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Short Communication

Mapping herpes simplex virus type 1 latency-associated transcript sequences that protect from apoptosis mediated by a plasmid expressing caspase-8

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LAT (latency-associated transcript) is the only herpes simplex virus type 1 (HSV-1) transcript abundantly expressed during neuronal latency. LAT expression is required for the high reactivation phenotype of HSV-1 and this phenotype correlates with LAT's anti-apoptosis properties. LAT nucleotides 1 to 1499 inhibit caspase-8 (death receptor apoptotic pathway), but not caspase-9 (mitochondrial apoptotic pathway), –induced apoptosis as efficiently as larger LAT fragments. LAT sequences important for inhibiting caspase-8–induced apoptosis were also localized. The ability of LAT nucleotides 1 to 1499 to efficiently inhibit caspase-8–induced apoptosis correlates with the high reactivation phenotype of a mutant virus expressing just the first 1.5 kb of LAT (nucleotides 1 to 1499). *Journal of NeuroVirology* (2004) **10,** 260–265.

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Herpes simplex virus type 1 (HSV-1) is a leading cause of both infectious corneal blindness (Zhang *et al*, 1998) and viral induced encephalitis (Kennedy and Chaudhuri, 2002). Following infection, HSV-1 establishes a lifelong latent infection within infected neurons of the host. Periodically, the virus reactivates, leading to the clinical manifestations of disease. As such, elucidation of the molecular mechanisms behind HSV-1 latency, reactivation, and virulence should lead to better strategies for reducing the incidence of HSV-1–induced disease.

During neuronal latency, abundant HSV-1 gene expression is restricted to expression of the latency-associated transcript (LAT) (Rock *et al*, 1987). The primary LAT transcript is 8.3 kb (Wagner *et al*, 1988; Zwaagstra *et al*, 1990); however, it is unstable and is spliced to produce a readily detectable 2.0-kb LAT intron (Farrell *et al*, 1991; Krummenacher *et al*, 1997; Wagner *et al*, 1988). The LAT locus is situated in the viral long repeats and is therefore present in two copies. LAT increases the spontaneous reactivation phenotype in the rabbit ocular model (Perng *et al*, 1994, 1996) and the induced reactivation phenotype in rabbits (Hill *et al*, 1990; Perng *et al*, 1994) and mice (Leib *et al*, 1989). It is currently not known whether LAT's main influence on the reactivation phenotype occurs during (1) establishment of latency, (2) maintenance of latency, or (3) reactivation from latency. In fact, it would not be surprising to find that LAT functions play a crucial role in one or more of the respective steps of latency.

We have previously shown that LAT can protect HSV-1–infected neurons from dying by blocking apoptosis (Perng *et al*, 2000), and this has been independently confirmed (Ahmed *et al*, 2002). This anti-apoptosis function may increase the pool of infected

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neurons that survive during the establishment of latency (i.e., when expression of the other HSV-1 anti-apoptotic genes, except for LAT, are turned off). LAT might also protect neurons from death during maintenance of latency, thereby maximizing the number of latently infected neurons available for subsequent reactivation. LAT's anti-apoptosis function could also play a direct role during reactivation from latency by preventing neurons in which reactivation is occurring from premature death, and/or inhibiting apoptosis pathways that stimulate reactivation. As such, modulation of neuronal apoptosis by LAT may be critical for several steps in the HSV-1 latency-reactivation cycle.

Apoptosis is a form of physiological cell death in which individual cells are eliminated or removed from tissue in a temporal manner or in response to a specific signal without inducing an inflammatory response. It is also a common response to viral infection (Hay and Kannourakis, 2002; Teodoro and Branton, 1997). In general, apoptosis sacrifices the individual infected cell but reduces the overall virus load in the host by killing infected cells before virus replication is complete.

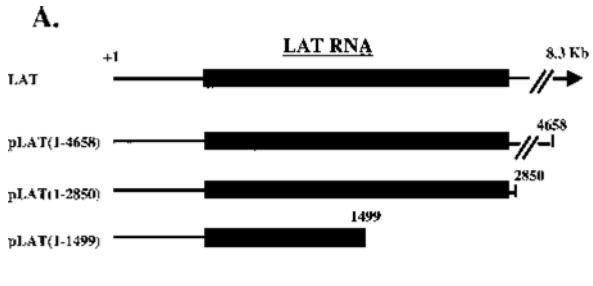
Cytotoxic T lymphocytes can also kill infected target cells by activating apoptosis by two mechanisms: the death receptor pathway (Kagi *et al*, 1995) and/or perforin assisted granzyme B pathways (Medema, 1997). Activated T cells up-regulate the cell surface ligand CD95L/FasL and bind the cognate receptor CD95/Fas on target cells (Nagata and Golstein, 1995). Receptor engagement leads to oligomerization of the receptor and recruitment of procaspase-8 to the death-inducing signaling complex (DISC) through the adaptor molecule FADD (Boldin *et al*, 1996; Muzio *et al*, 1996). Procaspase-8 is activated by proteolysis and active caspase-8 cleaves various proteins within the cell including procaspase-3, resulting in activated caspase-3 and execution of apoptosis. Caspase-8 is also central to the granzyme B pathway of immune cell killing. Perforin aids delivery of granzyme B into the target cell where it directly cleaves and activates caspase-8. Finally, caspase-8 is an essential component of the TNF-R1, DR3, TRAIL-R1, and TRAIL-R2 death receptor pathways of apoptosis induction (Varfolomeev *et al*, 1998). The importance of this caspase family member in immune cell function is further evidenced by the recent description of an essential role for caspase-8 in antigen-induced activation of T cells (Salmena *et al*, 2003).

HSV-1 infection protects cells from cytotoxic T lymphocyte–induced apoptosis (Jerome *et al*, 1998) and inhibits caspase-3 activation in response to both granzyme B or Fas ligation (Jerome *et al*, 2001). Previously, following transient plasmid based expression, the HSV-1 immediate early US5 gene product was found to protect from Fas-induced apoptosis but not, however, from apoptosis induction by granzyme B (Jerome *et al*, 2001). We and others have shown that plasmids expressing LAT protect a variety of cell types from apoptosis induction by several different inducers of apoptosis (Ahmed *et al*, 2002; Henderson *et al*, 2002; Inman *et al*, 2001; Jin *et al*, 2003; Perng *et al*, 2000). Because rabbits infected with a LAT mutant appear to have a higher degree of immune infiltration in trigeminal ganglia compared to rabbits infected with wild-type HSV-1 (Perng *et al*, 2000), it is reasonable to predict that LAT may influence cytotoxic T lymphocyte–induced apoptosis following infection of sensory neurons.

Here, we have undertaken a study to map regions of LAT involved in suppression of caspase-8–induced apoptosis. We previously showed that the ability of plasmids expressing different regions of LAT to inhibit apoptosis correlates well with the ability of LAT mutant viruses expressing the corresponding LAT regions to support the high reactivation phenotype in animal models. For example, expression of just the first 1.5 kb of the 8.3-kb LAT gene is sufficient for both inhibition of apoptosis following transient expression *in vitro* (Inman *et al*, 2001) and the high spontaneous reactivation phenotype in vivo (Perng *et al*, 1996), suggesting that both functions are linked. We also demonstrated that a plasmid expressing nucleotides 1 to 4658 protected neuro-2A cells from caspase-8– and caspase-9–induced apoptosis (Henderson *et al*, 2002). Finally, an independent study showed that a plasmid containing a 2.8-kb *PstI-MluI* LAT fragment (LAT nucleotides 68 to 2850) was highly efficient in blocking anti-Fas–mediated apoptosis in HeLa cells (Ahmed *et al*, 2002). Taken together, these observations suggested that using LAT plasmids to identify regions of LAT that interfere with caspase-8–induced apoptosis is a useful approach to identify regions of LAT that are relevant to LAT's ability to support the high reactivation phenotype.

In an attempt to localize regions of LAT that interfered with caspase-8–mediated pathway of apoptosis induction, we evaluated the ability of plasmids expressing different LAT regions to protect against enforced expression of caspase-8. Neuro-2A cells were cotransfected with a plasmid expressing β-galactosidase (β-gal; an indicator of which cells were transfected), a plasmid expressing caspase-8 or caspase-9 (to induce apoptosis), and the indicated LAT plasmid (Figure 1A). In this assay system, LAT expression is under the control of the LAT promoter. The human cytomegalovirus (CMV) promoter drives expression of caspase-8 and β-gal. Previous studies showed that LAT does not affect activity of the CMV promoter (Inman *et al*, 2001).

Cultures were stained for β-gal expression 72 h post transfection, and the number of β-gal–positive cells was counted. Consistent with previous results (Henderson *et al*, 2002), pLAT(1–4658), a LAT plasmid expressing the first 4.6-kb of LAT protected from caspase-8–induced apoptosis (approximately 60% survival). Progressive removal of sequences from the 3′ end of LAT in pLAT(1–4658) did not dramatically affect the ability of LAT to protect from



B.

	Caspase 8	Caspase 9
pLAT(1-4658)	62±13	47±11
pLAT(1-2850)	57+8	54+12
pLAT(1-1499)	65±14	22±5
Blank vector	9±4	4±3

Figure 1 Comparison of LAT's ability to inhibit caspase-8– or caspase-9–induced apoptosis in neuro-2A cells. (**A**) LAT, shown at the top, indicates the relative location of the LAT promoter, the primary 8.3-kb LAT RNA, and the stable 2-kb LAT intron (black box). The region downstream of the 2-kb LAT contains a break and is not drawn to scale. Plasmid pLAT(1–4658) contains sequences encoding the stable 2-kb LAT and additional sequences up to nucleotide 4658 that are downstream of the stable 2-kb LAT. (**B**) Neuro-2A cells were transfected with the designated plasmids. At 72 h after transfection, the number of β-gal+ cells was counted. For all transfections, 2 μg of pCMV–β-gal, 2 μg of the caspase-8 plasmid (casp-8) or the caspase-9 plasmid (casp-9), and 4 μg of the designated LAT constructs were used. The number of β-gal+ cells that were obtained when neuro-2A cultures were cotransfected with pcDNA3.1 (6 μg DNA) + pCMV–β-gal (2 μg DNA) was set at 100%. The results are from three independent studies.

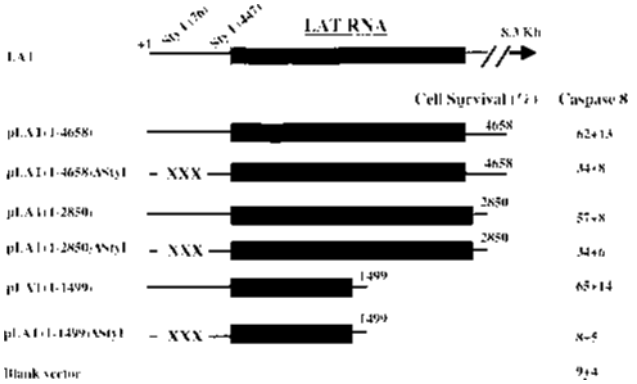


Figure 2 Inhibition of caspase-8–induced apoptosis by plasmids expressing various portions of LAT. Neuro-2A cells were cotransfected with 4 μg of the indicated LAT plasmid (or empty plasmid), 2 μg of a plasmid expressing caspase-8 (Perng *et al*, 2002), and 1 μg of a plasmid expressing β-galactosidase (pCMV–β-gal). All of the LAT fragments are expressed in these plasmids from the LAT promoter. Expression of caspase-8 and β-galactosidase are from the CMV promoter. As previously described, at 72 h after transfection the cells were processed for detection of β-gal activity and the number of β-gal–positive (blue) cells were counted (Henderson *et al*, 2002). The number of β-gal–positive cells present in control cultures (1 μg of pCMV–β-gal, 6 μg empty plasmid, no caspase 8 plasmid) was set to 100%. The values shown are the means of three different experiments. The values for the results in Figures 1 and 2 were performed in the same experiments.

caspase-8–induced apoptosis. Thus pLAT(1–4658), pLAT(1–2850), and pLAT(1–1499) all suppressed caspase-8–induced apoptosis with similar high efficiencies. This delineates a smaller region of LAT (LAT nucleotides 1 to 1499) that is capable of the same inhibition of caspase-8–induced apoptosis as LAT nucleotides 1 to 4658. As shown here for purposes of comparison (Figure 1B), we previously reported that pLAT(1–4658) and pLAT(1–2850) also efficiently inhibited caspase-9–induced apoptosis. However, pLAT(1–1499) inhibited caspase-9–induced apoptosis with only about 50% the efficiency of the larger plasmids (Jin *et al*, 2003). As expected, cells cotransfected with an empty vector (LAT−) plasmid showed approximately 9% or 4% survival in response to caspase-8 or caspase-9 expression, respectively. Thus, in transiently transfected neuro-2A cells pLAT(1–1499) inhibited caspase-8–, but not caspase-9–, induced apoptosis as efficiently as the two larger constructs pLAT(1–2850) and pLAT(1–4658).

A previous study demonstrated that sequences encompassing the StyI-StyI fragment (nucleotides 76 to 447) were crucial for the ability of pLAT(1–1499) to inhibit caspase-9–induced apoptosis (Jin *et al*, 2003). As a comparison, we tested the ability of the respective LAT ΔStyI plasmids to inhibit caspase-8–induced apoptosis. With respect to pLAT(1–4658) or pLAT(1–2850), deletion of the StyI fragment reduced the ability of LAT to inhibit caspase-8–induced apoptosis (Figure 2). However, both of the larger plasmids with ΔStyI deletions, pLAT(1–4658)ΔStyI or pLAT(1–2850)ΔStyI, retained substantial anti-apoptosis activity relative to a blank expression vector. Cell survival with pLAT(1–1499) ΔSty (Figure 2) was comparable to that with the empty vector (LAT−) plasmid (Figure 2). Thus, deletion of the StyI-StyI region (LAT nucleotides 76 to 447) from pLAT(1–1499) eliminated the ability of this 1.5-kb region to suppress caspase-8–induced apoptosis. The difference between pLAT(1–1499) and pLAT(1–1499) ΔSty was dramatic and suggests that a function completely or partially within LAT sequences 76 to 447 was important for protection from caspase-8–induced apoptosis by LAT. These results are similar to what we previously found with caspase-9–induced apoptosis (Jin *et al*, 2003), and suggest that LAT sequences between nucleotides 1499 and 2850 can partially compensate for deletion of the StyI-StyI region (LAT nucleotides 76 to 447). This putative functional domain may be completely within the nucleotides 1499 to 2850 region or may partially overlap the region of LAT from nucleotides 448 to 1499.

Additional studies were performed to localize sequences in the first 1499 bp of LAT that were necessary for inhibiting caspase-8–induced apoptosis (Figure 3). A plasmid expressing 1338 nucleotides of LAT, pLAT(1–1338), inhibited caspase-8–induced apoptosis with similar efficiency as pLAT(1–1499), whereas pLAT(1–1091) had approximately 40% less

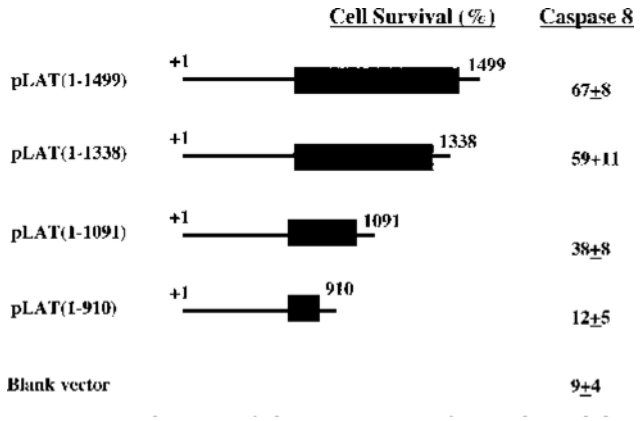


Figure 3 Localization of the 3′ terminus of LAT that inhibits caspase-8–induced apoptosis. Neuro-2A cells were cotransfected with 4 μg of the indicated LAT plasmid (or empty plasmid), 2 μg of a plasmid expressing caspase 8 (Henderson *et al*, 2002), and 1 μg of a plasmid expressing β-galactosidase (pCMV–β-gal). All of the LAT fragments are expressed in these plasmids from the LAT promoter. Expression of caspase 8 and β-galactosidase are from the CMV promoter. As previously described, at 72 h after transfection the cells were processed for detection of β-gal activity and the number of β-gal–positive (blue) cells were counted (Henderson *et al*, 2002). The number of β-gal–positive cells present in control cultures (1 μg of pCMV–β-gal, 6 μg empty plasmid, no caspase 8 plasmid) was set to 100%. The values shown are the means of four different experiments. The values for pLAT(1–1499) are slightly different than those in Figures 1 and 2 because the results for all of the plasmids shown in Figure 3 were performed together in the same experiments.

activity. A Tukey-Kramer multiple comparisons post NOVA test revealed that the differences between pLAT(1–1499) and pLAT(1–1388) to inhibit caspase-8–induced apoptosis were not significantly different ($P > .05$). However, there was a significant difference between the ability of pLAT(1–1499) or pLAT(1–1388) to inhibit caspase-8–induced apoptosis relative to the plasmid pLAT(1–1091) ($P < .001$). A plasmid expressing the first 910 nucleotides of LAT inhibited caspase-8–induced apoptosis with similar efficiency as a blank vector ($P > .05$). As expected, there was a significant difference among pLAT(1–1499), pLAT(1–1388), and pLAT(1–1091) to inhibit caspase-8–induced apoptosis compared to pLAT(1–910) ($P < .001$). Thus, the first 1338 nucleotides of the 3′ end of LAT were sufficient for efficient inhibition of caspase-8–induced apoptosis. Deletion of LAT nucleotides 1091 to 1338 (pLAT1–1091) reduced LAT's ability to inhibit caspase-8–induced apoptosis in neuro-2A cells, suggesting that the minimal 3′ end of LAT sufficient to provide LAT's full efficiency to inhibit caspase-8–induced apoptosis lies somewhere between LAT nucleotides 1 to 1091 and 1 to 1338.

When compared to our previous report detailing the ability of LAT to inhibit apoptosis induced by the initiator caspase of the mitochondrial apoptosis pathway, caspase-9, the results reported for caspase-8–induced apoptosis are indistinguishable with one notable exception, pLAT(1–1499) (Figure 1). We previously showed that pLAT(1–1499) was only half as efficient at inhibiting caspase-9–induced apoptosis compared to both pLAT(1–2850) and pLAT(1–4658) (Jin *et al*, 2003). In contrast, the results presented here indicate that pLAT(1–1499) and pLAT(1–1338) each inhibited caspase-8–induced apoptosis as efficiently as the larger LAT plasmids. This is similar to our previous studies in which pLAT(1–1499) blocked apoptosis induced by etoposide or sodium butyrate in neuro-2A cells and sodium butyrate in CV-1 cells, as efficiently (60% to 70%) as APALAT, a plasmid expressing LAT nucleotides 301 to 2659 (Inman *et al*, 2001). This suggests that different regions of LAT may be involved in suppressing caspase-8– and/or caspase-9–mediated apoptosis or that two separate anti-apoptosis domains are located within the LAT locus.

When the results presented here detailing the ability of plasmids expressing LAT fragments to protect against caspase-8–induced apoptosis are compared with the ability of such LAT fragments to protect against caspase-9–induced apoptosis and the previously reported reactivation phenotypes of HSV-1 mutants expressing similar LAT regions, a strong correlation between LAT's ability to block caspase-8–induced apoptosis and LAT's ability to support the high reactivation phenotype is evident (summarized in Table 1). For example, the plasmid pLAT(1–1499) efficiently blocked caspase-8–induced apoptosis, whereas LAT3.3A, an HSV-1 mutant that expresses the identical LAT region, has a wild-type high reactivation phenotype. The first 1.5 kb of LAT in pLAT(1–1499) and LAT3.3A contains just the first 838 nucleotides of the stable 2-kb LAT and the complete 660-nucleotide exon upstream of the stable 2-kb LAT. As expressed in pLAT(1–1499) and LAT3.3A, the first 1.5 kb of LAT does not retain any of the stability of the stable 2-kb LAT intron (Perng *et al*, 1996). Because LAT3.3A's spontaneous reactivation phenotype is indistinguishable from its parental wild-type virus HSV-1 McKrae, and because pLAT(1–1499) retains LAT's full anti-apoptosis activity against caspase-8–induced apoptosis, neither of these phenotypes requires expression of the complete 2-kb LAT intron or the stability of the 2-kb LAT intron.

In contrast, pLAT(1–1499)ΔSty does not block caspase-8–induced apoptosis, whereas LAT2.9A, an HSV-1 mutant that expresses the same LAT nucleotides (1 to 76 plus 447 to 1499) has the low-reactivation phenotype of a LAT-null mutant (Table 1). Finally, pLAT(1–2850)ΔSty and pLAT(1–4658)ΔSty, both appeared to have 50% of the protective ability of plasmids pLAT(1–1499), pLAT(1–2850), and pLAT(1–4658) towards caspase-8–induced apoptosis. Interestingly, the corresponding LAT deletion virus (dLAT371), which contains a deletion in both copies of LAT corresponding to the *Sty*I-*Sty*I restriction fragment (LAT nucleotides 76 to 447) but expresses normal amounts of the remainder of LAT in animal models, exhibits a

Table 1 Summary of anti-apoptosis activity of various LAT plasmids and reactivation phenotype of corresponding HSV-1 mutants

Virus	*LAT nucleotides expressed*	*Reactivation phenotype*
dLAT2903	None	Low
Wild-type	1–8324	High
LAT3.3A	1–1499	High
dLAT371	1–76 + 447–8324	High
LAT2.9A	1–76 + 447–1499	Low

Plasmid	*LAT nucleotides expressed*	*Blocking of caspase-8–induced apoptosis*	*Blocking of caspase-9–induced apoptosis*
PcDNA3.1	None	None	None
pLAT(1–4658)	1–4658	High	High
pLAT(1–2850)	1–2850	High	High
pLAT(1–1499)	1–1499	High	Moderate
pLAT(1–4658)ΔSty	1–76 + 447–4658	Moderate	Moderate
pLAT(1–2850)ΔSty	1–76 + 447–2850	Moderate	Moderate
pLAT(1–1499)ΔSty	1–76 + 447–1499	None	None

wild-type spontaneous reactivation phenotype in animal models. This suggests that greater than a 50% reduction in the ability of LAT to suppress caspase-8–mediated apoptosis is necessary to produce a detectable change in the reactivation phenotype of LAT mutants following infection of mice or rabbits.

We had previously demonstrated that a plasmid expressing only the first 811 nucleotides of LAT, pLAT(1–811), was able to partially inhibit apoptosis induced by etoposide or sodium butyrate (Inman *et al*, 2001). In contrast, pLAT(1–910), which expresses the first 910 nucleotides of LAT, did not inhibit caspase-8–induced apoptosis (Figure 3). We believe that overexpressing caspase-8 may alter certain aspects of the apoptosis pathway relative to inducing apoptosis with chemicals like sodium butyrate or etoposide. It is also possible that these chemicals have other activities. For example, sodium butyrate can repress histone deacytlase activity (Bernhard *et al*, 1999), which would lead to induction of gene expression and consequently could influence apoptosis. In summary, these results highlight the complexity of apoptosis, in particular the possibility that different apoptosis inducers have subtle differences on the various pathways that lead to cell death.

As a whole, the study presented here points to an important role for LAT in suppression of caspase-8–induced apoptosis and indicates that the ability of LAT to protect against caspase-8–rather than caspase-9–induced apoptosis may be more critical and reflective of events that occur in animal models of neuronal latency and reactivation. Indeed, the trigeminal ganglia where HSV-1 resides during neuronal latency are sites where elevated levels of tumor necrosis factor (TNF) and infiltrating lymphocytes are detected (Halford *et al*, 1996; Liu *et al*, 1996; Nash *et al*, 1987; Shimeld *et al*, 1996, 1999; Simmons *et al*, 1992) and as such, efficient viral suppression of extrinsic or receptor-mediated apoptosis may help contribute to long-term HSV-1 survival within such an environment.

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