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# Physical Characterization of the Herpes Simplex Virus Latency-Associated Transcript in Neurons

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**RNA transfer (Northern) blot analysis was used to perform the physical characterization of the transcript expressed in murine sensory nerve ganglia latently infected with herpes simplex virus type 1. Most of this latency-associated transcript (LAT) was isolated in the poly(A)$^-$ fraction from ganglia. A smaller RNA species was also detected at less than 10% the abundance of the major one. LAT was not detected with probes from DNA outside the limits of the larger species. In situ hybridization data correlated well with Northern blot analysis; however, low levels of hybridization were seen with probes immediately outside the region of viral DNA giving positive Northern blot signals. S1 nuclease and primer extension mapping were used to locate the 5′ end of the LAT 510 bases to the left of a *KpnI* site at 0.783 map units. The 3′ end of the major latency-associated species was mapped to just within a 310-base-pair *SmaI* fragment located 660 to 970 base pairs to the right of the *SalI* site at 0.790 map units. These data were correlated with an analysis of the sequence of the DNA encoding this transcript and its possible function in the latent phase of infection.**

Latency is a hallmark of herpesvirus infection. In this state, viral genomes are available for reactivation of productive infection, but the viral products diagnostic of an active infection are difficult if not impossible to detect (9, 27). For herpes simplex virus type 1 (HSV-1), the molecular investigation of viral genes expressed during the latent state has been quite difficult since only a minority of cells in neural tissue harbor latent virus.

Despite such technical difficulties, it has been clear for some time that during the latent phase, antigens characteristic of productive infection are not reproducibly detectable (29). Further, the viral genome appears to be present in circular or concatenated forms rather than the linear molecules found in the intact virion (21). These data indicate that the latent phase is one in which viral gene expression is quite limited, a situation analogous to that seen in latent infections with the related Epstein-Barr herpesvirus (11).

These indications have recently been confirmed by our finding an abundant and previously unreported transcript in latently infected mouse sensory nerve ganglia (29). This latency-associated transcript (LAT) was estimated to be about the same size as, and partially complementary to, the α transcript encoding ICP0, one of the transcriptional activators expressed at the earliest phases of productive infection by HSV-1.

Since our initial communication, two other groups have detected and partially characterized HSV RNA in latently infected cells, and this RNA was found to be expressed from the same general region of the genome we are studying (2, 22, 24, 26). Both generally confirm our earlier results and those reported here. It should also be noted that Puga and Notkins (20) used a probe made by oligo(dT)-primed cDNA synthesis of latently infected neuronal RNA and reported a weak signal hybridizing to the same general genomic position as observed for LAT. This may reflect a poly(A)$^+$ RNA species of very low abundance that we detected with difficulty in the present study.

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We now present the precise physical characterization and genomic location of the HSV-1 LAT expressed in latently infected murine sensory nerve ganglia and correlate this with the sequence of the viral DNA encoding it. Interestingly, the most abundant species of this transcript (>95%) was recovered in the poly(A)$^-$ fraction; like many other HSV transcripts, it lacks large introns. Our findings suggest that this gene encodes a protein; however, the data obtained to date also suggest that a protein is not expressed by the major LAT during the latent phase of infection.

## MATERIALS AND METHODS

**Establishment of latent infection in mice and processing of spinal ganglia.** Six-week-old outbred male Swiss-Webster mice (Simonson Laboratories, Gilroy, Calif.) were used in all experiments. They were inoculated in each rear foot with $10^8$ PFU of HSV-1 strain KOS(M). The derivation of this virus strain and methods used for inoculation have been described previously (29, 32). Three or more weeks later, after latent infections had been established in lumbosacral spinal ganglia (28), ganglia were dissected from the animals, quick frozen in liquid nitrogen, and either cut on a cryostat or stored at −70°C until used.

**RNA isolation and fractionation.** RNA was isolated from latently infected ganglia by the guanidinium isothiocyanate-hot phenol method as described previously (29). Poly(A)-containing RNA was selected by oligo(dT)-cellulose (Collaborative Research, Inc., Waltham, Mass.) chromatography (4). RNA was size fractionated by electrophoresis with 1.4% agarose gels containing 10 mM methyl mercury hydroxide as previously described (4, 29); RNA was transferred by electrophoresis onto nylon membranes (Gene Screen; New England Nuclear Corp., Boston, Mass.). In some experiments, RNA was fractionated on 1.2% agarose gels containing 6% formaldehyde and blotted onto membranes (13).

**Primer extension.** Primer extension experiments were done with a synthetic 20-nucleotide oligomer (5′-dTGG TGTGCTGTAACACGAGC-3′). The location of the oligomer within the sequence of the DNA encoding the LAT

transcript is shown in the Results. Samples of 15 ng of 5′-$^{32}$P-labeled primer were annealed with RNA from 15 to 20 latently infected mouse ganglia. Extension was done at 50°C with 15 to 20 U of reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) in 15 μl of a buffer (pH 8.3) containing 50 mM KCl, 35 mM Tris, 5 mM $MgCl_2$, and 400 μM each deoxynucleoside triphosphate as described in references 10 and 15. Sequences obtained by the dideoxy chain termination method (see below) from the same primer annealed to DNA were cofractionated with the primer-extended cDNA as an absolute size marker.

**Recombinant DNA and sequence analysis.** All recombinant DNA was from the KOS(M) strain of HSV-1; fragments were cloned into either pUC or M13 vectors as described previously (13, 16). Restriction endonucleases were purchased from either Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). For synthesis of $^{32}$P-labeled DNA probes, the HSV-specific DNA fragment was isolated by digestion of the total plasmid with appropriate restriction enzymes, followed by electrophoresis and electroelution. Synthesis of uniformly labeled probes was accomplished by nick translation with [α-$^{32}$P]dCTP (3 Ci/μmol; Amersham Corp., Arlington Heights, Ill.). For 5′ end labeling of DNA for S1 nuclease analysis, the *Kpn*I site at 0.783 map units (m.u.) was cut with *Asp* 718 to provide a 5′ overhang. The 5′ phosphate residue was then removed with bacterial alkaline phosphatase (Bethesda Research Laboratories) and $^{32}$P labeled with [γ-$^{32}$P]ATP (7 Ci/μmol; ICN Pharmaceuticals, Inc., Irvine, Calif.) and bacteriophage T4 kinase (Bethesda Research Laboratories) (4, 13). The appropriate fragment was then released by digestion with *Pst*I.

Sequence analysis was done with convenient restriction sites within the DNA region of interest by the general methods of Maxam and Gilbert (14). Sequences determined in this way were confirmed by the dideoxy chain termination method of Sanger et al. (24) with single-stranded DNA cloned in M13.

**Hybridization conditions.** RNA transfer blots were hybridized with $40 \times 10^6$ cpm (Cerenkov) of radiolabeled DNA in the presence of 50% formamide, 0.4 M $Na^+$, 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8.0), 0.005 M EDTA, and Denhardt solution (3) at 49°C for 40 h. Details and procedures for rinsing have been described previously (4, 29). For S1 nuclease mapping with double-stranded DNA under R-looping conditions, hybridization was in the presence of 80% formamide in the same buffer without Denhardt solution for 12 h at 68°C. Before the hybridization, the 5′-end-labeled DNA was denatured with alkali and neutralized before addition of RNA. Conditions for S1 nuclease digestion and fractionation of material on acrylamide gels containing 8 M urea have been described previously (1, 6).

**In situ hybridization methods.** Appropriate cloned DNA fragments were nick translated with [$^3$H]deoxynucleotides to specific activities of $2 \times 10^7$ to $3 \times 10^7$ cpm/μg of DNA (29). Each probe was used on cryostat-cut sections of lumbosacral spinal ganglia taken from uninfected, latently infected, or acutely infected mice. In the latter instance, mice had been infected 4 days previously in rear footpads, and at the time of sacrifice, virus was replicating to high levels in the ganglia (32). Methods for preparation of slides and execution of RNA-specific hybridization reactions were a modification of those described by Haase et al. (8). In the present experiments, probes were prehybridized and the stringency of the hybridization method was increased as described by Stroop et al. (31). After a 2-week exposure, the slides were developed, stained with Giemsa, and observed.

## RESULTS

**Mapping LAT mRNA.** Our previous report established the direction of transcription of the HSV LAT transcript as opposite that of ICP0 (29). In this study, we used a number of subclones of DNA in the region of the HSV genome around the ICP0 gene and RNA transfer (Northern) blots of RNA from latently infected ganglia to establish its extent. Our data are summarized in Fig. 1A, and representative blots are shown in Fig. 1B.

The transcript from latently infected cells migrated marginally faster than did ICP0 mRNA fractionated in parallel on methyl mercury gels. This difference in migration rates was accentuated when formaldehyde gels were used (data not shown). Therefore, we concluded that the upper limit in size was on the order of 2.3 kilobases. Using total RNA, a readily detectable transcript of this size was seen only with probes made with DNA spanning the 2,500 bases between the *Sma*I sites at 0.777 and 0.794 m.u. (Fig. 1B, lanes ii and iv).

To determine precisely the maximum extent of the 3′ end of LAT complementary to ICP0, we used small DNA probes, and some of these data are shown in Fig. 2. Northern blots hybridized with the 660-base-pair *Sal*I-*Sma*I fragment between 0.790 and 0.794 m.u. gave a strong positive signal. In contrast, the 375-base-pair *Sma*I fragment immediately to the right of this (0.794 to 0.796 m.u.) gave a weak positive signal for HSV-1 LAT. This signal is clearly seen in long exposures (Fig. 2). DNA probes made from fragments immediately to the right of 0.796 m.u. were negative (Fig. 1B, lane v). Such data located the 3′ end of the transcript to be 660 to 970 bases to the right of the *Sal*I site at 0.790 m.u. The low intensity of the signal obtained with this nick-translated *Sma*I fragment suggested that the transcript extends only a short way (20 to 50 bases) 3′ of the *Sma*I site nearest the *Sal*I site at 0.790 m.u.

A second RNA band migrating with a size corresponding to 1.8 to 2 kilobases was seen on most gels with probes which hybridized to the major LAT species. Examples are seen in Fig. 1B (lanes ii and iv) and in Fig. 2. This transcript was found to be encoded by the same DNA strand as that encoding the major LAT by use of strand-specific probes (data not shown). It was not detected with any probes beyond the limits of the LAT, and we suggest that it represents a processed form of the major species (see Discussion).

Although RNA of the size of the major and minor HSV-1 LAT species was only detected with probes mapping within 0.777 and 0.793 m.u., DNA probes encompassed by the *Sma*I fragment spanning 0.770 to 0.777 m.u. and probes containing DNA to the right of the *Sma*I site at 0.796 m.u. did hybridize to RNA migrating diffusely with a size of no more than 300 to 350 bases on some Northern blots (Fig. 1B, lanes i, v, and vi). This result implies that some transcription does occur beyond the nominal 5′ and 3′ ends of the LAT transcript in latently infected cells. Based on exposure density of the autoradiographs, the amount of RNA responsible for such diffuse signals was less than 5% that of the 2.3-kilobase LAT transcript.

**In situ hybridization of latently infected ganglia.** Independent data supporting the Northern blot mapping were obtained by in situ methods with the same probes as those used above. Examples of the in situ data with both latently

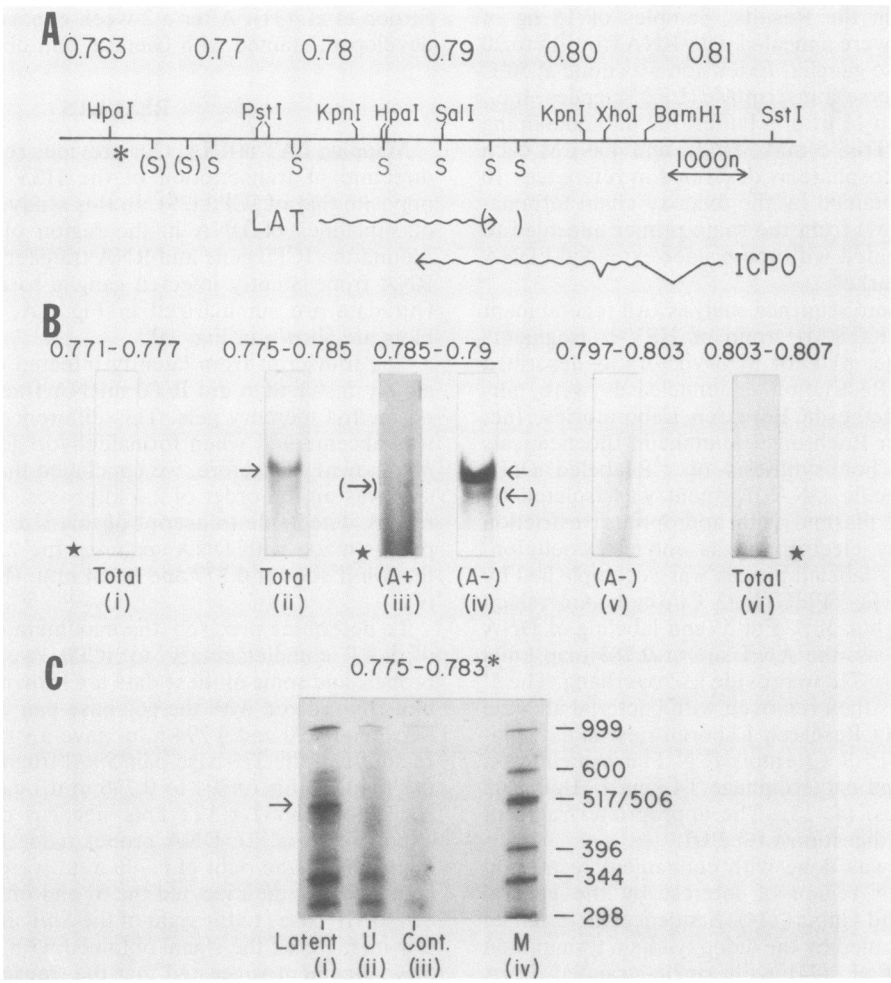


FIG. 1. Precise location of the LAT transcript on the prototypical arrangement of the HSV-1 strain KOS(M) genome. (A) Summary restriction map of the region encoding the transcript. Unique restriction sites are indicated. *Sma*I sites (S) shown in parentheses are approximate locations, and no *Sma*I sites to the right of the *Kpn*I site at 0.802 m.u. are included. The asterisk (*) indicates the location of the beginning of the long internal repeat region of the genome. (B) Northern blot analysis of RNA isolated from latently infected mouse sensory nerve ganglia. Details of the methods used are described in the Materials and Methods and Results. RNA from 25 ganglia were used for each Northern blot shown. The arrows indicate the size expected for the LAT transcript; arrows in parentheses indicate minor species; the stars indicate the presence of small diffusely migrating RNA discussed in the Results. Total ganglion RNA was used except for lanes iii and iv, in which poly(A)$^+$ and poly(A)$^-$ fractions of the same ganglion RNA preparation were used. Probes used were: 0.771–0.777, *Sma*I to *Sma*I; 0.775–0.785, *Pst*I to *Hpa*I; 0.785–0.79, *Hpa*I to *Sal*I; 0.797–0.803, *Sma*I to *Xho*I; 0.803–0.807, *Xho*I to *Bam*HI. Exposure of autoradiographs was for 16 h at −70°C with Cronex intensifying screens, except for lane iii, which was exposed for 6 days at this temperature. (C) S1 nuclease mapping of the 5′ end of LAT from latently infected ganglion RNA (lane i). A DNA probe extending from the *Pst*I site to the *Kpn*I site (the latter cleaved with *Asp* 718) selectively 5′ end labeled at the *Kpn*I site was hybridized under R-looping conditions as described in the text. S1 nuclease-resistant material was fractionated on a denaturing 8% acrylamide-urea gel versus size standards derived from *Eco*RI-*Hin*fI-digested pBR322 DNA (lane M). The sizes are shown to the right of lane M (nucleotides). The specific band of material migrating with a size of approximately 490 bases is indicated by the arrow. Other bands were also seen in a control tracks of material hybridized with uninfected-cell RNA (lane ii) and with commercial *Escherichia coli* tRNA (lane iii).

infected and uninfected ganglia are shown in Fig. 3. Strong, infected-cell-specific nuclear hybridization was observed with probes made from DNA fragments contained in the *Pst*I-*Hpa*I (0.775 to 0.785 m.u.; Fig. 3A), *Hpa*I-*Sal*I (0.785 to 0.790 m.u.; data not shown), and *Sal*I-*Bam*HI (0.790 to 0.807 m.u.; Fig. 3B) clones. No hybridization was seen in the uninfected controls (Fig. 3E and F). Positive signals were also obtained with probes made from the *Hpa*I-*Pst*I (0.764 to 0.774 m.u.; Fig. 3C) and the *Bam*HI-*Sst*I (0.807 to 0.815 m.u.; Fig. 3D) DNA fragments. However, the signal from these was less intense, and with the latter probe, fewer than 50% of the nuclei were labeled than were detected with the *Hpa*I-*Sal*I or the *Pst*I-*Hpa*I probes. With the *Hpa*I-*Pst*I probes, there was also a high background in uninfected cells (Fig. 3G). This background, and the positive nuclear signals seen, may relate to the cross-hybridization reported between this fragment and ribosomal or other cellular genes (17, 19, 25). Any specific hybridization detected with this probe and the positive nuclei observed with the *Bam*HI-*Sst*I probe may represent the small RNA detectable in some Northern blots. Alternatively, it may reflect a low level of transcription of other genes that are below the level of detection by Northern blot analysis (see Discussion).

**Distribution of HSV-1 LAT between poly(A)$^+$ and poly(A)$^-$ fractions.** Most of the LAT transcript was recovered in the fraction lacking poly(A) upon oligo(dT)-cellulose chromatog-

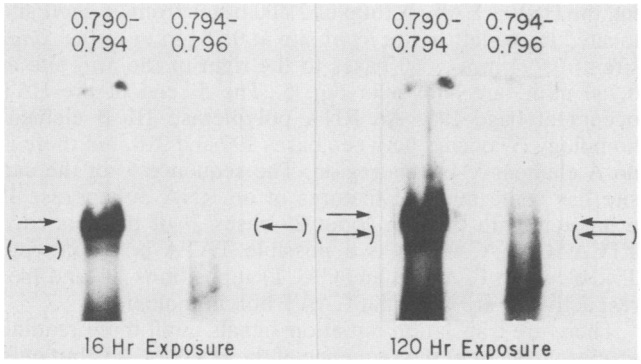


FIG. 2. Northern blot analysis of the 3′ end of the HSV-1 LAT. RNA samples from latently infected mouse ganglia were fractionated as described in the legend to Fig. 1C and electrophoretically transferred to filter membranes. The blots shown in the figure were hybridized with $28 \times 10^6$ of $^{32}$P-labeled nick-translated probe made from either the *Sal*I-*Sma*I fragment (0.790 to 0.794 m.u.) or the *Sma*I-*Sma*I fragments (0.794 to 0.796 m.u.) directly to the right of this as shown in Fig. 1A. Exposure times shown were 16 and 120 h with intensifying screens as described in the legend to Fig. 1. Both the major and minor LAT transcripts are detectable with difficulty with the probe covering 0.794 to 0.796 m.u.

raphy of RNA preparations from whole ganglia (Fig. 1B). When the poly(A)$^+$ fraction of total ganglion RNA was fractionated in parallel with the poly(A)$^-$ fraction and hybridized with the same probe, a faint band of RNA with a migration rate similar to that of the minor poly(A)$^-$ LAT species could be detected with long exposures. An example of such an experiment is shown in lanes iii and iv of Fig. 1B. Here, the exposure time for the poly(A)$^+$ fraction (lane iii) was approximately 10 times longer than for the poly(A)$^-$ fraction (lane iv). We also found some diffusely migrating RNA of small size in the poly(A)$^+$ fraction.

**Precise location of contiguous 5′ end of the LAT transcript.** We located the contiguous 5′ end of the LAT transcript using S1 nuclease analysis of hybrids between latently infected neuronal RNA and DNA selectively 5′ end labeled at the *Kpn*I (*Asp* 718) site at 0.783 m.u. The results of one such experiment are shown in Fig. 1C. The probe was isolated as a 1,073-base-pair *Pst*I-*Kpn*I fragment (0.775 to 0.783 m.u.) cloned in pUC18, and hybridization of the DNA to latently infected ganglion RNA was done as described in Materials and Methods. S1-resistant material was fractionated by denaturing acrylamide gel electrophoresis with *Eco*RI-*Hin*fI-digested pBR322 DNA as a size standard. An S1-resistant fragment migrating with a size corresponding to 500 ± 20 bases was seen in the sample hybridized with latently infected RNA (Fig. 1C, lane i). This band was completely absent in parallel tracks of DNA hybridized with uninfected HeLa cell RNA and with *Escherichia coli* tRNA, which served as controls (Fig. 1C, lanes ii and iii). We further confirmed this location by using a probe 5′ end labeled at a *Hin*fI site that is located 150 bases 3′ of the cap site (data not shown). There were other bands of radioactive DNA resistant to S1 digestion generated under the conditions of hybridization used (Fig. 1C). In each case, however, these bands were seen in both the control and ganglion RNA tracks; therefore, they were not specific for latently infected ganglion RNA. Although the reason for the occurrence of such nonspecific S1-resistant material has not been fully investigated, it may relate to the high G+C content of HSV DNA from this region of the genome. Whatever the source of this background, its existence precluded the use of larger

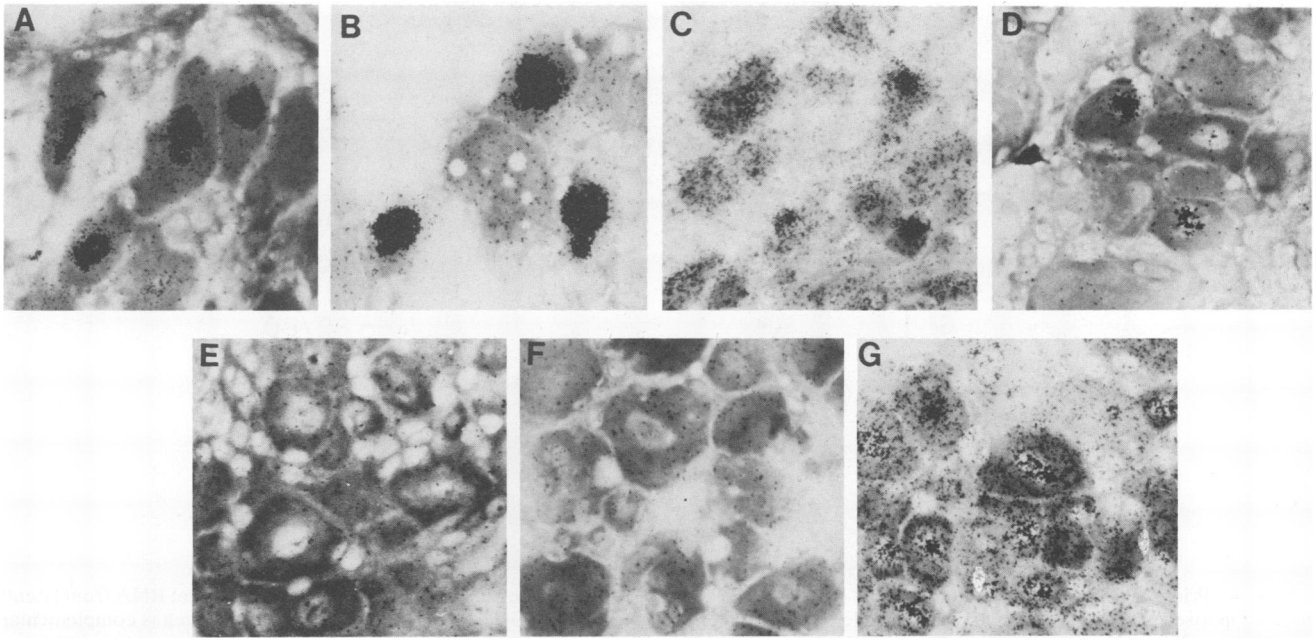


FIG. 3. Hybridization in situ of $^3$H-labeled HSV-1 DNA probes to RNA in latently infected and uninfected murine spinal ganglia. Probes with the map positions noted, prepared and hybridized to tissue sections as described in the Materials and Methods, were used to detect virus-specific RNA in ganglionic neurons. (A) Probe, *Pst*I-*Hpa*I (0.775 to 0.785 m.u.), latently infected ganglia. (B) Probe, *Sal*I-*Bam*HI (0.79 to 0.807 m.u.), latently infected ganglia. (C) Probe, *Hpa*I-*Pst*I (0.764 to 0.775 m.u.), latently infected ganglia. (D) Probe, *Bam*HI-*Sst*I (0.807 to 0.815 m.u.), latently infected ganglia. (E) Probe, *Pst*I-*Hpa*I (0.775 to 0.785 m.u.), uninfected ganglia. (F) Probe, *Sal*I-*Bam*HI (0.79 to 0.807 m.u.), uninfected ganglia. (G) Probe, *Hpa*I-*Pst*I (0.764 to 0.775 m.u.), uninfected ganglia.

probes for carrying out a complete S1 analysis of the HSV-1 LAT transcript.

The location of the 5′ end of the HSV-1 LAT was precisely established by primer extension. Here, a 20-base primer complementary to the direction of transcription of the LAT corresponding to a position from 379 to 399 bases to the right (5′) of the *Kpn*I (*Asp* 718) site at 0.783 m.u. was synthesized by using the sequence data discussed in the next section. This primer was annealed with total latently infected ganglion RNA, and reverse transcriptase was used to synthesize cDNA with the 5′ end of the LAT as its template. A typical experiment is shown in Fig. 4; the sequence seen is complementary to the mRNA coding (sense) strand. The only cDNA species of any intensity consistently migrated at a position which located the cap site at the first G within the trinucleotide AGG. As shown in the sequence data discussed below, this places the LAT cap site 510 bases to the left of the *Kpn*I site at 0.783 m.u. This result is in excellent agreement with that determined by S1 analysis. Further, the lack of any larger species confirmed our conclusion that there is no readily detectable splice at the 5′ end of the HSV-1 from latently infected ganglia. Preliminary RNA-primed dideoxy sequence data are fully consistent with the location of the cap site shown in Fig. 4 (M. Flanagan and E. Wagner, unpublished data).

**Analysis of sequence of HSV-1 DNA encoding the LAT.** We next determined the nucleotide sequence of the DNA encoding the HSV-1 LAT. A total of 2,400 bases from an *Sph*I site located to the left of the *Kpn*I site at 0.783 m.u. to the *Sma*I site at 0.794 m.u., 660 bases to the right of the *Sal*I site at 0.790 m.u., are shown in Fig. 5. The 5′ end of the LAT occurs at base 195. An RNA polymerase III B element homology (7) occurs between bases 199 and 210, but there is no A element 5′ of this region. The sequence 5′ of the cap site has some minimal features of an RNA polymerase II promoter, with the cap about 25 bases 3′ of the sequence TTCAACAAA, which is a possible TATA box homolog. Two elements (CAGTA and CACT) at positions 109 and 145, respectively, are potential CAAT box homologies.

There are translation initiation signals in all three reading frames throughout the sequence of the HSV-1 LAT, but only two display codon usage patterns which are at all consistent with their encoding a polypeptide, based on the analysis method of Fickett (5). One is the open reading frame (ORF) initiated at base 625 with the nominal eucaryotic initiation codon GATATGG, which remains open for 207 bases (67 amino acids). A second ORF encoding 305 amino acids begins with the ATG at base 1160, 50 bases to the left of the *Hpa*I site at 0.785 m.u. This ORF extends to base 2074, 334 bases to the right of the *Sal*I site at 0.790 m.u.; therefore, the 3′ portion of this ORF is complementary to the 3′ 360 bases of the ORF for ICP0 (18). The Northern blot data of Fig. 1 and 2 indicate that the 3′ end of the HSV-1 LAT extends as many as 50 bases to the right of the *Sma*I site at 0.794 m.u.

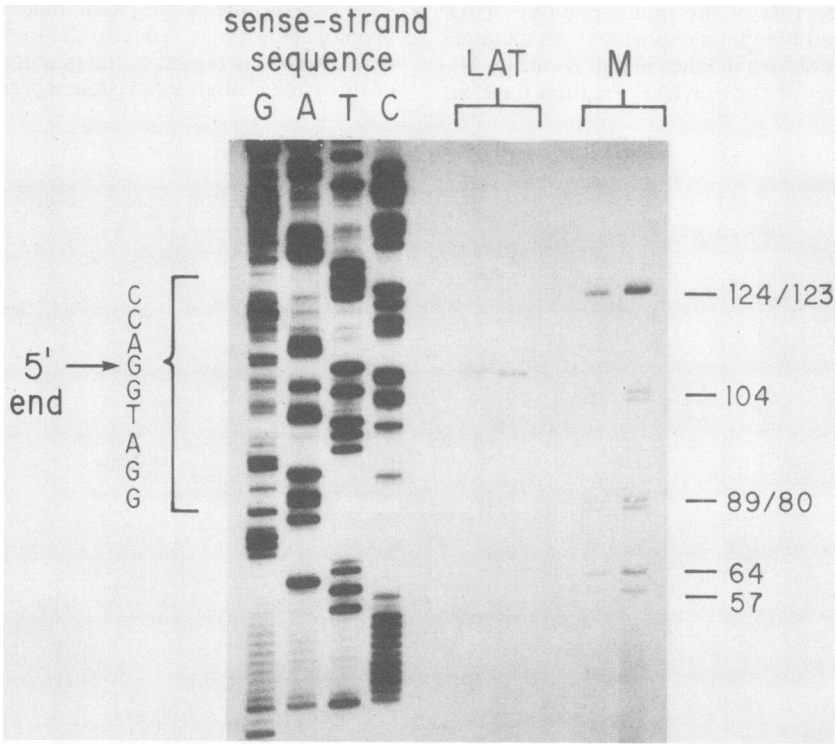


FIG. 4. Primer extension localization of the 5′ end of the HSV-1 LAT. As described in the text, a total of 20 μg of total RNA from latently infected mouse sensory nerve ganglia was annealed with a primer of the sequence TGGTGTGCTGTAACACGAGC which is complementary to a location 395 to 415 bases to the left of the *Kpn*I site at 0.783 m.u. based on sequence data shown in Fig. 5. Reverse transcriptase was used to synthesize a cDNA fragment which was fractionated on a denaturing sequencing gel next to a dideoxy sequence run from the same primer annealed to the sense (coding) strand of the DNA encoding the 5′ end of the HSV-1 LAT. Two different preparations of RNA are shown in the two lanes indicated LAT. The four lanes of DNA sequence terminating at G, A, T, and C are shown. *Hae*III-digested pBR322 was included as a rough size marker in lanes M; note that in the gels shown here, these sizes only approximately correspond with the length of the single-stranded DNA fragments. Numbers to the right show sizes in nucleotides. The sequence of the sense (coding) DNA strand near the LAT cap is also indicated.


FIG. 5. Nucleotide sequence of the DNA encoding the HSV-1 LAT. The sequence of the KOS(M) strain of HSV-1 between an *Sph*I site 708 bases to the left of the *Kpn*I site at 0.783 m.u. and an *Sma*I site 660 bases to the right of the *Sal*I site at 0.790 m.u. is shown. The cap site of the HSV-1 LAT is at base 195. A number of other features discussed in the text are indicated. These include long stretches of pyrimidines (18 to 45, 69 to 109, and 114 to 137), a polymerase III B element (199 to 210), several possible CAAT box homologies, and a weak TATA box homology. The sequence complementary to the primer used for primer extension corresponds to bases 293 to 312; the *Hin*fI site used for S1 analysis is at base 349. The *Kpn*I site (0.783 m.u.) is at base 708, and there are *Hpa*I sites at bases 1037 and 1210, the latter being at 0.785 m.u. The *Sal*I site at 0.790 m.u. is at base 1740. The two potential ORFs are between bases 625 and 831 and 1160 and 2074. There are 15 repeats of the sequence GCACCCCCACTCCCAC between bases 1233 and 1472. The translational frame for ICP0 ends at base 1720 on the opposite strand. Differences between the sequences of the KOS(M) and 17 *syn*$^+$ strains of HSV-1 (Table 1) are underlined.

TABLE 1. Strain differences in the DNA sequence encoding the 3′ portion of HSV-1 LAT

| Difference[a] | Position[b] | | Effect on encoding protein | |
|---|---|---|---|---|
| | KOS(M) | 17 *syn*$^+$ | ICP0 | LAT ORF-2[c] |
| 15(GCAC$_5$ACTC$_3$A)→9 | 1233–1472 | 4097–4280 | | 5(TPTPTHPHSHAPPLPR)→3 |
| C:G→T:A | 1476 | 4131 | | T→I |
| C:G→T:A | 1622 | 3987 | | P→S |
| A:T→C:G | 1624 | 3985 | | |
| G:C→A:T | 1898 | 3711 | T→M | V→I |
| C:G→A:T | 2062 | 3547 | T→T | I→I |
| A:T→C:G | 2182 | 3427 | W→C | |
| T:A→C:G | 2307 | 3302 | T→A | |
| T:A→C:G | 2319 | 3290 | T→A | |
| A:T→G:C | 2332 | 3277 | T→T | |
| G:C→A:T | 2356 | 3253 | A→A | |

[a] The base in the HSV-1 LAT mRNA sense (coding) strand is shown for both strains; the base in KOS(M) is shown first.
[b] The positions of the indicated nucleotides for the KOS(M) strain are as shown in Fig. 5; the position in 17 *syn*$^+$ is as shown in reference 18.
[c] Indicates changes in predicted amino acid encoded by HSV-1 KOS(M) to 17 *syn*$^+$.

Since the sequence of the ICP0 gene extending through the *Hpa*I site at 0.785 m.u. has been published for HSV-1 strain 17 *syn*$^+$ (18), some comparisons are of interest and are summarized in Table 1. The same ORF complementary to that encoding the C-terminal region of ICP0 is seen, and except where 9 sets of the repeat motif TGCGTGG GAGTGGGGG are seen in strain 17 *syn*$^+$, while 15 occur in KOS(M), the homology between the strains is high. Ten single-base differences occur: seven are transitions and three are transversions. Five of the changes are unique to the C-terminal portion of the ICP0-coding region, while three are unique to the second ORF contained in the DNA encoding the major LAT. Two base changes occur in the 360-base complementary overlap between the ICP0 and the second LAT ORFs. Interestingly, these results in more conservative changes in the amino acid sequence of the second LAT ORF than in the ICP0 ORF. The same level of homology is seen between these two strains of HSV-1 to the left of the *Hpa*I site at 0.785 m.u., based on unpublished sequence data (D. McGeoch, personal communication).

## DISCUSSION

In this communication, we defined several properties of the LAT which we previously detected in murine sensory neurons latently infected with the HSV-1 strain KOS(M). Of particular interest in the present study was a determination of LAT map coordinates on the viral genome, the detailed characterization of the 5′ end of the molecule, and the analysis of the DNA sequence encoding the transcript.

Our precise location of the 5′ end of the transcript combined with our high-resolution Northern blot analysis indicate that the major HSV-1 LAT which is no more than 2.3 kilobases from its migration on high-resolution gels is readily detected only with probes extending from bases 195 to 2400 (Fig. 5) and is weakly detected with the DNA fragment immediately to the right of this (Fig. 2). The use of S1 analysis to locate precisely the 3′ end of the transcript as described in previous publications (4, 6) was not particularly fruitful since we did not obtain probes with very high specific activities. Preliminary analysis, however, does indicate that probes 3′ end labeled at the *Sal*I site at 0.790 m.u. do not protect more than 700 bases of DNA (G. B. Devi-Rao and E. Wagner, unpublished data). These considerations strengthen our impression based on Northern blot data such as shown in Fig. 2 that the 3′ end of the major LAT maps quite close to the *Sma*I site at 0.794 m.u. The close correlation between transcript size and sequence data suggests that any intron within the major HSV-1 LAT must be quite small.

The strong in situ hybridization in latently infected neurons obtained with probes that detect the LAT transcript on Northern blots confirms our earlier conclusion that the major LAT species is the most abundant transcript expressed in latent infection (29). A smaller and less abundant LAT species was detected only with probes of the same sense and position of the viral genome as those used to detect the major species. We suggest that this smaller species is a processed form of the major one. The fact that a very faint band of RNA of this approximate size is also seen in the poly(A)$^+$ fraction (Fig. 1B, lane iii) tends to support this impression, but the small amount of this smaller species precluded further characterization at this time. We have no evidence that any other viral transcript is expressed during the latent phase of infection in murine sensory nerve ganglia. Obviously, however, low levels of transcription cannot be excluded, particularly if these molecules are metabolically rapidly degraded after synthesis.

With respect to the cellular abundance of LAT, our RNA samples were derived from 20 ganglia that contained a total of $10^7$ cells. Of these cells, roughly $3 \times 10^5$ were latently infected neurons. The intensity of the Northern blot signals was comparable to that seen for abundant HSV transcripts, such as VP5, isolated from $10^6$ cultured cells [1 μg of infected-cell poly(A)-containing RNA]. Therefore, the LAT is present at an average level of abundance comparable to that of abundant viral transcripts isolated during productive infection or to $2 \times 10^4$ to $5 \times 10^4$ molecules per infected cell. This estimate was further confirmed by our in situ hybridization experiments. In these, equivalently labeled gene-specific DNA probes used to detect LAT in latent infection and VP5 mRNA in productive infection demonstrated equivalent signals (data not shown).

The high levels of the HSV LAT seen in latently infected murine sensory nerve ganglia are also seen in latently infected human (J. Stevens, L. Haar, D. Porter, M. L. Cook, and E. Wagner, submitted for publication) and rabbit (J. Stevens, J. Hill, G. B. Devi-Rao, and E. Wagner, unpublished data) trigeminal ganglia. In contrast to the abundance of the LAT transcript in these cells, recent work has established that the HSV-1 LAT is expressed at very low levels during productive infection of cultured rabbit skin cells (E. Wagner, G. B. Devi-Rao, Y.-F. Zhang, and J. Stevens, manuscript in preparation). Since only LAT of all potential HSV transcripts is expressed in the latent phase,

promoter-control regions mediating its expression may have neuronal specificity. In this regard, further characterization of the 5′ end of the LAT will be required to identify fully the DNA sequences controlling its expression. However, it may be that the poor TATA and CAAT box homologies noted in the sequence shown in Fig. 5 have a role in the unusual expression of this transcript. Also, the several long stretches of pyrimidines seen (bases 16 to 43 and 59 to 135) may have some role in tissue specificity.

Although we do not know the role of HSV LAT in latent infection, a mutant of HSV-1 (X10-13) with a deletion in the 5′ end of the HSV-1 LAT and its control sequences has recently been characterized (R. Javier, V. Dissette, E. Wagner, and J. Stevens, manuscript in preparation). This virus establishes latent infections in mice yet expresses no detectable LAT or any other viral transcript, including the less abundant in situ signals mapping 3′ to the LAT (Fig. 3D). This suggests that all RNA species seen in the latently infected neurons are related to the expression of the major LAT species and are not due to independent transcription units. It also demonstrates that expression of the LAT gene is not required to establish the latent state in mouse neurons. This does not, of course, exclude its acting to augment the normal restriction of the HSV lytic cycle in neurons.

As discussed in reference 29, one possible role of the major LAT species is to suppress the expression of the α ICP0 transcript to which it is partially complementary by an antisense mechanism. The ICP0 protein is a transcriptional activator, and it was recently reported that HSV mutants lacking this gene replicate poorly at low multiplicities of infection (23, 30). Of equal interest, Schaffer and colleagues have found that two ICP0 deletion mutants cannot reactivate from the latent state in murine trigeminal ganglia even though viral DNA is present (D. M. Coen, D. M. Knipe, K. L. Taylor, and P. A. Schaffer, manuscript in preparation).

The abundance and nuclear localization of the major HSV-1 LAT is consistent with its functioning as an antisense regulator. An experimental system involving antisense regulation which demonstrates most of the properties described here has been reported (12). There thymidine kinase mRNA was associated with the antisense RNA and restricted to the nucleus when RNA complementary to the 3′ end of thymidine kinase mRNA was present. Unlike the results reported with that model, however, we did not detect any of the sense transcript (ICP0) in latently infected mice.

We consider it unlikely that the major species of the LAT simply encodes a protein functioning in the latent phase of infection. The physical location of the major LAT in the latent phase argues against its being translated. The positions of the identified ORFs vis-a-vis the 5′ end of the transcript, however, suggest that the major LAT could be spliced to generate a readily translatable mRNA. It should be noted that there is no identifiable polyadenylation signal within 900 bases of the 3′ end of the major transcript (18; Y.-F. Zhang and E. Wagner, unpublished data). Although the smaller and poly(A)$^+$ LAT species may be products of splicing, their low abundance has made full characterization impossible at this time. In preliminary attempts to identify an LAT-encoded translation product, however, we have synthesized a 14-amino-acid peptide on the basis of a predicted antigenic sequence within the second HSV-1 LAT ORF, and antiserum was produced in rabbits. Although the antibodies were highly reactive against the peptide in enzyme-linked immunosorbent assays (titer, >1:10,000), we have not yet detected antigens in immunohistochemical tests performed on acutely or latently infected murine spinal ganglia (J. Stevens, M. Cook, and V. Dissette, unpublished data).

In conclusion, whatever function the HSV-1 LAT performs in latent infection, it is clear that it is abundantly expressed in neurons. We have recently found that this transcript is expressed only at low levels in infected cultured cells (Wagner et al., in preparation). A definition of those elements of the LAT promoter-control region which mediate cellular specificity will be of great interest. In addition to these studies, we are currently investigating the expression of this transcript under various conditions of infection and, perhaps most importantly, studying its function in the latent state.

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