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Jin, Ling; Peng, Weiping; Perng, Guey-Chuen; Brick, David J.; Nesburn, Anthony B.; Jones, Clinton J.; and Wechsler, Steven L., "Identification of Herpes Simplex Virus Type 1 Latency-Associated Transcript Sequences That both Inhibit Apoptosis and Enhance the Spontaneous Reactivation Phenotype" (2003). *Papers in Veterinary and Biomedical Science*. 59.
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Identification of Herpes Simplex Virus Type 1 Latency-Associated Transcript Sequences That both Inhibit Apoptosis and Enhance the Spontaneous Reactivation Phenotype

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Received 5 November 2002/Accepted 6 March 2003

The herpes simplex virus type 1 (HSV-1) latency-associated transcript (LAT) gene is essential for the high spontaneous and induced reactivation phenotype of HSV-1 in the rabbit ocular model and for the high induced reactivation phenotype in the mouse ocular model. Recently we showed that LAT has an antiapoptosis function, and we hypothesized that LAT's ability to inhibit apoptosis played an important role in LAT's ability to enhance the reactivation phenotype. Expression of just the first 1.5 kb of the 8.3-kb LAT gene is sufficient for both inhibition of apoptosis in an *in vitro* transient-transfection assay and the high spontaneous reactivation phenotype *in vivo*. Here we show the results of more complex mapping studies in which inhibition of apoptosis and the enhanced spontaneous reactivation phenotype also appear to be linked. The HSV-1 mutant virus dLAT371 has a high spontaneous reactivation phenotype in rabbits, suggesting that the LAT region deleted in this mutant (LAT nucleotides 76 to 447) is not required for this phenotype. The LAT3.3A viral mutant (which expresses LAT nucleotides 1 to 1499) also has a high spontaneous reactivation phenotype, suggesting that the region of LAT not expressed by this mutant (LAT nucleotide 1500 to the end of LAT) is also not required for this phenotype. Surprisingly, LAT2.9A, which is a combination of dLAT371 and LAT3.3A (i.e., it expresses LAT nucleotides 1 to 76 and 447 to 1499), has a low spontaneous reactivation phenotype indistinguishable from that of LAT null mutants. We report here that consistent with the low spontaneous reactivation phenotype of LAT2.9A, a plasmid expressing the identical LAT RNA did not inhibit caspase 9-induced apoptosis. In contrast, plasmids containing the same deletion but able to transcribe up to or past LAT nucleotide 2850 (rather than just up to LAT nucleotide 1499) inhibited caspase 9-induced apoptosis, consistent with the high spontaneous reactivation phenotype of dLAT371. Thus, LAT2.9A may have a low spontaneous reactivation phenotype because the LAT RNA that is made cannot block apoptosis, and dLAT371 apparently has a high spontaneous reactivation phenotype because the LAT RNA made has significant antiapoptosis activity. Furthermore, LAT appeared to have at least two regions capable of interfering with caspase 9-induced apoptosis. One region partially overlaps LAT nucleotides 76 to 447. The second region is partially (or completely) downstream of LAT nucleotide 1499.

Following ocular herpes simplex virus type 1 (HSV-1) infection, the virus travels up nerves to the trigeminal ganglia (TG), where it establishes a latent infection lasting for life. A hallmark of HSV-1 latency is sporadic reactivation and the return of the virus to the original peripheral site of infection, where recurrent disease may result. When HSV-1 is latent in the TG, the recurrent disease following reactivation can lead to scarring of the cornea and loss of sight. In developed nations, HSV-1 is the most common cause of corneal blindness due to an infectious agent (9). How HSV-1 establishes, maintains, and reactivates from latency remains to be fully elucidated.

During neuronal latency, a single HSV-1 gene is abundantly transcribed. This gene, designated the latency-associated transcript (LAT), is located in the long repeat region of the viral genome and is thus diploid (i.e., present in two copies per genome). The primary LAT transcript is 8.3 kb and gives rise

to a family of LAT RNAs, including the very stable 2-kb LAT (15, 18, 19), which appears to be an intron produced by splicing (4).

HSV-1 LAT null mutants that are unable to transcribe any of the LAT gene have reduced reactivation phenotypes. Thus, LAT transcription-negative mutants reactivate poorly by induced reactivation in the mouse (7, 8, 16), by induced reactivation in the rabbit (2, 17), and by spontaneous reactivation in the rabbit (11, 12). Thus, a high reactivation phenotype requires LAT. The term reactivation phenotype is used here because these experiments use reactivation as a phenotype without regard to whether LAT enhances this phenotype directly (i.e., affects the reactivation process), indirectly (i.e., affects establishment or maintenance of latency, thereby increasing the pool of latently infected neurons), or both.

LAT3.3A, an HSV-1 strain McKrae mutant expressing just the first 1.5 kb of the primary 8.3-kb LAT has a high, McKrae-like spontaneous reactivation phenotype in rabbits (3, 12). Thus, the first 1.5 kb of LAT alone is sufficient for high levels of spontaneous reactivation in the rabbit (12). Comparative sequence analysis of this region suggests that there is no well-

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conserved LAT open reading frame (3). Thus, unless unreported splicing occurs in this region, it is unlikely that LAT's function is due to a LAT protein. The primary LAT completely overlaps the important immediate-early gene ICP0 in an antisense direction (20), suggesting an antisense regulatory role for LAT. However, the first 1.5 kb of LAT does not overlap any of ICP0. Thus, antisense repression of ICP0 by LAT is not essential for LAT's ability to enhance the reactivation phenotype in the rabbit ocular model.

Recently we showed that rabbits ocularly infected with the LAT null mutant dLAT2903 had significantly increased apoptosis in their TG compared to rabbits ocularly infected with wild-type or marker-rescued virus (10). This difference was observed on days 7 and 10 postinfection, at the end of the acute infection (establishment of latency), but not on day 3 postinfection, during peak acute infection. This suggests that LAT, the only viral gene remaining highly active following the acute infection, was protecting against virus-induced apoptosis. No difference was seen between LAT⁻ and LAT⁺ viruses on day 3 postinfection, because HSV-1 has several antiapoptosis genes that are expressed at this time. We have also shown that plasmids expressing LAT have antiapoptosis activity in vitro (6, 10). LAT's antiapoptosis activity both in vitro and in mice has been confirmed by others (1; S. E. Straus, personal communication).

We also showed that a plasmid expressing just the first 1.5 kb of LAT can efficiently block apoptosis induced by a variety of chemicals (6). This is consistent with the wild-type reactivation phenotype of LAT3.3A, which also expresses just the first 1.5 kb of LAT, and strengthens the hypothesis that LAT's antiapoptosis activity is functionally related to its ability to enhance the reactivation phenotype.

In this report we try to shed some light on an unexplained anomaly that occurred during our spontaneous reactivation phenotype mapping in rabbits. dLAT371 expresses the entire 8.3-kb primary LAT except for a 371-nucleotide deletion from LAT nucleotides 76 to 447. In rabbits this mutant has a wild-type spontaneous reactivation phenotype, suggesting that the region of LAT from nucleotides 76 to 447 is not required for this phenotype (13). LAT3.3A expresses just the first 1.5 kb of LAT from an ectopic location in the genome of an otherwise LAT null mutant. This mutant also has a wild-type spontaneous reactivation phenotype (12). A third mutant, LAT2.9A (14), is identical to LAT3.3A except that LAT nucleotides 76 to 447 are deleted as in dLAT371. Since the dLAT371 data indicated that LAT nucleotides 76 to 447 are not required and since the LAT3.3A data suggested that LAT nucleotides downstream of nucleotide 1500 are not required, it was expected that LAT2.9A, like LAT3.3A and dLAT371, would have a wild-type spontaneous reactivation phenotype. Instead, LAT2.9A has a significantly reduced spontaneous reactivation phenotype, indistinguishable from that of the LAT null mutant dLAT2903 (14).

We report here that a plasmid expressing the identical region of LAT expressed in LAT2.9A (nucleotides 1 to 76 plus 447 to 1,499) had no significant antiapoptosis activity in transient-transfection assays. In contrast, plasmids similar to the LAT expressed by dLAT371 (containing the same deletion but also containing at least 1.3 kb of additional LAT coding sequences) had significant antiapoptosis activity. These results

suggest that LAT2.9A has a LAT null spontaneous reactivation phenotype because the LAT RNA made cannot block apoptosis, while dLAT371 has a wild-type spontaneous reactivation phenotype because the LAT RNA made has significant antiapoptosis activity. These results strengthen the mapping correlation between LAT's antiapoptosis activity and its ability to enhance the reactivation phenotype and therefore add additional strong support for the hypothesis that LAT's antiapoptosis activity is functionally related to its ability to enhance the reactivation phenotype. These results also suggest that LAT contains at least two regions capable of inhibiting apoptosis and that sequences between nucleotides 76 and 447 are important for the antiapoptotic activity when only the first 1,500 nucleotides are expressed.

Structures of LAT in mutant viruses referred to in this study. The viruses referred to in this report are shown schematically in Fig. 1. McKrae is the wild-type parent for all of the mutants. dLAT2903 is a true LAT null mutant that makes no LAT RNA. Both copies of LAT (one in each viral long repeat) have the core LAT promoter, a putative secondary LAT promoter just upstream of the 2-kb LAT, and the first 1,667 nucleotides of the primary LAT RNA deleted (11). This mutant has a significantly reduced spontaneous reactivation phenotype, indicating that LAT is essential for the high wild-type spontaneous reactivation phenotype in the rabbit infection model. dLAT371 contains a deletion in both copies of LAT corresponding to a *SylI-SylI* restriction fragment from LAT nucleotides 76 to 447. This mutant expresses normal amounts of the remainder of LAT and has a wild-type spontaneous reactivation phenotype (13). This suggests that LAT nucleotides 76 to 447 are not required for the high spontaneous reactivation phenotype. LAT3.3A contains a LAT restriction fragment inserted into the UL region of dLAT2903 between the UL37 and UL38 genes. The LAT insert contains the entire LAT promoter and the first 1.5 kb of the primary LAT RNA. LAT3.3A effectively has LAT nucleotides 1500 to 8324 deleted, as it expresses just the first 1.5 kb of the primary LAT (LAT nucleotides 1 to 1499). This region contains approximately 660 nucleotides upstream of the 2-kb LAT and the first 838 nucleotides of the 2-kb LAT. This mutant has a high spontaneous reactivation phenotype, demonstrating that the first 1.5 kb of LAT is sufficient to produce a wild-type spontaneous reactivation phenotype and that LAT nucleotides 1500 to 8324 are not required (12). LAT2.9A is identical to LAT3.3A except that the LAT insert contains the same *SylI-SylI* deletion (LAT nucleotides 76 to 447) as dLAT371. Since the wild-type spontaneous reactivation phenotypes of dLAT371 and LAT3.3A showed that LAT nucleotides 76 to 447 and 1500 to 8324 are not required for the wild-type spontaneous reactivation phenotype, it was expected that LAT2.9A, which contains both deletions, would have a wild-type spontaneous reactivation phenotype. However, unexpectedly, LAT2.9A has a reduced spontaneous reactivation phenotype indistinguishable from that of dLAT2903 (14).

LAT-containing plasmids used in this study. The regions of LAT expressed by the viruses and plasmids relevant to this report are shown schematically in Fig. 2. Figure 2A shows the full-length 8.3-kb primary LAT expressed by wild-type McKrae. Figure 2B shows the LAT RNAs expressed by the dLAT371 mutant and by two corresponding plasmids that have the same *SylI-SylI* deletion. pLAT(1-4658) Δ Syl expresses the

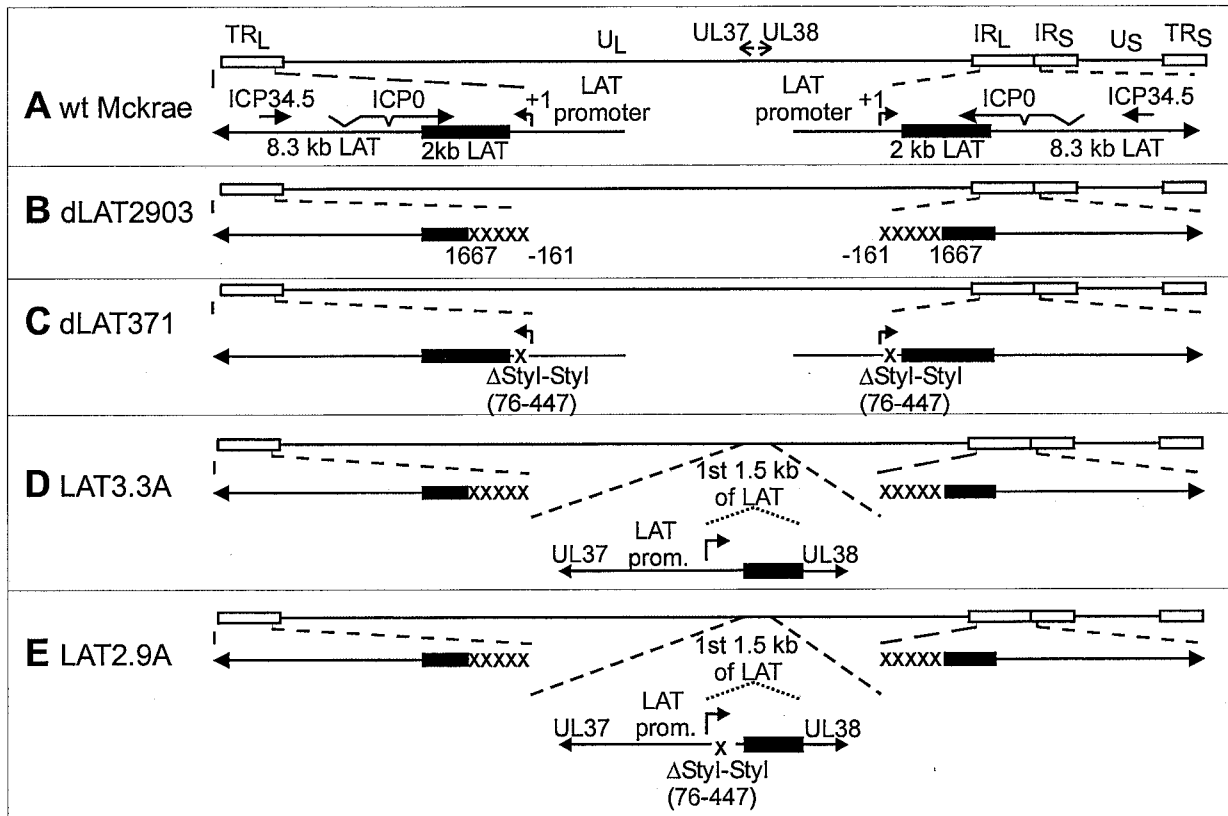


FIG. 1. Schematic representation of wild-type and mutant viruses. (A) The prototypic HSV-1 genomic structure of wild-type (wt) McKrae is shown at the top. The viral repeat regions are shown as open rectangles. TR_L, terminal long repeat; IR_L, internal (or inverted) long repeat; TR_S, terminal short repeat; IR_S, internal (or inverted) short repeat. The unique long (U_L) and the unique short (U_S) regions are each represented by a solid line. Below the genomic structure, the LAT region (one in each long repeat) is shown in expanded form. The primary 8.3-kb LAT is shown as a long arrow. The stable 2-kb LAT is shown as a solid rectangle. +1 and the small arrow indicate LAT nucleotide +1, the start of LAT transcription. The relative locations of the ICP0 and ICP34.5 mRNAs are shown for reference. (B) dLAT2903 is a LAT null mutant. It contains a deletion (xxxxxx) in both copies of LAT from LAT nucleotide -161 to +1667 and is therefore missing the core LAT promoter, a putative secondary LAT promoter just upstream of the 2-kb LAT, and the first 1,667 nucleotides of the primary LAT RNA (11). (C) dLAT371 contains a deletion in both copies of LAT corresponding to a *StyI-StyI* restriction fragment from LAT nucleotides 76 to 447 (13). (D) LAT3.3A contains a LAT restriction fragment inserted into the U_L region of dLAT2903 between the UL37 and UL38 genes. The LAT insert contains the entire LAT promoter and the first 1.5 kb of the primary LAT RNA. This virus transcribes only the first 1.5 kb of LAT (12). (E) LAT2.9A is identical to LAT3.3A except that the LAT insert contains the same *StyI-StyI* deletion (LAT nucleotides 76 to 447) as dLAT371 (14).

first 4,658 LAT nucleotides except for nucleotides 76 to 447 (*StyI-StyI*). pLAT(1-2850) Δ Sty expresses the first 2,850 LAT nucleotides except for nucleotides 76 to 447. Figure 2C shows the LAT RNA expressed by the LAT3.3A virus and the identical LAT RNA expressed by the plasmid pLAT(1-1499). Figure 2D shows the LAT RNA expressed by the LAT2.9A virus and the identical LAT RNA expressed by the plasmid pLAT(1-1499) Δ Sty (LAT nucleotides 1 to 76 plus 447 to 1499). Figure 2E shows plasmids corresponding to those in Fig. 2B, except that they do not contain the *StyI-StyI* deletion.

Antiapoptosis activities of the LAT plasmids. Neuro-2A cells were cotransfected with a plasmid expressing caspase 9 (to induce apoptosis), a plasmid expressing β -galactosidase (an indicator of which cells were transfected), and the indicated LAT plasmid (Fig. 3). Previous studies have demonstrated that a LAT plasmid expressing the first 4.8 kb of LAT inhibits caspase 9-induced apoptosis in transiently transfected Neuro-2A cells (5). The cultures were stained for β -galactosidase 72 h later, and the number of β -galactosidase-positive cells were

counted. The number of β -galactosidase-positive cells obtained when cells were transfected with the empty plasmid plus the β -galactosidase plasmid was set to 100%. pLAT(1-1499), which expresses the same LAT RNA as the virus LAT3.3A with a high spontaneous reactivation phenotype, significantly protected against caspase 9-induced apoptosis. This mapped a LAT function capable of inhibiting caspase 9-induced apoptosis to within the same 1.5-kb region capable of producing a wild-type spontaneous reactivation phenotype in rabbits. This was consistent with our previous study in which we mapped a LAT function capable of inhibiting sodium butyrate-, etoposide-, and Bax-induced apoptosis to the same location (6).

The large plasmids containing the same *StyI-StyI* deletion as the dLAT371 virus with a wild-type spontaneous reactivation phenotype [pLAT(1-4658) Δ Sty and pLAT(1-2850) Δ Sty] also provided significant protection against caspase 9-induced apoptosis. There was no significant difference among these three plasmids (Fig. 3) ($P > 0.05$). This showed another correlation between LAT RNA's antiapoptosis activity and its ability to

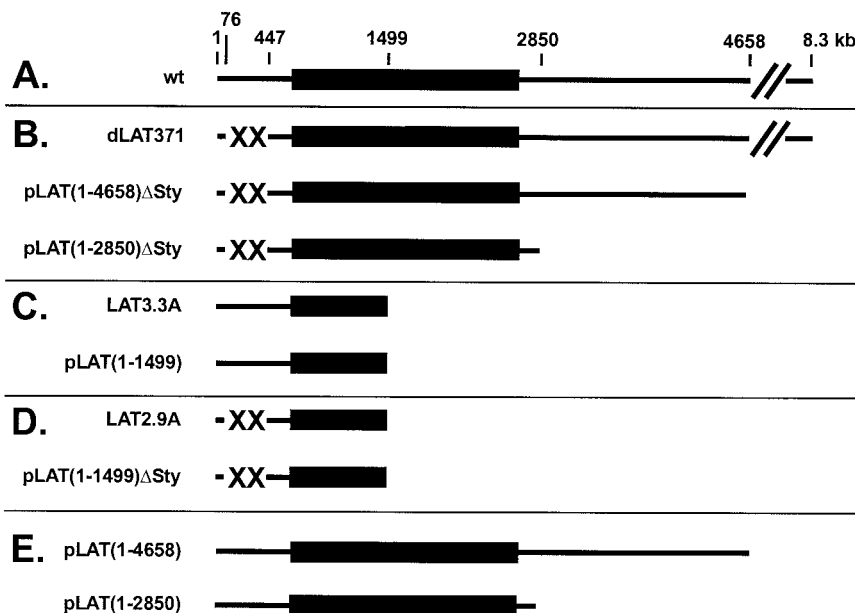


FIG. 2. Schematic representation of LAT-containing plasmids. (A) Wild-type (wt) 8.3-kb primary LAT. The numbers show relative LAT nucleotide locations. The pair of diagonal lines between 4658 and 8.3 kb depicts a break in the sequence, and thus this region is not drawn to scale. The solid rectangle indicates the location of the stable 2-kb LAT. (B) dLAT371 indicates the RNA made by this mutant virus. This RNA has LAT nucleotide 76 to 447 deleted (xx) but is otherwise full length. pLAT(1-4658) Δ Sty indicates the LAT RNA expressed by this plasmid. The same *SlyI-SlyI* region is deleted, and the transcript terminates at LAT nucleotide 4658. pLAT(1-2850) Δ Sty indicates the LAT RNA expressed by this plasmid. This contains the same *SlyI-SlyI* deletion, and the RNA terminates at LAT nucleotide 2850. (C) LAT3.3A indicates the LAT RNA (LAT nucleotides 1 to 1499) made by this mutant virus. pLAT(1-1499) indicates the identical RNA made by this plasmid. (D) LAT2.9A indicates the LAT RNA (LAT nucleotides 1 to 76 plus 447 to 1499) made by this mutant virus. pLAT(1-1499) Δ Sty indicates the identical LAT RNA expressed by this plasmid. (E) pLAT(1-4658) and pLAT(1-2850) are the same as pLAT(1-4658) Δ Sty and pLAT(1-2850) Δ Sty in panel B, except that these plasmids do not have the *SlyI-SlyI* region (LAT nucleotides 76 to 447) deleted.

support the high spontaneous reactivation phenotype. In contrast, pLAT(1-1499) Δ Sty, which expresses the same LAT RNA as the poorly reactivating LAT2.9A virus, did not provide significant protection against caspase 9-induced apoptosis ($P < 0.05$ compared to the first three plasmids; $P > 0.05$ compared to empty plasmid). This showed a correlation between an inability to block apoptosis and an inability to support the high spontaneous reactivation phenotype for this LAT RNA.

LAT expression in all of the LAT-containing plasmids was driven by the LAT promoter. Therefore, similar amounts of LAT RNA should be transcribed following transfection. In the caspase 9 and β -galactosidase plasmids used here, expression was driven by the cytomegalovirus (CMV) promoter. We previously showed that LAT does not alter activity of this promoter (6). Thus, the amounts of caspase 9 and β -galactosidase were not affected by the LAT plasmids.

The mutant viruses LAT3.3A and LAT2.9A express LAT RNAs identical to those of pLAT(1-1499) and pLAT(1-1499) Δ Sty, respectively, from an ectopic insert. We previously showed that the steady-state levels of LAT(1-1499) RNA and LAT(1-1499) Δ Sty RNA made by these viruses in tissue culture are indistinguishable (14). We also found similar steady-state levels of these transcripts in TG of rabbits latently infected with LAT3.3A or LAT2.9A (14). Thus, the *SlyI-SlyI* deletion (Δ LAT 76-447) does not appear to significantly alter the transcription or stability of these LAT RNAs. To confirm that the same would be true for the corresponding plasmids used above, we transfected Neuro-2A cells with either pLAT(1-

1499) or pLAT(1-1499) Δ Sty as was done for the transient-transfection assays. We then performed semiquantitative reverse transcriptase PCR (RT-PCR) with the same primers and conditions that we previously used with the LAT3.3A and LAT2.9A viruses discussed above. These primers amplify a 160-bp product specific for a region located downstream of the *SlyI-SlyI* deletion and upstream of the start of the 2-kb LAT (LAT nucleotides 471 to 631) (14). The amounts of the RT-PCR product were similar with both plasmids (Fig. 4, lanes 1 and 2). No product was detected when RT was left out of the reaction (Fig. 4, lanes 3 and 4). The RT-PCR product comigrated with the PCR products obtained from the pLAT(1-1499) plasmid and from wild-type virus (lanes 5 and 6). The internal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RT-PCR control was similar in both extracts (lanes 7 and 8). Similar results were obtained in three separate experiments. Thus, the reduced LAT antiapoptosis activity seen with pLAT(1-1499) Δ Sty was unlikely to be the result of reduced levels of LAT RNA compared to pLAT(1-1499).

Two additional plasmids, pLAT(1-2850) and pLAT(1-4658) (Fig. 2E), were included in these experiments. The LAT regions in these plasmids correspond to the LAT regions in pLAT(1-2850) Δ Sty and pLAT(1-4658) Δ Sty, except that the *SlyI-SlyI* region (LAT nucleotides 76 to 447) is not deleted. Both of these plasmids had similar antiapoptosis activity in this assay, and this activity was significantly higher than that of the corresponding Δ Sty plasmids (Fig. 3).

Thus, the plasmids used here fell into three distinct groups

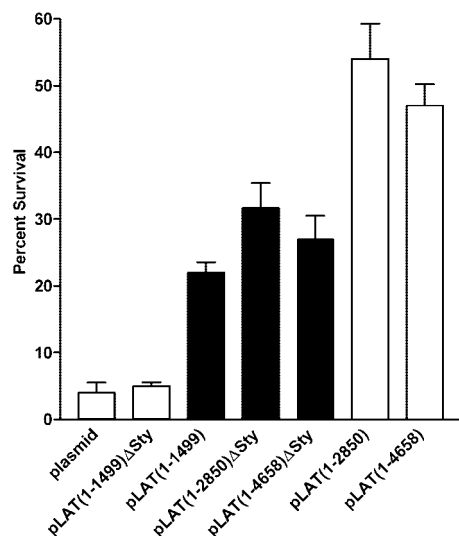


FIG. 3. Inhibition of caspase 9-induced apoptosis by plasmids expressing various portions of LAT. Neuro-2A cells were cotransfected with 4 μ g of the indicated LAT plasmid (or empty plasmid), 2 μ g of a plasmid expressing caspase 9 (5), and 1 μ g of a plasmid expressing β -galactosidase (pCMV- β -Gal). All of the LAT fragments are expressed in these plasmids from the LAT promoter. Expression of caspase 9 and β -galactosidase is from the CMV promoter. As we previously described, at 72 h after transfection the cells were processed for detection of β -galactosidase activity and the number of β -galactosidase-positive (blue) cells were counted (5). The number of β -galactosidase-positive cells present in control cultures (with 1 μ g of pCMV- β -Gal, 6 μ g of empty plasmid, and no caspase 9 plasmid) was set to 100%. The values shown are the means and standard deviations from three different experiments. The results shown by the solid bars were all similar to each other ($P > 0.05$). The results shown by the open bars were also similar to each other ($P > 0.05$), as were the results shown by the gray bars ($P > 0.05$). Each of the solid bars was significantly different from each of the open bars and each of the gray bars ($P < 0.05$).

regarding their ability to block caspase 9-induced apoptosis (Fig. 3). (i) The empty plasmid and pLAT(1-1499) Δ Sty had background activity (approximately 5% cell survival). (ii) pLAT(1-1499), pLAT(1-2850) Δ Sty, and pLAT(1-4658) Δ Sty had significant activity compared to the first group of plasmids (approximately 25 to 30% cell survival). (iii) pLAT(1-2850) and pLAT(1-4658) had significantly higher activity than the second group of plasmids (approximately 45 to 50% cell survival). The plasmids within each group were significantly different from each of the plasmids in the other two groups ($P < 0.05$).

Our previous studies demonstrated that LAT has antiapoptosis activity (6, 10). Despite some initial controversy, LAT's antiapoptosis activity has now been confirmed by two additional groups (1; Straus, personal communication). We hypothesize that LAT's antiapoptosis activity may play a key role in the ability of LAT to support the high spontaneous reactivation phenotype. This hypothesis was supported by our finding that expression of just the first 1.5 kb of the primary 8.3-kb LAT transcript is sufficient to both support the wild-type spontaneous reactivation phenotype (12) and to interfere with apoptosis in the same transient-transfection assay reported here (6). Thus, LAT's ability to support wild-type levels of sponta-

neous reactivation and a LAT antiapoptosis function both map to the same 18% of LAT. This comapping is consistent with a functional relationship between these two LAT functions and supports our hypothesis that LAT's antiapoptosis activity is important in its ability to enhance the spontaneous reactivation phenotype.

We previously found that in HSV-1 strain McKrae, deletion of LAT nucleotides 76 to 447 (*StyI-StyI*) in the context of the entire LAT gene (dLAT371) (13) does not reduce spontaneous reactivation. In addition, we showed that insertion of the LAT promoter and the first 1.5 kb of LAT (LAT3.3A) into an ectopic location in the LAT null mutant dLAT2903 restored the wild-type spontaneous reactivation phenotype (12). Since LAT2.9A is identical to LAT3.3A except that it contains the *StyI-StyI* deletion, it was expected that LAT2.9A would have a wild-type spontaneous reactivation phenotype. However, reactivation of LAT2.9A was greatly reduced and indistinguishable from that of the LAT null mutant dLAT2903 (14). At that time we speculated that the LAT null mutant spontaneous reactivation phenotype of LAT2.9A might be due to the lack of a portion of LAT from 1.5 to 8.3 kb. Thus, the *StyI-StyI* region may not be required in the context of the otherwise complete LAT gene but is needed when only the first 1.5 kb of LAT is present. This would suggest that there is at least one additional functional region that is located either partially or completely downstream of the first 1.5-kb region. Furthermore, the functional region within the first 1.5 kb and the functional region(s) spanning or downstream of nucleotide 1499 would each have to be able to produce the wild-type spontaneous reactivation phenotype, but the effects on spontaneous reactivation could not be additive. This could occur if each functional region resulted in the maximum spontaneous reactivation possible in the rabbit model.

The spontaneous reactivation phenotypes of the above-described mutants presented us with the opportunity to further test our hypothesis that LAT's antiapoptosis activity is related to its ability to enhance the spontaneous reactivation phenotype. Our hypothesis predicted that consistent with the reduced spontaneous reactivation phenotype of LAT2.9A, a

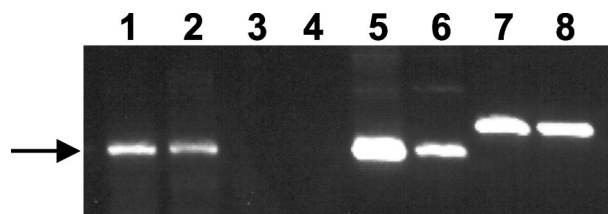


FIG. 4. Similar steady-state levels of pLAT(1-1499) and pLAT(1-1499) Δ Sty RNA following transfection of Neuro-2A cells. Neuro-2A cells were transfected with the indicated LAT plasmid as described in the legend to Fig. 3. At 48 h after transfection, total cell lysates were prepared and semiquantitative RT-PCR was performed as we previously described (14). The primers used amplify a 160-bp product corresponding to LAT nucleotides 471 to 631 (14). Lane 1, pLAT(1499) Δ Sty with RT; lane 2, pLAT(1499) with RT; lane 3, pLAT(1499) Δ Sty with no RT; lane 4, pLAT(1499) with no RT; lane 5, marker [PCR of purified pLAT(1499) plasmid]; lane 6, marker [PCR of purified HSV-1 virion DNA]; lane 7, RT-PCR of GAPDH internal standard from an aliquot of pLAT(1499) Δ Sty-transfected cell extract; lane 8, RT-PCR of GAPDH internal standard from an aliquot of pLAT(1499)-transfected-cell extract.

plasmid expressing LAT RNA identical to that expressed by LAT2.9A would have little or no antiapoptosis activity. In support of this prediction, pLAT(1-1499) Δ Sty did not inhibit caspase 9-induced apoptosis. Our hypothesis also predicted that consistent with the wild-type spontaneous reactivation phenotype of dLAT371, a plasmid containing the same Δ Sty deletion (LAT nucleotides 76 to 447) but expressing a longer LAT fragment more consistent with dLAT371 would efficiently block apoptosis. In support of this prediction, both pLAT(1-2850) Δ Sty and pLAT(1-4658) Δ Sty inhibited caspase 9-induced apoptosis as efficiently as the positive control plasmid pLAT(1-1499). These results strongly supported our hypothesis that LAT's antiapoptosis activity plays an important role in its spontaneous reactivation phenotype, since the ability to enhance the spontaneous reactivation phenotype and the ability to block apoptosis correlated even with this rather complex set of mutants and plasmids.

These results also strongly suggest that a LAT antiapoptosis function that we previously mapped to completely within the first 1.5 kb of LAT (6) completely or partially overlaps LAT nucleotides 76 to 447 (the region deleted in LAT2.9A). Our results also strongly suggest that there is a second LAT antiapoptosis region that maps somewhere between LAT nucleotides 447 and 2850, with at least part of this functional region being located between LAT nucleotides 1500 and 2850. The latter region includes approximately 58% of the 3' end of the LAT intron (also known as the stable 2-kb LAT), and the location of the second LAT antiapoptosis region is therefore consistent with the previously suggested hypothesis that the LAT intron may play a role in LAT's antiapoptotic activity (1). The McKrae-based LAT mutants LAT3.3A (12) and dLAT371 (13), which express LAT RNA corresponding to the first and second LAT antiapoptosis regions, respectively, both have wild-type spontaneous reactivation phenotypes in the rabbit. Thus, the results reported here also suggest that each of the two LAT antiapoptosis regions alone may be sufficient for the high spontaneous reactivation phenotype in rabbits. However, it is likely that both regions are important in the latency-reactivation cycle in humans.

L.J. and W.P. made equal contributions to this paper.

This work was supported by Public Health Service grants EY12823, EY13191 (to S.L.W.), and 1P20RR15635 (to C.J.); the Discovery Fund for Eye Research; and the Skirball Program in Molecular Ophthalmology.

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