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The Abundant Latency-Associated Transcripts of Herpes Simplex Virus Type 1 Are Bound to Polyribosomes in Cultured Neuronal Cells and during Latent Infection in Mouse Trigeminal Ganglia

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During herpes simplex virus type 1 (HSV-1) latency, limited viral transcription takes place. This transcription has been linked to the ability of the HSV-1 genome to reactivate and consists of abundant 2.0- and 1.5-kb collinear latency-associated transcripts (LATs), spanned by minor hybridizing RNA (mLAT). The 1.5-kb LAT is derived from the 2.0-kb LAT by splicing, and both transcripts contain two large overlapping open reading frames. The molecular action mechanisms of the latency-associated gene expression are unknown, and no HSV-1 latency-encoded proteins have been convincingly demonstrated. We have cloned the entire latency-associated transcriptionally active HSV-1 DNA fragment (10.4 kb) under control of a constitutive promoter and generated a neuronal cell line (NA4) stably transfected with the viral LAT's region. NA4 cells produced the 2.0- and the 1.5-kb LATs. Northern blotting and reverse transcription-PCR analysis of RNA from NA4 cells and from trigeminal ganglia of mice latently infected with HSV-1 revealed that the two abundant LAT species were present in the polyribosomal RNA fractions. After addition of EDTA, which causes dissociation of mRNA-ribosome complexes, both LATs could be detected only in subpolyribosomal, but not in polyribosomal fractions. These results show that (i) HSV-1 LATs are bound to polyribosomes during latency in vivo, as well as in neuronal cells in vitro, and therefore might be translated, and that (ii) the NA4 cell line is a suitable tool with which to look for HSV-1 latency-encoded gene products.

Following primary infection, herpes simplex virus type 1 (HSV-1) colonizes the human peripheral sensory ganglia (PSG), establishes latent infection, and can undergo periodic reactivations to produce recurrent mucocutaneous lesions at the peripheral sites innervated by the infected ganglia (for reviews, see references 42, 48, and 52). Viral nucleic acids but not mature virions are detected in latently infected human PSG throughout life (9, 51). The only HSV-1 genes expressed during latency are localized within the repeat segments of the viral genome (Fig. 1A to C). In vivo, they synthesize two types of RNAs: two collinear latency-associated transcripts (LATs), 2.0 and 1.5 kb in size, detectable by Northern blot analysis and by in situ hybridization (41, 45, 51, 53). These transcripts are spanned by minor hybridizing RNA (mLAT), identified only by in situ hybridization (10, 34). The most abundant LAT, 2.0-kb LAT, is also detectable during lytic infection of tissue culture cells by Northern blot analysis (45). The 1.5-kb LAT is derived from the 2.0-kb LAT by an unusual splicing, since the 5′ splice junction is GC instead of the consensus GT (46). The LATs are either poorly polyadenylated (45) or not polyadenylated at all (59), while the mLAT is polyadenylated (11). Investigation of the cellular localization of the HSV-1 latent RNAs by in situ hybridization has revealed that although most of the signal was observed within the nucleus, it was also present in the cytoplasm of the neuronal cells within PSG (51).

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Experiments with LAT-negative mutants showed that the LATs are not required for HSV-1 lytic replication and establishment or maintenance of latency (4, 19, 21, 26, 50), but suggested that they are necessary for efficient reactivation from mouse and rabbit trigeminal ganglia (TG) (3, 18, 55). The molecular action mechanisms of the latency-associated genes are unknown. The idea that the LATs may encode gene products has attracted much interest. Analysis of the relevant DNA sequences in several HSV-1 strains has revealed two large overlapping open reading frames (ORFs) within the 2.0-kb LAT, which potentially encode a 30-kDa polypeptide (ORF1) and a 12-kDa (ORF2) polypeptide (Fig. 1C). It has been shown that the splicing to produce the 1.5-kb LAT removes an intron that inhibits translation of these ORFs in a transcription-translation system in vitro (46).

LATs' protein products have been examined both in vivo and in vitro. Hitherto, the in vivo studies have yielded negative results. (i) Antibodies raised against synthetic peptides which were generated on the basis of the DNA sequence coding for the LATs failed to identify LAT products in latently infected tissue (J. Stevens, personal communication in reference 58 and J. G. Spivack, personal communication in reference 46). (ii) Analysis of the differences in protein profiles between HSV-1 strains capable of expressing the LATs and mutant viruses devoid of LATs did not enable the identification of specific LAT-encoded proteins (60). (iii) An 80-kDa protein was detected in an in vitro latency model (12) and was presumed to be coded by ORF1, although this ORF would be expected to encode for a much smaller protein. This result was not reproduced. Therefore, one of the key questions, which has yet to be

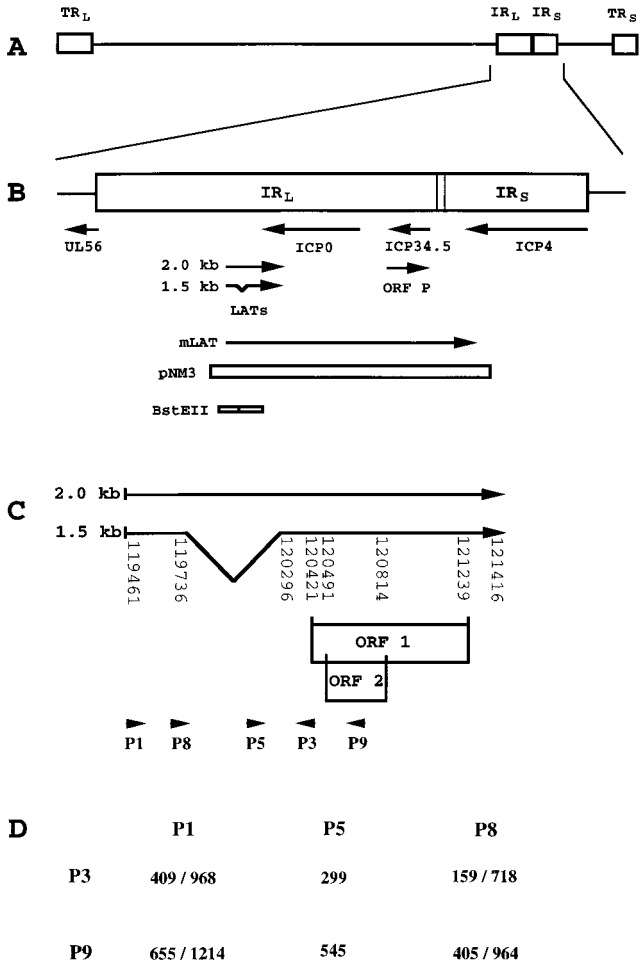

FIG. 1. Map of the HSV-1 genome region expressing the LATs and locations of primers used for RT-PCR analysis. (A) Schematic structure of the HSV-1 genome. Inverted repeats (IR, internal; TR, terminal) flanking unique long (TR_L and IR_L) and unique short (TR_S and IR_S) genome regions are indicated. (B) Enlarged joint region of HSV-1 genome. Arrows indicate the HSV-1 transcripts. mLAT and two collinear, 2.0- and 1.5-kb LATs are the only viral RNAs produced during latency. The open box shows the HSV-1 genome region that has been cloned into the plasmid pNM3. Two joined open bars indicate the two *Bst*EII DNA fragments used as a hybridization probe. (C) The 2.0- and 1.5-kb LATs and their two large ORFs. The nucleotide numbers of the 5′ and the 3′ ends of the LATs, both ORFs, and of the splicing boundaries are provided according to the complete HSV-1 DNA sequence in GenBank (accession number X14112). The location and orientation of the primers P1, P3, P5, P8, and P9 used in this study are indicated by arrows. (D) Sizes of DNA fragments produced by PCR with the appropriate pairs of primers and cDNA from the LATs as a template (spliced 1.5-kb and unspliced 2.0-kb LATs). Intron-specific primer P5 is complementary only to 2.0-kb LAT.

resolved, is whether these transcripts leave the nucleus during latent infection and/or reactivation and are transported to the cytoplasm where they might be translated. It was shown recently that the 2.0-kb LAT is present in the cytoplasm of acutely infected tissue culture cells and productively infected SCID mouse brain stem and that it comigrates with ribosomal subunits in polysome separation gradients (36). However, no information is available about the localization of HSV-1 LATs on polysomes during latency in vivo or in neuronal cells in vitro.

In this study, we have examined the hypothesis that some fraction of the LATs is present in the cytoplasm and is bound to polyribosomes in TG tissue of mice latently infected with HSV-1 in vivo and in a neuronal cell line expressing the LATs in vitro. For in vitro studies, we have generated a neuronal cell line stably transfected with the entire HSV-1 genome region (10.4 kb), which is transcriptionally active during latency. For in vivo studies, we used mice latently infected with HSV-1 strain F. Northern and reverse transcription-PCR (RT-PCR) analyses of RNA from polyribosomal fractions, isolated from our neuronal cell line as well as from TG of latently infected mice, demonstrated that HSV-1 LATs are present in the cytoplasm and are bound to polyribosomes in both our in vivo and in vitro systems.

MATERIALS AND METHODS

Cell lines and viruses. NA cells (32), a derivative of the mouse neuroblastoma neuronal cell line Neuro-2a (22) (a variant of the cell line C1300) were obtained from A. McMorris, Wistar Institute, Philadelphia, Pa. The neuronal nature of the NA cells is supported by their morphology, electrical excitability, acetylcholine sensitivity, and expression of a number of neuron-specific proteins (32). Cells were maintained in MEME (minimal essential medium Eagle) with 10% heat-inactivated fetal bovine serum. HSV-1 strain F was obtained from B. Roizman, University of Chicago. The virus was propagated and titrated on CV-1 cells. Viral stocks were stored at −80°C.

Generation of a stable cell line expressing the LATs. NA cells (2×10^6 per plate) were seeded in MEME medium with 10% fetal calf serum, which was then replaced by low-glucose (1 g/liter) Dulbecco's modified Eagle's medium. A solution containing 10 μg of pNM3 DNA (29), linearized by *Sca*I, 0.5 μg of SV_2Neo DNA, 62.5 μl of 2 M $CaCl_2$, and H_2O in total volume of 0.5 ml was added gradually to a solution containing 0.49 ml of 2× HBS buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 280 mM NaCl [pH 7.1]) and 0.01 ml of 70 mM Na_2PO_4 (pH 6.8). The final solution, containing the DNA and the calcium phosphate precipitate, was added to the cells, and this mixture was incubated overnight. On the following day, the medium was replaced by MEME. Every 3 to 5 days, fresh neomycin G418 (0.3-mg/ml final concentration) was added to the plates. After 2 weeks of selection, each of the surviving colonies was transferred to a separate plate and checked for expression of LATs by Northern blotting and RT-PCR analysis of total cellular RNA. One representative clone expressing the LATs, NA4, was used for this study.

Establishment of latent HSV-1 infection in mice TG. Inbred BALB/c female mice were infected by HSV-1 strain F via the cornea as reported (49, 50). Confirmation of latent HSV-1 infection in TG tissue was performed by explant reactivation (49). Latently infected mice used in this study were sacrificed 30 or more days after infection.

Preparation of total RNA from cell culture and murine TG. Total RNA was isolated from cells or TG with Tri-reagent (Molecular Research Center, Inc.) according to the manufacturer's instructions, treated with RQ1 DNase (Promega), extracted with phenol-chloroform, and precipitated by ethanol.

Isolation of polyribosomal RNA from cell culture and murine TG. Fractionation of polyribosomes was done according to the protocol of O. Meyuhas (33) with some modifications. Shortly, before being harvested, cells were treated for 15 min with cycloheximide (final concentration of 90 μg/ml) to arrest protein synthesis and to fix polysomal structures. After trypsinization, cells were diluted with ice-cold phosphate-buffered saline (PBS) and pelleted at 4°C. The cell pellet was resuspended in a small volume of polysomal buffer (25 mM Tris [pH 7.5], 25 mM NaCl, 10 mM $MgCl_2$, 0.14 M sucrose, 0.05% Triton X-100, 0.5 mg of heparin per ml) and lysed by addition of 1/10 volume of the lysis buffer (10% Triton X-100, 10% deoxycholate). After cell lysis and centrifugation for 2.5 min at 13,000 × *g* (4°C) to remove nuclei, the supernatant (cytoplasmic fraction) was transferred to another tube containing the same volume of polysomal buffer and loaded on a linear sucrose gradient (15 to 45% in buffer containing 25 mM Tris [pH 7.5], 25 mM NaCl, 10 mM $MgCl_2$, and 0.1 mg of heparin per ml). After centrifugation for 3 h 45 min at 27 krpm (4°C, rotor TST28), fractions were collected, and their optical density at 260 nm (OD_{260}) profile was analyzed with a flow spectrophotometry device. Typically, five fractions were collected from each gradient: three from the polysomal area, including disomes, and two from the subpolysomal area (peak of monosomes-60S ribosomal subunit, and the lightest fraction [see Fig. 2A, for example]). RNA was precipitated by 0.1 M NaCl and ethanol, dissolved in guanidinium thiocyanate, and pelleted through a CsCl cushion according to standard protocol (section 4.2.1 in reference 1).

In the case of mice TG, the critical step has been to snap-cool the exposed TG, which was done by rinsing the opened skull, after removal of the brain, with 10 ml of ice-cold sterile PBS. This treatment lowers the temperature of the TG to less than 10°C, thus avoiding the dissociation of the polysomes. The cold TG were quickly removed, washed in a large volume of ice-cold PBS, and frozen in liquid nitrogen. After thawing in polysomal buffer on ice, the TG from 15 to 20 mice were crushed with a Polytron at 4°C, transferred to a Dounce homogenizer (Wheaton; 7 cm^3), and lysed by addition of 1/10 volume of lysis buffer, followed by 10 to 12 strokes with tight pestle. After lysis, the cellular extract was processed similar to the extract obtained from cultured cells.

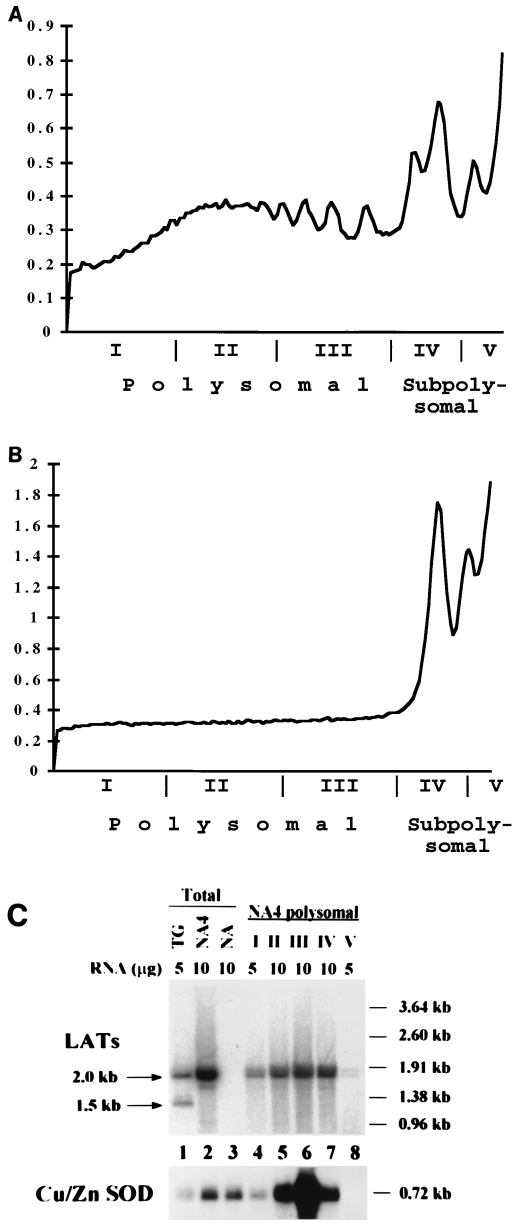

FIG. 2. Fractionation and analysis of cytoplasmic RNA from NA4 cells. (A and B) Separation analysis by flow spectrophotometry of polyribosomes in the cytoplasmic fractions from NA4 cells after centrifugation through a sucrose gradient. Vertical axis, OD_{260}; horizontal axis, fractions that were usually collected starting from the bottom of the tube. (A) Typical profile of cells that were treated with cycloheximide (90 μg/ml, 15 min before harvesting). (B) Cytoplasmic extract, prepared as described for panel A and treated with EDTA (15 mM final concentration) before being loaded on a sucrose gradient containing 15 mM EDTA. (C) Northern blot analysis of total RNA from NA and NA4 cells and separate polysomal fractions from NA4 cells. Lanes: 1, total RNA from TG of mice latently infected with HSV-1 strain F; 2, total RNA from NA4 cells; 3, total RNA from NA cells; 4 to 8, polysomal fractions I to V from NA4 cells (Fig. 2A); 1, 4, and 8, 5 μg of RNA; 2, 3, 5, 6, and 7, 10 μg of RNA per lane. At the bottom is shown the same blotted membrane, hybridized with a DNA probe specific to the housekeeping Cu/Zn SOD gene.

RT and PCR. Typically, 3 μg of RNA was subjected to RT with avian myeloblastosis virus reverse transcriptase (Promega) in a reaction volume of 25 μl for 1 h at 42°C. After inactivation of the enzyme (5 min at 95°C), the reaction mixtures were treated with RNase A (0.1 mg/ml, 20-min incubation at 37°C) to destroy all RNA and to significantly decrease the background in subsequent PCRs. For PCR, 3 μl of the RT mixture was diluted into the volume of 50 μl. PCR was done with the *Taq* polymerase of Appligene as described earlier (29). The following DNA primers were used for PCR of DNA fragments from the HSV-1 LAT region (Fig. 1C and D):

P1, 5′-GGTAGGTTAGACACCTGCTTCTCC (HSV-1 bases 119461 to 119484)
P3, 5′-GAAAGCATCCTGCCACTGGCATGGA (bases 120428 to 120404)
P5, 5′-CTCTGGCTGCACCGCATTCTTG (bases 120130 to 120151)
P8, 5′-GCCAATGACCCACGTACTCCAAG (bases 119711 to 119733)
P9, 5′-CTTTTCCCTGTCTGTGTTGGATG (bases 120674 to 120652)

RT was done with primer P3 or primer P9.

For RT-PCR analysis of the mouse Cu/Zn superoxide dismutase (Cu/Zn SOD) gene (2) the following primers were used:

22424, 5′-GAAAGCGGTGTGCGTGCTGAAG (SOD exon 1)
23321, 5′-GAGTGAGGATTAAAATGAGGTCC (SOD exon 3)

RT was done with primer 23321.

The primers were chosen according to the following criteria: (i) location in an AT-rich region, (ii) GC contents of the primer not less than 50%, (iii) absence of significant homology with all rodent and human sequences present in GenBank or with other HSV-1 sequences, and (iv) a PCR product size of between 0.2 and 1 kb.

Southern blot analysis. DNA fragments were separated on a 2% agarose gel and transferred onto GeneScreen Plus membrane (NEN Research Products) by downward capillary transfer (section 2.9.7 in reference 1) by the alkaline transfer protocol of the manufacturer. Hybridization was also done according to the manufacturer's instructions.

Northern blot analysis. Northern blot analysis was performed according to the method of Spivack and Fraser (45) with a Bio-Rad model 785 vacuum blotter. RNA markers (281 to 6583 bases; catalog no. G3191) were purchased from Promega Corporation.

Preparation of radioactively labeled DNA probes. The *Bst*EII DNA probe consisting of two equimolar fragments, 897 bp and 977 bp, covering the HSV-1 genomic region from bp 119194 to 121068 (Fig. 1B), was cut from plasmid pNM3 (29). The probe to the housekeeping Cu/Zn SOD gene (2), consisting of a 198-bp DNA fragment, was prepared by RT-PCR of RNA from TG of mice with primers 22424 and 23321. Both probes were purified from agarose gel by the Geneclean II kit (BIO 101, Inc.) and labeled by random priming with the "rediprime" or "multiprime" DNA labeling systems of Amersham. The labeled probes were purified on a Sephadex G-50 column. The specific activities of the probes were typically about 10^8 cpm/μg of DNA.

RESULTS

Construction of a neuronal cell line stably expressing HSV-1 LATs. In order to analyze the cellular localization of HSV-1 LATs in vitro, we have constructed a neuronal cell line stably expressing these transcripts. The HSV-1 (strain F) DNA fragment, 10.4 kb in size, which covers the entire region of the viral genome that is transcriptionally active during latency, was cloned under control of an immediate-early (IE) cytomegalovirus (CMV) promoter (plasmid pNM3 [Fig. 1B]) (29). In HSV-1, transcription of the LATs is driven by two latency-associated promoters (LAPs), LAP1 and LAP2 (8). Only the weaker promoter, LAP2 is present in pNM3, and the entire transcriptional unit present in this plasmid is driven by the strong IE CMV promoter. The neuronal cell line NA (32), a derivative of the mouse neuroblastoma cell line Neuro-2a (22), was stably transfected with pNM3. The selected clone, NA4, was found to produce both the 2.0- and the 1.5-kb LATs: the 2.0-kb LAT was produced at amounts comparable to those present during latent HSV-1 infection in murine TG (Fig. 2C, lane 2), and the 1.5-kb LAT was produced at relatively low levels, observed in Northern blots as a faint band only after overexposure of the film (data not shown) but easily detectable by RT-PCR analysis (Fig. 3B, lane 2).

Mapping of the 3′ end of the LATs by PCR indicated that the size of the 2.0-kb LAT is between 1,876 and 1,956 bases, and the size of the 1.5-kb LAT is between 1,317 and 1,397 bases (46). By our measurements, these two RNAs were about 1,760 and about 1,230 bases, respectively, but in order to correspond to the current nomenclature, we will refer in the text to the LATs as 2.0 and 1.5 kb in size. Two additional RNAs

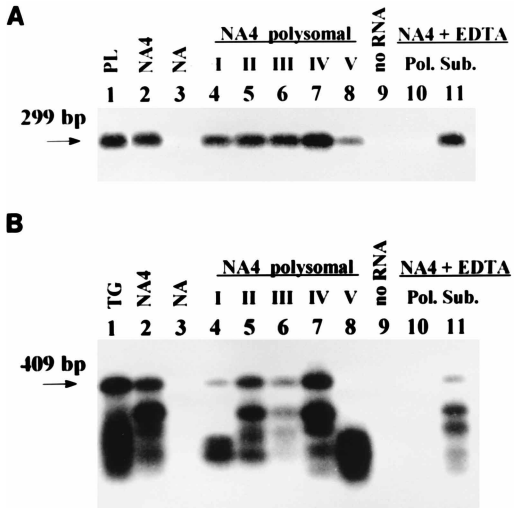

FIG. 3. Southern blot analysis of RT-PCR products from RNA of separate polysomal and subpolysomal fractions from NA4 cells. RT was done with primer P3. (A) PCR with primers P3 and P5. The DNA fragment with a size of 299 bp is representative of the 2.0-kb LAT. Lanes: 1, PCR with plasmid pNM3 as a template; 2, total RNA from NA4 cells; 3, total RNA from NA cells; 4 to 8, polysomal fractions I to V, respectively, of RNA from NA4 cells; 9, RT-PCR without RNA template; 10 and 11, pooled polysomal (I, II, and III) and subpolysomal (IV and V) fractions, respectively, of RNA from NA4 cells, treated with EDTA prior to being loaded on a sucrose gradient. (B) PCR with primers P3 and P1. The DNA fragment with a size of 409 bp is representative of the 1.5-kb LAT. Lanes: 1, total RNA from TG of mice latently infected with HSV-1; 2 to 11, same as in panel A.

could be detected by Northern blot analysis of total RNA from NA4 cells: a weak band about 250 bases smaller than the 2.0-kb LAT (Fig. 2C, lane 2) and a faint band about 300 bases smaller than the 1.5-kb LAT, visible only after overexposure. The first of these additional bands is also present in all cell lines tested (NA, CV-1, and 293) that had been infected by HSV-1 strain F (our unpublished data). These additional bands could represent the products of alternative splicing or other modifications of the abundant LATs in cell lines.

Analysis of polyribosomal RNA fractions in NA4 cells. Polyribosomal RNA from NA and NA4 cells was obtained by fractionation of cytoplasmic extracts through a sucrose gradient followed by RNA extraction with guanidinium thiocyanate and centrifugation through a CsCl cushion. Before being harvested, cells were treated for 20 min with cycloheximide in order to enable fixation of the polyribosomes. A typical profile of sucrose gradients with fractionated polysomes is shown in Fig. 2A. As a control, EDTA, which causes dissociation of RNA-polyribosome complexes, was added to the cytoplasmic extract (15 mM final concentration) prior to being loaded on the gradient (Fig. 2B). Usually, five fractions were collected (Fig. 2A); in some experiments, fractions from I to III were combined into a single polysomal fraction, and fractions IV and V were combined to produce one subpolysomal fraction, including monosomes, separate subunits, and soluble fractions.

Northern blot analysis of RNA obtained from separate polysomal fractions of NA4 cells is shown in Fig. 2C. Total RNA from TG of mice latently infected with HSV-1 (strain F) and from NA4 and NA cells (lanes 1, 2, and 3, respectively) served as controls. The 2.0-kb LAT was present in total (lane 2), polysomal (lanes 4 to 6), and subpolysomal (lanes 7 and 8) RNA fractions from NA4 cells, together with a bigger transcript (1,890 bases in our measurements, while the 2.0-kb LAT moved as 1,760 bases). The 1.5-kb LAT, clearly observed in RNA obtained from latently infected TG (lane 1), could not be detected in RNA from NA4 cells by Northern blotting. Nevertheless, the 1.5-kb LAT was readily revealed in RNA from NA4 cells by RT-PCR analysis (see Fig. 5). To validate the separation of polysomal and subpolysomal RNA fractions, we also examined the fractions for the presence of a housekeeping gene product, Cu/Zn SOD mRNA, which is intensively translated (2). Rehybridization of the same blotted membrane with a specific DNA probe to this gene's exons demonstrated that almost all spliced Cu/Zn SOD mRNA was located in our polysomal fractions (Fig. 2C, bottom). The short Cu/Zn SOD mRNA was located mostly in the lightest polysomal fraction (lane 6, fraction III), whereas the larger 2.0-kb LAT was present in significant amounts, also in the heavy polysomal fractions (lanes 4 and 5, fractions I and II).

To detect the LATs and to characterize the effect of EDTA on their cellular localization, RT-PCR analysis of RNA obtained from separate polysomal fractions of NA4 cells and from combined polysomal and subpolysomal fractions in the presence and absence of EDTA, was carried out. Southern blotting of the RT-PCR products of NA4 RNA from these fractions is shown in Fig. 3. The 2.0- and 1.5-kb LATs were detected by RT-PCR with primer pairs P5/P3 and P1/P3, respectively (Fig. 1C). Theoretically, RT-PCR of LATs with primers P1/P3, flanking the splicing region, should produce two DNA fragments: 409 bp from the 1.5-kb LAT and 968 bp from the 2.0-kb LAT (Fig. 1D). However, as reported by us earlier (29), the PCR product from the 2.0-kb RNA was undetectable at the level of Southern blot analysis, probably because of the secondary structure of the unspliced RNA and/or competition between the smaller spliced fragment and the bigger unspliced fragment. In order to detect the 2.0-kb LAT, we used the P5 intron-specific primer in PCR following RT with primer P3.

A 299-bp DNA fragment representative of the unspliced 2.0-kb LAT, produced by primers P5 and P3, was detected in total RNA from NA4 cells (Fig. 3A, lane 2) as well as in all five separate polysomal fractions of RNA from NA4 cells (lanes 4 to 8). A 409-bp DNA fragment representative of the spliced 1.5-kb LAT, produced by primers P1 and P3, was detected in total RNA from NA4 cells (Fig. 3B, lane 2) and in all three polysomal fractions (lanes 4 to 6), as well as in a monosomal fraction (lane 7). After treatment of the cytoplasmic extract from NA4 cells with EDTA (15 mM final concentration [Fig. 2B]), DNA fragments representative of both spliced and unspliced LATs were detected only in the subpolysomal fraction (Fig. 3A and B, lane 11), but not in the polysomal fraction (Fig. 3A and B, lane 10), showing that the RNAs detected by our assay indeed comigrated with polysomes and were dissociated from the RNA-ribosome complexes by EDTA. For each reaction, appropriate controls without RT did not identify any DNA band (data not shown). Likewise no RT-PCR products were present following the reactions with no RNA template (Fig. 3A and B, lane 9). The origin of the smaller bands, present in most of the RNA fractions from NA4 cells, is unknown. To confirm the detection of the 1.5-kb LAT in NA4 cells by RT-PCR, the same analysis was carried out with another pair of primers, P8 and P9, also flanking the splicing region but covering another sequence in the LAT gene (see Fig. 1C). Similarly, the 1.5-kb LAT was detected in the polysomal fractions of RNA from NA4 cells (data not shown). These results demonstrate that both LATs produced in NA4 cells are bound to polysomes and suggest that they may be intensively translated, since they are present in all, even the heaviest, polysomal fractions.

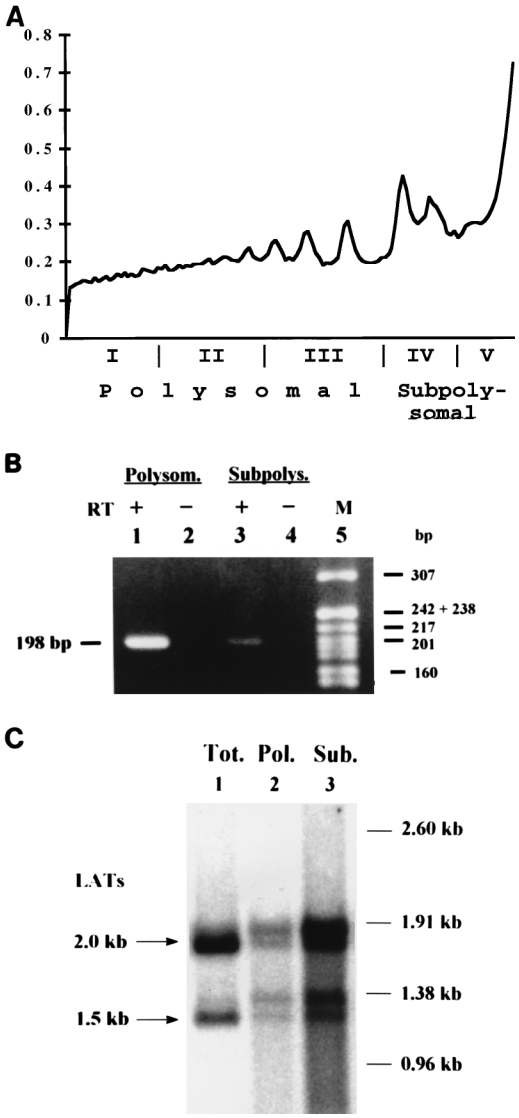

FIG. 4. Analysis of polyribosomal RNA from TG of mice. (A) Separation analysis by flow spectrophotometry of polyribosomes in the cytoplasmic fractions from TG of mice latently infected with HSV-1 strain F after centrifugation through a sucrose gradient. Vertical axis, OD_{260}; horizontal axis, fractions that were usually collected starting from the bottom of tube. (B) RT-PCR analysis of polysomal (fractions I, II, and III) and subpolysomal (fractions IV and V) RNAs obtained from mice TG with primers specific to the housekeeping Cu/Zn SOD gene. Lanes: 1 and 2, polysomal RNA with and without RT enzyme in the reaction mixture, respectively; 3 and 4, subpolysomal RNA with and without RT enzyme in the reaction mixture, respectively; 5, DNA marker (pBR322 DNA digested by *Msp*I). (C) Northern blot analysis of RNA from TG of mice latently infected with HSV-1 strain F. Lanes: 1, total RNA; 2, pooled polysomal RNA (fractions I, II, and III); 3, pooled subpolysomal RNA (fractions IV and V).

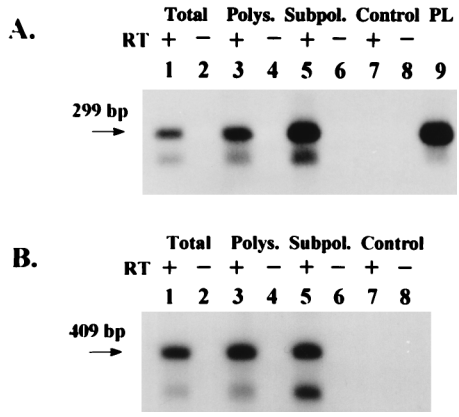

FIG. 5. Southern blot analysis of the RT-PCR products of RNA from TG of mice latently infected with HSV-1 strain F. RT was done with primer P3. Polysomal (Polys.) RNA included fractions I, II, and III; subpolysomal (Subpol.) RNA included fractions IV and V (Fig. 4A). For each sample, a control PCR without RT enzyme in the reaction mixture is presented (lanes 2, 4, 6, and 8). Lanes: 1, 3, and 5, total, polysomal, and subpolysomal RNAs, respectively, from TG of mice latently infected with HSV-1 strain F; 7, RT-PCR without an RNA template. (A) PCR with primers P3 and P5. The 299-bp DNA fragment is representative of the 2.0-kb LAT. Lane 9, PCR with plasmid (PL) pNM3 as a DNA template. (B) PCR with primers P3 and P1. The 409-bp DNA fragment is representative of the 1.5-kb LAT.

Analysis of polyribosomal RNA fractions in TG. In order to investigate whether binding of the LATs to polysomes, observed in the neuronal NA4 cell line, also takes place in vivo, we fractionated cytoplasmic extracts from TG of mice latently infected with HSV-1 strain F. Preparation of polysomal fractions from murine TG was difficult because of the rapid dissociation of the polyribosomes in the process of TG explantation, and was achieved by rapid rinsing of the TG with ice-cold PBS after removal of the brain. The typical profile of cytoplasmic extracts from murine TG, fractionated by centrifugation through sucrose gradient, is shown in Fig. 4A. The obtained pooled polysomal (fractions I, II, and III) and subpolysomal (fractions IV and V) RNAs were examined for the presence of the housekeeping Cu/Zn SOD RNA by RT-PCR analysis with primers specific to this gene's exons. As can be seen in Fig. 4B, most of the spliced Cu/Zn SOD mRNA (represented by the 198-bp RT-PCR product) was located, as expected, in the polysomal fraction (compare lanes 1 and 3).

The results of Northern blot analysis of pooled polysomal and subpolysomal fractions of RNA from TG of mice latently infected with HSV-1 (F) are shown in Fig. 4C. It can be seen that the 2.0- and the 1.5-kb LATs were present in the polysomal fraction (lane 2). The same result was obtained with RNA from separate polysomal fractions of infected TG (data not shown). In each polysomal and subpolysomal fraction, two additional bands were noticed, each one about 130 bp bigger than the 1.5- and the 2.0-kb LATs (calculated sizes of the detected bands: 1,890, 1,760, 1,340 and 1,230 bases). The reason for these doublets is not known, but they could be explained by the recent finding that both LATs exist in linear and in circular forms (61).

To further investigate the localization of the LATs in TG by a different approach, we applied the same RT-PCR analysis that was done for RNA obtained from NA4 cells. The results of Southern blot analysis of the RT-PCR products of total, polysomal, and subpolysomal RNAs from TG of latently infected mice with primer pairs P5/P3 and P1/P3 are shown in Fig. 5. Both the 299-bp DNA fragment, representative of the 2.0-kb LAT, and the 409-bp DNA fragment, representative of the spliced 1.5-kb LAT, were detected in total, polysomal, and subpolysomal fractions of RNA from TG of latently infected mice (Fig. 5A and B, lanes 1, 3, and 5, respectively). One smaller band of unknown origin was detected in all three RNA fractions with each pair of primers (lanes 1, 3, and 5). The same RT-PCR analysis of total, polysomal, and subpolysomal RNAs from TG of noninfected mice did not produce any band (not shown). Similarly to the situation in cell culture, separate polysomal and subpolysomal fractions were isolated from TG of latently infected mice, and RT-PCR analysis of RNA from these fractions was carried out with primers P8 and P9. South-

ern blot analysis of these RT-PCR products revealed the presence of the spliced 1.5-kb LAT in each separate RNA fraction from latently infected TG (data not shown). Thus, the results of all of the in vivo tests (Fig. 4 and 5) clearly indicate that both the 2.0- and 1.5-kb HSV-1 LATs are present in the cytoplasm of latently infected TG and are bound to all tested polysomal fractions.

DISCUSSION

The role of the LATs' expression has become one of the central issues of HSV-1 latency research. While experiments with different LAT-negative mutants have suggested that the LATs are necessary for efficient in vivo reactivation from TG of latently infected mice and rabbits (4, 5, 19, 21, 27, 37, 50, 55), the stage of latent infection that they modulate (establishment of latency versus reactivation), their molecular action mechanisms, and the exact sequences responsible for the reactivation phenotype have not been determined.

Do the LATs have a role in reactivation? The genetic approach. The use of several animal and in vitro models, different HSV-1 strains, various sites of latent infection, and different reactivation techniques makes it difficult to compare the results obtained by independent research groups (16) and to draw any definite conclusions. While Fareed and Spivack demonstrated that the first 1,186 bp starting from the 5′ end of the 2.0-kb LAT could be deleted without significant effect on viral explant reactivation from mice TG (14), two other groups showed recently that the first 1.5 kb from the transcription start of LAP1 (which includes about 660 bp between the start sites of the mLAT and the 2.0-kb LAT and about 840 bp of the 2.0-kb LAT coding sequence) are sufficient for spontaneous (38) and induced (6) HSV-1 reactivation from TG of rabbits. Further deletion analysis demonstrated that a 371-bp region between nucleotides 76 and 447 from the transcription start of LAP1 could also be removed without any effect on spontaneous reactivations (39). The same deletion also had no effect on viral explant reactivation from mice TG (30). Based on these findings, only nucleotides 1 to 76 and/or 447 to 1499 can harbor the HSV-1 reactivation function. In the rabbit, the reactivation function was mapped to a 348-bp region between nucleotides 205 and 553 from the LAP1 start (6). The conclusion from these results is that the reactivation function of HSV-1 is localized between nucleotides 447 and 553 from the start of LAP1 transcription. However, deletion of this area, as well as two other subdeletions inside the 348-bp area, had no effect on virus-induced reactivation from rabbit TG (6).

Thus, while the genetic approach seems to leave no place for the 2.0- and the 1.5-kb LATs, and especially for their large ORFs, in the viral reactivation process, the data to back this conclusion are contradictory and conflicting. Moreover, the relevance of the findings in laboratory animals and in vitro to the native human HSV-1 infections is questionable, since, for example, a set of viral genes that are "nonessential" in vitro, were still preserved in nature under selection pressure (reviewed in reference 42).

Do the LATs have a role in reactivation? The molecular/cellular approach. By in situ hybridization, the LATs could be visualized mainly, but not exclusively, on and around the nucleus of the latently infected neuron (9, 51, 53). Several studies also suggested that some fraction of the LATs is polyadenylated. (i) mRNA complementary to ICP0 mRNA of HSV-1 was detected by RT of poly(A)$^+$ mRNA isolated from TG of latently infected mice (40). (ii) The 1.5- and 2.0-kb LATs were detected in poly(A)$^+$ mRNA isolated from the same tissue, and the 1.5-kb/2.0-kb LAT ratio in this fraction was higher than those in the total or poly(A)$^-$ fractions (45). (iii) Some of the cDNA clones used to determine the 2.0-kb LAT sequence were obtained from poly(A)$^+$ fractions of RNA from TG of latently infected mice (46). It is of note that the LATs obtained from latently infected dorsal root ganglia, where they seem not to play an important role during reactivation, are not polyadenylated (11). Moreover, in the case of bovine herpesvirus 1 latency-related gene, whose gene product was recently identified in cell culture in vitro (20), only a small fraction of LAT RNA is polyadenylated and alternatively spliced (20).

The only attempt to examine whether some fraction of HSV-1 LATs is indeed present in the cytoplasm was performed with nonneuronal cells during acute infection (36). This experiment demonstrated that the 2.0-kb LAT is present in the cytoplasm of acutely infected tissue culture cells and productively infected SCID mouse brain stem and that it comigrates with ribosomal subunits in polysome profiles (36).

We therefore decided to study the possible function of the LATs by a cellular and molecular rather than a genetic approach, especially since most of the attempts to examine LATs' function and their potential ORFs applied the latter approach (4, 14, 15, 19, 26, 35, 43, 50, 55). Since nonpolyadenylated RNA may be transported to the cytoplasm and translated (e.g., the histone mRNAs [13]), and polyadenylated RNA that is transported to the cytoplasm might be either poorly translated (e.g., the human CMV [HCMV] IE2 2.2-kb mRNA [47]) or not translated at all (e.g., the H19 RNA [7]), we did not address the question of polyadenylation in this work. We therefore asked a different, more direct question—whether LATs are bound to polyribosomes regardless of their possible polyadenylation. For this purpose, we used a neuronal cell line expressing the LATs and the in vivo latency model in TG of mice.

The LATs are associated with polyribosomes in vitro and during latent in vivo infection. In this study, we show that in NA4 cells (a neuronal cell line stably transfected with the entire 10.4-kb DNA fragment from HSV-1 that is transcriptionally active during latent infection and expresses the 2.0- and the 1.5-kb LATs), both of these RNA species are present in the cytoplasm and are bound to polyribosomes (Fig. 2 and 3). More important, we were able to demonstrate that both LATs are present in the cytoplasm and are bound to polyribosomes in TG of mice latently infected with HSV-1 (Fig. 4 and 5). The following control experiments were performed and confirm our findings. (i) Confirmation was obtained in terms of the quality of separation of the polyribosomal RNA fractions (Fig. 2A and B and 4A), and the distribution of the Cu/Zn SOD RNA, visualized by Northern blot analysis for cells (Fig. 2C, bottom part) and by RT-PCR analysis for TG (Fig. 4B). (ii) In the case of cellular RNA, dissociation of cytoplasmic RNA-ribosome complexes by EDTA revealed that the LATs could be identified in the polyribosomal fractions only when the polyribosomes were intact (Fig. 3). (iii) In all cases of RT-PCR analysis, controls without RT enzyme in the reaction followed by the same PCR demonstrated that the samples in the reactions were RNA and not DNA. (iv) Finally, in parallel analysis of RNA from brain stems of mice latently infected with HSV-1, all LATs were detected exclusively in the subpolysomal but not in the polysomal RNA fractions (15a), proving that in our experimental system, there was no leakage of the LATs' molecules from the nuclear or subpolysomal to the polysomal area.

The relative levels of 1.5-kb/2.0-kb LAT in NA4 cells are significantly lower than those in latently infected mouse TG, similar to the situation in transient transfection assays of neuronal cells (29). This may be the result of a less efficient splicing of the 2.0-kb LAT in neuroblastoma cells compared to that in neurons in vivo. The additional bands in our RT-PCR assays

(Fig. 3B, lanes 2, 4 to 7, and 11; Fig. 5A and B, lanes 1, 3, and 5) could be either artifacts of the RT-PCR or products of some, yet unidentified, alternatively spliced LAT derivatives, as has been noted earlier (11). A detailed RT-PCR analysis of the LAT region with a set of different primers may reveal all variants of the LATs that are bound to polyribosomes.

The LATs' large ORFs—do they code for gene products? Although no HSV-1 latency-associated proteins have yet been convincingly demonstrated and antibodies raised against synthetic fragments of the LATs' two ORFs did not identify proteins of the expected size (see reference 12 and Fareed and Spivack, personal communication in reference 14), several arguments suggest that the large LATs' ORFs may indeed synthesize polypeptides. (i) The sizes of both ORFs (272 and 107 codons), are too large for random ORFs. (ii) The structures of the LATs' ORFs (the smaller one inside the bigger, close to its start, and in the +1 frame) are typical for a number of animal viruses, which have a bicistronic mRNA (24), and provide efficient translation for both ORFs (17). (iii) Translation initiation regions of both ORFs are typical for some viral bifunctional mRNAs. Usually, in such mRNA, the first AUG codon has a poor surrounding context, while the second AUG has a more favorable one. This results in "leaky scanning": part of the 40S ribosomes bypass the first AUG codon, and initiation occurs on both AUGs (24). This may also be the situation in the case of the genes expressed by HSV-1 during latency: the sequence at ORF2 start site CCC**A**AC<u>AUG</u>**G** better fits the consensus of vertebrate mRNA's start site (GCC(**A**/**G**)CC<u>AU</u>G**G**) (23) than does the sequence at the ORF1 start site GGC **A**GG<u>AUG</u>**C** (the most conserved consensus positions are in boldface). (iv) The splicing producing the 1.5-kb LAT removes an intron that inhibits translation of these ORFs in a transcription-translation system in vitro (46). Finally, the identification in vitro of the BHV-1 LAT's ORF2-encoded polypeptide (20) and the detection of its association with cyclin A in transfected cells (44) make it possible that HSV-1 LATs' large ORFs may also code for a protein.

The possible functional meaning of smaller ORFs within the latency-active region of HSV-1. The attempts to identify possible latency-encoded proteins have focused on the largest ORFs. Antibodies were raised against synthetic peptides of the two large ORFs of the LATs (see reference 12 and personal communications from J. Stevens in reference 58 and J. G. Spivack in reference 46), and tagging of the five large ORFs of the mLAT was intended to reveal whether they are expressed during HSV-1 latency. However, this region contains 16 ORFs that are longer than 50 codons and many more short ORFs (25). There are many examples of translation regulation by small peptides (reviewed in reference 28). Recently, it was found that *Escherichia coli* 23S rRNA codes for a pentapeptide (54). Overexpression of 23S rRNA fragments containing this minigene renders cells resistant to the ribosome-inhibiting antibiotic erythromycin. Therefore, the possibility that the function of the latency-association gene of HSV-1 may be mediated by a small peptide that affects the function of cellular ribosomes cannot be ruled out. Analysis of all regions of the LATs that contain acceptable ribosome-binding sites may enable examination of this hypothesis.

Regulation of LATs' turnover—another possible function for their association with polyribosomes. While the association of the LATs with polyribosomes is highly suggestive of translation and synthesis of a functional protein, another possibility should also be considered. There are examples of mRNAs that are bound to polyribosomes but that do not code for a functional protein. One such example is the RNA of the U22 host gene (UHG), whose introns code for eight different small nucleolar RNAs (56). The polyadenylated spliced UHG RNA, consisting of nonfunctional exons, is found on polyribosomes, where it is rapidly degraded. The UHG RNA does not contain long ORFs and has multiple termination signals in all three frames. This may cause RNA degradation, since a nonsense codon can activate specific pathways of RNA decay (31). However, the presence of the long ORFs in the LATs' coding sequence differs from what is found in the UHG RNAs.

Another example are the defective interfering (DI) RNAs of the mouse hepatitis virus (57), which contain large ORFs, one of which spans almost the entire length of the RNA. Mutagenesis of this ORF revealed that the translated sequence is irrelevant, indicating that translation per se, but not a gene product, plays a critical role in DI virus propagation. Probably the synthesis or degradation of DI RNAs is affected by translation (57). Therefore, in the case of HSV-1, the association of the LATs with polyribosomes may be connected to the regulation of their metabolism, but not to their function.

Further investigation of the function of the association of the LATs with polyribosomes, as well as the localization of the exact region of LATs' sequences that bind to ribosomes and the search for potential LAT proteins can be performed with the NA4 cell line as constructed and characterized for this study.

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