Region of Herpes Simplex Virus Type 1 Latency-Associated Transcript Sufficient for Wild-Type Spontaneous Reactivation Promotes Cell Survival in Tissue Culture

MELISSA INMAN,[1] GUEY-CHUEN PERNG,[2] GAIL HENDERSON,[1] HOMAYON GHIASI,[2,3]
ANTHONY B. NESBURN,[2,3] STEVEN L. WECHSLER,[2,3] AND CLINTON JONES[1]*

*Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, Nebraska 68583-0905[1];
Ophthalmology Research Laboratories, Cedars-Sinai Medical Center Burns & Allen Research Institute, Los Angeles,
California 90048[2]; and Department of Ophthalmology, UCLA School of Medicine, Los Angeles, California 90024[3]*

The latency-associated transcript (LAT) is the only abundant herpes simplex virus type 1 (HSV-1) transcript expressed during latency. In the rabbit eye model, LAT null mutants do not reactivate efficiently from latency. We recently demonstrated that the LAT null mutant dLAT2903 induces increased levels of apoptosis in trigeminal ganglia of infected rabbits compared to LAT⁺ strains (G.-C. Perng, C. Jones, J. Ciacci-Zarella, M. Stone, G. Henderson, A. Yokht, S. M. Slanina, F. M. Hoffman, H. Ghiasi, A. B. Nesburn, and C. S. Wechsler, Science 287:1500–1503, 2000). The same study also demonstrated that a plasmid expressing LAT nucleotides 301 to 2659 enhanced cell survival of transfected cells after induction of apoptosis. Consequently, we hypothesized that LAT enhances spontaneous reactivation in part, because it promotes survival of infected neurons. Here we report on the ability of plasmids expressing different portions of the 5′ end of LAT to promote cell survival after induction of apoptosis. A plasmid expressing the first 1.5 kb of LAT (LAT nucleotides 1 to 1499) promoted cell survival in neuro-2A (mouse neuronal) and CV-1 (monkey fibroblast) cells. A plasmid expressing just the first 811 nucleotides of LAT promoted cell survival less efficiently. Plasmids expressing the first 661 nucleotides or less of LAT did not promote cell survival. We previously showed that a mutant expressing just the first 1.5 kb of LAT has wild-type spontaneous reactivation in rabbits, and a mutant expressing just the first 811 nucleotides of LAT has a reactivation frequency higher than that of dLAT2903 but lower than that of wild-type virus. In addition, mutants reported here for the first time, expressing just the first 661 or 76 nucleotides of LAT, had spontaneous reactivation indistinguishable from that of the LAT null mutant dLAT2903. In summary, these studies provide evidence that there is a functional relationship between the ability of LAT to promote cell survival and its ability to enhance spontaneous reactivation.

Following ocular, oral, or intranasal infection, herpes simplex virus type 1 (HSV-1) establishes latent infection in trigeminal ganglia (TG) (3, 4). The only abundant viral transcript expressed in latently infected neurons is the latency-associated transcript (LAT) (11, 12, 32, 48, 51, 54). The primary 8.3-kb primary LAT transcript is unstable and is spliced, yielding an abundant stable 2-kb LAT (12, 48, 53, 56) that is a stable intron (17, 33). LAT is antisense to ICP0 and is primarily localized in the nucleus. This has led to the suggestion that LAT represses ICP0 expression by an antisense mechanism (48), which in turn represses productive infection (8, 20). However, we previously showed that the first 1.5 kb of the primary LAT is sufficient for spontaneous reactivation from latency (45). Since this region does not overlap ICP0, antisense repression of ICP0 expression by LAT is not required for spontaneous reactivation in the rabbit model. Although LAT is important for latency in small-animal models (27, 52), its functional roles during the latency-reactivation cycle are not understood.

In transient—transfection assays, a LAT fragment (LAT nucleotides 301 to 2659) encompassing the stable 2-kb LAT derived from strain KOS enhanced cell survival following an apoptotic insult (42). The same study also demonstrated that a McKrae LAT⁻ mutant (dLAT2903) had increased levels of apoptosis in rabbit TG. These findings suggested that LAT is important for latent infections because it promotes survival of infected neurons.

HSV-1 can induce or inhibit apoptosis (programmed cell death) in a cell type-dependent manner after infection of cultured cells (1, 2, 18, 19, 36). Several antiapoptotic genes have been identified (1, 2, 18, 42), suggesting that regulation of apoptosis is crucial for the virus's life cycle. Trauma, stress, or other imbalances of growth factors or cytokines can induce neuronal apoptosis, and neuronal apoptosis is linked to neurodegenerative disorders (7, 21, 23, 26, 35, 38–40). HSV-1 replication and productive gene expression can occur in sensory ganglia during acute infection and reactivation from latency (29–31, 50), which can lead to apoptosis in TG during acute infection (42). Thus, the ability of HSV-1 to minimize neuronal apoptosis may be an important aspect of the latency cycle.

In this study, we demonstrate that pLAT3.3, a plasmid expressing the first 1.5 kb of the 8.3-kb primary HSV-1 McKrae LAT, promotes cellular survival in CV-1 and neuro-2A cells following apoptosis induction. This region does not overlap ICP0 and contains only 838 nucleotides of the 5′ end of the stable 2-kb LAT intron. We also demonstrate that pLAT3.3 inhibited Bax-induced apoptosis in CV-1 cells. Additional plas-

mids expressing smaller regions of LAT were constructed and tested. The ability of these plasmids to promote cell survival correlated with the ability of recombinant viruses expressing the corresponding LAT fragments to spontaneously reactivate in the ocular rabbit model of HSV-1 latency. This suggests that the ability of LAT to promote cell survival after induction of apoptosis plays an important role at one or more steps in the HSV-1 latency cycle.

MATERIALS AND METHODS

Cells and viruses. Cells were plated at a density of 5×10^5 cells/100-mm plastic dish in Earl's modified Eagle's medium supplemented with 5% fetal bovine serum. All medium contained penicillin (10 U/ml) and streptomycin (100 μg/ml). CV-1 cells were split at a 1:5 ratio every 4 or 5 days. Neuro-2A cells were obtained from the American Type Culture Collection (Rockville, Md.) and split in a 1:4 ratio every 3 to 5 days.

All parental and mutant viruses were plaque purified three times and passaged only one or two times prior to use. Wild-type McKrae, dLAT2903, dLAT2903R, LAT3.3A (originally designated LAT1.5a), and LAT2.6A have been described previously (16, 44, 45). Rabbit skin (RS) cells, used for preparation of virus stocks and for culturing rabbit tear films, were grown in Eagle's minimal essential medium supplemented with 5% fetal calf serum (FCS).

Bluo-gal cotransfection and analysis of cell death. Cells were plated at a density of 2×10^5/well in six-well plastic plates (35 mm²/well) 12 to 16 h prior to the transfection. Cells were transfected with a human cytomegalovirus (CMV) expression vector containing the β-galactosidase gene (pCMV–β-Gal) and one of the plasmids described below by the calcium phosphate method. For each 35-mm well, 250 μl of a calcium phosphate solution (125 mM CaCl₂, 25 mM HEPES-NaOH [pH 7.1], 0.75 mM Na₂HPO₄-NaH₂PO₄ [pH 7.0], 125 mM NaCl) and the designated amount of plasmid DNA mixture were added to the cells. Five hours after transfection, cells were shocked with 20% glycerol–phosphate-buffered saline (PBS) solution for 4 min and then washed with PBS twice. Fresh medium containing 5% FCS and 5 mM sodium butyrate was added to the cells to enhance expression of genes encoded by plasmids, because sodium butyrate can inhibit histone deacetylase activity (5). Long-term treatment of cell lines with sodium butyrate can lead to apoptosis (22, 49). When CV-1 cells are treated with 5 mM sodium butyrate for 16 h, we have seen an approximately twofold increase in apoptosis, as judged by measuring ONA content (data not shown). When CV-1 cells are treated for 40 to 48 h with sodium butyrate, we can routinely detect 7 to 12 times more apoptosis than in control cultures. The cells from each well were incubated at 37°C for 16 h and then subcultured at a 1:5 ratio onto a 24-well dish. These cells were treated with 15 μM etoposide or 5 mM sodium butyrate (37°C for 48 h), and β-galactosidase expression was analyzed as described previously (9, 10, 25, 42). Briefly, cells were rinsed with calcium- and magnesium-free PBS (CMF-PBS), fixed with 2% formaldehyde–0.2% glutaraldehyde in PBS for 5 min, and then washed twice with CMF-PBS. Fixed cells were stained for 6 to 24 h with 0.1% Bluo-gal (Gibco) in a PBS solution which contained 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM MgCl₂. After rinsing in PBS, positive cells were observed microscopically, and the average number of blue-stained cells was counted. At least five fields per plate were counted, and the average number of cells per field was calculated.

The Bax expression plasmid used in this study was purchased from Upstate Biotechnology (Lake Placid, N.Y.). The Bcl-2 expression plasmid was obtained from John Reed (San Diego, Calif.).

Construction of LAT2.5A and LAT1.8A. LAT1.8A contains the LAT promoter plus the first 76 nucleotides of the primary LAT RNA in the unique long region between UL37 and UL38. LAT2.5A contains the LAT promoter and the first 661 nucleotides of the primary LAT RNA in the same novel location. The parental virus for both constructs was dLAT2903, a mutant of HSV-1 strain McKrae containing a 1.8-kb (EcoRV-HpaI) deletion in both copies of LAT that removed 0.2 kb of the LAT promoter and 1.6 kb of the 5′ end of the primary 8.3-kb LAT transcript (44). dLAT2903 transcribes no LAT RNA and reactivates poorly relative to LAT⁺ viruses (wild type or dLAT2903R). These mutants were constructed as previously described for LAT3.3A (originally designated LAT1.5a) and LAT2.6A (16, 43, 44, 46) except for the size of the structural portion of the LAT gene included with the LAT promoter (shown schematically in Fig. 5). Briefly, an HSV-1 McKrae restriction fragment containing LAT nucleotides −1793 to +76 (the LAT promoter plus the first 76 nucleotides of the primary LAT) or LAT nucleotides −1793 to +661 (the LAT promoter plus the first 661 nucleotides of the primary LAT) was inserted into a uniquely constructed PacI

site between the HSV-1 McKrae genes UL37 and UL38 contained in plasmid pV375Pac (45). LAT1.8A and LAT2.5A were each generated by cotransfection and homologous recombination of infectious dLAT2903 viral DNA with the LAT1.8A or LAT2.5A plasmid as we previously described (45). Following cotransfection, isolated plaques were picked and screened for the proper insertion between UL37 and UL38 by restriction digestion and Southern analysis. Selected plaques were plaque purified three times and grown into virus stocks. Virus stocks were analyzed by restriction enzyme digestion and Southern blots to confirm that the correct LAT fragment was present between UL37 and UL38. This analysis also verified that both long repeats retained the original deletion (the 1.8-kb LAT promoter and the first 1.6 kb of the 5′ end of the primary LAT transcript) in both viral long repeats.

Rabbits. New Zealand White male rabbits (Irish Farms), 8 to 10 weeks old, were used for all experiments. Rabbits were treated in accordance with the Association for Research in Vision and Ophthalmology, American Association for Laboratory Animal Care, and National Institutes of Health guidelines.

Rabbit model of ocular HSV-1 infection, latency, and spontaneous reactivation. Rabbits were bilaterally infected without scarification or anesthesia by placing 2×10^5 PFU of HSV-1 per eye into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye for 30 s (16, 42, 44, 45, 47). At this dose of HSV-1 McKrae, virtually all of the surviving rabbits harbor a bilateral latent HSV infection in both TG, resulting in a high group rate of spontaneous reactivation. Latency is assumed to be established by 28 days postinfection. Acute ocular infection of all eyes was confirmed by HSV-1-positive tear film cultures collected on day 3 or 4 postinfection.

Detection of spontaneous reactivation by ocular shedding. Beginning on day 30 postinfection, tear film specimens were collected daily from each eye for 26 days as previously described (16, 42, 44, 45, 47), using a nylon-tipped swab. The swab was then placed in 0.5 ml of tissue culture medium and squeezed, and the inoculated medium was used to infect RS cell monolayers. These monolayers were observed in a masked fashion by phase light microscopy for up to 7 days for HSV-1 cytopathic effects (CPE). All positive monolayers were blind passaged onto fresh cells to confirm the presence of virus. Viral DNA was purified from randomly selected positive cultures derived from latently infected rabbits and analyzed by restriction enzyme digestion and Southern blots to confirm that the CPE was due to reactivated HSV-1 and that the reactivated virus was identical to the input virus.

Statistical analyses were performed using Instat, a personal computer software program. Results were considered statistically significant when the P value was <0.05.

RESULTS

Localization of McKrae LAT sequences that promote cell survival. Our previous study demonstrated that in transient-transfection assays, a plasmid expressing LAT nucleotides 301 to 2659 from HSV-1 KOS promoted cell survival after induction of apoptosis and that the HSV-1 McKrae LAT reduces apoptosis frequency in TG of infected rabbits (42). To confirm that McKrae LAT would also promote cell survival after apoptosis induction in cultured cells and to further map the involved LAT region, LAT plasmids were constructed from McKrae (Fig. 1). These plasmids contain the LAT promoter but do not possess a strong exogenous poly (A) addition site at their 3′ terminus, suggesting that transcripts synthesized from these constructs would not have polyadenylated tails. The ability of LAT fragments to inhibit apoptosis was measured by cotransfecting cells with a β-Gal expression plasmid (pCMV–β-Gal) and the designated LAT constructs (9, 10, 25, 34, 42). Apoptosis was then induced by treating the cultures with 5 mM sodium butyrate or 15 μM etoposide. Both chemicals induce apoptosis in a variety of cells (5, 6, 22, 37, 49). Monkey kidney (CV-1) or mouse neuroblastoma (neuro-2A) cells that are transfected with pCMV–β-Gal have fewer blue cells when apoptosis is induced (9, 10, 47). If the LAT plasmid inhibits apoptosis, the number of β-Gal⁺ cells will be higher than in the empty vector control.
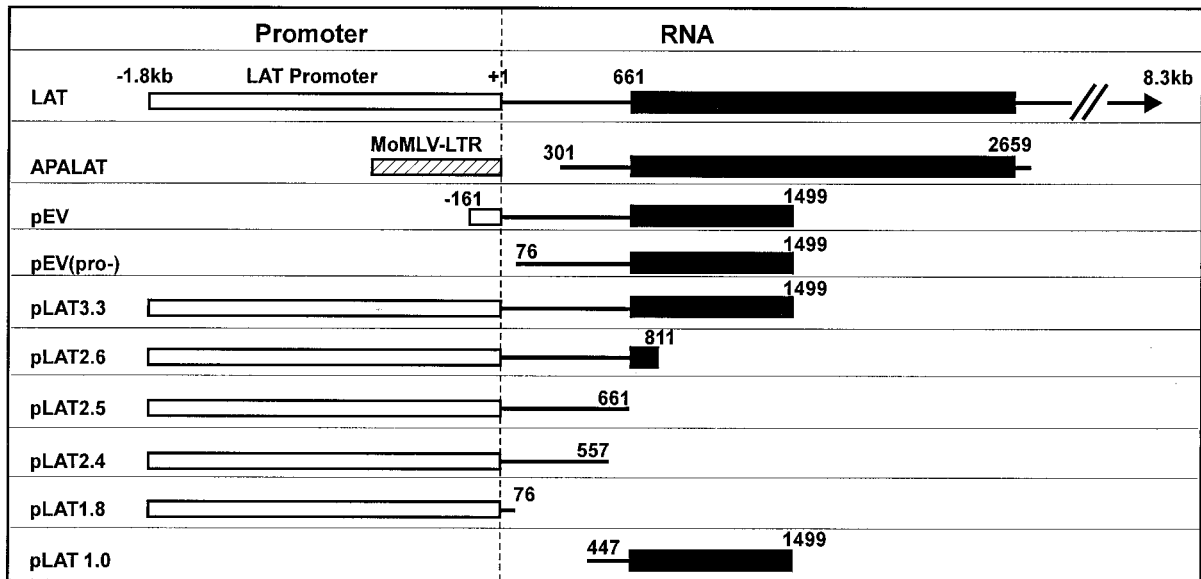
FIG. 1. Schematic representation of plasmid inserts tested for antiapoptosis activity in transient-transfection assays. LAT, shown at the top, indicates the relative location of the LAT promoter, the primary 8.3-kb LAT RNA, and the stable 2-kb LAT intron (black box). The region downstream of the 2-kb LAT contains a break and is not drawn to scale. APALAT contains the Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) driving expression of LAT (nucleotides 301 to 2659) from HSV-1 strain KOS. The remaining LAT fragments are all derived from HSV-1 strain McKrae. The portion of the LAT promoter in pEV (−161 to +76) is sufficient for high transcription levels in transient-transfection assays. pEV(pro−) is identical to pEV but without the promoter. The LAT fragments in pLAT3.3, pLAT2.6, pLAT2.5, and pLAT1.8 correspond to the UL37-UL38 LAT inserts in the LAT3.3A, LAT2.6A, LAT2.5A, and LAT1.8A mutant viruses shown in Fig. 5, respectively.

We have previously characterized the effects that several chemicals have on apoptosis using CV-1 cells (9, 10, 42) and have shown that the APALAT fragment (Fig. 1) protects CV-1 and neuro-2A cells from apoptosis (42). Since the latency model used for McKrae is the rabbit, rabbit skin cells could have been used to examine apoptosis. However, we thought that for the purposes of mapping the region within LAT that protects against apoptosis, it was logical to employ the cells we used previously. In addition, CV-1 cells efficiently support HSV-1 productive infection and LAT transcription. Neuro-2A cells retain many neuronal characteristics, and there are no available neuron-like cell lines derived from rabbits.

Following treatment with etoposide or sodium butyrate, APALAT (KOS nucleotides 301 to 2659) and the antiapoptotic baculovirus gene cpIAP both had a higher frequency of cell survival than a blank expression vector (pcDNA3.1) (Fig. 2). Neuro-2A cells transfected with pLAT3.3 also had increased cell survival relative to cells transfected with pcDNA3.1 following treatment with sodium butyrate or etoposide. pLAT3.3 also enhanced cell survival in CV-1 cells treated with sodium butyrate. In both cases, the increased survival with pLAT3.3 was similar to that seen with APALAT. This confirms that McKrae LAT and KOS LAT can both enhance cell survival after apoptosis induction with similar efficiency. Furthermore, it suggests that the cell survival domain resides in the region common to both plasmids, which is bounded by LAT nucleotides 301 to 1499.

Etoposide did not consistently induce apoptosis in CV-1 cells and thus was not used for this study. Cultures of CV-1 and neuro-2A cells transfected with pLAT2.5, pLAT2.4, or pLAT1.8, had levels of cell survival similar to those in cultures

transfected with pcDNA3.1 after apoptosis induction. This suggested that expression of LAT nucleotides 1 to 661 (pLAT2.5) or less (pLAT2.4 and pLAT1.8) was unable to protect cells from apoptosis. As expected, the promoterless
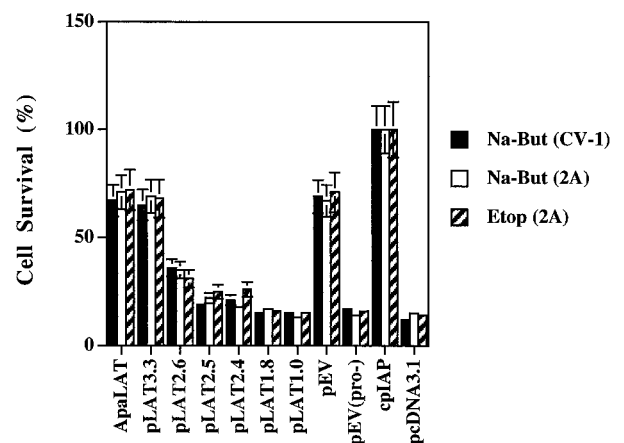

FIG. 2. Efficiency of cell survival after transfection with LAT constructs. Deletion plasmids of LAT are shown in Fig. 1. CV-1 and neuro-2A (2A) cells were transfected with LAT constructs (4 μg of DNA) and pCMV–β-Gal (1 μg of DNA). Cells were treated with sodium butyrate (Na-But, 5 mM) or etoposide (Etop, 15 μM) to induce apoptosis. Cell survival was measured by counting β-Gal+ cells at 48 h after transfection as described previously (9, 10, 42). The number of β-Gal+ cells that were present in cultures transfected with pCMV–β-Gal plus pcDNA3.1 without etoposide or sodium butyrate treatment was set at 100%. The values are the means of five different experiments.

LAT fragment pLAT1.0 also was not able to promote cell survival.

Interestingly, pLAT2.6, expressing LAT nucleotides 1 to 811, had cell survival values that were lower than those for pLAT3.3 and APALAT but slightly higher than those for the other deletion constructs or the empty plasmid. This suggested that LAT nucleotides 1 to 811 retained a small amount of activity but that additional sequences were required for efficient cell survival, as seen with the larger LAT plasmids (i.e., LAT nucleotides 1 to 1499 or 301 to 2659). CV-1 and neuro-2A cells transfected with pEV, a construct containing the first 1.5 kb of LAT and the minimal LAT promoter, also exhibited enhanced cell survival relative to those transfected with pcDNA3.1. The enhanced cell survival was similar to that of both pLAT3.3 and APALAT, consistent with this promoter having high activity in these cells (56). When the promoter was deleted [pEV(pro−)], cell survival was not enhanced, demonstrating that expression of LAT was necessary for its cell survival activity.

Although the data in Fig. 2 suggested that LAT products inhibited apoptosis in transiently transfected cells, one could argue that LAT transactivated the CMV immediate-early promoter in the CMV–β-Gal plasmid and that this resulted in increased numbers of β-Gal⁺ cells. To eliminate this possibility, the pEV construct was cotransfected with a CMV-chloramphenicol acetyltransferase (CAT) construct, and CAT activity levels were measured. In three independent experiments, pEV did not activate the CMV promoter (Fig. 3A). The intensity of the blue cells and the number of blue cells were similar when LAT (pEV) or a blank expression vector (pNEB193) (Fig. 3B) was cotransfected with pCMV–β-Gal, which was consistent with the inability of pEV to transactivate the CMV promoter. After etoposide treatment, the number of β-Gal⁺ cells decreased less when CV-1 cells were cotransfected with pEV in addition to pCMV–β-Gal. Since etoposide induces apoptosis in a variety of mammalian cells (5, 22, 49), a reduction in β-Gal⁺ cells after etoposide treatment is indicative of apoptosis, as we concluded previously (9, 10, 42).

LAT sequences inhibit Bax-induced apoptosis. Bax is an important proapoptotic protein that interacts with the mitochondrial membrane, promotes cytochrome *c* release, and thus activates Apaf-1 (apoptosis-activating factor-1) (41, 55). Activated Apaf-1 initiates a caspase cascade that precedes the cytological and biochemical events leading to apoptosis. The Bcl-2 protein interacts with Bax and thus inhibits apoptosis. A CMV expression vector containing Bax reduced the number of β-Gal⁺ CV-1 cells (Fig. 4A) and neuro-2A cells (data not shown). To determine if LAT could inhibit Bax-induced apoptosis, cells were cotransfected with Bax, pEV, pEV(pro−) and pCMV-β-Gal. A dose-dependent increase in β-Gal⁺ cells was observed when increasing amounts of the pEV construct were cotransfected with Bax. In contrast, the pEV (pro−) construct had no effect on Bax-induced apoptosis, indicating that expression of LAT was necessary for promoting cell survival. As expected, Bcl-2 interfered with Bax-induced apoptosis and was more efficient than the pEV construct.

The β-Gal⁺ cells (blue cells) transfected with Bax were compared to those cotransfected with Bax and APALAT or Bax and pEV by specifically staining DNA with Hoescht 33342. This procedure allows one to identify β-Gal⁺ cells using phase contrast microscopy and then visualize chromatin by fluorescence. β-Gal⁺ cells transfected with Bax had condensed chromatin and more frequently contained apoptotic bodies (Fig. 4B) compared to cells transfected with the empty vector (Fig. 4C). In contrast, β-Gal⁺ cells from cultures cotransfected with Bax plus APALAT (Fig. 4D) or Bax plus pEV (Fig. 4E) had less condensed chromatin and had nuclear morphology similar to that of normal cells. Of 200 β-Gal⁺ cells that were transfected with Bax alone, 86% had condensed chromatin and thus were apoptotic. Only 20% of the β-Gal⁺ cells transfected with Bax and pEV or Bax and APALAT appeared to be apoptotic. Thus, LAT (both pEV and APALAT) interfered with Bax-induced apoptosis.

Lack of spontaneous reactivation by LAT1.8A and LAT2.5A. To determine if the ability of LAT to interfere with apoptosis correlated with spontaneous reactivation, additional LAT deletion mutants were constructed and tested in the rabbit ocular latency model. The genomic structures of wild-type HSV-1 McKrae, *d*LAT2903, *d*LAT2903R, LAT3.3A, LAT2.6A, LAT2.5A, and LAT1.8A are shown in Fig. 5. All viruses were derived from HSV-1 strain McKrae. The construction and properties of *d*LAT2903, its marker-rescued virus *d*LAT2903R, LAT3.3A, and LAT2.6A have been described previously (16, 44, 45). Wild-type McKrae contains two copies of LAT, one in each viral long repeat. *d*LAT2903 contains a deletion in both copies of LAT from −161 to +1677 relative to the start of the primary LAT transcript (Fig. 5B, indicated by XXXXX). This virus is missing key promoter elements, makes no LAT RNA, and is a true LAT null mutant. Insertion of 1.8 kb of the LAT promoter and different lengths of LAT into a unique *Pac*I site that was constructed between UL37 and UL38 (Fig. 5C, D, E, and F) gave LAT3.3A, LAT2.6A, LAT2.5A, and LAT1.8A from *d*LAT2903. Due to the complete deletion of the LAT promoter and the first 1.67 kb of the primary LAT transcript, none of these mutants are capable of transcribing any LAT RNA from either copy of LAT in the long repeats (16). As previously shown for LAT3.3A and LAT2.6A (46, 47), these mutants do, however, transcribe the expected region of LAT from their ectopic insert. LAT3.3A, LAT2.6A, LAT2.5A, and LAT1.8A make the first 1,499, 811, 661, and 76 nucleotides of the primary LAT, respectively. Additional details of the construction of these viruses are given in Materials and Methods.

As with the LAT null mutant *d*LAT2903 (44), LAT1.8A and LAT2.5A were wild type for replication in tissue culture, replication in rabbit eyes, induction of eye disease in rabbits, and neurovirulence, as judged from rabbit survival (data not shown). To examine spontaneous reactivation, 18 rabbits/group were infected with 2 × 10⁵ PFU of LAT1.8A, *d*LAT2903, *d*LAT2903R, or LAT3.3A per eye. In a separate experiment, 28 or 29 rabbits/group were similarly infected with LAT2.5A, *d*LAT2903, or LAT3.3A. Beginning 30 days postinfection (at which time latency had already been established), the eyes from all surviving rabbits were swabbed once a day for 26 days to collect tear films for analysis of reactivated virus as described in Materials and Methods. The cumulative number of virus-positive tear film cultures is shown in Fig. 6A and B. Because there were minor, nonsignificant differences in the numbers of surviving rabbits in the different groups within each experiment, the data were standardized to represent cumula-
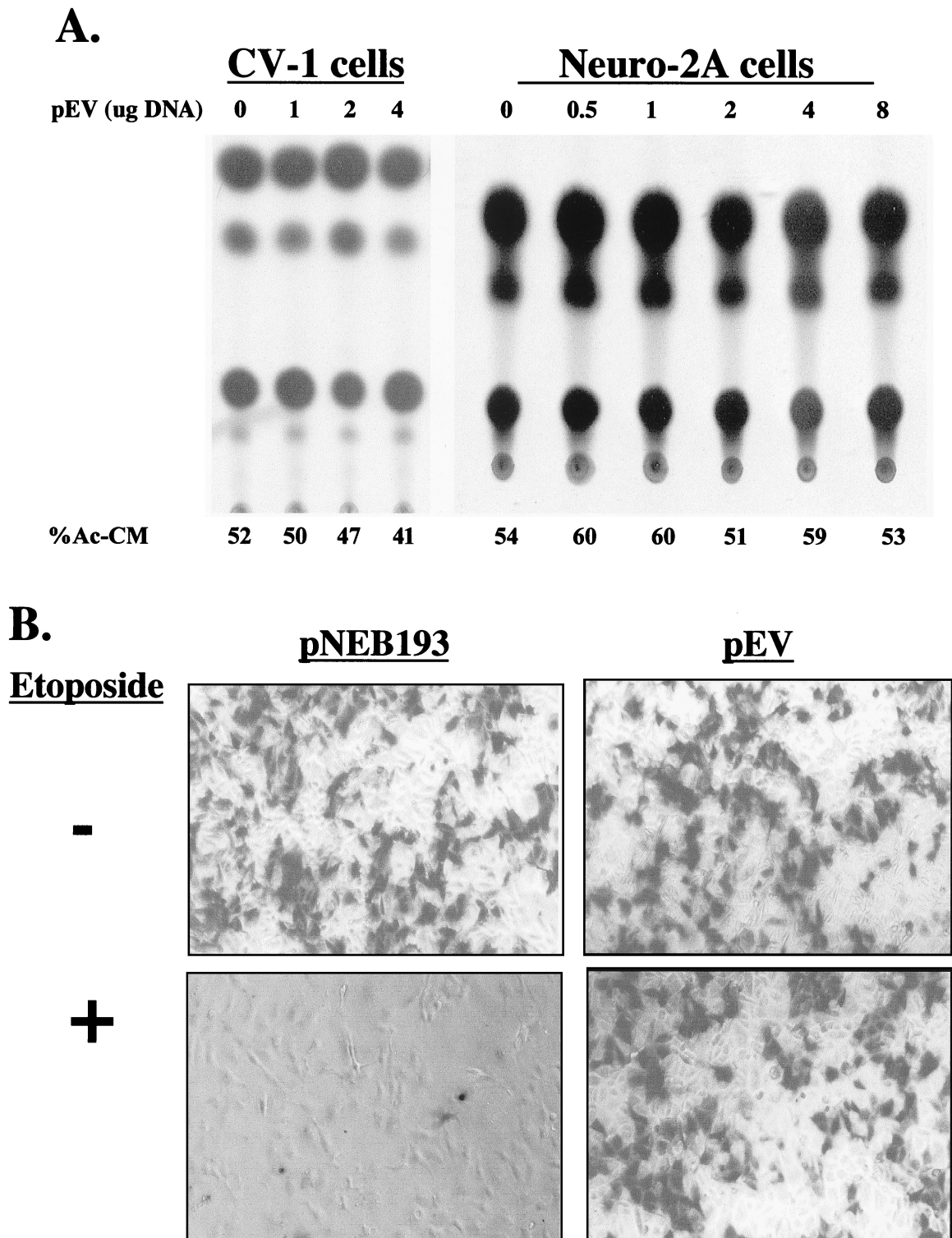
A.

B.

FIG. 3. LAT does not activate the CMV promoter. (A) CV-1 and neuro-2A cells were transfected with pCMV cat (2 μg) and the indicated amounts of plasmid pEV (Fig. 1). Blank expression vector (pNEB193) was used to maintain the same amounts of plasmid for each transfection. At 48 h after transfection, CAT enzymatic activity was measured as described previously (13, 14, 28). The percent acetylated chloramphenicol (%Ac-CM) was quantified using a PhosphoImager. (B) CV-1 cells were cotransfected with pCMV–β-Gal (1 μg of DNA) and pNEB193 (4 μg of DNA) or pEV (4 μg of DNA). Some cultures were treated with etoposide (15 μM) at 16 h after transfection to induce apoptosis. At 48 h after transfection, cells were stained to detect β-Gal^{+} cells. Representative panels are shown.
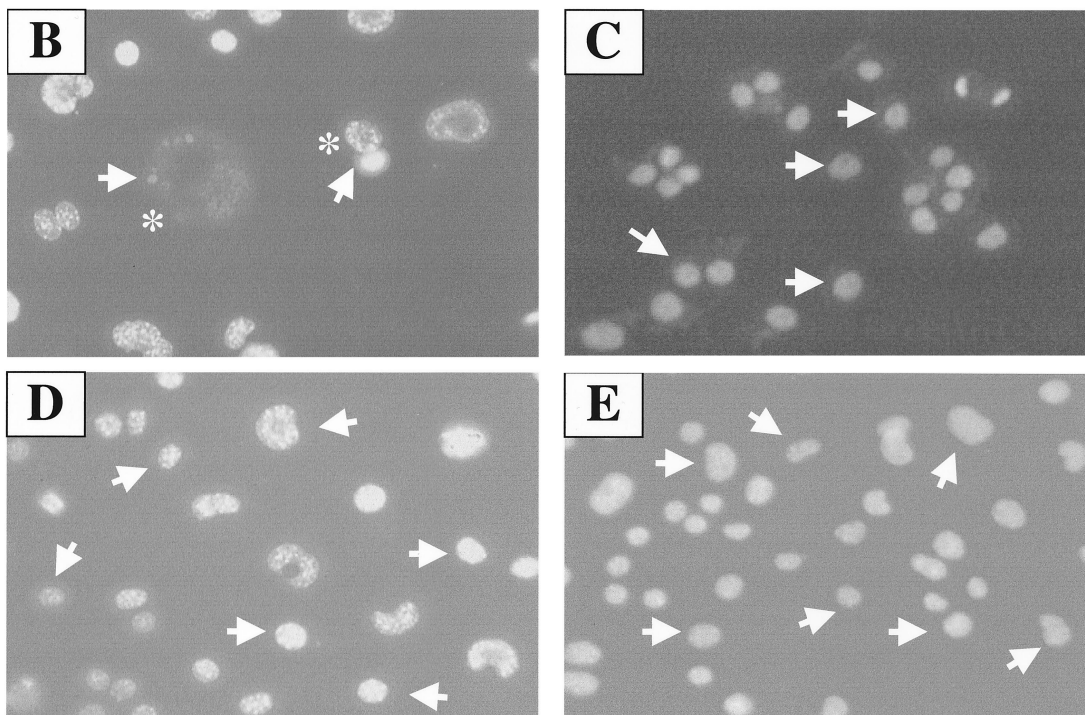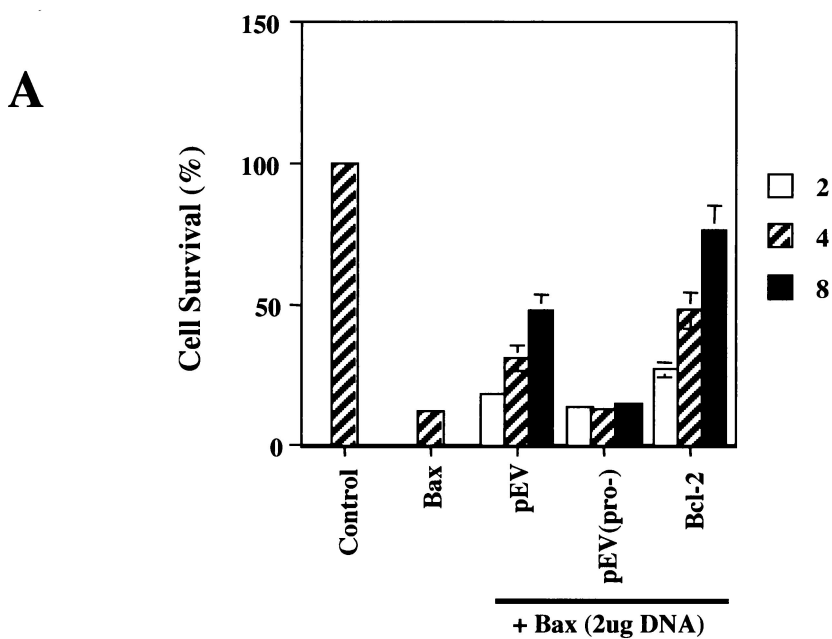
FIG. 4. LAT interferes with Bax-induced apoptosis. (A) CV-1 cells were cotransfected with the designated LAT construct or a CMV expression vector containing Bcl-2 (2, 4, or 8 μg of DNA), Bax (2 μg of DNA), and pCMV–β-Gal (1 μg of DNA). To maintain constant amounts of DNA, a blank expression vector (pcDNA3.1) was added to the mixture. At 48 h after transfection, the number of β-Gal$^+$ cells was counted. The number of β-Gal$^+$ cells present in control cultures (1 μg of pCMV–β-Gal and 9 μg of pcDNA3.1) was set at 100%. The values are the means of four different experiments. (B to E) After β-Gal staining, cellular DNA was stained with Hoescht 3342 as described previously (13). β-Gal$^+$ cells were identified using phase contrast microscopy, and DNA staining of the same cell was visualized by fluorescence. Arrows denote β-Gal$^+$ cells. (B) CV-1 cells transfected with Bax (2 μg of DNA). (C) CV-1 cells transfected with a blank expression vector, pcDNA3.1. (D) CV-1 cells transfected with Bax (2 μg of DNA) and APALAT (8 μg of DNA). (E) CV-1 cells transfected with Bax (2 μg of DNA) and pEV (8 μg of DNA). For panels B to E, pCMV–β-Gal (1 μg of DNA) was included in each transfection. A blank expression vector (pcDNA3.1) was added to the mixture to maintain the same amount of DNA (8 μg of DNA for each sample). An asterisk denotes an apoptotic cell, as judged by the presence of condensed chromatin and apoptotic bodies.
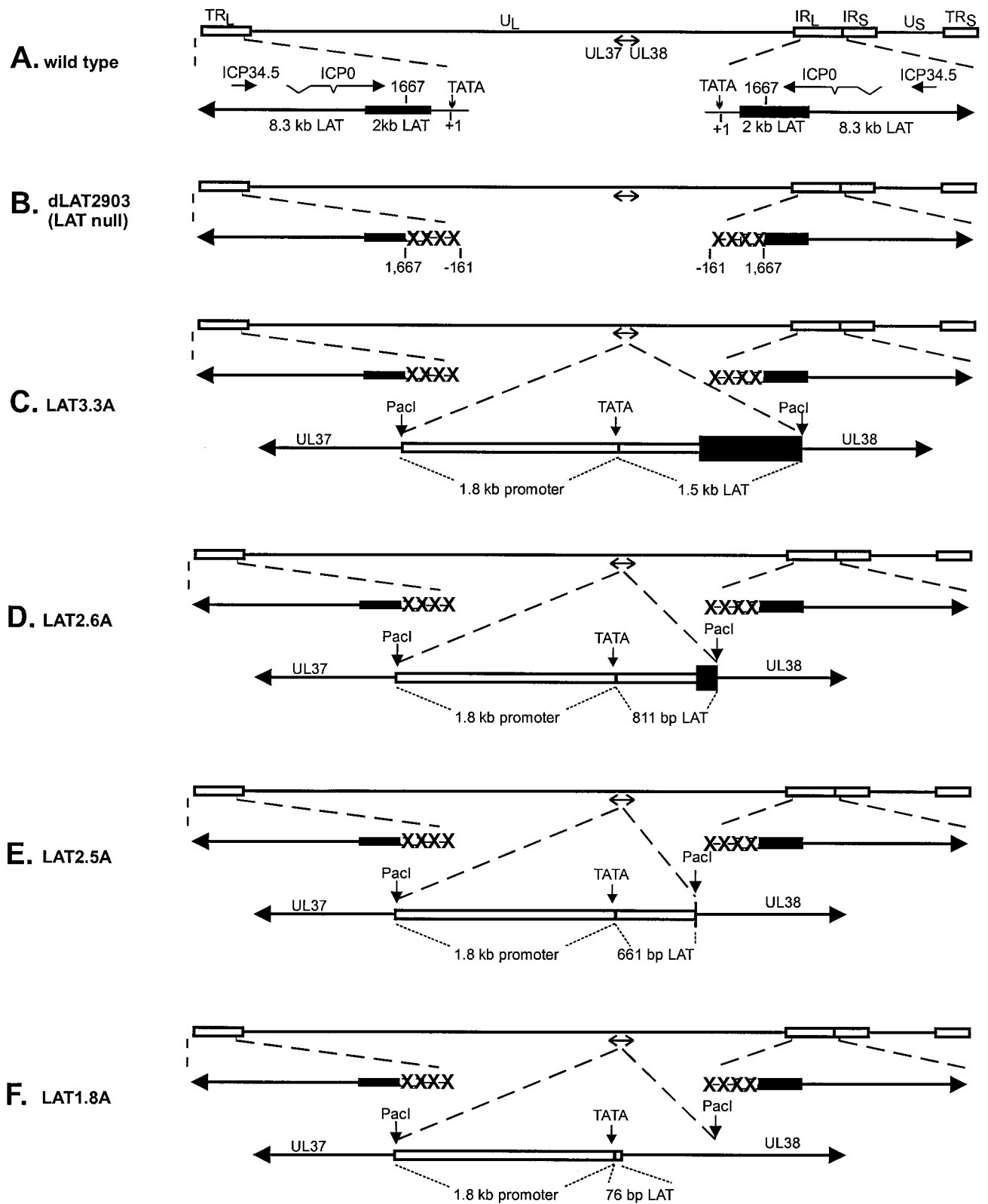
FIG. 5. Structures of LAT mutant viruses. (A) Schematic representation of wild-type HSV-1 and marker-rescued dLAT2903R. The prototypic orientation of HSV-1 shown here contains a unique long (U_L) region and a unique short (U_S) region (solid lines), each bounded by inverted repeats (open rectangles). TR_L, long terminal repeat; IR_L, long internal repeat; TR_S, short terminal repeat; IR_S, short internal repeat. The dashed lines under the genome indicate a blow up of the repeat regions. Arrows indicate the locations and directions of the LAT, ICP34.5, and ICP0 transcripts. The solid rectangle within the primary 8.3-kb LAT transcript indicates the location of the stable 2-kb LAT intron. TATA indicates the location
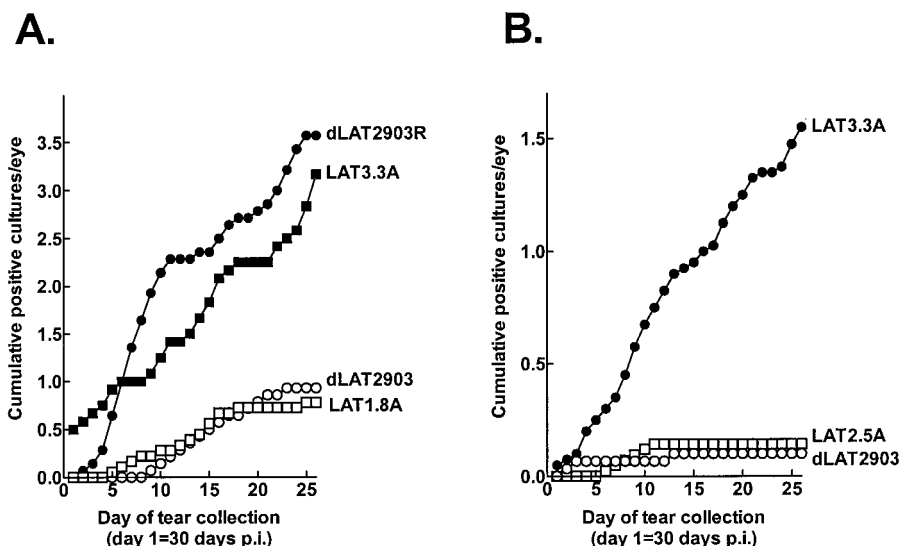
FIG. 6. Cumulative spontaneous reactivation of LAT2.5A and LAT1.8A. Rabbits were ocularly infected with dLAT2903, dLAT2903R, LAT3.3A, LAT1.8A, or LAT2.5A. Beginning on day 30 postinfection (p.i.) (day 1 of tear film collection), at which time latency had been established, tear films were collected daily for 26 days. These samples were then plated on RS cell monolayers and observed for the presence of CPE, which is indicative of spontaneously reactivated virus in the tears. Positive cultures were passaged, and DNA was analyzed. Southern analysis confirmed that the CPE was due to HSV-1 and that the mutant spontaneously reactivated viruses retained their deletions. The y axis represents the cumulative number of HSV-1-positive cultures for each virus group divided by the number of eyes in the group. Panels A and B show results from separate experiments. Statistical analyses are shown in Table 1.

tive positive cultures per eye. The cumulative spontaneous reactivation rate in rabbits latently infected with LAT1.8A (Fig. 6A, approximately one positive culture per eye on day 26) appeared to be less than that in rabbits infected with dLAT2903R or LAT3.3A (Fig. 6A, approximately 3 to 3.5 per eye on day 26) and similar to that in rabbits infected with dLAT2903 (Fig. 6A, approximately 1 per eye). Similarly, cumulative spontaneous reactivation of LAT2.5A appeared to be less than that of LAT3.3A, which has wild-type spontaneous reactivation (45), and similar to that of dLAT2903 (Fig. 6B).

In the first experiment, the number of positive eye cultures (Table 1) indicated that only 3% of the tear film cultures from rabbits latently infected with LAT1.8A contained spontaneously reactivated virus. This was similar to the number with dLAT2903 (3.6%) but significantly less than that with dLAT2903R (13.7%) and LAT3.3A (12.2%) (Table 1). In the second experiment, only 0.6% of the tear film cultures from rabbits latently infected with LAT2.5A contained spontaneously reactivated virus (Table 1). This was similar to the number with dLAT2903 (0.4%) and significantly less than that with LAT3.3A (6%) in this experiment (Table 1). Because the above analyses do not take into account the number of eyes in each of the groups, the data were also analyzed as follows. The

percentage of days on which virus-positive cultures were obtained for each eye in each group (i.e., the percentage of time that each eye was positive for spontaneous reactivation) was calculated, plotted as scattergrams, and analyzed by analysis of variance (ANOVA) (Fig. 7). This is a very powerful and stringent analysis because it takes into account both the number of eyes in each group and the duration of the study in one calculation. By this analysis, LAT1.8A reactivated similarly to dLAT2903 but less efficiently than dLAT2903R and LAT3.3A (Fig. 7A). This analysis also confirmed that LAT2.5A reactivated similarly to dLAT2903 but less efficiently than LAT3.3A (Fig. 7B). Thus, mutant viruses capable of expressing just the first 76 or 661 nucleotides of LAT from the LAT promoter had impaired spontaneous reactivation similar to the LAT null mutant. This correlates with the inability of these same LAT regions to promote cell survival after apoptosis in tissue culture, as described above.

In the experiments shown in Fig. 7 and Table 1, the spontaneous reactivation rates of the wild-type viruses LAT3.3A and dLAT2903R (Fig. 7A) are higher than that of the wild-type virus LAT3.3A (Fig. 7B). This type of variation between experiments is common and is believed to be due to undetermined environmental factors (unpublished observations).

(in the genomic DNA) of the LAT promoter TATA box. (B) Previously described LAT deletion mutant dLAT2903 (44), which contains a 1.8-kb deletion (−161 to +1667) in both copies of the LAT gene (one in each long repeat), indicated by XXXX, makes no LAT RNA, and reactivates poorly. (C) Mutant LAT3.3A, which was previously designated LAT1.5a (45). A blow up shows the 1.8-kb LAT promoter and the first 1.5 kb of the LAT transcript inserted between genes UL37 and UL38 in the unique long region of the LAT deletion mutant dLAT2903. LAT3.3A transcribes only the first 1.5 kb of LAT yet has wild-type spontaneous reactivation. (D, E, and F) LAT2.6A (16), LAT2.5A, and LAT1.8A, respectively, containing the LAT promoter and the first 811 nucleotides of the LAT transcript, the LAT promoter and the first 661 nucleotides of LAT, and the LAT promoter alone (up to LAT nucleotide +76), respectively, inserted between UL37 and UL38 of dLAT2903. LAT2.6A has a spontaneous reactivation midway between that of the wild type and dLAT2903 (16). LAT2.5A and LAT1.8A are described here for the first time and have spontaneous reactivation rates similar to that of dLAT2903.

TABLE 1. Spontaneous reactivation

Expt	Virus	No. of positive cultures/ no. tested (% positive)	Significance vs. control (P)
1	LAT1.8A (control)	14/468 (3.0)	
	dLAT2903	13/364 (3.6)	0.70
	dLAT2903R	50/364 (13.7)	<0.0001
	LAT3.3A	38/312 (12.2)	<0.0001
2	LAT2.5A (control)	6/1,092 (0.6)	
	dLAT2903	3/780 (0.4)	0.74
	LAT3.3A	62/1,040 (6.0)	<0.0001

However, within a single experiment, the relative spontaneous reactivation rates between wild-type and LAT⁻ viruses always remained similar. This illustrates the importance of including wild-type and LAT⁻ control viruses in each experiment and of housing all rabbits in each experiment in the same room.

DISCUSSION

The first 1.5 kb of the primary LAT (LAT3.3A) is sufficient to restore wild-type levels of spontaneous reactivation to a LAT null mutant (45). We show in this study that expression of the same fragment in a plasmid (pLAT3.3) promotes cell survival after apoptosis induction. The first 811 nucleotides of the primary LAT (LAT2.6A) only partially restore spontaneous reactivation to a LAT null mutant (16). We showed here that this LAT region (pLAT2.6) similarly only partially promoted cell survival after apoptosis induction. Further deletion of the LAT region (LAT2.5A and LAT1.8A) did not allow spontaneous reactivation or cell survival (pLAT2.5 and pLAT1.8). Thus, the results presented here show a correlation between the region of LAT capable of enhancing spontaneous reactivation in rabbits and the region of LAT capable of promoting cell survival after induction of apoptosis by chemicals or Bax. It should be pointed out that LAT's ability to inhibit apoptosis could enhance spontaneous reactivation by at least two distinct mechanisms. First, LAT could enhance spontaneous reactivation at the level of establishment or maintenance by enhancing neuronal survival during acute infection. The finding that a LAT null mutant (dLAT2903) has higher levels of apoptosis in TG during acute infection than LAT⁺ strains (42) supports a role for LAT in maximizing the number of neurons that survive acute infection. Second, it is also possible that LAT directly stimulates reactivation by prolonging survival of neurons as well as nonneuronal cells during spontaneous reactivation, thus maximizing virus production. Although the studies presented here correlate enhancement of cell survival with the ability of LAT to promote spontaneous reactivation in latently infected rabbits, they do not exclude the possibility that LAT has additional functions that are necessary for lifelong latency in humans.

Neuro-2A and CV-1 cells were used for these studies because we previously found that LAT had a positive effect on cell survival in these cell lines. Since there is a correlation between LAT's inhibiting apoptosis in these cell lines and promoting spontaneous reactivation in rabbits, there appears to be biological relevance to the findings that we observed in CV-1 and neuro-2A cells. In contrast, LAT had no effect on cell survival in two transformed cell lines, 293 (human epithelial cells immortalized by adenovirus) and COS-7 (CV-1 cells transformed by simian virus 40) (data not shown). These viruses encode oncogenes that interfere with the growth-suppressing properties of p53 and retinoblastoma protein and impair the proapoptotic functions of p53. In general, expression of these viral oncogenes confers resistance to apoptosis. We found that when a chemical agent was able to overcome these dominant antiapoptotic viral oncogenes and induce apoptosis in 293 and COS-7 cells, LAT was not able to promote survival.

Interestingly, in CV-1 and neuro-2A cells, the same chemicals had different effects on cell death. For example, etoposide did not effectively kill CV-1 cells but did induce apoptosis in neuro-2A cells. Furthermore, ceramide effectively induced apoptosis in CV-1 cells (9, 10, 42) but not in neuro-2A cells (data not shown). Several different apoptotic pathways are regulated
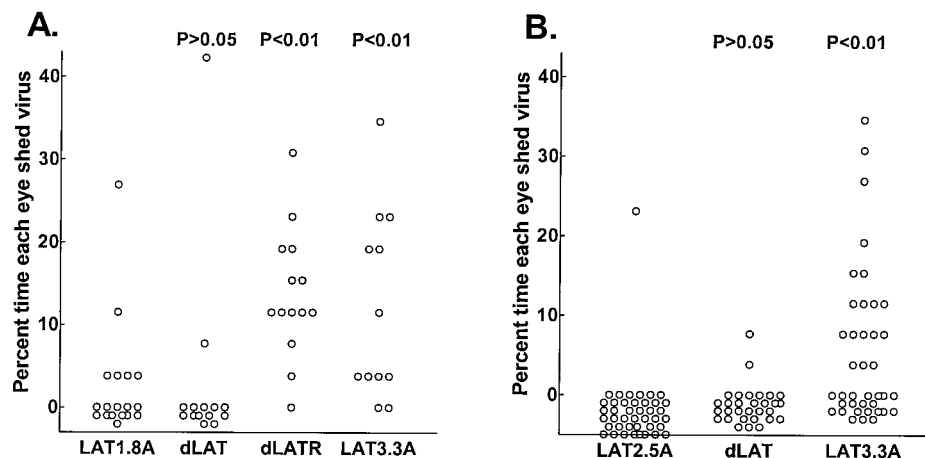

FIG. 7. Scattergram representation of spontaneous reactivation. Each data point represents the percentage of days during the 26-day observation period on which individual eyes were positive for spontaneously reactivated virus. To visually separate the individual data points, some of the zero points are plotted slightly below zero. The P values (ANOVA, Tukey post test) in panel A are relative to LAT1.8A. The P values in panel B are relative to LAT2.5A. dLAT, dLAT2903; dLATR, dLAT2903R.

by numerous factors. During immortalization or transformation of mammalian cells, the apoptotic pathways may be altered, resulting in the generation of long-lived cell lines that grow continuously. Thus, it is difficult to make sweeping conclusions about the ability of LAT to interfere with cell death induced by Bax or the chemicals used in this study. In spite of these shortcomings, one can conclude that LAT has the potential to inhibit cell death, which is consistent with previous findings (42). In particular, LAT can interfere with Bax-induced apoptosis in CV-1 cells.

Because the stable 2-kb LAT is easily detected during neuronal latency while the remaining LAT RNA is difficult to detect, the term LAT is sometimes used to refer to the 2-kb LAT rather than the primary 8.3-kb transcript. We showed here that a plasmid expressing the first 1.5 kb of the primary 8.3-kb LAT promotes cell survival as efficiently as a plasmid expressing the entire 2-kb LAT. This 1.5-kb region contains only the first 838 nucleotides of the 2-kb LAT, and none of this RNA has the stability of the intact 2-kb LAT (45). Thus, as we showed previously for spontaneous reactivation (45), neither the entire 2-kb LAT nor stability of the LAT RNA is required for promoting cell survival after apoptosis induction.

Although the 1.5-kb transcript contains several small open reading frames (ORFs), they are not well conserved among HSV strains KOS, McKrae, and 17syn+, even though LATs from all three of these strains can promote efficient spontaneous reactivation (15). This suggests that these ORFs are not expressed or not important or that their functional domains are small and not very obvious. The lack of an obvious poly(A) addition site in the pLAT3.3 plasmid, which nonetheless promoted cell survival, and the lack of obvious poly(A) addition sites within the insertion site in the LAT3.3A virus, which nonetheless reactivates efficiently from latency, appear to support the hypothesis that a LAT protein is not expressed. If a LAT protein does not exist, it would appear that LAT RNA sequences promote cell survival.

Approximately 90% of steady-state LAT (i.e., the stable 2-kb LAT) is localized in the nucleus (50, 56). Only 10% of LAT is found in the cytoplasm, some of which appears to be associated with polyribosomes (24). At first glance, this appears to be inconsistent with the ability of LAT to block apoptosis, as this function might be thought of as requiring a LAT product in the cytoplasm. Even if LAT were exclusively limited to the nucleus, it could inhibit apoptosis by regulating transcription of one or more cellular genes. The stable 2-kb LAT represents the overwhelming majority of LAT that is detected during acute and latent infection, suggesting that if other novel forms of LAT were expressed, they would be difficult to detect. Thus, studies indicating that LAT is localized in the nucleus are essentially referring to the 2-kb LAT. As discussed above, the stable 2-kb LAT is not required either for wild-type levels of spontaneous reactivation or for blocking apoptosis. These functions can both be accomplished by just the first 1.5 kb of the primary LAT, a fragment that includes only the first 838 nucleotides of the 2-kb LAT. This truncated LAT lacks the stability of the 2-kb LAT, and its subcellular location is unknown. Thus, it will be of interest to elucidate the structure and subcellular localization of transcripts that are expressed by LAT sequences which are capable of inhibiting apoptosis. Studies directed at pinpointing the sequences in LAT that

interfere with apoptosis and identification of cellular components affected by LAT will be pursued.

REFERENCES

1. **Asano, S., T. Honda, F. Goshima, D. Watanabe, Y. Miyake, Y. Sugiura, and Y. Nishiyama.** 1999. US3 protein kinase of herpes simplex virus type 2 plays a role in protecting corneal epithelial cells from apoptosis in infected mice. J. Gen. Virol. **80:**51–56.
2. **Aubert, M., and J. A. Blaho.** 1999. The herpes simplex virus type 1 regulatory protein ICP27 is required for the prevention of apoptosis in infected human cells. J. Virol. **73:**2803–2813.
3. **Baringer, J. R., and P. Swoveland.** 1973. Recovery of herpes simplex virus from human trigeminal ganglions. N. Engl. J. Med. **288:**648–650.
4. **Bastian, F. O., A. S. Rabson, C. L. Yee, and T. S. Tralka.** 1972. Herpesvirus hominis: isolation from human trigeminal ganglion. Science **178:**306–307.
5. **Bernhard, D., M. J. Ausserlechner, M. Tonko, M. Löffler, B. L. Hartmann, A. Csordas, and R. Kofler.** 1999. Apoptosis induced by the histone deacetylase inhibitor sodium butyrate in human leukemic lymphoblasts. FASEB J. **13:**1991–2001.
6. **Boesen-de Cock, J. G., A. D. Tepper, E. de Vries, W. J. van Blitterswijk, and J. Borst.** 1999. Common regulation of apoptosis signaling induced by CD95 and the DNA-damaging stimuli etoposide and gamma-radiation downstream from caspase-8 activation. J. Biol. Chem. **274:**14255–14261.
7. **Busser, J., D. S. Geldmacher, and K. Herrup.** 1998. Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. J. Neurosci. **18:**2801–2807.
8. **Chen, S. H., M. F. Kramer, P. A. Schaffer, and D. M. Coen.** 1997. A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus. J. Virol. **71:**5878–5884.
9. **Ciacci-Zanella, J., M. Stone, G. Henderson, and C. Jones.** 1999. The latency-related gene of bovine herpesvirus 1 inhibits programmed cell death. J. Virol. **73:**9734–9740.
10. **Ciacci-Zanella, J. R., and C. Jones.** 1999. Fumonisin B1, a mycotoxin contaminant of cereal grains, and inducer of apoptosis via the tumour necrosis factor pathway and caspase activation. Food Chem. Toxicol. **37:**703–712.
11. **Deatly, A. M., J. G. Spivack, E. Lavi, and N. W. Fraser.** 1987. RNA from an immediate early region of the type 1 herpes simplex virus genome is present in the trigeminal ganglia of latently infected mice. Proc. Natl. Acad. Sci. USA **84:**3204–3208.
12. **Deatly, A. M., J. G. Spivack, E. Lavi, D. R. O'Boyle 3rd, and N. W. Fraser.** 1988. Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissues of mice map to similar regions of the viral genome. J. Virol. **62:**749–756.
13. **Devireddy, L. R., and C. J. Jones.** 1999. Activation of caspases and p53 by bovine herpesvirus 1 infection results in programmed cell death and efficient virus release. J. Virol. **73:**3778–3788.
14. **Devireddy, L. R., and C. J. Jones.** 2000. Olf-1, a neuron-specific transcription factor, can activate the herpes simplex virus type 1-infected cell protein 0 promoter. J. Biol. Chem. **275:**77–81.
15. **Drolet, B. S., G. C. Perng, J. Cohen, S. M. Slanina, A. Yukht, A. B. Nesburn, and S. L. Wechsler.** 1998. The region of the herpes simplex virus type 1 LAT gene involved in spontaneous reactivation does not encode a functional protein. Virology **242:**221–232.
16. **Drolet, B. S., G. C. Perng, R. J. Villosis, S. M. Slanina, A. B. Nesburn, and S. L. Wechsler.** 1999. Expression of the first 811 nucleotides of the herpes simplex virus type 1 latency-associated transcript (LAT) partially restores wild-type spontaneous reactivation to a LAT-null mutant. Virology **253:**96–106.
17. **Farrell, M. J., A. T. Dobson, and L. T. Feldman.** 1991. Herpes simplex virus latency-associated transcript is a stable intron. Proc. Natl. Acad. Sci. USA **88:**790–794.
18. **Galvan, V., R. Brandimarti, and B. Roizman.** 1999. Herpes simplex virus 1 blocks caspase-3-independent and caspase-dependent pathways to cell death. J. Virol. **73:**3219–3226.
19. **Galvan, V., and B. Roizman.** 1998. Herpes simplex virus 1 induces and blocks apoptosis at multiple steps during infection and protects cells from exoge-

nous inducers in a cell-type-dependent manner. Proc. Natl. Acad. Sci. USA **95:**3931–3936.

20. **Garber, D. A., P. A. Schaffer, and D. M. Knipe.** 1997. A LAT-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. J. Virol. **71:**5885–5893.

21. **Gill, J. S., and A. J. Windebank.** 1998. Cisplatin-induced apoptosis in rat dorsal root ganglion neurons is associated with attempted entry into the cell cycle. J. Clin. Investig. **101:**2842–2850.

22. **Giuliano, M., M. Lauricella, G. Calvaruso, M. Carabillo, S. Emanuele, R. Vento, and G. Tesoriere.** 1999. The apoptotic effects and synergistic interaction of sodium butyrate and MG132 in human retinoblastoma Y79 cells. Cancer Res. **59:**5586–5595.

23. **Gobbel, G. T., M. Bellinzona, A. R. Vogt, N. Gupta, J. R. Fike, and P. H. Chan.** 1998. Response of postmitotic neurons to X-irradiation: implications for the role of DNA damage in neuronal apoptosis. J. Neurosci. **18:**147–155.

24. **Goldenberg, D., N. Mador, M. J. Ball, A. Panet, and I. Steiner.** 1997. The abundant latency-associated transcripts of herpes simplex virus type 1 are bound to polyribosomes in cultured neuronal cells and during latent infection in mouse trigeminal ganglia. J. Virol. **71:**2897–2904.

25. **Hsu, H., J. Xiong, and D. V. Goeddel.** 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell **81:**495–504.

26. **Hu, S., P. K. Peterson, and C. C. Chao.** 1997. Cytokine-mediated neuronal apoptosis. Neurochem. Int. **30:**427–431.

27. **Jones, C.** 1998. Alphaherpesvirus latency: its role in disease and survival of the virus in nature. Adv. Virus Res. **51:**81–133.

28. **Jones, C., G. Delhon, A. Bratanich, G. Kutish, and D. Rock.** 1990. Analysis of the transcriptional promoter which regulates the latency-related transcript of bovine herpesvirus 1. J. Virol. **64:**1164–1170.

29. **Knotts, F. B., M. L. Cook, and J. G. Stevens.** 1974. Pathogenesis of herpetic encephalitis in mice after ophthalmic inoculation. J. Infect. Dis. **130:**16–27.

30. **Kramer, M. F., S. H. Chen, D. M. Knipe, and D. M. Coen.** 1998. Accumulation of viral transcripts and DNA during establishment of latency by herpes simplex virus. J. Virol. **72:**1177–1185.

31. **Kramer, M. F., and D. M. Coen.** 1995. Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. J. Virol. **69:**1389–1399.

32. **Krause, P. R., K. D. Croen, S. E. Straus, and J. M. Ostrove.** 1988. Detection and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia. J. Virol. **62:**4819–4823.

33. **Krummenacher, C., J. M. Zabolotny, and N. W. Fraser.** 1997. Selection of a nonconsensus branch point is influenced by an RNA stem-loop structure and is important to confer stability to the herpes simplex virus 2-kilobase latency-associated transcript. J. Virol. **71:**5849–5860.

34. **Kumar, S., M. Kinoshita, M. Noda, N. G. Copeland, and N. A. Jenkins.** 1994. Induction of apoptosis by the mouse Nedd2 gene, which encodes a protein similar to the product of the *Caenorhabditis elegans* cell death gene ced-3 and the mammalian IL-1 beta-converting enzyme. Genes Dev. **8:**1613–1626.

35. **Le-Niculescu, H., E. Bonfoco, Y. Kasuya, F. X. Claret, D. R. Green, and M. Karin.** 1999. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. Mol. Cell. Biol. **19:**751–763.

36. **Leopardi, R., and B. Roizman.** 1996. The herpes simplex virus major regulatory protein ICP4 blocks apoptosis induced by the virus or by hyperthermia. Proc. Natl. Acad. Sci. USA **93:**9583–9587.

37. **Nip, J., D. K. Strom, B. E. Fee, G. Zambetti, J. L. Cleveland, and S. W. Hiebert.** 1997. E2F-1 cooperates with topoisomerase II inhibition and DNA damage to selectively augment p53-independent apoptosis. Mol. Cell. Biol. **17:**1049–1056.

38. **Park, D. S., S. E. Farinelli, and L. A. Greene.** 1996. Inhibitors of cyclin-dependent kinases promote survival of post-mitotic neuronally differentiated PC12 cells and sympathetic neurons. J. Biol. Chem. **271:**8161–8169.

39. **Park, D. S., B. Levine, G. Ferrari, and L. A. Greene.** 1997. Cyclin dependent kinase inhibitors and dominant negative cyclin dependent kinase 4 and 6 promote survival of NGF-deprived sympathetic neurons. J. Neurosci. **17:**8975–8983.

40. **Park, D. S., E. J. Morris, L. Stefanis, C. M. Troy, M. L. Shelanski, H. M. Geller, and L. A. Greene.** 1998. Multiple pathways of neuronal death induced by DNA-damaging agents, NGF deprivation, and oxidative stress. J. Neurosci. **18:**830–840.

41. **Pawlowski, J., and A. S. Kraft.** 2000. Bax-induced apoptotic cell death. Proc. Natl. Acad. Sci. **97:**529–531.

42. **Perng, G.-C., C. Jones, J. Ciacci-Zanella, M. Stone, G. Henderson, A. Yukht, S. M. Slanina, F. M. Hoffman, H. Ghiasi, A. B. Nesburn, and S. Wechsler.** 2000. Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript (LAT). Science **287:**1500–1503.

43. **Perng, G.-C., K. Chokephaibulkit, R. L. Thompson, N. M. Sawtell, S. M. Slanina, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1996. The region of the herpes simplex virus type 1 LAT gene that is colinear with the ICP34.5 gene is not involved in spontaneous reactivation. J. Virol. **70:**282–291.

44. **Perng, G.-C., E. C. Dunkel, P. A. Geary, S. M. Slanina, H. Ghiasi, R. Kaiwar, A. B. Nesburn, and S. L. Wechsler.** 1994. The latency-associated transcript gene of herpes simplex virus type 1 (HSV-1) is required for efficient in vivo spontaneous reactivation of HSV-1 from latency. J. Virol. **68:**8045–8055.

45. **Perng, G.-C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, and S. L. Wechsler.** 1996. The spontaneous reactivation function of the herpes simplex virus type 1 LAT gene resides completely within the first 1.5 kilobases of the 8.3-kilobase primary transcript. J. Virol. **70:**976–984.

46. **Perng, G.-C., S. M. Slanina, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 1996. A 371-nucleotide region between the herpes simplex virus type 1 (HSV-1) LAT promoter and the 2-kilobase LAT is not essential for efficient spontaneous reactivation of latent HSV-1. J. Virol. **70:**2014–2018.

47. **Perng, G.-C., S. M. Slanina, A. Yukht, H. Ghiasi, A. B. Nesburn, and S. L. Wechsler.** 2000. The latency-associated transcript gene enhances establishment of herpes simplex virus type 1 latency in rabbits. J. Virol. **74:**1885–1891.

48. **Rock, D. L., A. B. Nesburn, H. Ghiasi, J. Ong, T. L. Lewis, J. R. Lokensgard, and S. L. Wechsler.** 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J. Virol. **61:**3820–3826.

49. **Soldatenkov, V. A., S. Prasad, Y. Voloshin, and A. Dritschilo.** 1998. Sodium butyrate induces apoptosis and accumulation of ubiquitinated proteins in human breast carcinoma cells. Cell Death Differ. **5:**307–312.

50. **Speck, P. G., and A. Simmons.** 1991. Divergent molecular pathways of productive and latent infection with a virulent strain of herpes simplex virus type 1. J. Virol. **65:**4001–4005.

51. **Stevens, J. G., E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman.** 1987. RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. Science **235:**1056–1059.

52. **Wagner, E. K., and D. C. Bloom.** 1997. Experimental investigation of herpes simplex virus latency. Clin. Microbiol. Rev. **10:**419–443.

53. **Wagner, E. K., W. M. Flanagan, G. Devi-Rao, Y. F. Zhang, J. M. Hill, K. P. Anderson, and J. G. Stevens.** 1988. The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. J. Virol. **62:**4577–4585.

54. **Wechsler, S. L., A. B. Nesburn, R. Watson, S. Slanina, and H. Ghiasi.** 1988. Fine mapping of the major latency-related RNA of herpes simplex virus type 1 in humans. J. Gen. Virol. **69:**3101–3106.

55. **White, E.** 1996. Life, death, and the pursuit of apoptosis. Genes Dev. **10:**1–15.

56. **Zwaagstra, J. C., H. Ghiasi, S. M. Slanina, A. B. Nesburn, S. C. Wheatley, K. Lillycrop, J. Wood, D. S. Latchman, K. Patel, and S. L. Wechsler.** 1990. Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript. J. Virol. **64:**5019–5028.