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Relationship between Polyadenylated and Nonpolyadenylated Herpes Simplex Virus Type 1 Latency-Associated Transcripts

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RNA from the region of the genome encoding herpes simplex virus type 1 latency-associated transcripts (LATs) expressed during lytic infection yields low abundances of both polyadenylated and nonpolyadenylated forms. As has been previously shown for latent infection (A. T. Dobson, F. Sedarati, G. Devi-Rao, W. M. Flanagan, M. J. Farrell, J. G. Stevens, E. K. Wagner, and L. T. Feldman. J. Virol. 63:3844–3851, 1989), all lytic-phase expression of such transcripts requires promoter elements situated approximately 600 bases 5′ of the previously mapped 5′ end of the poly(A)$^-$ forms of LAT. Transient expression experiments revealed no other clear promoter elements within this region, and relatively small amounts of latent-phase transcripts initiating at the same site as observed for lytic-phase LAT could be detected by RNase protection assays. In the lytic phase of infection, the most abundant forms of polyadenylated LAT extended 1,600 bases from the initiation site near the LAT promoter to a potential splice donor site. Poly(A)$^-$ LAT species were not recovered in significant amounts from lytically infected neuroblastoma cells, but such RNA from lytically infected rabbit skin cells comapped with poly(A)$^-$ LAT from latently infected sensory neurons. Both map between canonical 5′ splice donor and 3′ splice acceptor sites 1,950 bases apart. Poly(A)$^-$ LAT cochromatographed with uncapped rRNA on *m*-aminophenyl boronate agarose under conditions in which capped mRNA was bound. All of these data confirm the previously presented scheme for the expression of poly(A)$^-$ LAT as a stable intron derived from the splicing of a large primary transcript; however, we were unable to detect the spliced polyadenylated product of this splicing reaction.

During the latent phase of infection of sensory nerve ganglia, only one region of the herpes simplex virus type 1 (HSV-1) genome is expressed as RNA (see reference 38 for a review). Expression of latency-associated transcripts (LATs) is not important for the establishment or maintenance of the latent phase of infection in laboratory animals (19, 20, 31, 36), but we have recently reported that LAT expression is strongly correlated with efficient induced HSV reactivation in the rabbit eye model (18). Other laboratories have reported that LAT$^-$ viruses are not efficiently recovered from latently infected murine trigeminal ganglia in an in vitro virus recovery assay (23, 36); however, we have not seen any differences in kinetics or efficiency of virus recovery with use of latently infected dorsal root ganglia (31).

To further characterize the biological role and mode of action of latent-phase transcription, a full characterization of LAT expression is essential; this has been a continuing effort by both us and other groups (7–11, 16, 21, 29, 34, 35, 37, 39–43). Using RNA transfer (Northern) blot and nuclease protection analysis, LATs can be detected in latently infected ganglia as two partially colinear abundant poly(A)$^-$ species approximately 2,000 and 1,250 bases in length which are related by splicing and are antiparallel to and overlap the 3′ end of the mRNA encoding the regulatory protein ICP0. In situ hybridization has shown that these poly(A)$^-$ transcripts are nuclear. Less abundant colinear poly(A)$^+$ transcripts have also been detected; these minor transcripts extend from 650 bases upstream to at least 5 kb downstream of the abundant LATs (11, 39–41).

These properties suggest a situation in which LATs are expressed from a single long primary transcription unit and the abundant poly(A)$^-$ species are derived by processing of this precursor. Consistent with this model, the 5′ end of the abundant, poly(A)$^-$ LAT from latently infected neurons maps at a canonical splice signal (40), and it has been recently shown that latent-phase transcription is controlled by recognizable promoter elements within two *Pst*I sites 880 and 600 bp 5′ of the abundant LATs (11). During lytic infection a ca. 9-kb poly(A)$^+$ transcript has been detected which extends from this putative promoter to a site near a canonical polyadenylation signal near the 3′ end of the transcription unit encoding ICP4. Finally, smaller poly(A)$^+$-containing RNA species colinear with the large transcript were also detected during lytic infection, suggesting that some processed forms of LAT are expressed as cytoplasmic forms.

Despite their consistency, these observations cannot formally rule out the possibility that some or all poly(A)$^-$ LATs as well as some of the poly(A)$^+$ species are expressed under the control of additional downstream promoter sequences which require some DNA elements within the *Pst*I sites for activity during latent infections in neurons. Further, the exact relationship of the poly(A)$^-$ LAT species to the poly(A)$^+$ species observed in lytic infection has not been defined. To further investigate such problems, we have continued our analysis and comparison of LATs expressed in both latent and lytic phases of infection by HSV-1. Here

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we show that the DNA sequences controlling latent-phase LAT expression are also required for expression during lytic infection and that transcripts initiating at the cap site for the primary poly(A)$^+$ transcript seen in lytic infection can be isolated from latently infected neurons. We have used RNase protection assays to quantitate LAT expression under different conditions of infection and have precisely located the 3′ end of the poly(A)$^-$ species to a canonical splice acceptor site 1,950 bases 3′ of its 5′ end. Such assays have also been used to identify a major species of poly(A)$^+$ LAT which only partially comaps with the abundant poly(A)$^-$ species. Finally, we find that a significant proportion of LAT cochromatographs with (uncapped) rRNA on an *m*-aminophenyl boronate agarose column which fractionates capped and uncapped RNA (44). All of these data confirm our model that all latent-phase transcription is via a single highly processed transcript which is spliced to yield the abundant poly(A)$^-$ species—the LAT intron or introns.

MATERIALS AND METHODS

Virus and sources of RNA. Methods for latent infection of 6-week-old outbred Swiss-Webster mice (Simonson Laboratories, Gilroy, Calif.), isolation of dorsal root ganglia, virus infection of cultured rabbit skin cells, and isolation of RNA by use of guanidinium isothiocyanate-hot phenol have been described (40, 41). Rabbit skin and murine neuroblastoma cells were infected at a multiplicity of infection of 10 PFU per cell, and infected cell RNA was generally isolated at 5 to 6 h after infection. RNA from infected cells and latently infected ganglia was fractionated into poly(A)$^+$ and poly(A)$^-$ fractions by use of oligo(dT)-cellulose (Collaborative Research, Inc., Waltham, Mass.) chromatography (40). The 17*syn*$^+$ strain of HSV-1 was used as wild-type (wt) virus, and all genomic positions for restriction and other sites are referred to the corresponding positions in the published sequence (reviewed in reference 25). Three recombinant viruses were also used. (i) Δ*Pst* [derived from the KOS(M) strain of HSV-1] contains a deletion of LAT promoter elements between *Pst*I sites located at sites corresponding to bases 118,559 and 118,862 of the IR_L and in the equivalent position in the TR_L of the 17*syn*$^+$ strain; this virus expresses no detectable LAT in latent-phase infection of murine sensory nerve ganglia (11). (ii) Virus 8117, has a deletion of DNA between *Bst*EII sites at bases 119,194 and 120,091, retains the LAT promoter but lacks the sequences upstream of and including the 5′ end of the stable poly(A)$^-$ LAT intron. It does not express stable poly(A)$^-$ LAT during latent infection (19). (iii) Virus RHA6 (derived from a 17*syn*$^+$ parent) contains a 442-bp simian virus 40 DNA fragment containing both polyadenylation sites (14, 33) substituted for the HSV-1 DNA between two *Hpa*I sites at bases 120,299 and 120,467 of the IR_L and the equivalent positions in the TR_L. This recombinant was generated and screened, and the genomic structure was confirmed as described previously (11, 13). RHA6 virus expresses a truncated poly(A)$^+$ LAT derivative during lytic-phase infection (see below) and no stable poly(A)$^-$ LAT during latent infection of murine sensory nerve ganglia (31a).

RNA. Poly(A)$^+$ RNA from 2×10^6 infected cells (10 μg) or from 40 latently infected murine dorsal root sensory nerve ganglia was used for size fractionation and RNase protection assays. Similarly, poly(A)$^-$ RNA from 10^6 infected cells (40 μg) or from 10 to 12 ganglia was used. For Northern blot analysis, RNA was size fractionated on 1.4% agarose gels containing 6% formaldehyde; it was then transferred by capillary blotting onto nylon membranes (Biotrans, ICN, Irvine, Calif.) (11, 24, 40, 41). Blots were probed with appropriate cloned DNA fragments uniformly labeled with [α-^{32}P]dCTP (3 Ci/mol; Amersham Corp., Arlington Heights, Ill.) by random hexamer priming (11, 24). Generally, the LAT-specific probe was DNA contained within the *Sph*I site at base 119,287 and a *Kpn*I site found in the KOS(M) strain corresponding to base 119,968 of the 17*syn*$^+$ strain. The LAT/ICP0 probe covered the *Hpa*I site corresponding to base 120,467 and the *Sal*I site corresponding to base 120,902 or this *Sal*I site and the *Bam*HI site at base 123,460. An ICP0-specific synthetic oligonucleotide probe covering bases 124, 174 to 124, 193 (58 to 76 bases 3′ of the mRNA cap site) was 5′ end labeled to a specific activity of 5×10^5 ^{32}P cpm/ng, using [γ-^{32}P]ATP (7 Ci/mol; ICN) and bacteriophage T4 kinase (Bethesda Research Laboratories, Gaithersburg, Md.) by standard methods (11, 40, 41).

RNase protection assays have been described elsewhere (11, 15). The RNA probes were made by using pGEM templates with Promega Biotec (Madison, Wis.) T7 or SP6 polymerase according to the instructions supplied. Probe was labeled with 100 μCi of [^{32}P]UTP (800 Ci/mmol; Amersham) per μg of template DNA. Templates are described at appropriate points in the text. Full-length probe was fractionated on a 6% acrylamide–8 M urea sequencing gel and eluted for hybridization. A total of 10^6 cpm of purified RNA probe was hybridized with appropriate RNA samples in 50 μl of 80% formamide–40 mM piperazine-*N*,*N*′-bis(ethanesulfonic acid) (PIPES; pH 6.4)–400 mM NaCl–1 mM EDTA at 56°C for 16 h. The mixture was then digested by addition of 300 μl of RNase A (40 μg/ml; Sigma)-RNase T_1 (2 μg/ml; Sigma) in 10 mM Tris (pH 7.5)–300 mM NaCl–5 mM EDTA for 1 h at 30°C. The digestion mix was then treated with 50 μg of proteinase K (Sigma) at 37°C for 15 min and phenol-chloroform extracted. RNase-resistant material was fractionated on an 8% acrylamide–8 M urea sequencing gel, using DNA and occasionally RNA size standards. This concentration of acrylamide was found to eliminate the differences in migration rates for RNA and DNA size standards noted previously (11).

RNA was fractionated into capped and uncapped species according to the basic methods of Wilk et al. (44). A 3-ml bed volume of *m*-aminophenyl boronate agarose (Amicon Corp., Danvers, Mass.) was washed with 20 bed volumes of H_2O and then with 10 bed volumes of 0.1 M sodium acetate. The column was equilibrated with 7 to 10 bed volumes of binding buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 8.5], 1 M NaCl, 100 mM $MgCl_2$). RNA (500 μg) was resuspended in 0.5 ml of H_2O and brought to 1× binding buffer in a final volume of 1 ml. Each sample was reloaded 10 times through the column and then rinsed with 7 to 10 bed volumes of binding buffer. RNA was eluted with a 10-ml linear 1 to 0.2 M NaCl gradient in 50 mM sodium acetate, and 0.5 ml fractions were collected. Yeast tRNA was added as a carrier (20 μg), and samples were ethanol precipitated overnight.

Transient expression assays. Transient expression vectors containing β-galactosidase as a reporter enzyme were constructed by using appropriate portions of the LAT promoter and sequences 3′ of this region ligated into the pBR322-based PSVOdLacZ vector described previously (3, 14). For LAT promoter derivatives (pSVOdLacZ-LAT), we replaced the 40-base *Sal*I-*Hin*dIII polylinker of pSVOdLacZ with a synthetic adaptor (GTCGACCGGCCGTGCCGATAAGC

TT) that contains a *Sal*I site, bases 118,791 through 118,804 of HSV-1 DNA, and a *Hin*dIII site. Thus, it inserts the HSV-1 *Eag*I site (−11 from the LAT cap) and HSV-1 sequence to +3 of the LAT cap. Then the 352-base DNA fragment spanning the *Eag*I site at nucleotides 118,439 to 118,791 was inserted and screened for proper orientation. For transient expression vectors incorporating the 640-odd bases to the 5′ end of the poly(A)$^-$ LAT species, we converted the *Pss*I site at base 119,499 [37 bases 3′ of the 5′ end of poly(A)$^-$ LAT] to a *Hin*dIII site and cloned either the *Pst*I-*Hin*dIII fragment spanning bases 118,863 to 119,499 into pSVOdLacZ or the *Eco*RV-*Hin*dIII fragment spanning bases 118,638 to 119,499 into the pSVOdLacZ-LAT vector. This resulted in incorporation of the entire LAT promoter and sequences upstream of the poly(A)$^-$ LAT.

For transient assays, rabbit skin cells were seeded at 6×10^5 cells per 60-mm plate and then grown for 18 h to 80% confluence (1×10^6 to 1.4×10^6 cells). At 4 h prior to transfection, the culture medium was replaced with new culture medium. Cultures were then transfected with 10 μg of form I plasmid DNA. Transfection was by $CaPO_4$ precipitation as described previously (3, 14, 32). At 5 h following transfection, the cells were subjected to shock with 15% glycerol and washed with saline and 3 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Fresh medium was then added, and after 24 h cells were infected with HSV-1 at a multiplicity of 1 to 2 PFU per cell and overlaid with medium containing 50 μg of thymidine arabinoside. Uninfected cultures were also treated with fresh medium at 24 h following transfection. After another 18 h cells were harvested, extracts were prepared (17), and protein concentration in the extracts was determined by using a commercial (Bio-Rad) protein assay kit with bovine serum albumin as a standard. β-Galactosidase activity was assayed as described previously (14, 32). Specific activity of β-galactosidase was expressed as units of enzyme activity per milligram of protein, where 1 U of β-galactosidase cleaves 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per min at 30°C and 1 nmol of the product (*o*-nitrophenol) per ml has an A_{414} of 0.0045. All data were normalized to the amount of reporter plasmid recovered from the transfected cultures as determined by quantitative Southern blot hybridization. This final normalization was extremely important, since we routinely observed that murine neuroblastoma cells were as much as 10-fold more efficient in incorporating transfecting plasmids containing the LAT promoter region than were rabbit skin cells.

RESULTS

The latent-phase promoter is required for LAT expression during the lytic phase of infection. The promoter located within the two *Pst*I sites (nucleotides 118,659 and 118,863) has been shown to be necessary for the latent-phase expression of all detectable LAT. But, as pointed out in the introduction, while this result is consistent with the existence of a single LAT promoter, we could not formally exclude an independent promoter for the abundant poly(A)$^-$ LAT which requires elements within this upstream DNA sequence for its activity. We excluded this possibility in three separate ways: (i) analysis of LAT expression in recombinant viruses containing defined modifications of the LAT transcription unit; (ii) transient expression analysis of putative LAT promoter sequences; and (iii) comparative mapping of the initiation of transcription of lytic and latent-phase LAT RNA species.

(i) LAT expression in recombinant viruses. We compared lytic-phase LAT expression by wt virus and a LAT promoter deletion recombinant virus (Δ*Pst*). In addition to the major 2-kb poly(A)$^-$ species, lytic infection with wt virus results in expression of LAT poly(A)$^+$ RNA with sizes ranging from 2 to ca. 9 kb hybridizing to the LAT probe (Fig. 1A, tracks i and ii; 11). The RNA species seen in the poly(A)$^+$ fraction were not significantly reduced by a second round of oligo(dT)-cellulose chromatography (track iii). These results are in contrast to those seen when RNA was isolated from latently infected mouse sensory nerve ganglia, in which case the major species is the 2 kb poly(A)$^-$ RNA and no poly(A)$^+$ RNA was detectable (tracks iv and v). No RNA the size of the stable poly(A)$^-$ or poly(A)$^+$ LAT was expressed during lytic infection with the Δ*Pst* recombinant, and although some diffuse radioactivity migrating with a size greater than 2 kb was observed in some blots (Fig. 1B, tracks i to iv). Controls using an ICP0-specific probe indicated that normal amounts of ICP0 were expressed by the mutant virus compared with wt (compare Fig. 1A, track iiic, with Fig. 1B, tracks ic and iic).

Infection with recombinant RHA6, which contains the simian virus 40 polyadenylation sequences inserted between the two *Hpa*I sites at nucleotides 120,299 and 120,467, resulted in the expression of a single poly(A)$^+$ LAT derivative migrating with a size of ca. 2 kb (Fig. 1B, track v). This RNA did not extend beyond the *Hpa*I site at nucleotide 120,299, since a probe spanning this *Hpa*I site and the *Sal*I site at nucleotide 120,902 hybridized only to ICP0 mRNA (Fig. 1B, track vii). No poly(A)$^-$ LAT derivatives in the size range of 1,000 to 1,200 bases were expressed by this virus; such RNA would be expected if the poly(A)$^-$ LAT species had an independent transcription initiation site at its 5′ end.

(ii) Transient expression assays. As described in Materials and Methods, we placed the β-galactosidase gene under control of portions of the LAT promoter and DNA sequences 5′ of the poly(A)$^-$ LAT species in transient expression vectors in order to assess potential promoter activity. Typical data are shown in Fig. 2; parallel experiments were carried out with a β-galactosidase reporter construct containing the HSV dUTPase (U_L50) promoter. This latter control construct expressed 8 ± 3 U of β-galactosidase per mg in uninfected cells and 380 ± 80 U/mg in infected rabbit skin cells. The construct containing 360 bases upstream of the LAT cap (bases 118,439 to 118,805) expressed somewhat more β-galactosidase activity in uninfected rabbit skin cells (10 ± 5 U/mg); however, levels of indicator enzyme activity increased only approximately 3-fold in infected cells (35 ± 10 U/mg), compared with a better than 50-fold increase with dUTPase promoter. This result is consistent with those reported by others (45).

Several constructs containing small deletions within the LAT promoter region showed slightly higher activities in uninfected rabbit skin cells and in cells after HSV infection. We also carried out transient expression assays in uninfected A2 murine neuroblastoma cells to gauge the possibility that the primary and any secondary LAT promoters might evidence some neuronal specificity. Constructs containing DNA spanning bases 118,439 to 119,499 [40 bases 3′ of the 5′ end of the poly(A)$^-$ LAT species] or containing DNA between bases 118,863 and 119,499 induced no appreciable β-galactosidase activity. The control dUTPase promoter construct had very low activity in these cells, while normal-

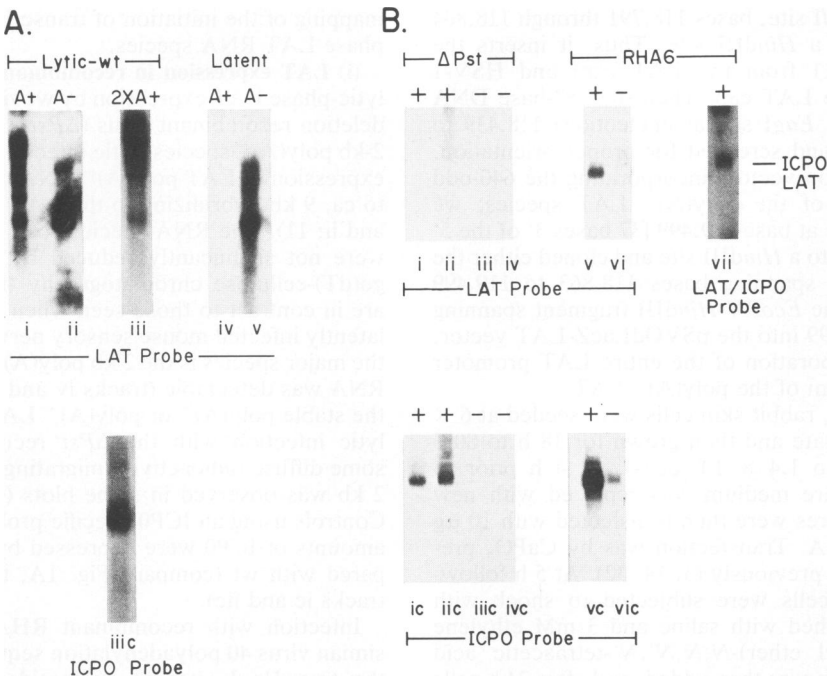


FIG. 1. Northern blot analysis of LAT expressed following lytic infection by wt virus and by Δ*Pst* and RHA6 recombinants of HSV-1. RNA was isolated from lytically infected rabbit skin cells or latently infected murine sensory nerve ganglia, fractionated into poly(A)$^+$ and poly(A)$^-$ fractions, size fractionated, blotted, and then hybridized with a LAT-specific probe spanning bases 119,287 to 119,968. (A) Tracks i, ii, and iii represent RNA isolated from cells lytically infected with wt HSV-1; track iii contains RNA refractionated through a second oligo(dT)-cellulose column to confirm its poly(A)$^+$ character. Tracks iv and v contain RNA from neurons latently infected with wt virus. The blots of track iii was then stripped and rehybridized with an oligonucleotide probe specific for ICP0 (track iiic). (B) Tracks i and ii contain 10 and 20 μg, respectively, of poly(A)$^+$ RNA from cells lytically infected with the Δ*Pst* recombinant, and tracks iii and iv contain 40 and 80 μg, respectively, of poly(A)$^-$ RNA from the same cells. Tracks v and vii contain 10 μg of poly(A)$^+$ RNA from RHA6-infected cells, while track vi contains 40 μg of poly(A)$^-$ RNA from such cells. The LAT/ICP0 probe used here was the *Hpa*I-*Sal*I fragment spanning bases 120,466 to 120,902. Blots were stripped and hybridized with ICP0-specific probe (tracks ic to vic).

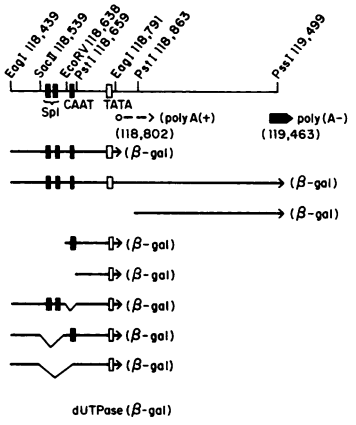
ized values for LAT promoter-controlled reporter enzyme activity were equivalent to those seen in uninfected rabbit skin cells. In contrast to the situation with rabbit skin cells, expression of this promoter in neuroblastoma cells required sequences between the *Pst*I site at base 118,659 and the *Sac*II site at base 118,539, since deletions within this region significantly lowered activity in these cells while increased activity was observed in transfected rabbit skin cells. Despite the differences between transient expression in the two cell types, no significant promoter activity was found by using DNA sequences immediately upstream of the 5′ end of the poly(A)$^-$ LAT. These results again indicate that no secondary promoter exists 5′ of the poly(A)$^-$ LAT species.

(iii) 5′ end mapping of latent- and lytic-phase LATs. We used RNase protection analysis to compare the 5′ ends of the putative primary LAT species expressed during lytic and latent phases of infection. A ca. 63-base RNA fragment corresponding to the 5′ end of the primary LAT transcript was protected when poly(A)$^+$ but not poly(A)$^-$ RNA from lytically infected cells was hybridized with a complementary RNA probe spanning 72 bp of DNA between the *Eag*I site at base 118,791 and the *Pst*I site at base 118,863 (Fig. 3A, tracks i to iii; 11). A protected RNA species of the same size was seen with use of poly(A)$^-$ RNA from latently infected sensory nerve ganglia (track v).

We also used poly(A)$^+$ and total RNA isolated from cells lytically infected with the 8117 virus to investigate the role of any sequences between the LAT promoter and the 5′ region of the stable poly(A)$^-$ LAT species in expression of the primary transcript. A protected fragment of the same size was obtained with this RNA as with infections with wt virus (Fig. 3B, tracks i and ii). This finding confirms our transient expression data which indicated that sequences 3′ of the LAT cap have no major role in LAT lytic-phase expression.

Effect of complementary overlap on the ratio of LAT to ICP0 expressed during lytic infection. We used RNAse protection assays to compare the steady-state level of poly(A)$^+$ LAT expressed during lytic infection with that of ICP0 mRNA. This latter transcript was detected by using a complementary RNA probe spanning an *Nco*I site at base 124,106 and a *Sph*I site at base 124,419. This DNA fragment overlaps the 5′ end and 145 bases of the ICP0 transcript. With the same amount of infected cell poly(A)$^+$ RNA hybridized to each probe (10 μg), the amount of protected radioactivity was at least 20-fold greater for ICP0 than for the LAT (Fig. 3A; compare tracks i, ii, and viii).

We used the 203-bp DNA fragment spanning the *Pst*I sites at nucleotides 118,659 and 118,863 to generate RNase protection probes complementary to LAT to confirm that the 5′ end of poly(A)$^+$ LAT expressed in lytically infected neuroblastoma cells maps to the same site on the HSV genome as observed in infected rabbit skin cells (Fig. 4, tracks i and iii). The *Pst*I probe also yields a larger additional protected RNA



	β-gal (U/mg prot/ng DNA)		
	Neuro 2A	Rabbit Skin	
	Uninfected	Uninfected	Infected
	15 ± 6	10 ± 5	35 ± 10
	5 ± 0.5	2 ± 1	3
	0.4 ± 0.2	ND	ND
	1.7 ± 0.2	12 ± 2	55
	1.6 ± 0.2	ND	ND
	9 ± 3	16 ± 5	68 ± 18
	2 ± 0.3	27 ± 7	77 ± 26
	2.5 ± 0.5	24 ± 7	66
	1.4 ± 0.2	8 ± 3	380 ± 80

FIG. 2. Transient expression of β-galactosidase under the control of the LAT promoter. The locations of the 5′ end of the primary poly(A)$^+$ and poly(A)$^-$ LATs are indicated in relation to specific promoter features. Nucleotide numbers are from McGeoch (reviewed in reference 25). Transient expression vectors were constructed; details of transfection and subsequent infection or mock infection of transfected rabbit skin or murine neuroblastoma (Neuro 2A) cells with HSV-1 are described in Materials and Methods. Specific activity of β-galactosidase (β-gal) induced in uninfected or infected cells is expressed as units of enzyme activity per milligram of protein; 1 U of β-galactosidase cleaves 1 nmol of ONPG per min at 30°C and one nmol of the product (*o*-nitrophenol) per ml has an A_{414} of 0.0045. All data were normalized to the amount of reporter plasmid recovered from the transfected cultures as determined by quantitative Southern blot hybridization. Error ranges are averages of four to five separate transfection assays; ND indicates not determined; values with no error ranges are results of single transfection assays.

fragment derived from the 3′ end of a low-abundance lytic-phase poly(A)$^+$ RNA terminating 5′ of the LAT promoter and utilizing the polyadenylation signal occurring near base 118,710 (31b).

Since the RHA6 virus expresses a truncated LAT which does not overlap the 3′ end of ICP0, we were able to directly assess the effect of the normal complementary overlap on the steady-state levels of these transcripts during lytic infection. Although the total amount of ICP0 and LAT recovered from infected cells varied somewhat between experiments, we found no significant difference between the ratio of LAT to ICP0 in RNA recovered from cells infected with RHA6 virus and that isolated from wt infections. We also observed no differences in the kinetics of accumulation of LAT in infections with these viruses (Fig. 4, tracks v to xiii). We did similar experiments using RNA from infected neuroblastoma cells, and again no differences in the levels or ratios of these two RNA species were seen (data not shown).

The proportions of poly(A)$^+$ and poly(A)$^-$ LATs expressed in lytic infection depends on the type of cell infected. The data described in the preceding sections demonstrate that LAT is a low-abundance RNA whose level of expression is not particularly sensitive to the type of cell infected or to complementary overlap with the much more abundant ICP0 mRNA. Although poly(A)$^-$ LAT is a major component of LAT expressed during lytic infection of many cultured cells, we found that it is not present in high proportions in infected murine or human neuroblastoma cells. Poly(A)$^+$ and poly(A)$^-$ LATs were readily detectable in Northern blots of RNA from infected murine 3T12 cells hybridized with a LAT-specific probe spanning the *Sph*I site at base 119,287 and the *Kpn*I site at base 119,968 (Fig. 5, tracks i and ii). In contrast, only poly(A)$^+$ LAT was seen in RNA isolated from infected murine neuroblastoma (2A) cells (tracks iii and iv); similar results were seen with infected human neuroblastoma (SKNMC) cells (data not shown). A *Sal*I-*Bam*HI probe (bases 120,903 to 123,460) hybridized to poly(A)$^-$ LAT and poly(A)$^+$ ICP0 mRNA from lytically infected rabbit skin cells (tracks v and vi) but only to poly(A)$^+$ ICP0 mRNA isolated from infected neuroblastoma cells (tracks vii and viii). Poly(A)$^+$ LAT expressed in neuroblastoma cells has the same general size distribution as that expressed in infected rabbit skin and other cells.

Mapping the contiguous portions of poly(A)$^+$ and poly(A)$^-$ LATs. We used a number of specific DNA fragments encompassing the LAT transcription unit to generate strand-specific RNA probes for RNase protection mapping of poly(A)$^+$ and poly(A)$^-$ LATs to further define the relationship between these species. Poly(A)$^+$ RNA from infected neuroblastoma cells served as a control to obviate contamination of poly(A)$^+$ RNA with the poly(A)$^-$ species, and poly(A)$^+$ RNA from cells infected with the RHA6 recombinant virus provided an unprocessed RNA control. The data (Fig. 6) clearly indicate that the 5′ portion of abundant poly(A)$^+$ LAT maps between the transcript cap site at base 118,802 and the *Hpa*I site at 120,467, while the poly(A)$^-$ LAT maps between the previously identified canonical splice donor site at base 119,463 and a nominal splice acceptor site at base 121,416. Further, our data indicate that the spliced poly(A)$^+$ product of the primary transcript which also is generated by the formation of the LAT intron is present in very much lower abundance than the major spliced poly(A)$^+$ species in lytic infection. Our interpretations of these data are as follows.

(i) Poly(A)$^+$ LAT mapping upstream of the 5′ end of the poly(A)$^-$ LAT intron can be isolated from lytically infected cells. Proceeding 3′ of the LAT cap site at base 118,802, it is seen that poly(A)$^+$ but not poly(A)$^-$ RNA from infected cells protects a large RNA fragment of a LAT-complementary RNA probe synthesized from the cloned 425-bp fragment of DNA contained within the *Pst*I site at nucleotide 118,863 and the *Sph*I site at base 119,287 (Fig. 6A, tracks ii and iii). Although full-length protected probe was difficult to recover, a protected fragment of the same size was recovered when poly(A)$^+$ RNA from RHA6-infected cells was hybridized (data not shown), and we conclude that secondary structure of the probe interferes with its full annealing with homologous RNA.

(ii) Abundant poly(A)$^+$ LAT overlaps the 5′ portion of poly(A)$^-$ LAT. Previous data located the 5′ end of poly(A)$^-$ LAT at base 119,463 within the canonical splice donor sequence AGGT (40). In agreement with this result, a ca. 150-base fragment was protected from RNase digestion when RNA from latently infected murine sensory nerve ganglia was hybridized with a LAT-complementary RNA probe synthesized from a 166-bp DNA fragment encompassing the previously located 5′ end of latent-phase poly(A)$^-$ LAT (between the *Aha*II site at base 119,447 and the *Hin*fI site at base 119,613) (Fig. 6B, track xiii). A fragment this size was also the major one protected with poly(A)$^-$ RNA from lytically infected rabbit skin cells (track iii), while essentially no protection was obtained with poly(A)$^-$ RNA from lytically infected neuroblastoma cells (track vi).

Poly(A)$^+$ RNA produced a different pattern of protected fragments. A significant amount of full-length probe was obtained with poly(A)$^+$ RNA from rabbit skin cells or murine neuroblastoma cells lytically infected with wt virus

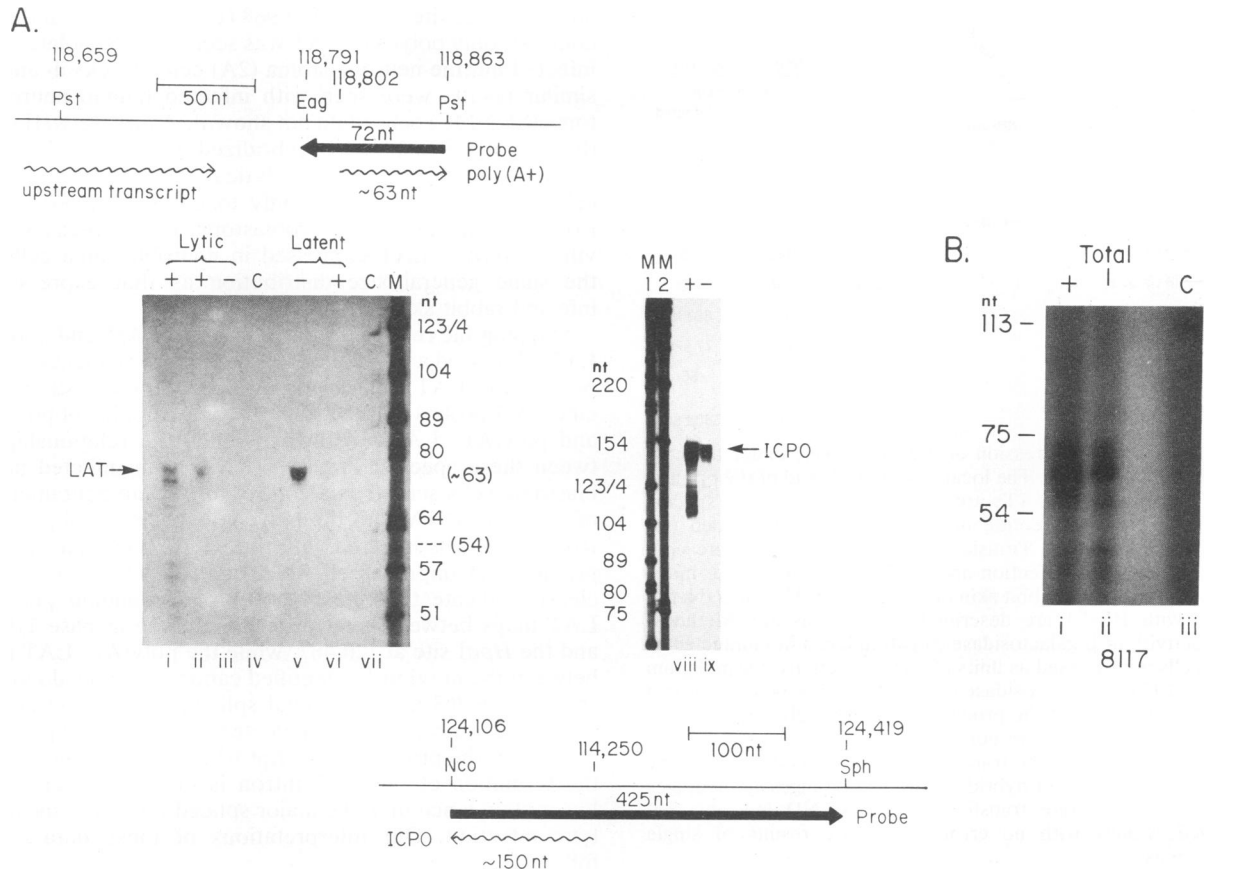


FIG. 3. Location of the 5′ end of the primary LAT transcript expressed during latent infection. A complementary RNA probe spanning 72 bp of DNA between the *Eag*I site at base 118,891 and the *Pst*I site at base 118,862 cloned in pGEM1 was linearized by digestion with *Eco*RI, and an RNA probe complementary to LAT was synthesized by using T7 RNA polymerase. (A) Infection with wt virus. A schematic representation of probes, the protected fragments produced, and the 5′ end of the primary LAT transcript is shown. A probe was hybridized with 10 μg of poly(A)$^+$ RNA (tracks i and ii) or 40 μg of poly(A)$^-$ RNA (track iii) from lytically infected rabbit skin cells. A probe was also hybridized with poly(A)$^+$ and poly(A)$^-$ RNA from 12 latently infected murine sensory nerve ganglia (tracks v and vi). Following hybridization and RNase digestion, the protected material was fractionated on a 12% denaturing acrylamide gel, using *Hin*fI-digested pBR322 DNA fragments as size markers (lane M1). The size of the protected material is ca. 63 bases as a result the anomalous migration of RNA fragments in gels of this concentration (11). Track vii contains material obtained when a probe was hybridized with 50 μg of total RNA from uninfected rabbit skin cells and then digested with RNase. Tracks viii and ix contain protected fragments following hybridization of 10 or 40 μg of the same infected rabbit skin cell poly(A)$^+$ and poly(A)$^-$ RNA used in tracks i to iii to an ICP0-complementary RNA probe spanning an *Nco*I site at base 124,106 and a *Sph*I site at base 124,419. These fragments were fractionated on an 8% gel, which minimizes differences between RNA and DNA migration rates. (B) Poly(A)$^+$ and total RNA from cells lytically infected with mutant virus 8117 hybridized with the 5′ LAT probe. nt, Nucleotides.

was hybridized (tracks ii, iv, x, and xi). Different preparations of poly(A)$^+$ RNA provided variable amounts of the smaller protected (150-base) fragment (compare tracks ii, x, and xi); we suggest that this is a result of occasional incomplete fractionation of the poly(A)$^-$ and poly(A)$^+$ species, since poly(A)$^+$ RNA from cells infected with the RHA6 recombinant virus protected only the full-length probe (track i). Neither poly(A)$^+$ nor poly(A)$^-$ RNA isolated from rabbit skin cells infected with the Δ*Pst* recombinant virus protected this probe (tracks vii and viii). This result was expected from our Northern blot data indicating that this recombinant expresses no LAT in lytic infection (cf. Fig. 1B).

(iii) Abundant poly(A)$^+$ LAT does not extend beyond the *Hpa*I site at base 120,467, while poly(A)$^-$ LAT terminates at a splice acceptor site 500 bases 3′ of the *Sal*I site at base 120,903. As expected from previously reported Northern blot analyses (11, 41), various RNA probes complementary to LAT mapping from the 5′ end of the intron to the *Hpa*I site at base 120,467 were protected from RNase digestion by hybridization with both poly(A)$^+$ and poly(A)$^-$ RNA from lytically infected cells and RNA from latently infected murine sensory ganglia (data not shown). Poly(A)$^-$ RNA isolated from rabbit skin cells lytically infected with wt virus protected the 147-base LAT-complementary probe spanning the *Hpa*I site at base 120,299 and the *Sma*I site at base 120,448, while the major protection product of poly(A)$^+$ RNA from such cells was a 116-base fragment (Fig. 6C, tracks i and ii), although a small amount of full-length protected probe can be observed in longer exposures of the autoradiographs (not shown). RNA from RHA6-infected cells provided no protection, as expected since this region is replaced with the simian virus 40 polyadenylation signals in the recombinant (tracks iv and v). RNA from latently infected murine ganglia also protected the whole fragment

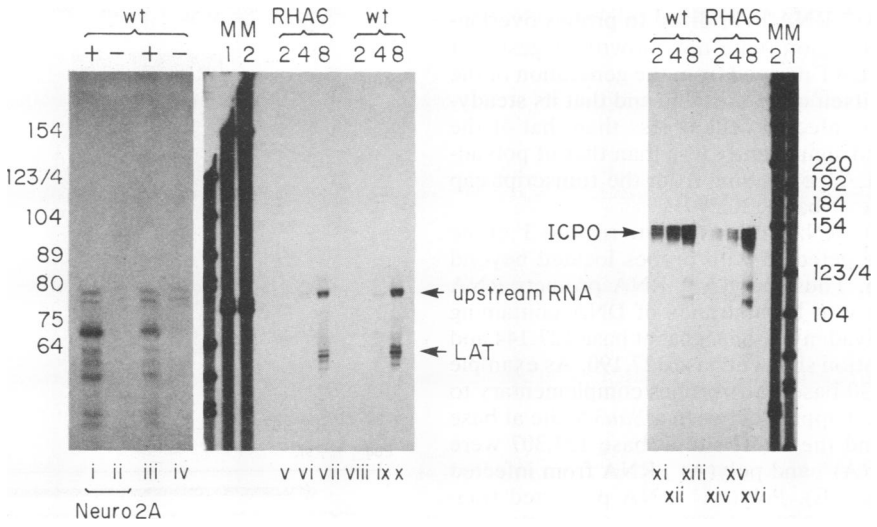


FIG. 4. Comapping of the 5′ ends of poly(A)$^+$ LATs expressed in wt infections of rabbit skin and murine neuroblastoma cells (Neuro 2A) and from RHA6-infected rabbit skin cells. Probes for RNase protection were LAT-complementary RNA transcribed from DNA between *Pst*I sites at nucleotides 118,659 and 118,862. Gels shown are 8%, and *Hin*fI-digested pBR322 DNA fragments (sizes shown in nucleotides) are included as a second size marker (lane M2). As described in the text, the larger protected fragment is generated by the presence of a low-abundance transcript mapping 5′ of LAT which is expressed in lytic but not latent infection. Also shown is a comparison of the steady-state levels of LAT and ICP0 in total RNA isolated at 2, 4, and 8 h following lytic infection of rabbit skin cells with wt or RHA6 virus; 40 μg of RNA was used with the LAT probe, and 10 μg was used with the ICP0 probe.

(track viii). Use of a probe spanning the *Nar*I site at base 120,246 and the *Sma*I site with poly(A)$^+$ RNA resulted in the protection of an RNA fragment approximately 50 bases larger (data not shown); therefore, a major portion of poly(A)$^+$ LAT expressed in lytic infection locally terminates at a site between bases 120,408 and 120,418. Although the sequence of this region has elements of a splice donor, it is not a canonical one (27).

Poly(A)$^+$ RNA from lytically infected cells provided no protected fragments other than a very small amount of full-length probe when hybridized to LAT-complementary probes located between the *Sal*I site at base 120,903 and the *Sma*I site at base 121,562 (Fig. 6D, track vii). Thus, as predicted from the Northern blot analysis shown in Fig. 3, abundant poly(A)$^+$ LAT is not transcribed from DNA between the *Sal*I site at base 120,903 and the *Bam*HI site at base 123,460. In contrast to the results with poly(A)$^+$ RNA, poly(A)$^-$ RNA from infected rabbit skin cells and poly(A)$^-$ or total RNA from latently infected murine sensory nerve ganglia protected a 190-base fragment when hybridized with a LAT-complementary RNA probe spanning bases 121,223 to 121,562 synthesized from a cloned *Sau*3A-*Sma*I DNA fragment (tracks ii, vi, and viii); this probe was not protected by poly(A)$^-$ RNA from neuroblastoma cells infected with wt virus (track iv). This result locates the 3′ end of the abundant poly(A)$^-$ LAT near base 121,416 or within the canonical splice acceptor sequence TCTCCCTCCCAGGGCA. Finally, poly(A)$^-$ RNA from lytically infected cells protected an approximately 500-base fragment of a LAT-complementary RNA probe made from cloned DNA spanning the *Sal*I site at 120,903 and the *Sma*I site at 121,562 (data not shown).

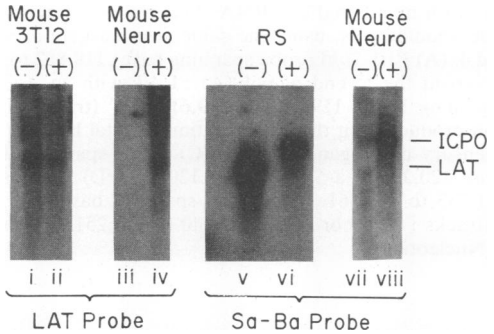


FIG. 5. Expression of only a small amount of poly(A)$^-$ LAT in lytically infected murine neuroblastoma cells. Samples of poly(A)$^+$ and poly(A)$^-$ RNAs from lytically infected murine 3T12 or neuroblastoma (Neuro) cells were fractionated, blotted, and hybridized with a LAT-specific probe or a probe spanning the *Sal*I site (base 120,902) and the *Bam*HI site (base 123,459) which hybridizes both to poly(A)$^-$ LAT and to ICP0 mRNA (Sa-Ba probe).

Poly(A)$^+$ RNA hybridizes to LAT-complementary probes 3′ of the LAT intron. Many preparations of poly(A)$^+$ RNA from lytically infected cells protected small amounts of LAT-complementary probes 3′ of the local terminus of the relatively abundant poly(A)$^+$ RNA species (Fig. 6D, track vii); we suggest that this is due to the presence of the ca. 9-kb poly(A)$^+$ LAT species observable in Northern blots (Fig. 1A, tracks i and iii). Splicing of the poly(A)$^+$ LAT primary transcript should produce a poly(A)$^+$ LAT species containing RNA homologous to the region between the splice acceptor at base 121,416 and the *Sma*I site at base 121,562. However, despite our ability to locate the 3′ end of the poly(A)$^-$ LAT intron in RNAse protection assays and the slight protection of the full-length probe by poly(A)$^+$ RNA, the *Sau*3A-*Sma*I probe never yielded the expected 140-base fragment or any smaller fragments when hybridized to poly(A)$^+$ RNA from lytically infected cells. This and the fact that no appropriate-size RNase-protected fragments were

obtained with poly(A)$^+$ RNA hybridized to probes overlapping the splice donor region (data not shown) suggest that the spliced poly(A)$^+$ LAT derived from the generation of the stable LAT intron is itself quite unstable and that its steady-state level in lytically infected cells is less than that of the primary transcript and significantly less than that of polyadenylated species of LAT extending from the transcript cap through the *Hpa*I site at base 120,299.

LAT sense poly(A)$^+$ RNA homologous to regions 3′ of the LAT intron could be detected with probes located beyond the IR_L-IR_S junction. Thus, poly(A)$^+$ RNA protects RNA probes complementary to both strands of DNA containing the putative LAT polyadenylation signal at base 127,144 and the ICP4 polyadenylation signal at base 127,190. As example of such protection, 530-base RNA probes complementary to both strands of DNA mapping between a *Sau*3A site at base 126,776 in the IR_S and the *Sac*II site at base 127,307 were hybridized with poly(A)$^+$ and poly(A)$^-$ RNA from infected rabbit skin cells (Fig. 6E). Poly(A)$^+$ RNA protected fragments migrating between 150 and 400 bases in length when hybridized to a LAT-complementary RNA probe (track i); this same RNA hybridized with the ICP4-complementary RNA probe provided protected fragments migrating with a size of approximately 130 to 150 bases (track iv). Poly(A)$^-$ RNA provided relatively little protection to either probe (tracks ii and v). Such results place the 3′ end of ICP4 mRNA essentially 30 bases 3′ of the ICP4 polyadenylation signal. Similar precision was not possible with the LAT-complementary RNA probe; however, the data are consistent with poly(A)$^+$ RNA extending 3′ of the putative LAT polyadenylation signal at base 127,144. Further, approximately 100 bases of a LAT-complementary probe spanning *Taq*I sites at bases 127,140 and 127,252 was also weakly protected with poly(A)$^+$ RNA (track viii).

Poly(A)$^-$ LAT is uncapped. In a final set of experiments to characterize poly(A)$^-$ LAT, we examined its behavior upon chromatography using *m*-aminophenyl boronate agarose, which has been shown to selectively bind capped RNA species under conditions of moderate ionic strength (44). Our basic procedure was to bind total RNA from lytically infected cells to such columns in 1 M salt, followed by gradient elution with 1.0 to 0.2 M salt. Fractions were collected and subjected to denaturing gel electrophoresis, and rRNA was visualized by staining (Fig. 7A). Although selective binding was not absolute, the experiments demonstrated that the bulk of the 5.2- and 2-kb rRNA was eluted in the 0.8 to 0.7 M salt range, while the bulk of diffusely migrating poly(A)$^+$ RNA was eluted in fractions containing 0.4 to 0.2 M salt.

To determine the behavior of the 2-kb LAT species, infected cell RNA was fractionated on these columns and subjected to Northern blot analysis. Hybridization of these blots with a probe specific for LAT RNA indicated that the bulk of this material eluted in the unbound fraction along with rRNA (Fig. 7B). In contrast, an ICP0 probe detected significant 2.8-kb ICP0 mRNA in the bound fraction.

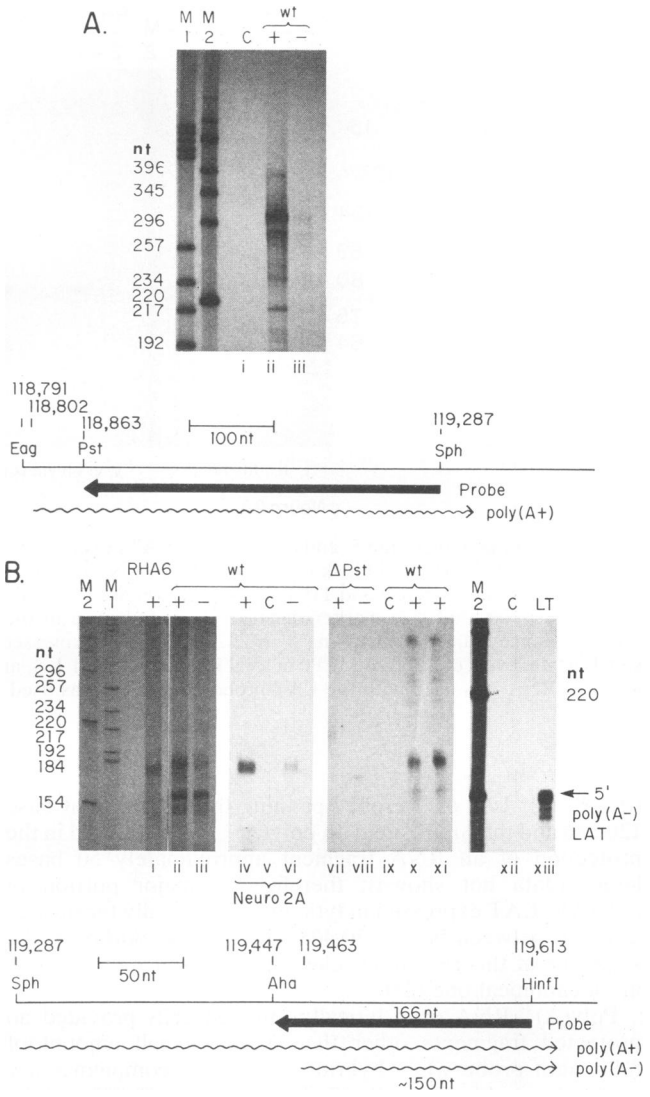


FIG. 6. RNase mapping of poly(A)$^+$ and poly(A)$^-$ LATs. RNase protection assays were carried out as described in the text and in the legend to Fig. 3. Schematic locations of RNA probes and the major protected species are indicated in each panel. Types and sources of RNA are indicated on the appropriate tracks. Lanes C, Control digestions with uninfected cell RNA. Material was fractionated on 8% polyacrylamide gels, using the same size markers as shown in Fig. 3 and 4. (A) *Pst*I-*Sph*I probe spanning bases 118,862 to 119,286. (B) Analysis at the 5′ end of poly(A)$^-$ LAT with an *Aha*II-*Hin*fI probe spanning bases 119,446 to 119,612. LT (track xiii) is the protection product from the hybridization of total latently infected murine sensory nerve ganglia RNA. (C) Probe spanning the *Hpa*I site at base 120,298 to a *Sma*I site at 120,561. (D) Probe spanning bases 121,233 to 121,561. (E) Probes spanning bases 126,775 and 127,307 (tracks i to vi) or bases 127,140 to 127,251 (tracks vii and viii). nt, Nucleotides.

DISCUSSION

A summary of lytic-phase transcription in the long repeat region of HSV-1 is shown in Fig. 8. Our data clearly show that all LAT expression in lytic as well as latent infection is a function of transcription of a primary poly(A)-containing transcription unit initiating at the previously identified upstream promoter. No other transcriptional control elements having a significant role in the lytic-phase expression of this primary transcript are evident from the experiments reported here. Thus, the LAT promoter is active in transient expression assays, while sequences downstream of the primary transcript cap site are not (Fig. 2). Further, although deletion of this promoter results in a virus (Δ*Pst*) that expresses no poly(A)$^+$ or poly(A)$^-$ LAT in lytic-phase

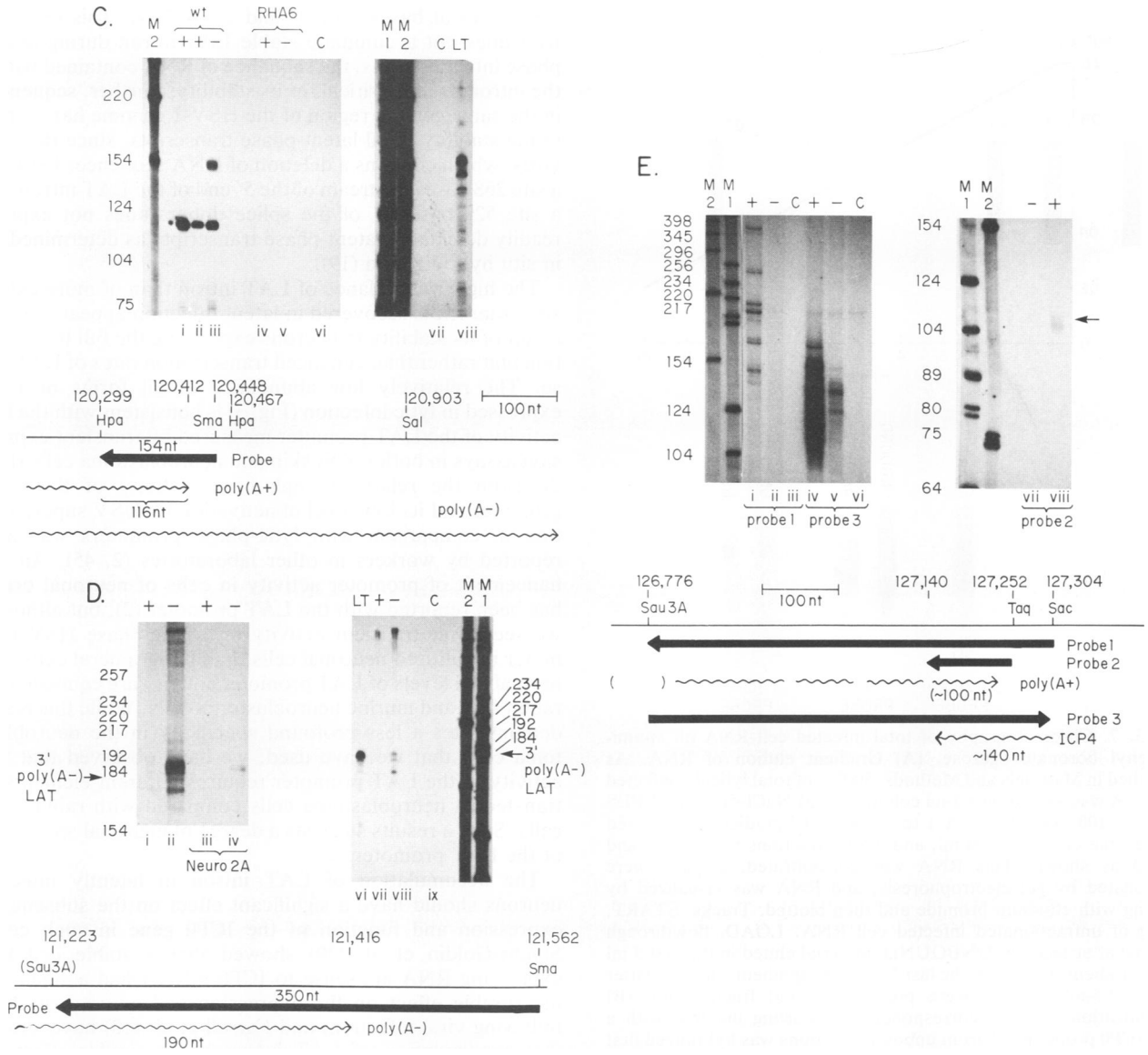


FIG. 6—*Continued.*

infection (a result consistent with the earlier finding that this promoter is required for latent phase transcription), a virus with a deletion of sequences downstream of the transcript start site (8117) still expresses the primary transcript, as evidenced by the RNase protection results shown in Fig. 3B. In the lytic phase of infection, a number of partially colinear transcripts are derived from the primary latent-phase transcription unit; the most readily detectable ones are indicated in Fig. 8. The locations of the 5′ and 3′ ends of the abundant poly(A)$^-$ LAT (Fig. 6B and D) and the fact that it is uncapped (Fig. 7) demonstrate that it is a stable intron processed from the primary transcript. These results are in complete agreement with recent experiments of Farrell and colleagues (12), who have shown that expression of recombinant transcripts containing the 5′ and 3′ LAT splice sites yields an LAT intron identical to that isolated from latent or lytic infection.

Although spliced poly(A)$^+$ LAT species can be readily detected in lytic-phase infection by using RNase protection assays, the spliced species resulting from generation of the LAT intron is not present at high enough levels to be detected. Regardless of the low abundance of this and other individual species, the expression of processed polyadenylated LATs is fully consistent with a situation in which the expression of one or more latent-phase HSV proteins mediates the reactivation-positive phenotype associated with LAT expression. A full characterization of cDNA generated from polyadenylated LAT species will be necessary to reliably identify potential translational reading frames for such a protein or proteins, since (to date, at least) comparative analysis of other neurotropic herpesvirus latent-phase transcription has not provided any meaningful illumination concerning possible latency proteins. Thus, two other alphaherpesviruses, bovine herpesvirus and pseudorabies virus, express latent-phase RNA which could encode reasonable-size proteins, but despite their being highly basic, little

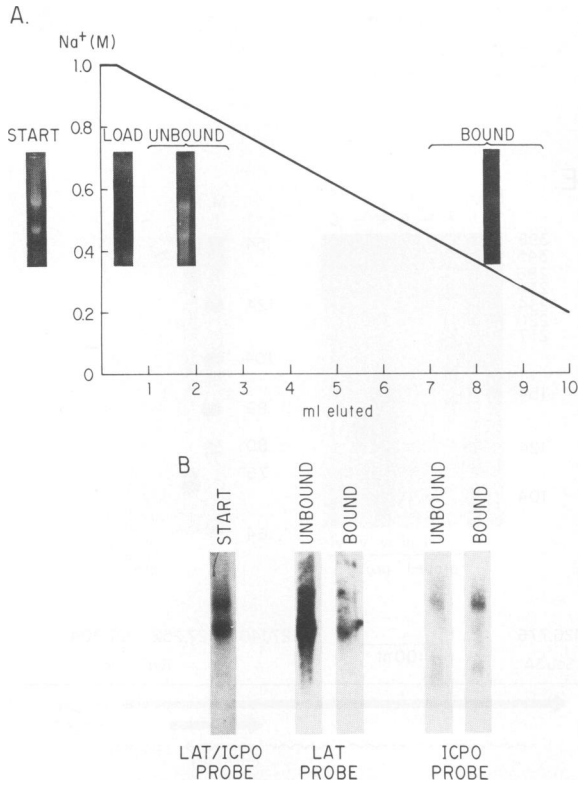


FIG. 7. Chromatography of total infected cell RNA on *m*-aminophenyl boronate agarose. (A) Gradient elution of RNA. As described in Materials and Methods, 500 μg of total lytically infected cell RNA was loaded on a 3-ml column in 1 M NaCl–50 mM HEPES (pH 8.5)–100 mM $MgCl_2$. A 1 to 0.2 M NaCl gradient was passed through the column in 10 ml, and 0.5-ml fractions were taken and pooled as shown. This RNA was concentrated, aliquots were fractionated by gel electrophoresis, and RNA was visualized by staining with ethidium bromide and then blotted. Tracks: START, 50 μg of unfractionated infected cell RNA; LOAD, flowthrough material after loading; UNBOUND, material eluted in the first 3 ml of the gradient; BOUND, the last 3 ml of the gradient. In these latter tracks, 0.5-ml aliquots were precipitated and fractionated. (B) Hybridization of RNA corresponding to starting material with a LAT/ICP0 probe. RNA from unbound fractions was hybridized first with a LAT probe. Following this, the blot was stripped and rehybridized with an ICP0 probe.

homology is seen with the largest potential translational products expressed by HSV-1 (22, 28, 28a). Further, the 5′ regions of the HSV-1 and HSV-2 LAT transcription units lack any striking homology in DNA sequence between the promoter region and the 3′ end of the ICP0 translational reading frame (1a, 26).

The stability of the LAT intron derived from the primary latent-phase transcription product is clearly unusual. Even more unusual is the fact that this intron itself can be further processed during latent-phase infection (41), although the precise mechanism of this processing is unclear. Interestingly, accumulation of readily detectable levels of the LAT intron is not a priori a requirement of LAT expression in the lytic phase, since it is not abundant in at least some lytically infected neuroblastoma cells. Those features of the LAT intron responsible for its stability are not yet determined, but Block et al. have recently described a recombinant virus with an insertion of bacteriophage lambda DNA within the *Hpa*I sites at bases 120,299 and 120,467 (4). This recombinant does not accumulate stable LAT intron during latent-phase infection; thus, the sequence of RNA contained within the intron itself is critical for its stability. Further, sequences in the same general region of the HSV-1 genome have a role in the stability of all latent-phase transcripts, since the 8117 virus, which contains a deletion of DNA sequences between a site 268 bases upstream of the 5′ end of the LAT intron and a site 529 bases 3′ of the splice donor, does not express readily detectable latent-phase transcripts as determined by in situ hybridization (19).

The higher abundance of LAT intron than of more extensive transcripts recovered in latent infection appears to be a result of its stability in neurons expressing the full transcription unit rather than enhanced transcription rates of LAT per se. The relatively low abundance of all forms of LAT expressed in lytic infection (Fig. 3) is consistent with the low activity of the LAT promoter measured by transient expression assays in both rabbit skin and neuroblastoma cells (Fig. 2). Both the relatively high basal activity of the LAT promoter and its low level of activation by HSV superinfection in comparison with lytic-phase promoters was also reported by workers in other laboratories (2, 45). An enhancement of promoter activity in cells of neuronal origin has been reported with the LAT promoter (2), but although we see lower transient activity of a lytic-phase HSV promoter in cultured neuronal cells than in peripheral cells, the normalized levels of LAT promoter activity are equivalent in rabbit skin and murine neuroblastoma cells. While this result demonstrates a less profound specificity in the neuroblastoma cells that we have used, we have observed that full activity of the LAT promoter requires different elements in transfected neuroblastoma cells compared with rabbit skin cells. Such a results suggests a degree of neuronal specificity of the LAT promoter.

The accumulation of LAT intron in latently infected neurons should have a significant effect on the subsequent expression and function of the ICP0 gene in such cells. Sandri-Goldin et al. (30) showed that a stable cell line expressing RNA antisense to ICP0 mRNA had a small but measurable effect on the expression and activity of ICP0 following viral infection, and Farrell et al. (12) have shown that accumulation of LAT intron has a similar effect on ICP0-mediated transcriptional activation in transfected cells. Our present data with truncated LAT expressed by the RHA6 recombinant, however, show that antisense suppression of either LAT or ICP0 mRNA levels as a result of their complementary overlap cannot be observed during the normal course of lytic infection (Fig. 4). Thus, high levels of LAT intron such as accumulated in latently infected cells would appear to be essential for any antisense modulation of ICP0 activity in latently infected neurons.

In summary and as shown in Fig. 8, the data presented in this report indicate that processed polyadenylated variants of the primary LAT are of equivalent abundance to the LAT intron during lytic infection. A poly(A)$^+$ derivative of LAT expressed either during latency or during the initial phases of reactivation may have a role in reactivation as important as or more important than the presence of the stable LAT intron itself. The generation and complete characterization of appropriate modifications of all portions of the latent-phase transcription unit is the most direct approach to identification of those portions of the LAT which have biological function. Such experiments are currently in progress.

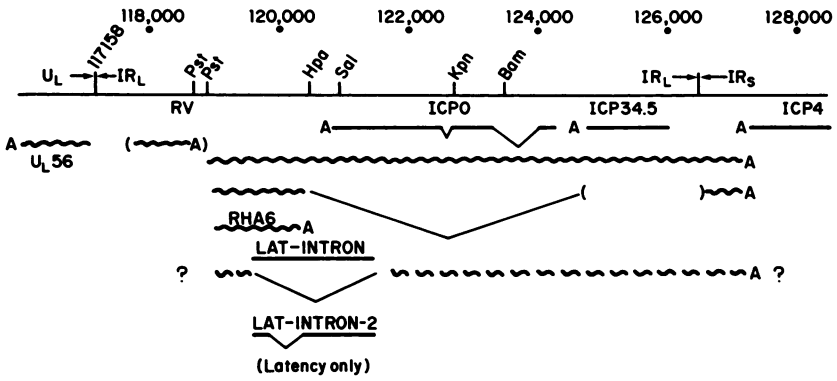


FIG. 8. Schematic representation of transcription within the IR_L and joint region during lytic infection by HSV-1. Selected restriction sites are shown, and the numbers at the top represent base numbers in the total DNA sequence (25). The position of the minor transcript 5′ of the LAT promoter is approximate and is based on unpublished data (31b). The location of the transcript encoding ICP34.5 is based on data of Chou et al. (1, 5, 6). The accumulation of a second (spliced) LAT intron is seen only in latent infection (41); as discussed in the text, the presence of spliced poly(A)$^+$ RNA derived by the generation of the LAT intron is below the limits of detection of the methods described here. The transcript indicated RHA6 is the truncated transcript generated by the recombinant virus containing a polyadenylation signal within the *Hpa*I sites at nucleotides 120,299 and 120,467.

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