Two Small RNAs Encoded within the First 1.5 Kilobases of the Herpes Simplex Virus Type 1 Latency-Associated Transcript Can Inhibit Productive Infection and Cooperate To Inhibit Apoptosis[∇]

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The herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) is abundantly expressed in latently infected trigeminal ganglionic sensory neurons. Expression of the first 1.5 kb of LAT coding sequences is sufficient for the wild-type reactivation phenotype in small animal models of infection. The ability of the first 1.5 kb of LAT coding sequences to inhibit apoptosis is important for the latency-reactivation cycle. Several studies have also concluded that LAT inhibits productive infection. To date, a functional LAT protein has not been identified, suggesting that LAT is a regulatory RNA. Two small RNAs (sRNAs) were previously identified within the first 1.5 kb of LAT coding sequences. In this study, we demonstrated that both LAT sRNAs were expressed in the trigeminal ganglia of mice latently infected with an HSV-1 strain that expresses LAT but not when mice were infected with a LAT null mutant. LAT sRNA1 and sRNA2 cooperated to inhibit cold shock-induced apoptosis in mouse neuroblastoma cells. LAT sRNA1, but not LAT sRNA2, inhibited apoptosis less efficiently than both sRNAs. When rabbit skin cells were cotransfected with plasmids that express LAT sRNA1 and HSV-1 genomic DNA, the amount of infectious virus released was reduced approximately 3 logs. Although LAT sRNA2 was less effective at inhibiting virus production, it inhibited expression of infected cell protein 4 (ICP4). Neither LAT sRNA had an obvious effect on ICP0 expression. These studies suggested that expression of two LAT sRNAs plays a role in the latency-reactivation cycle by inhibiting apoptosis and productive infection.

Most adults in the United States harbor latent herpes simplex virus type 1 (HSV-1) (48, 71) in sensory neurons located in trigeminal ganglia (TG) or sacral dorsal root ganglia (34, 68). Acute infection is typically initiated in the mucocutaneous epithelium. Despite a vigorous immune response during acute infection, HSV-1 establishes latency in sensory neurons. Latent HSV-1 periodically reactivates from latency, resulting in the shedding of infectious virus and various recurrent clinical disorders (reviewed in references 34 and 35).

Mice, rabbits, or humans latently infected with HSV-1 express abundant levels of the latency-associated transcript (LAT) in latently infected neurons (12, 14, 15, 38, 45, 62, 65, 69, 70). The primary LAT transcript is 8.3 kb, and splicing yields a stable 2-kb LAT and an unstable 6.3-kb LAT (14, 62, 73). The 2-kb LAT can be further spliced in infected neurons (43). The majority of the 2-kb LAT is not capped or polyadenylated and appears to be a stable intron (19, 40). In general,

HSV-1 LAT null mutants do not reactivate from latency as efficiently as LAT-expressing strains (reviewed in references 34, 35, and 68). Expression of the first 1.5 kb of LAT coding sequences (LAT nucleotides [nt] 1 to 1499) is crucial for wild-type (wt) levels of reactivation in small animal models (28, 33, 56).

LAT reduces apoptosis in infected tissue culture cells (32) and promotes neuronal survival in the TG of infected rabbits (53) and mice (1, 4). Plasmids expressing LAT interfere with caspase-8- and caspase-9-induced apoptosis (1, 27, 28, 33, 49, 53). Inhibiting apoptosis is an important function of LAT because three antiapoptosis genes (30, 31, 46, 52) restore the wt reactivation phenotype to that of a LAT null mutant. LAT also represses productive viral gene expression, in particular infected cell protein 4 (ICP4), in the TG of mice during acute infection (10, 23). Certain studies also concluded that LAT inhibits ICP0 expression (10, 23, 44), whereas others concluded that LAT does not influence ICP0 expression (5, 9). The ability of LAT to inhibit apoptosis and productive infection is likely to enhance the survival of infected neurons and promote the latency-reactivation cycle.

A recent study (67) concluded that the 8.3-kb LAT is a microRNA (miRNA) precursor that encodes four miRNAs and two miRNAs located in LAT promoter sequences. Another study identified an miRNA approximately 450 bases up-

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stream of the start site of LAT (13). We identified two small RNAs (sRNAs) encoded by the first 1.5 kb of LAT coding sequences (LAT sRNA1 and sRNA2) that are 62 nt and 36 nt long, respectively (51). LAT sRNA1 and sRNA2 do not appear to be miRNAs because the mature miRNA bands that migrate between 21 and 23 nt were not detected. LAT sRNA1 and LAT sRNA2 would not have been identified by using procedures previously described by Umbach et al. (67) because sRNA species migrating between 17 and 30 nt were size selected and then analyzed.

In this study, we demonstrated that LAT sRNA1 and LAT sRNA2 cooperated to inhibit apoptosis in transiently transfected cells. Cotransfection of a plasmid that expresses LAT sRNA1 with HSV-1 DNA inhibited the production of infectious virus by approximately 1,000-fold. LAT sRNA2, but not sRNA1, inhibited ICP4 protein expression and production of infectious virus by approximately fivefold.

MATERIALS AND METHODS

Cells and viruses. Mouse neuroblastoma cells (neuro-2A) were obtained from ATCC, and rabbit skin (RS) cells were from S. Wechsler (University of California, Irvine, CA). Cells were seeded in Earle's minimal essential medium supplemented with 10% (vol/vol) fetal bovine serum. neuro-2A cells were split at a 1:6 ratio every 2 or 3 days. All media contained penicillin (10 U/ml) and streptomycin (100 µg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Viruses were plaque purified three times and passed one or two times in RS cells prior to use. dLAT2903 (the LAT mutant) and dLAT2903R (the LAT rescued virus) were described previously (54–56) and were obtained from S. Wechsler (University of California, Irvine, CA).

Infection of mice with HSV-1. Eight to 10 week-old Swiss Webster female mice (Jackson Laboratory or Charles River Laboratory) were used. Viral infections were done without scarification as previously described (36, 54, 55, 57). TG were extracted during latency (at least 30 days after infection).

Preparation of sRNAs and reverse transcription-PCR (RT-PCR). sRNAs were prepared from the TG of latently infected mice or transfected cells using the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. sRNA samples were precipitated in 100% ethanol and suspended in 20 µl RNase-free water, which is referred to as the total sRNA fraction. LAT sRNA1 and sRNA2 in TG or transfected cells were detected using a previously described protocol (61). In brief, half of the total sRNA was polyadenylated at 37°C for 60 min in a 100-µl reaction mixture using a poly(A) tailing kit (Ambion). A volume equal for acid/phenol/chloroform was added, mixed, and centrifuged, and the aqueous phase was removed. The poly(A)-tailed sRNA was precipitated using 100% ethanol and suspended in 10 µl RNase-free water. To generate a library of cDNA from sRNA, polyadenylated sRNA and 2 µg of a cDNA adapter primer (5'-GCGAGCACAGAATTAATACGACTCACTATAGGT12VN-3') were mixed with 2 µl of 10 mM mixed deoxynucleoside triphosphates in a 13-µl reaction volume, incubated at 65°C for 5 min, and annealed at 4°C for 5 min. RT was carried out with 200 U of SuperScript III reverse transcriptase (Invitrogen), 1 µl of 0.1 M dithiothreitol, 4 µl of 5× buffer, and 2 U of RNase OUT in a final reaction volume of 20 µl at 50°C for 60 min. Reverse transcriptase was inactivated by incubation at 70°C for 15 min. An sRNA1-specific primer (5'-GCCTG TGT TTTTGTGCTGGCTC-3') or sRNA2-specific primer (5'-CATCTTGT TTCTAACTAGTTCCTG-3') and an adaptor-specific primer (5'-GCGAGC ACAGAATTAATACGACT-3') were used for amplification of the respective sRNAs. PCR was performed using GoTaq DNA polymerase (Promega), 1 µl of the synthesized cDNAs, and 10 µM of primers in a 50-µl reaction volume using MJ Research thermal cycler PTC-200. A four-step PCR protocol (95°C for 10 min and then 35 cycles of 95°C for 30 s, 57°C [sRNA1] or 53°C [sRNA2] for 30 s, and 72°C for 30 s, and 72°C for 10 min) was used. Nested PCR was performed using an inner adapter primer (5'-AATTAATACGACTCACTATAGGT-3') with the inner sRNA1 primer (5'-TATGCTTGGGTCTTACTGCTG-3') or the inner sRNA2 primer (5'-CTATGTTCTGTTCTGT-3'). PCR products were analyzed on a 2% agarose gel. The sequence of the sRNA was verified after cloning PCR products into a vector supplied in the TOPO cloning kit (Invitrogen).

To detect ICP4 and ICP0 RNA expression, random primers were used for

first-strand cDNA synthesis by using total RNA. RT was carried out with 200 U of SuperScript III reverse transcriptase (Invitrogen), 1 µl of 0.1 M dithiothreitol, 4 µl of 5× buffer, and 2 U of RNase OUT in a final reaction volume of 20 µl. This reaction mixture was incubated at 50°C for 60 min. Reverse transcriptase was inactivated by incubation at 70°C for 15 min. To amplify ICP4 cDNA, the following primers were used: GTGATCAGGGCGTACTGCTG (forward) and CCTTCTACGCGCTATC (reverse). The ICP4-amplified cDNA was 229 bp. PCR analysis was conducted as described above.

As a loading control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was amplified from total RNA. GAPDH primers were CCATGGAGAA GGCTGGGG (forward primer) and CAAAGTTGTCTATGGATGACC (reverse primer). The amplified product was 200 bp.

Analysis of the effects of LAT sRNAs on ICP4 or ICP0 expression. Cells were cotransfected with 2 µg of an ICP4 plasmid (PN11; obtained from P. Schaffer, University of Arizona) or 2 µg of an ICP0 expression plasmid (obtained from S. Silverstein, Columbia University) and 4 µg of sRNA expression plasmids. The empty vector or plasmid expressing the control small interfering RNA (siRNA) was also transfected with ICP0 or ICP4.

Transfections were performed in 60-mm dishes using cells seeded 24 h before. At the time of transfection, cultures were approximately 80% confluent. Cells were transfected using 3 µl of TransIT transfection reagent (Mirus) per microgram of DNA. After 24 or 48 h posttransfection, cells were harvested and total proteins were extracted with CellLytic MT mammalian reagent (Sigma). The efficiency of transfection was approximately 70%, as judged by the number of green fluorescent protein (GFP)-positive cells following transfection of a GFP expression vector. The lysis buffer contained one tablet of protease inhibitor cocktail (Sigma) for each 10 ml of buffer. Protein concentrations were measured using the Bradford assay (3).

Extracted proteins were loaded in a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, electrophoresis was performed, and proteins were transferred to a polyvinylidene difluoride membrane (Millipore). Western blotting was performed as previously described (64). Antiserum directed against ICP0 (H1A027-100) and ICP4 (H1A021-100) was purchased from Virusys Corp. The β-actin antibody was purchased from Santa Cruz Biotechnology.

Effects of LAT sRNAs on productive infection. RS cells were infected with dLAT2903 (LAT null mutant) (56) at a multiplicity of infection of 1.0. At 24 h after infection, the supernatant was collected and centrifuged at 7,000 rpm at 4°C for 20 min (10,000 × g). Virus particles were collected as a pellet by centrifugation (25,000 rpm for 3 h) in a 30% sucrose gradient. The pellet was suspended in Tris-EDTA buffer and treated with sodium dodecyl sulfate for 15 min at 37°C and with proteinase K for 30 min at 60°C. Samples were then extracted with phenol-chloroform-isoamyl alcohol (25:24:1) three times, and then three ether extractions were performed. Viral genomic DNA was then precipitated overnight at –80°C in 100% ethanol. The quality and quantity of viral DNA were determined by agarose gel electrophoresis (1%) and spectrophotometry (optical density at 260 nm).

RS cells were transfected with 2 µg of dLAT2903 genomic DNA and 4 µg of a plasmid expressing an sRNA or the designated negative controls. The efficiency of transfection was approximately 60%. Plates were subjected to three rounds of freezing and thawing (–80 to 37°C) at 48 h after transfection. Cellular debris was removed by centrifuging for 10 min (5,000 × g). The supernatant was used to perform plaque assays in RS cells.

Construction of plasmids that express LAT sRNAs. LAT sRNA sequences, wt or mutant, were synthesized by Integrated DNA Technology (Coralville, IA). The sRNAs contain BamHI and HindIII restriction enzyme sites at their 5' or 3' termini, respectively. The respective LAT sRNAs were cloned between the unique BamHI and HindIII sites of pSilencer 2.1-U6 neo (Ambion). The construct that contains wt LAT sRNA1 is referred to as pLATsRNA1; pLATsRNA2 contains the wt LAT sRNA2, pLAT1M contains the LAT sRNA1 with the ATG→TTG mutation, and pLAT2M contains the LAT sRNA2 with the ATG→TTG mutation (see Fig. 1B for the location of the ATG→TTG mutations). Sequencing of the respective inserts cloned into pSilencer 2.1-U6 neo confirmed that the expected LAT sRNA sequences were intact. An siRNA-expressing plasmid (Ambion) which expresses a hairpin siRNA with limited homology to known sequences in human, mouse, and rat genomes was used as a negative control.

Cold shock-induced apoptosis. neuro-2A cells were transfected with the designated plasmids using TransIT neural transfection reagent as described by the manufacturer (Mirus, Madison, WI). Approximately 75% of the cells were transfected when this protocol was performed with a GFP expression plasmid. For each experiment, a plate transfected with the GFP expression plasmid was included. If these cells were not efficiently transfected (as judged by estimating the percentage of GFP-positive cells), the cells were discarded and the experi-

FIG. 1. Schematic of HSV-1 and organization of the LAT locus. (A) The LAT locus is present in the unique long (UL) and unique short (US) regions. The primary 8.3-kb LAT is shown as a long arrow. The stable 2-kb LAT is shown as a solid rectangle. The LAT TATA box is denoted by TATA, and the small arrow and +1 indicate the start of LAT transcription (genomic nt 118801). The LAT promoter (LAP) is denoted by the gray rectangle. The relative locations of mRNAs encoding ICP0 and ICP34.5 are shown for reference. Expression of the first 1.5 kb of LAT coding sequences (+1,500) is sufficient for reactivation from latency (56). The relative locations of the two LAT sRNAs that were previously identified are denoted by asterisks (51). (B) Nucleotide sequences of the LAT sRNAs (51). The underlined ATGs correspond to the initiating ATGs of ORF4 (LAT sRNA1) or ORF8 (LAT sRNA2) (17). (C) Female Swiss Webster mice were infected with dLAT2903R (lane R) or dLAT2903 (lane 2903). At 30 days after infection, TG were extracted and sRNA was prepared by using the miRNA isolation kit (miVana; Ambion) according to the manufacturer's instructions. RT-PCR using adaptor primers and LAT-specific primers was used to examine sRNA expression, as described in Materials and Methods. (D) Double-stranded DNA corresponding to the respective sRNAs was synthesized and cloned into the siRNA expression plasmid, pSilencer 2.1-U6 neo (Ambion). neuro-2a cells were transfected with 5 μ g of a plasmid expressing LAT sRNA1 (lane 1), LAT sRNA2 (lane 2), or the empty pSilencer 2.1-U6 neo (lane E). At 40 h after transfection, sRNA was prepared using the mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions, and the expression of LAT sRNA was examined by RT-PCR. Following purification of sRNA by the mirVana kit, total sRNA (1 μ g) was electrophoresed on a 2% agarose gel to confirm that similar levels of RNA were used for RT-PCR amplification.

ment was repeated. Twenty-four hours prior to transfection, cells were plated at a density of 1×10^6 cells in complete growth medium in a T25 plastic flask. After transfection for 24 h, normal Earle's minimal essential medium was replaced with fresh medium containing 2% serum. After a 12-h incubation, cells containing 2% serum were incubated on ice (4°C) for 60 min with the caps of the flasks sealed using Parafilm. At the end of 1 h on ice, the caps were loosened, and flasks were incubated at 37°C for the designated amount of time. This protocol was modified slightly from our previous study (64) because we purchased a new viral cells from ATCC.

A total of 1×10^6 cells were collected, and apoptotic DNA was prepared as previously described (24, 32, 64). At the indicated times after cold shock-induced apoptosis, adherent and nonadherent cells were collected and pelleted by low-speed centrifugation ($2,000 \times g$ for 10 min at 4°C). Cell pellets were suspended in 400 μ l hypotonic buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 μ g/ml RNase, 2% Triton X-100) at 4°C for 2 h. Nuclei were removed by centrifugation ($10,000 \times g$ for 10 min at 4°C). Fragmented DNA in the supernatant was immediately loaded onto a DNA binding column (Sigma), centrifuged for 1 min at $12,000 \times g$, and washed with 500 μ l of wash solution (Sigma), and then the column was washed with 750 μ l of 70% ethanol. The bound DNA was eluted by adding 75 μ l Tris-EDTA buffer (pH 8.0) to the column and centrifuging the column at $12,000 \times g$ for 2 min.

Apoptotic DNA from 3×10^5 cells was loaded onto a 2% agarose gel. Using the Bio-Rad Molecular Imager FX, a high-resolution photograph of the ethidium bromide (EtBr)-stained gel was obtained. The intensity of the EtBr-stained DNA in each lane was measured using the Molecular Imager FX, and a raw value representing the intensity of the stained DNA was obtained. The values for the negative controls were set at 100%, and the values of other samples were normalized relative to the negative controls. When duplicate samples were examined using this protocol, we consistently observed the same levels of apoptotic DNA.

RESULTS

Expression of LAT sRNA in TG and cultured cells. A previous study identified two LAT sRNAs within the first 1.5 kb of LAT coding sequences (Fig. 1A and B) (51). To test whether these sRNAs were expressed in the TG of latently infected mice, female Swiss Webster mice were infected with a LAT null mutant that lacks the first 1.5 kb of the LAT coding sequences and the core LAT promoter (dLAT2903) or the

rescued virus (dLAT2903R) that has wt growth properties (56). A sRNA fraction was prepared at 30 days after infection as previously described (51). Thirty days after infection is operationally defined as latency because infectious virus was not detected from ocular swabs. LAT sRNA expression was analyzed by adding adapters to total sRNAs, and then LAT-specific cDNAs were amplified by using a primer that specifically amplifies LAT sRNA1 or sRNA2. This strategy was used to successfully clone cellular miRNAs (61), suggesting it would be useful for examining LAT sRNA expression in TG. LAT sRNA1 and sRNA2 were readily detected at 30 days after infection with dLAT2903R (Fig. 1C, lane R). Amplified products were the expected sizes based on the adapters and primers used to amplify the two sRNAs. LAT-specific bands were not detected following infection with dLAT2903 (lane 2903). Similar levels of RNA were used for RT-PCR, as judged by GAPDH levels in total RNA and by comparing total sRNA in the respective samples.

Double-stranded DNA oligonucleotides spanning LAT sRNA1 or sRNA2 were synthesized and cloned into pSilencer 2.1-U6 neo, an siRNA expression vector (Ambion). Expression of LAT sRNAs was examined in transiently transfected neuro-2A cells (Fig. 1D). Amplified LAT sRNA1 cDNA migrated as a 90-bp product (Fig. 1D, lane 1), and a LAT sRNA2 cDNA product migrated between 60 and 70 bp (Fig. 1D, lane 2). A LAT-specific amplified cDNA was not detected when the empty expression vector (pSilencer 2.1-U6 neo) was transfected into neuro-2A cells (Fig. 1D, lane E). Total sRNA levels used to detect LAT sRNAs were similar in the respective samples.

LAT sRNA2 inhibits ICP4 protein expression in transiently transfected cells. When the two LAT sRNAs were compared to the HSV-1 genome, ICP4 was predicted to be the most likely viral mRNA that would base pair with both LAT sRNAs (51), suggesting that LAT sRNA1 or sRNA2 inhibited ICP4 expression. To test whether LAT sRNAs inhibited ICP4 expression, RS cells (Fig. 2A) or neuro-2A cells (Fig. 2B) were cotransfected with an ICP4 expression plasmid and a plasmid expressing a LAT sRNA. Western blot analysis revealed that ICP4 protein levels were reduced following transfection of neuro-2A or RS cells with LAT sRNA2 (Fig. 2A and B, lane 2) relative to cells transfected with the plasmid expressing the negative control siRNA (Fig. 2A and B, lane NC) or the LAT sRNA1 expression plasmid (Fig. 2A and B, lane 1). In neuro-2A cells, we consistently observed that when wt LAT sRNA1 (lane 1), a mutant sRNA1 containing an ATG→TTG mutation (Fig. 2B, lane 1M), or a mutant LAT sRNA2 containing an ATG→TTG mutation (Fig. 2B, lane 2M) was included in the transfection mixture, ICP4 protein expression was slightly higher. For the location of ATG→TTG mutations in LAT sRNA1 or sRNA2, see Fig. 1B. LAT sRNA2 had no obvious effect on ICP4 RNA levels in RS cells (Fig. 2C, lane 2) or neuro-2A cells (data not shown), suggesting that LAT sRNA2 inhibited translation of ICP4 mRNA.

To test whether LAT sRNAs inhibited ICP0 protein expression, RS cells were cotransfected with an ICP0 expression plasmid and a plasmid expressing a LAT sRNA. LAT sRNA1 (Fig. 2D, lane 1) or LAT sRNA2 (Fig. 2D, lane 2) had no effect on ICP0 protein expression relative to the empty expression

FIG. 2. LAT sRNA2 inhibits ICP4 protein expression. Western blot analysis of ICP4 protein expression in RS cells (A), ICP4 protein expression in neuro-2A cells (B), or ICP0 expression in RS cells (D) was performed using commercially available antiserum, as described in Materials and Methods. Lane U, untransfected cells. Except for the U lane, all cells were transfected with 2 μ g of the PN11 plasmid expressing ICP4 (A to C) or the ICP0 expression plasmid (2 μ g plasmid) (D). Cultures were cotransfected with the following plasmids: 4 μ g of the empty siRNA vector (lane E), 4 μ g of plasmid expressing the negative control siRNA (lane NC), 4 μ g of the plasmid expressing LAT sRNA1 (lane 1), 4 μ g of the plasmid expressing LAT sRNA2 (lane 2), 2 μ g of each plasmid expressing LAT sRNA1 and LAT sRNA2 (lane B), 4 μ g of plasmid expressing mutant LAT sRNA1 (lane 1M), 4 μ g of plasmid expressing LAT sRNA1 and LAT sRNA2 (lane BM). β -Actin was used as a protein loading control (bottom). A total of 350 μ g of protein was loaded into each lane. As a positive control, RS cells were infected with dLAT2903 for 8 h, and total cell lysate was loaded (lane +). For panel C, total RNA was prepared and RT-PCR was performed using random primers. Amplification of ICP4 or GAPDH was performed using the primers described in Materials and Methods. Lane NT was a negative control that lacked template.

FIG. 3. LAT sRNA1 and sRNA2 inhibit productive infection in RS cells. RS cells were transfected with HSV-1 DNA (2 μ g of dLAT2903) and the designated plasmids that express LAT sRNAs. (A) Virus was collected from transfected cells at 48 h after transfection, and plaque assays were performed in RS cells. Bar NC, 4 μ g of a plasmid expressing the control siRNA; bar E, 4 μ g of the empty siRNA vector; bar 1, cells cotransfected with 4 μ g of the plasmid that expresses LAT sRNA1; bar 2, 4 μ g of the plasmid expressing LAT sRNA2; bar B, 2 μ g of each plasmid expressing LAT sRNA1 and LAT sRNA2; bar 1M, 4 μ g of the plasmid expressing mutant LAT sRNA1; bar 2M, 4 μ g of the plasmid expressing mutant LAT sRNA2; and bar BM, 2 μ g of each plasmid expressing mutant LAT sRNA1 and LAT sRNA2. The values are the averages of five independent experiments. Statistical analysis of the values was calculated using a *P* value unpaired test. (B) The designated samples were collected at 48 h after transfection, and Western blotting was performed using 600 μ g protein for ICP4 and β -actin. Lane U, cells not transfected with any plasmid or viral DNA; lane E, empty siRNA vector; lane 1, cells cotransfected with 4 μ g of the plasmid that expresses LAT sRNA1; lane 2, plasmid expressing LAT sRNA2; and lane +, cells infected with dLAT2903 (1 PFU/cell) for 8 h.

vector (Fig. 2D, lane E). Neither LAT sRNA had an effect on ICP0 RNA expression (data not shown).

LAT sRNA1 and sRNA2 inhibit productive infection. To test whether LAT sRNAs have an effect on productive infection, plasmids expressing LAT sRNA1 or sRNA2 were cotransfected with HSV-1 dLAT2903 genomic DNA, and the amount of virus produced in these cells was measured by plaque assays. dLAT2903 is a LAT null mutant virus (56), and consequently, the effects of each respective sRNA in the absence of endogenous LAT expression could be examined. Following transfection of RS cells with dLAT2903 and a plasmid expressing LAT sRNA1 (Fig. 3A, bar 1), the amount of infectious virus was reduced 2 to 3 logs at 48 h after transfection relative to the empty vector (Fig. 3A, bar E) or the plasmid expressing the control siRNA (Fig. 3A, bar NC). The difference between the amount of infectious virus in cells cotransfected with LAT sRNA1 and dLAT2903 genomic DNA was significant relative to that in cells cotransfected with dLAT2903 and a plasmid expressing the control siRNA or the empty vector ($P = 0.0001$). LAT sRNA2 reduced the amount of infectious virus

by three- to fivefold (Fig. 3A, bar 2), which was significantly different compared to the results obtained when the negative control was cotransfected with dLAT2903 ($P = 0.001$). Under the conditions of these studies, transfecting plasmids that express LAT sRNA1 and sRNA2 (Fig. 3A, bar B) with dLAT2903 did not have an additive or synergistic effect on the amount of infectious virus released relative to LAT sRNA1. However, the effect that both LAT sRNAs had on the amount of infectious virus released was significant relative to the two negative controls ($P < 0.0001$).

LAT sRNA1 containing the ATG→TTG mutation (Fig. 3A, bar 1M) and the empty vector had similar effects on the amount of infectious virus released ($P = 0.25$), but the difference between the mutant and wt sRNA1 was significant ($P = 0.0018$). There was no obvious difference between LAT sRNA2 and the mutated sRNA2 (Fig. 3A, bar 2M) ($P = 0.33$). Cotransfecting plasmids that express mutant LAT sRNA1 and sRNA2 (Fig. 3A, bar BM) did not have a significant effect on the amount of infectious virus released compared to the empty vector ($P = 0.33$). Similar trends were observed when these studies were performed in neuro-2A cells (data not shown).

Following transfection of the plasmid that expresses LAT sRNA2 with dLAT2903 (Fig. 3B, lane 2), ICP4 protein levels were reduced relative to samples cotransfected with the plasmid expressing LAT sRNA1 (Fig. 3B, lane 1) or the empty vector (Fig. 3B, lane E). Cells not transfected with dLAT2903 (Fig. 3B, lane U) do not express detectable levels of ICP4. As expected, β -actin protein levels were similar for all samples.

LAT sRNAs cooperate to inhibit cold shock-induced apoptosis. The first 1.5 kb of LAT coding sequences can inhibit apoptosis (28, 33, 50), suggesting that LAT sRNAs may influence apoptosis. To test this possibility, cold shock-induced apoptosis was used because this procedure reproducibly induces apoptosis in several different cell types (6, 25, 26, 39, 58, 59, 64). neuro-2A cells (Fig. 4A, lane 2A) or neuro-2A cells placed on ice for 1 h (Fig. 4A, lane 4°) contained no detectable DNA laddering (Fig. 4A). Within 2 h after returning cultures to 37°C, extensive DNA laddering was detected.

To test whether LAT sRNA1 and/or sRNA2 inhibited cold shock-induced apoptosis, apoptotic DNA levels were measured after cold shock-induced apoptosis. DNA laddering is a key biochemical hallmark of apoptosis, and we have developed a reproducible and quantitative assay to measure apoptotic DNA levels in agarose gels (32, 64). Relative to neuro-2A cells transfected with a vector that expresses a control siRNA supplied by Ambion (Fig. 4B, lane NC), DNA laddering was reduced approximately twofold when neuro-2A cells were cotransfected with plasmids that express LAT sRNA1 and sRNA2 (Fig. 4B, lane B). Cold shock-induced apoptosis was inhibited by LAT sRNA1 only when 6 μ g of the plasmid expressing LAT sRNA1 was transfected into neuro-2A cells (Fig. 4B, lane 6). Conversely, only 3 μ g of the plasmid encoding LAT sRNA1 was necessary to inhibit cold shock-induced apoptosis when cotransfected with the plasmid that expressed LAT sRNA2. The plasmid that expressed LAT sRNA2 (Fig. 4B, lane 2) was unable to inhibit apoptosis. As expected, the bovine herpesvirus 1 latency-related gene (Fig. 4B, lane LR) (11, 64) or Bcl-2 (Fig. 4B, lane Bcl) inhibited cold shock-induced apoptosis.

To confirm the results derived from DNA laddering assays

FIG. 4. Analysis of cold shock-induced apoptosis in neuro-2A cells. (A) neuro-2A cells were subjected to cold shock-induced apoptosis, as described in Materials and Methods. Apoptotic DNA was isolated from 3×10^5 cells/dish and electrophoresed in a 2% agarose gel. DNA was visualized after staining with EtBr and then photographed. Lane 2A represents actively growing neuro-2A cells and the 4° lane represents neuro-2A cells incubated at 4°C for 1 h. Cells were subjected to cold shock followed by incubation at 37°C for 1, 1.5, 2, 3, and 4 h, respectively, to induce apoptosis. The results are representative of more than 20 independent studies. (B) neuro-2A cells were transfected with pcDNA3.1 that expresses the BHV-1 LR gene (6 μ g plasmid) (lane LR), the Bcl-2 gene (6 μ g plasmid) (lane Bcl), plasmids expressing both LAT siRNAs (3 μ g of each plasmid) (lane B), increasing concentrations of the siRNA expression plasmid containing LAT sRNA1, or the plasmid expressing LAT sRNA2 (6 μ g plasmid DNA) (lane 6). neuro-2A cells transfected with pSilencer 2.1-U6 neo expressing the control siRNA were used as a negative control (6 μ g plasmid) (lane NC). To maintain equal amounts of plasmid DNA, certain cultures were cotransfected with the empty siRNA expression vector. The relative amount (Rel.) of apoptotic DNA in the respective lanes from panel B was measured using Bio-Rad Molecular Imager FX. The results are representative of at least five independent experiments.

(Fig. 4), the number of cells containing sub-G₁ DNA was measured by fluorescence-activated cell sorter analysis. Relative to mock-transfected cells or cells transfected with the plasmid expressing the control siRNA (Fig. 5A, bar M or NC, respectively), cotransfection with LAT sRNA1 and sRNA2 significantly decreased the number of cells containing sub-G₁

FIG. 5. LAT sRNAs inhibit cell death. neuro-2A cells (3×10^5) were transfected with the designated plasmids. (A) Following cold shock-induced apoptosis, nuclear DNA was stained with propidium iodide (50 μ g/ml) and fluorescence-activated cell sorter analysis was performed to measure the sub-G₁ levels of DNA. The values are the averages of five independent experiments. Column M, mock-transfected cells; bar NC, empty siRNA expression plasmid (6 μ g DNA); bar 1, 3 μ g of the plasmid expressing LAT sRNA1 and 3 μ g of the empty siRNA expression plasmid; bar 2, 3 μ g of the plasmid expressing LAT sRNA2 and 3 μ g of the empty siRNA expression plasmid; bar B, 3 μ g each of the LAT sRNA expression plasmids; and bar Bcl-2, 3 μ g of a Bcl-2 expression plasmid and 3 μ g of the empty siRNA expression plasmid. Asterisks denote significant differences ($P < 0.05$) from the NC and M values as determined by the Student *t* test. (B) Cold shock-induced apoptosis was performed at 40 h after transfection. After a 4-h incubation at 37°C, trypan blue exclusion was performed to identify dead cells. The number of cells that were not stained with trypan blue is shown. Bar E, empty siRNA expression plasmid (6 μ g DNA); bar NC, plasmid expressing the control siRNA (6 μ g DNA); bar 1, 3 μ g of the plasmid expressing LAT sRNA1 and 3 μ g of the empty siRNA vector; bar 2, 3 μ g of the plasmid expressing LAT sRNA2 and 3 μ g of the empty siRNA vector; bar B, 3 μ g each of the LAT sRNA expression plasmids; bar Bcl-2, 3 μ g of a Bcl-2 expression plasmid and 3 μ g of the empty siRNA vector; bar U, cells not cold shock treated. Asterisks denote significant differences ($P < 0.05$) from the NC and E values as determined by the Student *t* test.

levels of DNA after cold shock-induced apoptosis ($P < 0.05$) (Fig. 5A, bar B). This study also confirmed that both sRNAs cooperated to inhibit apoptosis compared to cells transfected with 3 μ g of LAT sRNA1 or sRNA2 (Fig. 5A, bar 1 or 2, respectively). As expected, Bcl-2 (Fig. 5A, bar Bcl-2) reduced the numbers of cells containing sub-G₁ levels of DNA.

Trypan blue exclusion studies were also performed after cold shock-induced apoptosis. Following cold shock-induced apoptosis, cells transfected with 3 μ g of LAT sRNA1 and

FIG. 6. Cold shock treatment of neuro-2A cells transfected with mutant LAT sRNAs. (A) neuro-2A cells were transfected with an empty cytomegalovirus (CMV) expression plasmid, pcDNA3.1 (6 μ g plasmid DNA) (bar E), 6 μ g of pSilencer 2.1-U6 neo expressing the control siRNA (bar NC), 6 μ g of the wt LAT sRNA1 (bar 1), plasmid expressing the mutated LAT sRNA1 (3 or 6 μ g plasmid) (bars 1M), 6 μ g of an siRNA expression plasmid expressing the mutant LAT sRNA2 (bar 2M), plasmids expressing both mutated LAT sRNAs (3 μ g of each plasmid) (bar BM), plasmids expressing both LAT siRNAs (3 μ g of each plasmid) (bar B), or 6 μ g of a CMV expression plasmid that contains the antiapoptosis Bcl-2 gene (bar Bcl). Bar 2A shows the results from actively growing neuro-2A cells. Cold shock-induced apoptosis, collection of apoptotic DNA, and analysis of apoptotic DNA were performed as described in Materials and Methods. The relative amount of apoptotic DNA shown by the respective lanes was measured using Bio-Rad Molecular Imager FX. The results are the mean of five independent experiments. Asterisks denote significant differences ($P < 0.05$) from the NC and E values as determined by the Student *t* test.

sRNA2 (Fig. 5B, bar B) contained more cells that excluded trypan blue staining than cells transfected with 3 μ g of the plasmid containing LAT sRNA1 (Fig. 5B, bar 1), 6 μ g of the empty vector (Fig. 5B, bar E), 6 μ g of the control siRNA plasmid (Fig. 5B, bar NC), or 3 μ g of the plasmid containing LAT sRNA2 ($P < 0.05$) (Fig. 5B, bar 2).

Mutagenesis of LAT sRNAs impairs their antiapoptosis activities. When the initiating ATGs of all eight open reading frames (ORFs) within the first 1.5 kb of LAT coding sequences are mutated to TTG, the antiapoptosis functions of LAT are reduced to background levels (7). LAT sRNA1 and LAT sRNA2 contain the initiating ATG of ORF4 or ORF8, respectively (17). To test whether the ATG→TTG mutations affected the antiapoptosis activities of the LAT sRNAs, the antiapoptosis activities of siRNA plasmids expressing the two mutated LAT sRNAs were compared to that of plasmids expressing wt sRNAs. Increasing concentrations of the mutated LAT sRNA1 (Fig. 6, bars 3 and 6 [1M]) had no inhibitory effect on cold shock-induced apoptosis compared to the siRNA vector expressing the nonrelevant siRNA (Fig. 6, bar NC). As expected, 6 μ g of the plasmid expressing wt LAT sRNA1 (Fig. 6, bar 1) inhibited cold shock apoptosis but not as efficiently as Bcl-2 (Fig. 6, bar Bcl).

Plasmids expressing the two mutant LAT sRNAs (Fig. 6, bar BM) or the mutant LAT sRNA2 (Fig. 6, bar 2M) had no

detectable antiapoptosis activity. As expected, plasmids expressing wt LAT sRNA1 and LAT sRNA2 (Fig. 6, bar B) protected neuro-2A cells from cold shock-induced apoptosis.

DISCUSSION

These studies suggested that expression of LAT sRNA1 and sRNA2 plays a role in the latency-reactivation cycle because the sRNAs inhibited productive infection and apoptosis. Both sRNAs are located within the first 1.5 kb of LAT coding sequences, which is required for and sufficient for producing high levels of the wt reactivation phenotype in small animal models (54–56). Additional genetic studies support a role for expression of the LAT sRNAs in the latency-reactivation cycle. For example, a recombinant virus that expresses just the first 811 bases of LAT coding sequences, and thus lacks the coding sequences for LAT sRNA1 and sRNA2, has reduced reactivation from latency (18, 28). Recombinant viruses containing more extensive LAT deletions reactivate from latency similar to a LAT null mutant (18, 28).

LAT sRNA2, but not LAT sRNA1, inhibited ICP4 protein expression in transiently transfected cells. Although sequences in LAT sRNA2 do not overlap ICP4 RNA sequences, extensive base pairing can potentially occur between LAT sRNA2 and the 3' terminus of ICP4 mRNA (51). In spite of the potential for LAT sRNA1 and sRNA2 to base pair with ICP0 mRNA (51), neither LAT sRNA inhibited ICP0 expression in the cell types examined. Since ICP4 is required for productive infection (16), it was not surprising to find that LAT sRNA2 inhibited productive infection. A LAT miRNA (miR-H6) located outside of the first 1.5 kb of LAT coding sequences also inhibits ICP4 protein expression, but not ICP4 mRNA levels (67). Reduction of ICP4 protein levels in the context of productive infection can have dramatic effects because an HSV-1 mutant that lacks both copies of ICP4 and has a defective Us3 gene (d120) induces high levels of apoptosis (21, 22, 41, 42, 47). When PC-12 cells are differentiated with nerve growth factor, the d120 mutant does not induce apoptosis and actually promotes cell survival relative to mock-infected cells (2). The ability of LAT sRNA2 and miR-H6 to inhibit ICP4 protein levels may help promote establishment or maintenance of latency by inhibiting lytic gene expression and protecting infected neurons from cell death.

Relative to LAT sRNA2, sRNA1 inhibited productive infection more efficiently, but had no obvious effect on ICP0 or ICP4 protein expression. LAT sRNA1 was also predicted to hybridize with VP16 (a tegument protein that stimulates immediate early gene expression) and UL8 (a protein necessary for viral DNA replication) mRNA, suggesting that expression of one or both of the associated viral genes may be reduced by LAT sRNA1 (51). LAT sRNA1 may also inhibit productive infection by inhibiting expression of a cellular protein that is necessary for productive infection. Finally, LAT sRNA1, due to its potential to form secondary structures that contain double-stranded RNA domains (51), may enhance innate immune responses. However, LAT sRNA1 does not appear to stimulate beta interferon promoter activity (T. Jaber, unpublished data). Studies designed to understand the mechanism by which LAT sRNA1 inhibits productive infection are currently under way.

The ability of the first 1.5 kb of LAT to promote reactivation from latency correlates with its ability to inhibit apoptosis because plasmids that express the first 1.5 kb of LAT coding sequences inhibit apoptosis (1, 27, 28, 33, 49, 53). Conversely, plasmids that express only the first 811 bases of LAT have reduced antiapoptosis functions, and furthermore, deletions of LAT have no antiapoptosis activity (28). Single-point mutations within LAT sRNA1 and sRNA2 reduced the ability of these sRNAs to inhibit apoptosis, which is in agreement with a previous study demonstrating that the same mutations in a large LAT fragment impacted its antiapoptosis functions (7). Although the single-point mutations appear to alter the putative secondary structure of these sRNAs (data not shown), it cannot be ruled out that these mutations had other effects. For example, the ATG-TTG mutations disrupt the 12-codon ORF within LAT sRNA1 and the 6-codon ORF within LAT sRNA2 (Fig. 1B).

At least three other viruses encode small noncoding RNAs with antiapoptosis activities. For example, a small non-protein coding RNA encoded by the Sendai virus inhibits apoptosis (29). Second, Epstein-Barr virus encodes sRNAs (EBER1 and EBER2) that are 167 or 172 nt, respectively. EBERs are abundantly expressed in latently infected B cells and can inhibit interferon-induced apoptosis (63). Finally, human cytomegalovirus encodes a non-protein coding RNA that inhibits cell death induced by mitochondria (60). A number of cellular sRNAs can also regulate cell death (8, 20, 37, 66, 72). In neuro-2A cells, cold shock-induced apoptosis is inhibited if caspase-9 or caspase-3 inhibitors, but not a caspase-8 inhibitor, are added to neuro-2A cells prior to shifting cells to 4°C (64). This suggested that LAT sRNAs inhibited the intrinsic pathway of apoptosis in neuro-2A cells following cold shock-induced apoptosis. Future studies will attempt to identify the precise step in the apoptosis signaling pathway that is inhibited by LAT sRNA1 and what role LAT sRNA2 plays in this process.

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