

# Herpes simplex virus latency-associated transcript is a stable intron

(antisense RNA/transactivation)

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**ABSTRACT** The latency-associated transcript (LAT) is the major viral transcript detected by *in situ* hybridization of mouse and human sensory ganglia latently infected with herpes simplex virus type 1. The last 750 bases of LAT are complementary to infected-cell polypeptide 0, a herpes simplex virus type 1 immediate-early gene that encodes a transactivating protein that may facilitate re-activation of the virus from the latent state. Several laboratories have shown that LAT accumulates in the nucleus and is not polyadenylated. Recently, we showed that the promoter for LAT lies 688 bases upstream from its 5' end. We report here that LAT is actually a uniquely stable intron. Furthermore, LAT effectively inhibits transactivation of gene expression by infected-cell polypeptide 0 in transient transfection assays.

The discovery of introns in eukaryotic genes explained the discrepancy in size between heterogeneous nuclear RNA and cytoplasmic mRNA. Although there has been a great deal of speculation about the persistence of introns in eukaryotic genomes, questions concerning their biological utility remain. Alternative splicing of a particular primary transcript can give rise to a variety of mRNA species, each encoding a different protein. Also, it has been suggested that the splicing of introns from primary transcripts may facilitate transport of the mRNA from the nucleus to the cytoplasm (1). However, since introns do not generally accumulate and have been assumed to be destroyed, until now it seemed unlikely that an intron would play a more general role in the regulation of gene expression.

The latency-associated transcript (LAT) is the major viral transcript detected by *in situ* hybridization of human or mouse sensory ganglia latently infected with herpes simplex virus type 1 (HSV-1) (2–4). LAT is a nonpolyadenylated 2.0-kilobase (kb) transcript (5, 6) that is restricted to nuclei (2). This transcript accumulates to high concentrations in neuronal cell nuclei during latency and in productively infected tissue culture cells. LAT lies within an 8.3-kb transcription unit present in the long repeats of HSV-1. The 5' end of LAT lies 688 base pairs (bp) downstream of its promoter (7) and has a sequence similar to the vertebrate splice donor consensus sequence (6). The last 750 bases of LAT are complementary, that is, antisense, to the HSV-1 immediate-early gene for infected-cell polypeptide 0 (ICP0). This protein is a potent transactivator of gene expression in transient assays (8–10); it has also been implicated in reactivation from the latent state in *in vivo* and *in vitro* latency systems (11–13).

Although strong evidence exists that antisense RNA is used to regulate gene expression in many prokaryotic systems (14), the use of natural antisense transcripts as inhibitors of gene expression in eukaryotic cells has only recently been elucidated. A number of antisense transcripts have now been

identified in eukaryotic systems (2, 15–20), but the biological significance of most has not been determined.

Two eukaryotic antisense transcripts appear to have important regulatory functions. Kimelman and Kirschner (21) have shown that *Xenopus* oocytes contain an antisense transcript to the mRNA for basic fibroblast growth factor (bFGF). This antisense transcript is present at 20-fold excess relative to the bFGF mRNA and is itself an mRNA capable of encoding a protein. Upon induction of meiosis in these oocytes, an RNA duplex modifying enzyme, first identified by Bass and Weintraub (22), is released from the nucleus into the cytoplasm. This modifying enzyme induces extensive covalent changes in the bFGF mRNA, leading to its deadenylation and possibly reducing its stability.

In addition, Khochbin and Lawrence (23) have shown that, in murine erythroleukemia cells chemically induced to differentiate, the initiation of production of a p53 antisense transcript coincides with a rapid decrease in the amount of p53 mRNA found in these cells. This p53 antisense transcript is confined to the nucleus and does not appear to encode a protein.

In this study, we show that LAT is a stable intron that can effectively inhibit transactivation by ICP0 in transient transfection assays. To our knowledge this is the first stable intron found within its natural biological context.

## MATERIALS AND METHODS

**Plasmid Constructions.** Plasmids were constructed as follows: (i) An HSV-1 *Apa* I DNA fragment encompassing the LAT was subcloned into the *Apa* I site of Bluescript (Stratagene) (pMF20). (ii) An *Asp*718–*Eco*RV DNA fragment derived from pMF20 was end-filled and subcloned into the *Eco*RV restriction site of pCH110 (Pharmacia) (pMF22). (iii) An HSV-1 *Pst* I–*Eco*RI DNA fragment derived from *Eco*RI DNA fragment J+K was subcloned into pUC19 (pMF37). (iv) A *Hind*III–*Eco*RI DNA fragment derived from pMF37 was subcloned into a *Hind*III–*Eco*RI DNA fragment derived from pCH110 (pMF39). All cloning procedures were performed as described (24).

**RNA Analysis.** Transfections were performed using Lipofectin (Bethesda Research Laboratories) as described by the manufacturer. RNA isolation and Northern blot analyses were performed as described (25). Ten micrograms of total RNA was electrophoresed in each lane of an agarose/formaldehyde gel at 20 V/cm.

**Isolation of a Spliced LAT Species.** Twenty-five micrograms of total RNA isolated from COS-7 cells transfected with pMF22 were added to the first-strand synthesis reaction mixture of the Pharmacia cDNA synthesis kit. The reaction

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Abbreviations: HSV-1, herpes simplex virus type 1; LAT, latency-associated transcript; ICP0, infected-cell polypeptide 0; PCR, polymerase chain reaction; TK, thymidine kinase; SV40, simian virus 40.

mixture was phenol-extracted, ethanol-precipitated, and resuspended in 100  $\mu$ l of 10 mM Tris-HCl, pH 8.0/1 mM EDTA.

The portion of the cDNA that spans the LAT splice junction was amplified by the polymerase chain reaction (PCR) (26) using primers that hybridized to either side of the stable intron. The oligonucleotide primers used were GT-GTCGTTCAACAAAGACGCC and CTCTTCCTCCTCT-GCTCTT. Five microliters of the cDNA was added to a reaction mixture that contained 0.9 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10% (vol/vol) dimethyl sulfoxide, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 50 pmol of the two primers, and 2.5 units of *Thermus aquaticus* polymerase (Perkin-Elmer/Cetus) in a total volume of 50  $\mu$ l.

The cycling reactions were performed with an Ericomp TwinBlock system thermal cycler. The first cycle was 94°C for 3 min, 52°C for 1 min, and 72°C for 10 min. This was followed by 40 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 2 min. One-tenth of the PCR product obtained was electrophoresed at 5 V/cm through a 4% NuSieve agarose gel containing ethidium bromide (1  $\mu$ g/ml). This gel was placed on a shortwave UV source to allow determination of the size and relative purity of the PCR product.

One-half of the PCR product was ethanol-precipitated, dried under vacuum, and resuspended in 100  $\mu$ l of restriction enzyme buffer L (Boehringer Mannheim). Ten units of restriction enzyme *Nci* I was added and the reaction was incubated for 2 hr at 37°C. The reaction mixture was ethanol-precipitated, dried under vacuum, resuspended in nick-translation buffer, and end-filled with [<sup>32</sup>P]dCTP (25). The DNA was then digested with *Sac* II and separated on a polyacrylamide gel.

The end-filled product was sequenced using the method of Maxam and Gilbert (27).

**Transient Assays.** Rabbit skin cells from 100-mm plates that were transfected 2 days earlier were washed with isotonic phosphate-buffered saline, scraped from the plate, pelleted, and resuspended in 100  $\mu$ l of 2 $\times$  luciferase buffer (200 mM KPO<sub>4</sub>, pH 8/2 mM dithiothreitol/300 mM MgSO<sub>4</sub>/10 mM ATP). Cells were lysed by a freeze-thaw procedure three times. The supernatant (4  $\mu$ l) was added to 96  $\mu$ l of 2 $\times$  luciferase buffer, 100  $\mu$ l of 1 mM luciferin was injected, and the luminescence was determined by a Monolight model 2010 luminometer (Analytical Luminescence Laboratories, San Diego). Transfections were carried out as described (28). Each plate was transfected with 1  $\mu$ g of p109, with 10  $\mu$ g of pCH110 or 10  $\mu$ g of pMF22, and with 1  $\mu$ g of pICP0 or 1  $\mu$ g of pBS. Each bar represents an average of the luminescence

obtained from luciferase assays on protein extracts from four transfection plates. Standard error is shown (see Fig. 5).

## RESULTS

**DNA Sequence Analysis of the LAT Region of HSV-1.** Since LAT is nuclear, nonpolyadenylated, and lies  $\approx$ 700 bp downstream of its promoter, we analyzed the DNA sequence of the LAT region for significant similarities to the vertebrate splice donor and acceptor consensus sequences.

The 5' end of LAT is an excellent match for the vertebrate splice donor consensus sequence. The best match for the splice acceptor sequence was found 1.95 kb downstream of the 5' end of LAT (Fig. 1). The predicted size of this potential intron is in good accordance with the 2.0-kb LAT detected by Northern blot analysis in total RNA isolated from productively infected HeLa cells.

**Production of the LAT in Transfected COS-7 Cells.** To determine if LAT is derived by utilization of these potential splicing signals, two vectors were created. A 2.4-kb HSV-1 DNA fragment encompassing the potential intron was subcloned into the  $\beta$ -galactosidase gene of pCH110 to create pMF22 (Fig. 2A). This plasmid contains a simian virus 40 (SV40) origin that allows it to replicate to high copy number in cell lines that express the SV40 large tumor antigen (29). pMF22 was transfected into COS-7 cells, which express the SV40 large tumor antigen, and total RNA isolated from these cells was analyzed by Northern blot hybridization. When this Northern blot was probed with a <sup>32</sup>P-labeled HSV-1 DNA fragment complementary to a portion of the 5' end of the LAT (ATD19), a single viral transcript was detected (Fig. 3A, lane A). A second plasmid, pMF39 (Fig. 2B), which contains the SV40 origin and a 13-kb HSV-1 DNA fragment that encompasses both ICP0 and LAT, was also transfected into COS-7 cells. Total RNA isolated from these cells was analyzed by Northern blot hybridization in a similar manner. Again a single viral transcript was detected (Fig. 3A, lane B). In both cases the transcript detected was indistinguishable from LAT detected by Northern blot hybridization of total RNA isolated from productively infected HeLa cells (Fig. 3B, lane A). Since COS-7 cells transfected with either plasmid produced the 2.0-kb LAT, it appears that LAT is generated using the splicing signals contained within the 2.4-kb HSV-1 DNA fragment in pMF22. LAT was not detected by Northern blot hybridization of total RNA isolated from mock-infected HeLa cells (Fig. 3B, lane B).

The same Northern blot, stripped and reprobed with a <sup>32</sup>P-labeled  $\beta$ -galactosidase fragment, revealed a 3.5-kb  $\beta$ -ga-

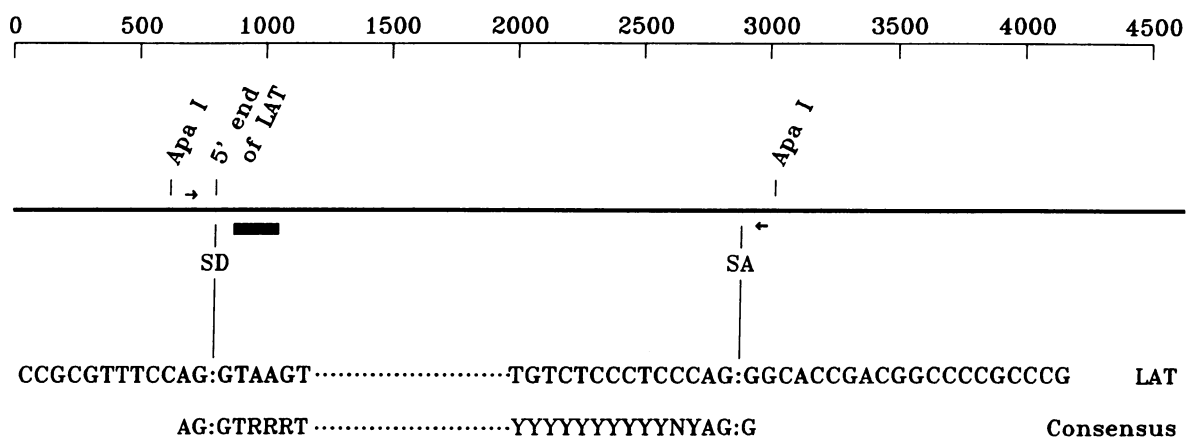


FIG. 1. Map of the *Apa* I fragment. DNA sequence analysis of the LAT region of HSV-1 revealed a potential splice donor (SD) and a potential splice acceptor (SA) within a 2.4-kb *Apa* I DNA fragment. The 5' end of the LAT (6) bears a striking similarity to the vertebrate splice donor consensus and an excellent match for the vertebrate splice acceptor consensus lies 1.95 kb downstream of the LAT 5' end. Arrows indicate locations of the oligonucleotide primers used in the PCR. The solid bar shows the location of ATD19.

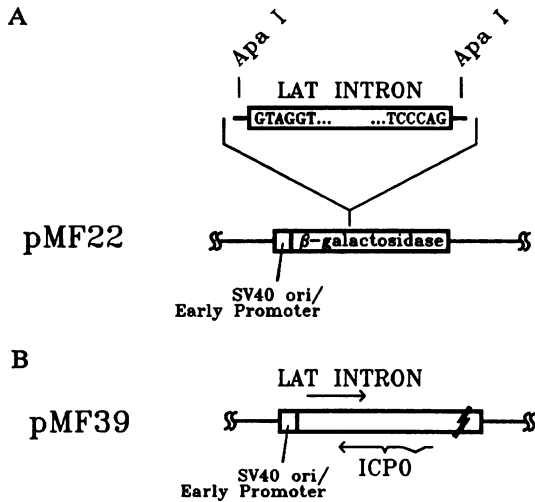


FIG. 2. LAT expression plasmid constructs. (A) The HSV-1 *Apa* I fragment that encompasses LAT was inserted into pCH110 (Pharmacia) at the *EcoRV* restriction enzyme site of the  $\beta$ -galactosidase gene. This plasmid contains an SV40 origin of replication and the *Escherichia coli*  $\beta$ -galactosidase gene whose expression is driven by the SV40 early promoter. (B) A 13-kb fragment that encompasses LAT and the genes for ICP0 and ICP4 of HSV-1 was inserted into a pCH110 deleted for the  $\beta$ -galactosidase gene. This plasmid also contains the SV40 origin of replication and LAT expression is driven by the SV40 early promoter.

lactosidase transcript in the lane loaded with total RNA isolated from pMF22-transfected COS-7 cells (Fig. 3A, lane C). This is the expected size of  $\beta$ -galactosidase mRNA, if LAT were spliced from the primary transcript and the  $\beta$ -galactosidase mRNA were rejoined. Since this 3.5-kb RNA was not detected with a probe that lies completely within LAT (Fig. 3A, lane A), a  $\beta$ -galactosidase RNA of this size could only result from splicing. As expected, no  $\beta$ -galactosidase transcript was detected by Northern blot hybridization of total RNA isolated from pMF39-transfected COS-7 cells (Fig. 3A, lane D).

No unspliced primary transcripts could be detected by Northern blot hybridization of total RNA isolated from pMF22-transfected COS-7 cells by using either the LAT probe or the  $\beta$ -galactosidase probe. This indicates that LAT is efficiently spliced from the primary transcript.

**PCR Analysis of the Spliced Product.** To determine the termini of the LAT species identified by Northern blot analysis, cDNAs produced from total RNA obtained from pMF22-transfected COS-7 cells were amplified by the PCR. The oligonucleotide primers used in this amplification spanned the LAT (Fig. 1). The PCR product obtained from this amplification was  $\approx$ 150 bp long (Fig. 4A), which corresponds well with the 147-bp product predicted. The DNA

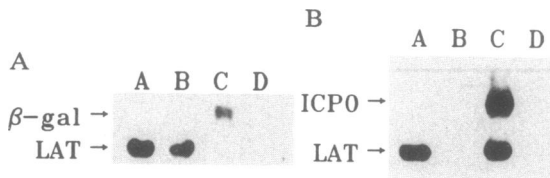


FIG. 3. Northern blot hybridization analysis of the LAT expression. (A) Northern blot analysis of total RNA isolated from COS-7 cells transfected with pMF22 (lanes A and C) or with pMF39 (lanes B and D) and probed with either  $^{32}$ P-labeled pATD19 (6) for LAT (lanes A and B; 12-hr exposure) or a  $^{32}$ P-labeled  $\beta$ -galactosidase ( $\beta$ -gal) DNA fragment (lanes C and D; 72-hr exposure). (B) Northern blot analyses of total RNA isolated from HeLa cells infected with HSV-1 (lanes A and C) or mock-infected (lanes B and D) and probed with pATD19 (lanes A and B) or with pICP0 (lanes C and D).

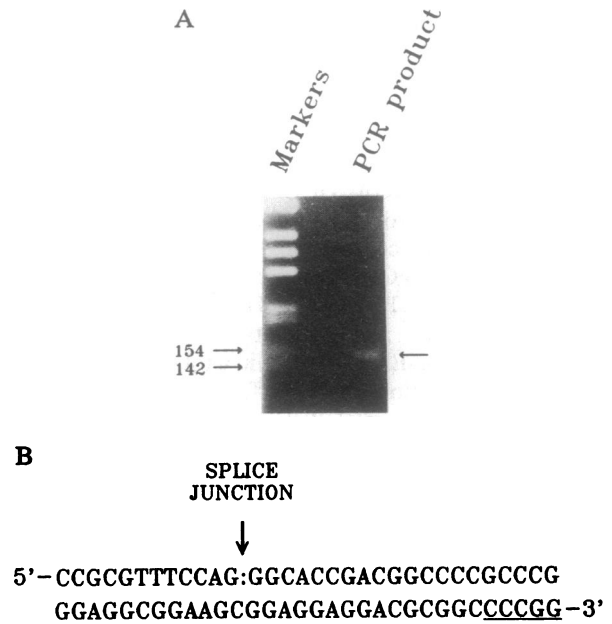


FIG. 4. PCR analysis of the LAT splicing. (A) PCR amplification of cDNAs obtained from pMF22-transfected COS-7 cells. The primers used in this amplification span the LAT. The product obtained was between 142 bp and 154 bp long. (B) DNA sequencing of the PCR product obtained was digested with *Nci* I (the underlined sequence) and end-filled with  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ . The DNA sequence was obtained by the method of Maxam and Gilbert (27). The lower part of Fig. 1 shows how the sequence of the PCR product fits into the genomic sequence to form consensus splice sites.

sequence determined for the portion of the PCR product that spans the splice junction is shown in Fig. 4B. Comparison of the sequence of the PCR product with the genomic sequence of the LAT region reveals that LAT was spliced from the  $\beta$ -galactosidase fusion transcript at the consensus splice donor and acceptor sites. These sequences, and the consensus splicing signals, are shown in Fig. 1. These results provide compelling evidence that LAT is an intron.

**Stability of LAT.** Extreme stability is not a general property of HSV-1 introns. Neither of the two introns of ICP0 can be detected by Northern blot hybridization of total RNA isolated from productively infected HeLa cells (Fig. 3B, lane C). Also, since the LAT can be detected in cells transfected with pMF22 or pMF39, as well as in latently infected neurons, HSV-1 infection is not necessary for the LAT stability. The quantities and specific activities of LAT and  $\beta$ -galactosidase probes used in the Northern blot analyses were similar and the autoradiograph of the  $\beta$ -galactosidase Northern blot was exposed six times as long as the autoradiograph of the LAT Northern blot. Although the definitive experiments have not been performed, comparison of the two lanes (Fig. 3A, lanes A and C) suggests that LAT is more stable than the  $\beta$ -galactosidase mRNA derived from the same primary transcript.

The stability of LAT can also be compared to that of the ICP0 mRNA in the Northern blot analysis of total RNA isolated from infected HeLa cells (Fig. 3B, lane C). The probe for this blot was complementary to 3 kb of the ICP0 mRNA and to only 750 bp of LAT. Clearly, under these conditions, LAT is approximately as abundant as the ICP0 mRNA.

**LAT Inhibits Transactivation by ICP0.** To study the effect of LAT on ICP0 transactivation, pMF22 was cotransfected into rabbit skin cells with a plasmid expressing ICP0 and another containing the reporter gene luciferase driven by the HSV-1 thymidine kinase (TK) promoter. We chose to study LAT in a heterologous construct to separate the effects of the intron from functions that may be encoded by an mRNA derived from the same region of the virus. Results of repeated

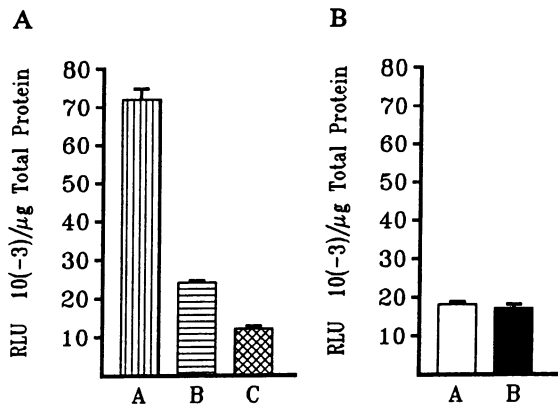


FIG. 5. (A) Antisense inhibition of ICP0 transactivation by the LAT intron. Luciferase assays of rabbit skin cells transfected with p109 (containing an HSV-1 TK promoter/luciferase gene construct), pCH110 (the COS vector plasmid alone), and pICP0 (bar A); with p109, pMF22, and pICP0 (bar B); or with p109, pCH110, and pBS (a Bluescript plasmid) (bar C). (B) Luciferase assays of rabbit skin cells transfected with p109, pMF22, and pBS (bar A) or with p109, pCH110, and pBS (bar B).

transfections show that, under the conditions studied, LAT inhibited ICP0 transactivation of the TK promoter by 50–80% (Fig. 5A). LAT had no effect on the baseline levels of transcription from the TK promoter (Fig. 5B) and cotransfection with a plasmid containing LAT with no promoter has no effect on transactivation of the TK promoter by ICP0. Although antisense inhibition of ICP0 gene function has been demonstrated (30) by driving the opposite strand of ICP0, it is shown here that the LAT intron itself can inhibit ICP0 function.

## DISCUSSION

LAT is a viral RNA that accumulates to high concentrations in the nuclei of latently infected ganglia as well as in cell culture late in infection. This RNA has been the subject of intense scrutiny. The DNA sequences that encompass LAT have been carefully analyzed for potential mRNA-generating signals. Two substantial open reading frames have been identified. In addition, the RNA stability, nuclear location, and lack of polyadenylation of LAT have all been closely examined (6, 15, 16, 31–33). Our results provide compelling evidence that LAT is a stable intron. This explains the many properties of LAT that are inconsistent with it being an mRNA, such as its lack of a cap, its nuclear location, and its lack of polyadenylation. However, the fact that this stable species is an intron does not preclude the possibility that alternative RNA splicing may allow open reading frames within LAT to encode proteins as part of less stable mRNA species.

Nuclear accumulation due to high stability is not a characteristic feature of introns, which are usually rapidly degraded after splicing of the primary transcript. An exception is an intron in the late region of SV40, which has been shown (34) to be stable. However, the stability of this intron is seen only in injected *Xenopus* oocytes, where it may be inefficiently debranched, and not in SV40-infected cells (34). LAT accumulates in infected neurons, in infected tissue culture cells, and in monkey kidney cells transfected with a LAT expression plasmid. This suggests that LAT has unique structural features that account for its relatively high stability. It is interesting to note that a 440-bp  $\lambda$  phage DNA insertion into LAT apparently destabilizes the intron (35). This virus reactivated normally in a cocultivation assay, a phenotype that is in agreement with our data, since protein functions for reactivation are unlikely to be encoded in an

intron. Instead of encoding a positive function for reactivation, it is more likely that the stability of this intron may allow it to function as a potent antisense inhibitor of ICP0 gene expression.

There are at least three mechanisms by which LAT might inhibit ICP0 gene expression. One possibility is that LAT sequesters ICP0 transcripts in the nucleus, making them unavailable for translation. A second possibility is that the RNA duplex modifying activity identified by Bass and Weintraub (22) induces adenosine to inosine transitions in the 3' end of ICP0 transcripts, making either the transcripts unstable or the protein product encoded by the altered transcripts nonfunctional. Everett (36) has shown that mutations or deletions in the 3' end of ICP0 can severely reduce its ability to transactivate. Finally, the RNA duplex formed by LAT and ICP0 may be targeted for degradation by double-strand RNA nucleases.

LAT is not required for the establishment of a latent infection (37, 38); nor could the nuclear LAT encode a protein involved in reactivation. However, since ICP0 has been implicated in reactivation of the virus from the latent state (11–13), it is reasonable to speculate that LAT modulates reactivation events by inhibiting ICP0 gene expression. In this respect, the partial inhibition of ICP0 function in our transient assays would not reflect the natural state. In human or mouse sensory ganglia, LAT accumulates to very high concentrations during latency whereas ICP0 and other immediate-early transcripts are apparently absent. Due to its prior accumulation in the neuronal cell nuclei, LAT would presumably be a much more potent inhibitor of ICP0 gene expression during reactivation events than it is in our transient assays. Complete loss of ICP0 function in this way would be phenotypically equivalent to an ICP0 deletion virus, which at low multiplicities of infection grows to titers 50–100 times lower in tissue culture cells than does wild-type virus (39). Conceivably this inhibition of viral growth could allow the preservation of a reservoir of latently infected neurons by preventing their destruction during reactivation.

Although a number of antisense systems have been described, important details about RNA stability, transport, and modification remain to be determined. The availability of a simple assay for ICP0 function and the ease of manipulation of both the LAT and ICP0 genes in the virus make this system an ideal one for exploring the basic mechanisms of antisense inhibition in eukaryotic organisms.

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