynthetic pathway. Is the 2-kb LAT a primary transcript, or is it the product of some form of splicing and/or processing reactions? Do the 2-kb LATs produced by the LAP deletion viruses have the same 5′ and 3′ ends? We mapped the 5′ ends of the 2-kb LAT molecules isolated from Vero cells infected with wt and LAP deletion viruses by primer extension. The primer used, LAT3b, is complementary to nt +66 to +27 relative to the previously mapped 5′ end of the 2-kb LAT (Fig. 1C) (47). Primer extension of infected-cell RNA with primer LAT3b detected the same 66-nt product in all infections (Fig. 3). This product was not detected in reactions with RNA from mock-infected cells. The same 5′ end was detected regardless of whether the infecting virus contained both, either, or neither of the LAP sequences. The 66-nt product maps to the same position as had been previously mapped for the 5′ end of the 2-kb LAT molecule (47). The yield of the 66-nt primer extension product closely correlated with the levels of 2-kb LAT detected by Northern blot analysis (Fig. 2A), confirming that the two detection methods gave comparable quantitative results. Since the 2-kb LATs produced by these viruses had the same 5′ ends and since they comigrated on agarose gels as detected by Northern blot (Fig. 2A), we infer that the 3′ ends of the RNAs are likely to be the same as well.

In addition to the major 5′ end detected with primer LAT3b, several minor primer extension products were detected. Reactions extended with primer LAT3b revealed the presence of two minor LAT region-specific RNAs with 5′ ends located at −15 and −180 relative to the major 2-kb LAT 5′ end. These termini map to sequences within LAP2 (Fig. 3). Consistent with this observation, these species were not detected in the LAP2 or LAP1+LAP2 deletion viruses. However, primer extension of RNA from cells infected with the LAP2 deletion mutant (*d*2) detected an RNA 5′ end 28 nt downstream of the TATA box within LAP1 (135-nt primer extension product). This result is consistent with an initiation site predicted to be used by the 8.5-kb mLAT described by others (10). A primer extension product (91 nt) with the 5′-end 71 nt downstream of the TATA box was also detected and may represent an additional 5′ end of the LAT RNAs or an incomplete reverse transcriptase product of the full-length 8.5-kb mLAT. No additional extension products were detected with the LAT3b primer when RNA from cells infected with the LAP1-LAP2 double-deletion mutant (*d*1-2) was used. Our results indicated that the predominant LAT RNA that accumulated during lytic infection was the 2-kb LAT. The 5′ end of the 2-kb LAT was surprisingly independent of the LAP sequences present. Other minor 5′ ends were detectable in lytic infections, and their significance is discussed below.

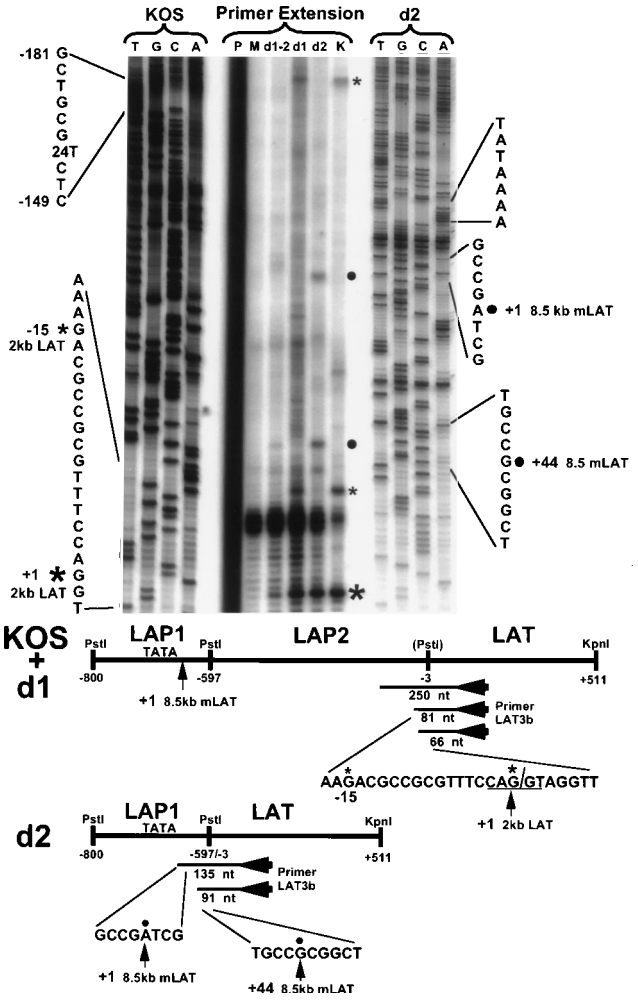

FIG. 3. 5′-end mapping of the 2-kb LAT by primer extension. Total RNA (75 μg) harvested from Vero cells mock infected or infected with *d*1-2, *d*1, *d*2, or wt KOS was hybridized to end-labeled primer LAT3b. Primer extension reaction products were resolved on a denaturing acrylamide gel. Sequencing reactions of plasmids bearing the wt, *d*1-2, or *d*2 sequence were included to serve as size standards. The bands representing the extension products from KOS and *d*1 are indicated by asterisks, and those from *d*2 are denoted by solid circles. A schematic indicating the locations of the extended products for KOS and *d*1 in relation to the 5′ end of the 2-kb LAT and the putative splice donor signal (CAG/GT) or for *d*2 versus the 5′ end of the 8.5-kb mLAT is displayed at the bottom of the figure.

2-kb LAT displays late-gene kinetics during lytic infection. HSV genes expressed at late times are dependent on viral DNA synthesis for expression (18, 29). Since the 2-kb LAT is expressed predominantly as a late gene following viral DNA synthesis (40) and LAP2 appears to be the principal promoter responsible for LAT expression in lytic infection, it might be

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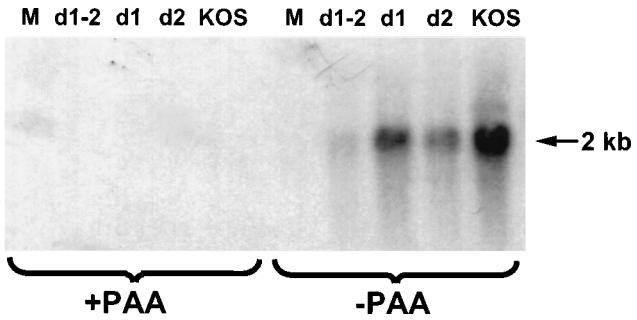

FIG. 4. Dependence of 2-kb LAT expression on viral DNA replication. Total RNA (20 μg) harvested 12 h p.i. from Vero cells mock infected or infected with the LAP deletion mutants or wt KOS virus was analyzed by Northern blot as in Fig. 2. Cells were treated with the viral DNA synthesis inhibitor PAA as indicated. The position of the 2-kb LAT as determined by RNA size standards is indicated by an arrow.

expected that LAP2-driven LAT expression would also depend on viral DNA synthesis.

To examine the dependence of 2-kb LAT expression on DNA synthesis, Vero cells were infected with the LAP deletion viruses in the absence or presence of the viral DNA polymerase inhibitor, PAA (27). Northern blot analysis was carried out with RNA harvested from Vero cells mock infected or infected with the different LAP deletion mutants or wt virus in the presence or absence of PAA (Fig. 4). In all cases, no LAT was detected in PAA-treated infections, indicating that LAT expressed by LAP1 or LAP2 is dependent upon viral DNA replication, as has been previously reported for wt virus (40). In the same assay, the expression of gC, encoded by a known late gene, was inhibited by PAA (data not shown). Thus, both LAP1- and LAP2-driven 2-kb LAT expression during lytic infection was dependent on viral DNA synthesis.

2-kb LAT expression during latency requires LAP1. To determine the relative contribution of the two LAPs to 2-kb LAT expression during latency, Northern blot analyses were carried out with RNA harvested from mouse TGs latently infected with the different LAP deletion mutants and wt virus at 42 days p.i. (Fig. 5). A LAT-specific probe (nt −3 to +511 relative to the 2-kb LAT 5′ terminus) was used to detect the 2-kb LAT. Deletion of LAP1 or deletion of both LAP1 and LAP2 completely abolished the accumulation of the 2-kb LAT. This finding is in agreement with earlier reports that first demonstrated the importance of LAP1 for LAT expression during latency (10, 30). Surprisingly, the LAP2 deletion mutant *d*2 expressed the 2-kb LAT at a level approximately two- to threefold lower than that of wt virus, indicating that LAP2 contributed in some fashion to the accumulation of the 2-kb LAT. At this level of detection, we concluded that LAP1 was essential for LAT expression during latency and that LAP2 sequences contributed to the accumulation of 2-kb LAT in a LAP1-dependent manner.

To detect the accumulation of LATs at a greater level of sensitivity, we used an RT-PCR assay. RNA isolated from latently infected TGs was subjected to RT, and aliquots from 10-fold serial dilutions of the cDNA were amplified by PCR with oligonucleotide primers LAT1 and LAT2 (Fig. 1C) (25). The amplification products were analyzed by Southern blot with LAT12, an internal oligonucleotide (Fig. 1C and 6). In this assay, LAT transcripts were readily detectable with RNA from wt infections but not from mock infections (Fig. 6E and A). Deletion of both LAP1 and LAP2 (*d*1-2) resulted in a complete loss of detectable LAT expression, demonstrating the essential nature of these *cis*-acting elements for LAT accumulation. Deletion of LAP1 alone resulted in a great decrease in LAT accumulation, consistent with the results obtained by Northern analysis. On the basis of the signal intensity from the 10^{-1} dilution of RT product derived from *d*1 (Fig. 6C) compared with that of wt KOS (Fig. 6E) at a 10^{-4} dilution, deletion of LAP1 resulted in an approximately 500-fold reduction in LAT accumulation relative to the level in wt KOS infection. Similarly, the level of LAT expression from *d*2 (Fig. 6D) was estimated to be approximately threefold lower than that of the wt virus. This estimate was also consistent with that derived from Northern blot analysis of RNA from mouse TGs latently infected with these different LAP deletion mutants (Fig. 5). In addition to the predicted 195-bp product, other minor amplification products (140 and 250 bp) were detected. Since these products are virus dependent and primer specific, they most probably represent mispriming as a result of the unusually high G+C content of this region of the HSV genome and have been routinely detected in other studies (12).

To ensure that all viruses had entered latency and had not reactivated, RT-PCRs were also performed with a pair of primers specific for the viral glycoprotein B (gB) gene (12). No gB-specific signal was detected in RNA samples from any of the LAP deletion mutants in mouse TGs (data not shown), confirming that in all cases, latency had been established and the LAT signal detected did not result from reactivated virus. Moreover, the LAT signals detected in RT-PCRs were not due to viral DNA contamination of the RNA samples, since PCRs carried out without prior RT showed no LAT-specific product (data not shown).

These results demonstrated that the deletion of LAP1 led to a significant decrease in the level of LAT accumulation during latency. In contrast, LAP2 deletion had only a minor effect on LAT accumulation during latency. Deletion of both LAP1-LAP2, however, resulted in complete loss of detectable LAT, even by RT-PCR, confirming that the LAT promoter complex is located within the LAP1-LAP2 region.

All LAP deletion mutants are capable of establishing and maintaining latency with an efficiency comparable to that of wt virus. Although most of the reports indicate that LAT expression is not required for the establishment or maintenance of latency (4, 10, 17, 20, 24, 42), one study reported that a LAT$^-$ mutant (KOS/62-3) established latency with reduced efficiency in the TGs (36). To determine whether the different levels of LAT accumulation detected from the various LAP deletion viruses during latency were due to different copy numbers of viral genomes harbored in the latently infected TGs, quantitative PCRs were carried out with DNA harvested from mouse TGs harboring wt or mutant latent virus (Fig. 7).

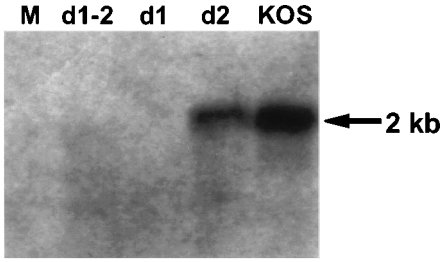

FIG. 5. Northern analysis of 2-kb LAT expression during latency. Total RNA (20 μg) harvested from TGs of mock-infected or *d*1-2, *d*1, *d*2, or wt KOS-infected mice at 42 days p.i. was resolved on a denaturing agarose gel, transferred to nylon membrane, and hybridized with a LAT-specific probe (−3 to +511). The position of the 2-kb LAT, as determined by RNA size standards, is indicated by an arrow.

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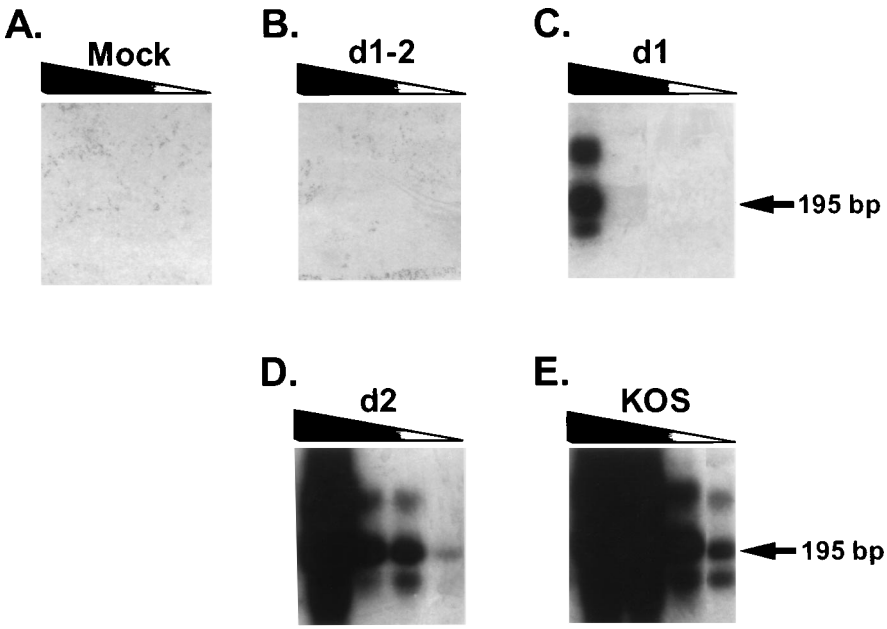

FIG. 6. Quantitative RT-PCR analysis of LAT expression during latency. Mice were mock infected (A) or infected with *d*1-2 (B), *d*1 (C), *d*2 (D), or wt KOS virus (E) by corneal scarification. At 42 days p.i., total RNA was harvested from latently infected TGs, and 3 μg of each sample was reverse transcribed with LAT1 primer. Tenfold serial dilutions of the cDNA products were PCR amplified with the LAT1/LAT2 primer pair. The reaction products were analyzed on Southern blots probed with radiolabeled oligonucleotide LAT12 (see Fig. 1C for locations of primers).

As a control for the efficiency of DNA extraction from mice TGs, 10-fold serial dilutions of TG DNA samples were amplified with a primer pair specific for the single-copy mouse cellular gene adipsin (21). Relatively similar signal intensities were detected from all TG DNA samples, including mock-infected TGs, indicating that approximately equal amounts of TG DNA were included in each reaction (Fig. 7A). When primers specific for the viral gB gene were used (12), relatively similar levels of gB signal were detected from TGs infected with all the LAP deletion mutants and the wt virus. No amplified products were detected from the mock-infected TGs (Fig. 7B). These results showed that latent viral DNA was present in all but the mock-infected TGs and that the large differences observed in LAT accumulation could not be accounted for by the relatively small variation observed in viral genome content. Furthermore, all of the LAP deletion mutants were capable of entering and maintaining latency with an efficiency comparable to that of wt virus.

DISCUSSION

The discovery of the HSV LATs (5, 6, 15, 33, 39, 43) has led to intensive studies to characterize the complex promoter elements which control latency gene expression (2, 10, 14, 50), to define the number and origin(s) of the LATs observed during both lytic (8) and latent (41, 47) infections, and ultimately to understand the function(s) of the LAT locus RNAs (13). Analysis of the latency RNAs and their transcriptional control has been difficult for several reasons. First, conditions under which the 2-kb LAT is expressed while the remainder of the viral genome is silent occur only in vivo, where the small number of infected cells makes it difficult to isolate sufficient quantities of RNA for detailed analyses. Second, the recent observation that only about 5% of neuronal cells harboring latent viral DNA express LAT abundantly as detected by in situ hybridization (31) has further complicated our understanding of the transcriptional control of LAT, since not all latently infected cells behave similarly with respect to LAT expression. For example, LAT expression may rely on transcription factors within a particular neuronal cell type or LAT production may be dynamic and changing within all latently infected cells, perhaps depending on available transcription factors and/or changes in splicing or other RNA-processing or transport activities. LAT expression may involve multiple promoter elements which switch on and off in response to changes in the transcriptional milieu of the latently infected cell. Finally, the inability of the virus to express LAT in immortalized cell culture systems in the absence of viral replication has impeded the usual kinds of genetic analyses to study LAT expression and function. While there have been reports of LAT expression in primary sensory ganglion cell culture systems (4), these cultures are difficult to prepare and maintain in substantial numbers in vitro, reducing their usefulness. Despite these obstacles, studies of the control of LAT expression have progressed and have yielded a complex pattern of gene regulation.

The experiments described in this report provide new information on the transcriptional control of LAT expression both during lytic infection in cell culture and during latency in animals. We wished to test directly the role played by two potential transcriptional promoters of LAT expression (10, 14). Our strategy was to utilize viruses with precise deletions of either LAP1, LAP2, or both promoter sequences and then test the ability of these viruses to express LAT under different circumstances.

Role of LAP1 and LAP2 sequences in LAT expression during lytic and latent infections. Earlier studies have demonstrated that the LAP1 sequences were essential for 2-kb LAT expression during latent infections (10) but unnecessary for expression in lytic infections (30). These earlier studies implied that there may be two or more distinct pathways for synthesis of the 2-kb LAT. Our studies reported here provide further evidence for this hypothesis. Deletion of LAP1 had little effect on 2-kb LAT expression in lytic infections (Fig. 2A) but greatly

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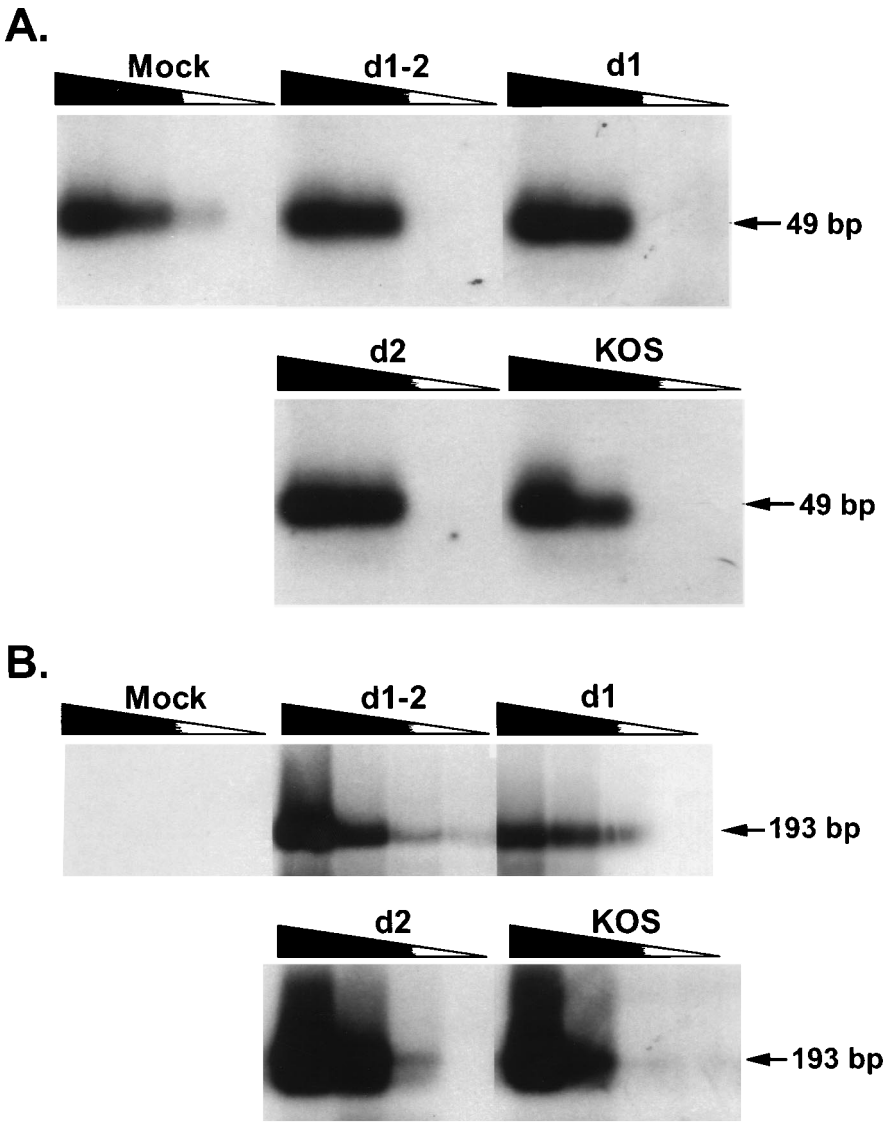

FIG. 7. Quantitative PCR of viral DNA in latently infected TGs. (A) Quantitation of mouse genomic DNA by PCR amplification of the mouse adipsin gene. DNA was isolated from the same TGs used for the experiments in Fig. 5 and 6. Tenfold serial dilutions of DNA samples (400 ng) were amplified with primer pair ADS1 and ADS2, specific for the single-copy mouse adipsin gene (21). The reaction products were analyzed on Southern blots probed with end-labeled oligonucleotide ADS12, which hybridizes to sequences internal to the PCR primers (21). The position of the 49-bp adipsin-specific product is indicated by an arrow. (B) Quantitation of viral DNA by PCR amplification with gB-specific primers. PCRs were carried out with fivefold serial dilutions of TG DNA samples (1 μg) with the gB-specific primer pair (12) and analyzed by Southern blot with the labeled oligonucleotide gB12. The position of the 193-bp gB-specific PCR product is indicated by an arrow.

reduced expression in latent infections as detected by Northern blot (Fig. 5) and RT-PCR (Fig. 6). These results and those reported earlier (10, 30) indicate that sequences in LAP1 are essential for 2-kb LAT expression but only during latent infections. The novel result reported here is the identification of the LAP2 sequences as being important for 2-kb LAT expression during lytic infections. The finding of promoter activity in the sequences just upstream of the 2-kb LAT (14) led us to test whether this region played any role in LAT expression in viral infections. By construction of viruses that completely lack LAP2 sequences, we were able to demonstrate that this region is needed for wild-type levels of expression of 2-kb LAT in lytic infections (Fig. 2) but is largely dispensable during latent infections (Fig. 5 and 6). Thus, LAP1 and LAP2 appear to play complementary roles during the viral life cycle. During lytic infections, LAP2 is the major control element, whereas in latent infections, LAP1 is the dominant control element.

A recent report by Maggioncalda et al. (26) has shown that a viral mutant (DSty) with a 370-bp deletion (*Sty*I-*Sty*I) in the LAP2 region expressed near wt level of the 2-kb LAT during both lytic and latent infections. However, the deletion in DSty removed sequences from −588 to −218 relative to the 5′ end of the 2-kb LAT, leaving the proximal 218 nt of LAP2 sequence intact. We detect two potential transcription initiation site within LAP2, mapping to −180 and −15 relative to the 2-kb LAT (Fig. 3). Thus, it is possible that the basal promoter element within LAP2 and some of the potential regulatory sequences either upstream or downstream were left intact and were sufficient for LAP2 activity from the natural LAT loci during viral infection.

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The need for two promoters capable of expressing LAT is unclear but might be explained on the basis of one possible LAT function. The overlap of the 2-kb LAT with the 3′ terminus of the ICP0 mRNA suggests that LAT may function as an antisense inhibitor of the translation of ICP0, an immediate-early gene critical for reactivation from latency. We suggest that LAP2-driven expression of LAT may play a role in suppressing ICP0 production during lytic infection, thereby assisting the virus in establishing latency. Once latency is established, LAP1 expresses LAT in order to maintain the latent state by inhibiting ICP0 protein synthesis. At least one report suggests that virus reactivation does not occur in latently infected neurons expressing high levels of LAT (11). Although such a suggested role for LAT has not been demonstrated in animal models, this does not rule out such a mechanism in the natural human host, particularly since the virus does not spontaneously reactivate in mice, the most frequently studied model of HSV latency. It will be of great interest to correlate the reactivation efficiency of these LAP deletion viruses with LAT expression during reactivation.

Structure of the 2-kb LAT in LAP1 and LAP2 deletion viruses. The finding that both the LAP1 and LAP2 deletion viruses make at least a detectable level of 2-kb LAT led us to characterize the structure of the LAT RNA in more detail. In particular, we were curious to determine whether the 2-kb LAT made from these viruses had the same 5′ ends as the wild-type virus. Using primer extension analysis, we determined that the 5′ ends of the 2-kb LAT were the same (±2 nt) regardless of whether it was made by wt, LAP1, LAP2 or the double LAP1+LAP2 deletion virus (Fig. 3). This finding leads to the hypothesis that the 5′ end of the 2-kb LAT may be synthesized by more than one processing and/or splicing pathway. It is possible that during latent infections, a primary transcript initiating from LAP1 is spliced to produce the 2-kb LAT. If this is true, one must also hypothesize that the primary transcript, perhaps the 8.5-kb mLAT (10), and other reaction products do not accumulate to a detectable level. The 2-kb LAT may be metabolically stable as a result of its predicted ability to form a complex secondary structure (4a). During lytic infections, 2-kb LAT synthesis is largely independent of LAP1 (Fig. 2) (30). Indeed, our primer extension experiments detected several 5′ ends within the LAP2 sequence (at −15 and −180 upstream of the 2-kb LAT 5′ end [Fig. 3]) in both wt and LAP1 deletion viruses. Although these sites may represent differences in processing and/or splice donor site usage, we suggest that they most probably represent transcription initiation within the LAP2 sequence, since the same primer extension product was detected at −180 in a recombinant virus that contained the simian virus 40 poly(A) site upstream of the putative LAT splice acceptor sequence (4b). It is possible that under these conditions, the 2-kb LAT initiates at or close to the 5′ end detected by primer extension. In this case, any processing and/or splicing would affect mainly the 3′ end of the molecule. However, this model does not explain the presence of the 2-kb LAT during lytic infection with the double LAP1+LAP2 deletion virus. Clearly, some other promoter element, perhaps read through from upstream transcription units, must be functioning in this virus. If the 2-kb LAT is stable in the infected nucleus, the 2-kb LAT could be generated as part of the processing of a larger primary transcript.

Evidence for initiation at LAP1. LAP1 sequences are essential for the expression of the 2-kb LAT in latent infections (Fig. 6) (10). It has been suggested that the 2-kb LAT is a stable intron derived from the 8.5-kb mLAT detected by Dobson et al. (10). We have not been able to detect the 8.5-kb mLAT by Northern blot (data not shown), nor have we or others detected what one might expect to be the mature 6.5-kb LAT from which the 2-kb LAT was spliced. However, in our primer extension experiments with RNA harvested from lytically infected cells, we were able to detect an extension product consistent with transcription initiation at LAP1. In this experiment, we used a primer complementary to sequences within the 2-kb LAT (+66 to +27) to extend RNA harvested from cells infected with the LAP2 deletion virus (Fig. 3). We detected an extension product whose size was consistent with an RNA whose 5′ end is 28 to 29 bp downstream from the TATA element in LAP1. This extension product was not detected in RNA samples from cells infected with the mutant virus deleted for LAP1 or with wt virus. Since LAP1 does contribute modestly to the 2-kb LAT accumulation under these conditions (compare *d*2 with *d*1-2 [Fig. 2]), it is conceivable that the extension product we detect is derived from a transcript that initiated at LAP1 but had not yet been processed to generate the mature 5′ end of the 2-kb LAT or represents a transcript that will not be properly processed to the 2-kb species.

The finding that LAP1 contributes to LAT expression during latent infection was consistent with transient-gene-expression studies which demonstrated that LAP1 contains enhancer elements which respond to neuronal cell transcriptional signals (2, 3, 52). We have observed that a replication-defective mutant virus deleted for the immediate-early gene, ICP4 (7), did not express the 2-kb LAT in cell culture unless the virus was able to replicate in a complementing cell line which provides ICP4 in *trans* (4b). In contrast, the defective mutant expressed LAT vigorously in the peripheral nervous system (9) and in the central nervous system in the absence of viral replication (38), again suggesting that LAP1 responds to neuron-specific signals. It might be expected, therefore, that LAP1 is not a major contributor to LAT expression in cell culture infections but requires a second promoter, presumably LAP2, to account for LAT synthesis during late times p.i. It remains to be demonstrated what specific elements in LAP1 account for neuron-specific expression during latency.

Functional independence and cooperativity of LAP1 and LAP2 sequences. While both LAP1 and LAP2 have been shown to be active in transient-reporter-gene-expression assays (2, 8, 14, 50), it was unclear whether they can function independently in the context of the viral genome in vivo. Our results indicate that both LAP1 and LAP2 can function independently to drive LAT expression, although their contributions in lytic and latent infections differ.

Previous studies have shown that LAP2 is active, albeit poorly, during latency in an ectopic locus (14). Surprisingly, LAP1 does not possess this capability when a transgene is inserted downstream of the putative LAT initiation site, replacing LAP2 and the 5′ end of the 2-kb LAT (28), or when LAP1 is moved to an ectopic site (24a). This observation suggests that LAP2 may influence the long-term activity of LAP1 during latency. Our current study shows that removal of LAP2 does not eliminate LAT expression during latency but, rather, reduces the level of LAT by two- to threefold. Thus, while LAP2 may influence the level of LAP1 activity, it is not required for LAT expression during latency.

One additional mechanism by which LAP2 may contribute to LAT expression would involve an effect of sequences within LAP2 on the efficiency of the splicing/processing event of a primary transcript from an upstream promoter (LAP1). There have been reports of sequences within the upstream leader/exon affecting the efficiency of RNA-processing/splicing events (1, 49). Such a mechanism would account for the decreased levels of LAT in the LAP2 deletion mutant (*d*2) during latency. However, further experiments to alter putative LAT splicing

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signals by site-directed mutagenesis are needed to address this issue.

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