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The Gene That Encodes the Herpes Simplex Virus Type 1 Latency-Associated Transcript Influences the Accumulation of Transcripts ($Bcl-x_L$ and $Bcl-x_S$) That Encode Apoptotic Regulatory Proteins

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The herpes simplex virus type 1 latency-associated transcript (LAT) inhibits apoptosis. We demonstrate here that LAT influences the accumulation of the $Bcl-x_L$ transcript versus the $Bcl-x_S$ transcript in Neuro-2A cells. $Bcl-x_L$ encodes an antiapoptotic protein, whereas $Bcl-x_S$ encodes a proapoptotic protein. Promoting the accumulation of $Bcl-x_L$ in neurons may inhibit apoptosis, thus enhancing the latency-reactivation cycle.

Herpes simplex virus type 1 (HSV-1) establishes latency in trigeminal ganglia (TG) following infection of the ocular, oral, or nasal cavity (6, 7). The latency-associated transcript (LAT) is the only viral transcript abundantly expressed in latently infected neurons (41). An unstable 8.3-kb LAT is spliced, yielding a stable 2-kb LAT intron (12, 28). Numerous researchers concluded that LAT promotes reactivation from latency by increasing the pool of latently infected neurons or directly stimulating the reactivation process (23, 46). The first 1.5 kb of the primary LAT is sufficient for spontaneous reactivation from latency in a rabbit ocular model of HSV-1 latency (40).

Plasmids expressing various LAT fragments enhance cell survival following an apoptotic insult (1, 17, 19, 38). The ability of these plasmids to promote cell survival correlates with the ability of viruses expressing the corresponding LAT fragments (LAT nucleotides 1 to 1499) to reactivate in the rabbit ocular model of HSV-1 latency (40). In the same rabbit ocular model of HSV-1 latency, a McKrae LAT null mutant (*dLAT2903*) had increased levels of apoptosis in rabbit TG (38). Furthermore, another LAT⁻ virus has increased neuronal apoptosis in acutely infected mice compared to a LAT⁺ virus (1). Although LAT may have several functions, its antiapoptosis activity may be important for the latency-reactivation cycle.

Mammals have two major apoptotic pathways: the death receptor-mediated pathway (for example, Fas or tumor necrosis factor receptor) and the mitochondrial pathway (27, 42, 47). The death receptor-mediated pathway activates caspase 8, which induces a caspase cascade including caspase 3. Activation of the mitochondrial pathway results in the release of important proapoptotic molecules, including cytochrome *c* and Smac/Diablo (47). Released cytochrome *c* associates with Apaf-1, leading to caspase 9 activation and then caspase 3 activation. Bcl-2 family proteins regulate cytochrome *c* (13, 24, 25, 48) and Smac/Diablo (11, 45) release. One of the Bcl-2

family proteins, $Bcl-x_L$, can also inhibit caspase 8-dependent apoptosis by sequestering caspase 8 at the mitochondrial membrane and inhibiting cleavage of downstream targets (43). Since HSV-1 encodes several antiapoptotic genes (ICP27, U_S3 , U_S5 , gD, and LAT) (1, 4, 5, 8, 14, 19–22, 32, 33, 37, 38), regulating apoptosis is clearly important for the viral life cycle.

One of our goals is to understand the steps in the apoptotic pathway that LAT inhibits. Henderson et al. have previously demonstrated that LAT inhibits caspase 8- and caspase 9-induced apoptosis (17). Since LAT can inhibit apoptosis of a proapoptotic Bcl-2 family member, Bax (19), and Bcl-2 family members regulate caspase 8- and caspase 9-induced apoptosis, we hypothesized that LAT might alter the expression of Bcl-2 family members. To test this hypothesis, we examined RNA expression of Bcl-2 family members in productively infected cells and transiently transfected Neuro-2A cells. Neuro-2A cells were chosen for these studies because they are mouse neuroblastoma cells derived from the peripheral nervous system, and LAT inhibits apoptosis in these cells (17, 19, 38). Similar end point titers of infectious virus were present in Neuro-2A cells that were infected with strain *dLAT2903*, the wild-type (wt) McKrae strain, or the rescued *dLAT2903* strain, which is consistent with previous studies using other cell lines (39). However, we have observed more apoptosis at 24 h after infection when Neuro-2A cells were infected with strain *dLAT2903* versus the wt McKrae strain (data not shown).

The mRNA that encodes the $Bcl-x_L$ protein can be alternatively spliced within exon 2, yielding a proapoptotic protein ($Bcl-x_S$) (31). The Bcl-x primers used here amplified a 250-bp product from $Bcl-x_L$ cDNA and a 64-bp product from the cDNA of the alternatively spliced $Bcl-x_S$ product (Table 1), as previously reported (31). Reverse transcriptase PCR (RT-PCR) of total RNA prepared from Neuro-2A cells productively infected with LAT⁻ strain *dLAT2903* for 24 h after infection produced a prominent 64-bp band corresponding to the $Bcl-x_S$ RNA (Fig. 1A, lane dLAT). In Neuro-2A cells infected with the wt McKrae strain (Fig. 1A, lane WT) or strain *dLAT2903*-rescued virus (data not shown), the 64-bp RT-PCR product was not readily detected. In some cultures of mock-

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TABLE 1. Primers used to amplify cDNA^a

Gene	Primer (type) ^b	PCR product(s) (bp)	Melting temp	No. of cycles	GeneBank accession no.
Bcl-2	GTTCGCTACCGTCGTGACTT (F) CAGCTCCGTTATCCTGGA (R)	267	60	35	NM 009741
Bcl-x	CCAGCTTACATAACCCAG (F) ATCCACAAAAGTGTCCACG (R)	250, 64 ^c	60	30–35	L35049
Casp 9	AAGACCATGGCTTTGAGGTG (F) AACAGCCAGGAATCTGCTTG (R)	333	60	35	AB01960
β-actin	GTGGGGCGCCCCAGGCACCA (F) C TCCTAATGTCACGCACGATTTC (R)	400	53	20	
VHS	TGCTACATTCCCACGATCAA (F) AGGTCCTCGTCGTCTCGTA (R)	346	60	30	AF007815
gC	AGGTCCTGACGAACATCACC (F) TAATACATTCCCTGGGTCGC (R)	208	60	30	JO2216
Bak	TTGCCCAGGACACAGAGGAGGT (F) GAATTGGCCCAACAGAACCACACC (R)	527	60	40	AF402617

^a The melting temperatures of the respective primers and the numbers of cycles used for PCR are given for each primer pair.

^b Forward (F) and reverse (R) primers were designed on the basis of the sequences of the respective genes. All primers are listed in a 5' to 3' direction.

^c The Bcl-x primers can amplify a 250-bp cDNA product (Bcl-x_L) and a 64-bp cDNA product (Bcl-x_S).

infected cells, low levels of the Bcl-x_S splice product were detected, which is consistent with these cells undergoing apoptosis after growth in subcultures. When 30 to 35 cycles of amplification were performed, the Bcl-x_S-amplified product was consistently detected when Neuro-2A cells were infected with strain *dLAT2903*. However, the Bcl-x_S-amplified product was not readily detected when fewer than 25 cycles were used for amplification. The sequence of the 64-bp RT-PCR product amplified in Neuro-2A cells infected with strain *dLAT2903* was identical to that of the Bcl-x_S spliced product.

Additional studies were performed to determine when the Bcl-x_S-amplified cDNA product was detectable in Neuro-2A cells that were infected with strain *dLAT2903*. In general, when Neuro-2A cultures were infected with *dLAT2903*, the Bcl-x_S spliced product was detectable between 6 and 8 h after infection (Fig. 1B). Cultures infected with the wt McKrae strain typically contained little or none of the Bcl-x_S-amplified product. In several experiments, Neuro-2A cultures infected with *dLAT2903* contained reduced levels of the Bcl-x_L-amplified product (in conjunction with the increased levels of the Bcl-x_S-amplified product). This was particularly evident when reduced numbers of cycles were used for amplification of the cDNA (35 cycles [Fig. 1A] versus 31 cycles [Fig. 1B]). In cultures infected with *dLAT2903*, since Neuro-2A cells must be split every 2 to 3 days or they begin to detach from the dish and undergo an apoptotic death, the condition of the cell cultures can influence Bcl-x_L levels and the appearance of the Bcl-x_S product. In summary, the results of these studies suggested that LAT either inhibited splicing of the primary Bcl-x transcript into Bcl-x_S or increased the ratio of Bcl-x_L to Bcl-x_S by stabilizing Bcl-x_L RNA levels and/or destabilizing Bcl-x_S RNA levels.

In contrast to the results obtained with Bcl-x, the levels of rRNA, Bcl-2, caspase 9, and β-actin did not change dramatically after infection (Fig. 2). In general, these results suggested that when Neuro-2A cells were infected with the wt McKrae strain of HSV-1 or the strain McKrae-based LAT⁻ mutant *dLAT2903*, the virus host shutoff (VHS) function was negligi-

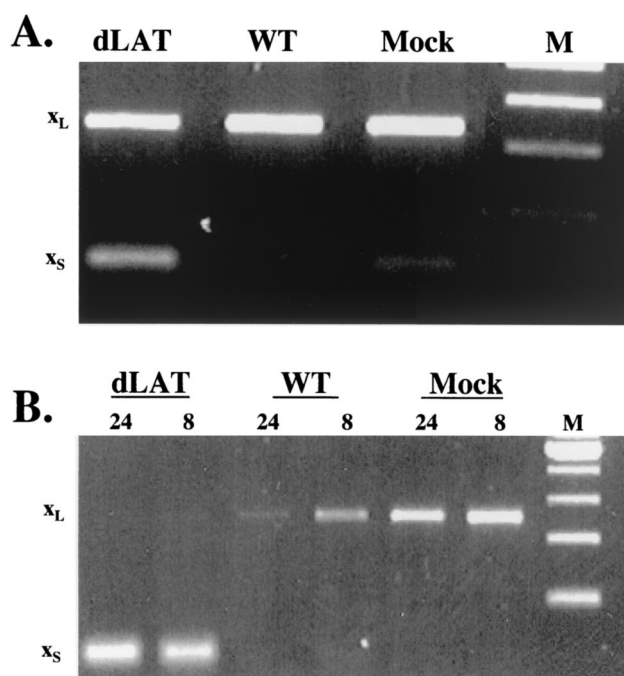


FIG. 1. Analysis of Bcl-x RNA expression in infected Neuro-2A cells. Using a multiplicity of infection of 4, Neuro-2A cells were infected with strain *dLAT2903* (*dLAT*) or wt HSV-1 (McKrae strain) (*WT*). As controls, cultures were mock infected (*Mock*). At 24 h after infection (*A*) or at 8 and 24 h after infection (*B*), RNA was prepared using Trizol reagent and the RNA was subsequently treated with RNase-free DNase I for 30 min at room temperature. First-strand cDNA was synthesized using 2 μg of total RNA as the template, 0.5 μg of oligo(dT) 12-18 as a primer, and a SuperScript preamplification kit (Stratagene, La Jolla, Calif.). For each sample, 1/10 of the cDNA reaction mixture was used with the indicated primers (Table 1). Using 35 (*A*) or 31 (*B*) cycles, amplification was conducted as described in Table 1. The positions of the Bcl-x_L (x_L) and Bcl-x_S (x_S) bands are indicated. Omitting reverse transcriptase from the reaction eliminated amplification of the specific bands (data not shown). A 100-bp ladder was used as a marker (*M*) to estimate the size of the amplified cDNA products. These results are representative of at least five independent experiments.

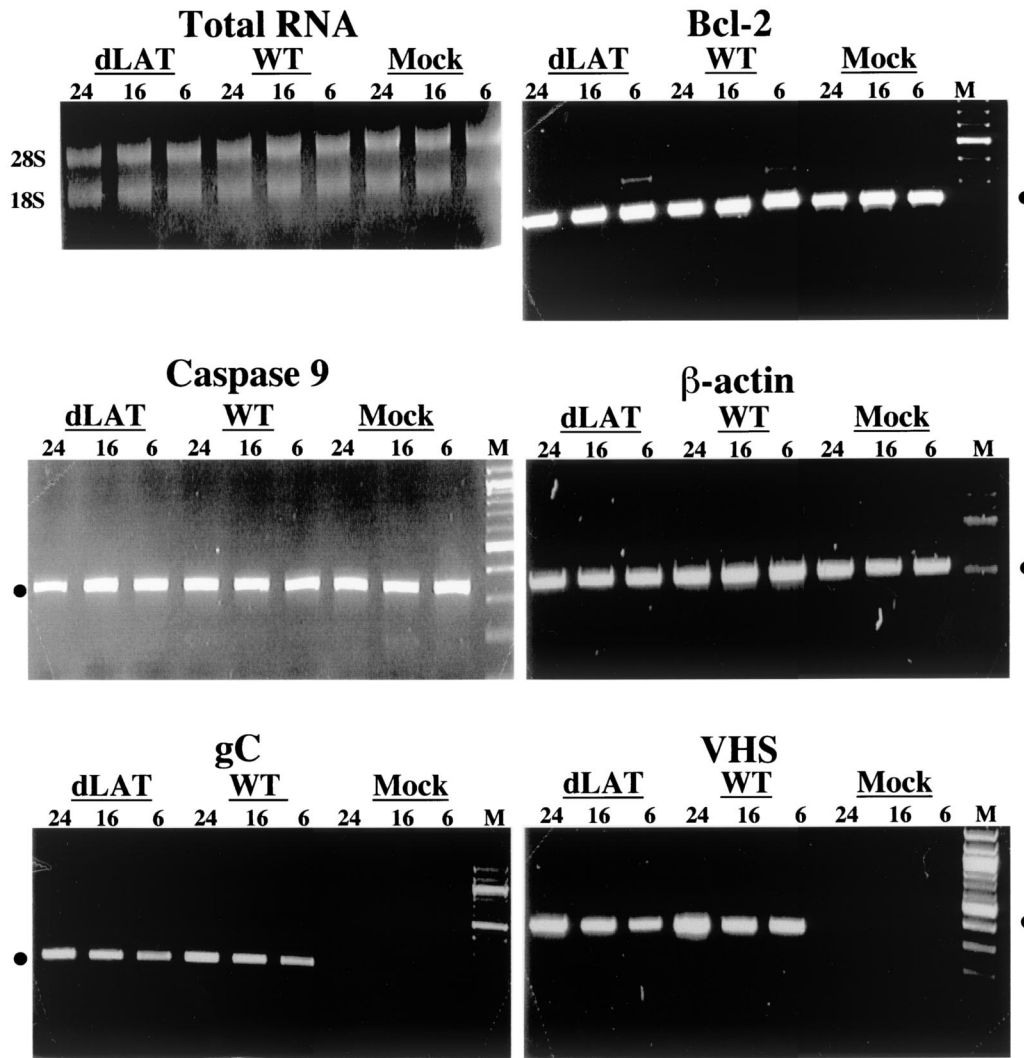


FIG. 2. Analysis of apoptosis regulatory genes in infected Neuro-2A cells. Using a multiplicity of infection of 4, Neuro-2A cells were infected with strain *dLAT2903* (*dLAT*) or wt HSV-1 (McKrae strain) (*WT*). As controls, cultures were mock infected (*Mock*). At 6, 16, or 24 h after infection, RNA was prepared using Trizol reagent and the RNA was subsequently treated with RNase-free DNase I for 30 min at room temperature. Samples containing total RNA (1 μ g) were electrophoresed on a 1.2% formaldehyde agarose gel, and the position of the rRNA was determined. First-strand cDNA was synthesized using 2 μ g of total RNA as the template, 0.5 μ g of oligo(dT)12-18 as a primer, and a SuperScript preamplification kit (Stratagene). For each sample, 1/10 of the cDNA reaction mixture was used with the indicated primers (Table 1). Amplification was conducted as described in Table 1. The closed circles denote the positions of amplified products. Omitting reverse transcriptase from the reaction eliminated amplification of the specific bands (data not shown). A 100-bp ladder was used as a marker (M) to estimate the size of the amplified cDNA products. These results are representative of at least four independent experiments.

ble. Since infection of sympathetic and sensory neurons with HSV has been reported to not elicit a potent VHS activity (34), this provided additional evidence that Neuro-2A cells have neuron-like properties. As expected, infection of nonneuronal cell types with the McKrae strain leads to reduction of the steady-state levels of β -actin RNA, indicating that the McKrae strain has a functional VHS activity (data not shown). Consistent with the finding that Neuro-2A cells infected with wt strain McKrae or strain *dLAT2903* yield similar amounts of virus, expression of VHS RNA or glycoprotein C (gC) RNA was the same when Neuro-2A cells were infected.

Since HSV-1 encodes several antiapoptotic genes that are expressed during productive infection (1, 4, 5, 8, 14, 19–22, 32, 33, 37, 38), we tested whether, in the absence of other viral

genes, LAT could inhibit accumulation of Bcl- x_s . Neuro-2A cells transfected with Bax (Fig. 3A, lane 3) and mock-transfected Neuro-2A cells (Fig. 3A, lane 4) both contained the 64-bp Bcl- x_s -amplified band. This was as expected, because Neuro-2A cells undergo apoptosis as a result of Bax expression (19) or when growth factors are depleted (17). In contrast, Neuro-2A cells transfected with LAT (Fig. 3A, lane 1) or the LAT plus Bax (lane 2) did not contain a prominent Bcl- x_s -amplified band, suggesting that in the absence of other viral genes, LAT inhibited accumulation of Bcl- x_s . Inclusion of reverse transcriptase in the cDNA reaction was required for detection of the 250-bp Bcl- x_L -amplified products and 64-bp Bcl- x_s -amplified products, confirming that cDNA, not contaminating DNA, was amplified (Fig. 3B). No dramatic changes

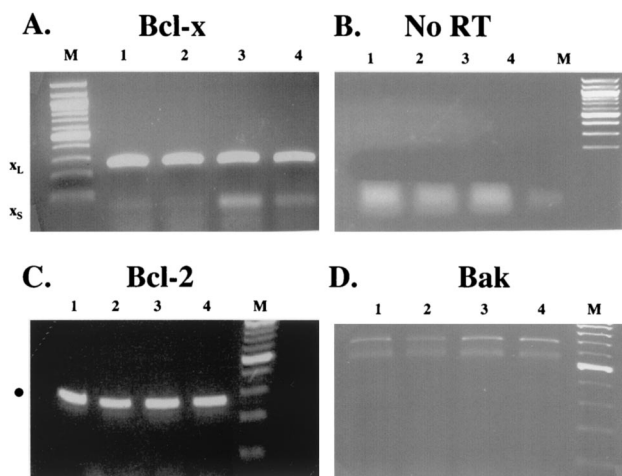


FIG. 3. LAT inhibits splicing of Bcl- x_S in transfected Neuro-2A cells. Neuro-2A cells were transfected with the plasmids described below. Lane 1, 4 μ g of LAT3.3–1 μ g of pcDNA3.1; lane 2, 4 μ g of LAT3.3–1 μ g of pCMVBax; lane 3, 4 μ g of pcDNA3.1–1 μ g of pCMVBax; lane 4, 5 μ g of pcDNA3.1. LAT3.3 contains the LAT promoter plus the first 1.5 kb of LAT coding sequences (19). pCMVBax contains the full-length Bax cDNA downstream of the human cytomegalovirus promoter; the plasmid was obtained from Upstate Biotechnology (Lake Placid, N.Y.). At 48 h after transfection, using primers that amplify Bcl-x (A), Bcl-2 (C), or Bak (D), RNA was prepared and RT-PCR was performed as described for Fig. 1. (B) cDNA reactions that did not contain reverse transcriptase (No RT) were amplified using the Bcl-X primers. In the No RT reactions, the Bcl-x primers did amplify the expected products. The No RT reaction also yielded no bands when the Bcl-2 or Bak primers were used (data not shown).

were detected in the levels or mobility of the Bcl-2-amplified product regardless of the treatment (Fig. 3C). Studies were also performed to test whether LAT altered splicing of Bak, because Bak is a proapoptotic gene that belongs to the Bcl-2 family. Interestingly, Bak can be alternatively spliced in neurons to yield an antiapoptotic protein rather than the proapoptotic protein seen in nonneuronal cells (44). In contrast to Bcl-x, LAT did not have a dramatic effect on Bak splicing patterns in transiently transfected Neuro-2A cells (Fig. 3D) or in productively infected Neuro-2A cells (data not shown).

This study demonstrated that LAT inhibited accumulation of Bcl- x_S . This suggests that LAT promoted splicing of Bcl-x to Bcl- x_L rather than to Bcl- x_S or that LAT stabilized Bcl- x_L steady-state RNA levels while at the same time destabilizing Bcl- x_S RNA levels. Since the levels of Bcl-2, caspase 9, and β -actin mRNA were not reduced dramatically in infected Neuro-2A cells (Fig. 2), the reduction of Bcl- x_L levels in cells infected with strain *d*LAT2903 was not merely the result of viral host shutoff activity. These results are important, because the Bcl- x_L protein has antiapoptotic activity whereas the Bcl- x_S protein is proapoptotic. Thus, increasing the ratio of Bcl- x_L to Bcl- x_S transcripts, as LAT appeared to do in these studies, should enhance cell survival (9). The ability of the LAT to interfere with caspase 8- and caspase 9-induced apoptosis (17) may be linked to influencing splicing of Bcl-x to Bcl- x_L rather than Bcl- x_S , since Bcl- x_L inhibits both caspase 8 (43)- and caspase 9-induced apoptosis (18, 35). Bcl- x_L , but not Bcl-2, is highly expressed in the central and peripheral nervous systems

of developing (26) and adult mice (15, 16, 30, 36), suggesting that Bcl- x_L is the most important Bcl-2 family protein expressed in TG.

Although LAT contains small open reading frames, a recent study concluded that LAT protein expression is not detected in infected cells or infected mice (29). The small open reading frames within the first 1.5 kb of LAT, a region of the LAT that promotes spontaneous reactivation, do not show a high degree of amino acid similarity between HSV-1 strains (10), suggesting that LAT RNA sequences, rather than a LAT protein, influences Bcl- x_S accumulation. The ability of LAT to interact with splicing factors (2) may influence splicing of certain cellular transcripts, including Bcl- x_L . Although caspase 9 (3) and Bak (44) are alternatively spliced, LAT expression did not dramatically alter the levels of these spliced products. Consequently, we conclude that LAT has the potential to influence expression of certain spliced cellular transcripts.

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