Journal of NeuroVirology, 10: 64–70, 2004
© 2004 Journal of NeuroVirology
ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280490261716

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Short Communication

The latency-related gene encoded by bovine herpesvirus 1 can suppress caspase 3 and caspase 9 cleavage during productive infection

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When the bovine herpesvirus 1 (BHV-1) latency-related (LR) gene is inserted into the latency-associated transcript (LAT) locus of a herpes simplex virus type 1 (HSV-1) LAT deletion mutant, high levels of spontaneous reactivation from latency and enhanced pathogenesis occur. The LR gene, but not LAT, inhibits caspase 3 cleavage during productive infection. Plasmids containing LAT or the LR gene inhibit caspase 3 activation in transiently transfected cells, suggesting productive infection blocks certain antiapoptotic properties of LAT. These studies demonstrate a correlation between the enhanced pathogenic potential of CJLAT and the LR gene inhibiting caspase 3 cleavage during productive infection. *Journal of NeuroVirology* (2004) **10,** 64–70.

Keywords: apoptosis; bovine herpesvirus 1 (BHV-1) latency related (LR) gene; caspase cleavage

Bovine herpesvirus 1 (BHV-1) infections cause a significant economic loss to the cattle industry. Clinical symptoms include abortions, encephalitis, conjunctivitis, respiratory disease, genital disease, and pneumonia (Li *et al*, 1997; Liu *et al*, 1996; Nicholson and Thornberry, 1997; Wolf and Green, 1999; Zou *et al*, 1999). BHV-1 also induces immunosuppression resulting in secondary bacterial infections, bronchopneumonia, and even death. Infection in cattle is initiated by productive infection on mucosal epithelium. During productive infection, 70 to 80 viral genes are temporally expressed. Like other members of the Alphaherpesvirinae subfamily, BHV-1 establishes and maintains a life long latent infection in sensory ganglionic neurons of its host.

The only BHV-1 gene expressed in latently infected neurons is the latency-related (LR) transcript that is encoded by the LR gene (Rock *et al*, 1987). A fraction of LR RNA is polyadenylated and alternatively spliced in bovine trigeminal ganglia (TG) (Devireddy and Jones, 1998; Hossain *et al*, 1995). A LR protein has been identified (Hossain *et al*, 1995) that associates with cdk2/cyclin complexes (Jiang *et al*, 1998). LR gene products inhibit S phase entry (Schang *et al*, 1996) and interfere with chemically induced apoptosis (Ciacci-Zanella *et al*, 1999). A stop codon mutation near the beginning of the LR protein–coding sequences interferes with virus shedding from the eye and TG during acute infection of calves. Furthermore, the LR mutant does not reactivate from latency following dexamethasone treatment (Inman *et al*, 2001a, 2002), indicating that the LR gene has several functions that are required for the latency-reactivation cycle.

Herpes simplex virus type 1 (HSV-1) encodes a latency-associated transcript (LAT) that is abundantly transcribed during latency and is antisense to ICP0 (Jones, 1998; Rock *et al*, 1987). LAT expression promotes establishment and reactivation from latency in mouse and rabbit models (Maggioncalda *et al*, 1996; Perng *et al*, 1994, 1996, 2000; Sawtell, 1997; Sawtell and Thompson, 1992; Thompson and Sawtell, 1997, 2001). LAT inhibits apoptosis (Ahmed *et al*, 2002; Inman *et al*, 2001b; Perng *et al*, 2000) and decreases expression of other viral genes in

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This study was supported by the Discovery Fund for Eye Research, the Skirball Program in Molecular Ophthalmology, Public Health Service grants to SW (EY11629, and EY12823) and CJ (1P20RR15635), and USDA grants to CJ (2000-02060, 2002-02450, and 2003-02213).

Received 2 July 2003; revised 27 August 2003; accepted 29 August 2003.

a neuronal-derived cell line (Mador *et al*, 1998), suggesting that LAT, like the LR gene, has several functions.

To test whether the LR gene could restore spontaneous reactivation to an HSV-1 LAT deletion mutant (dLAT2903), the LR gene was recombined into the LAT locus of dLAT2903, creating a recombinant virus with two copies of the BHV-1 LR gene (CJLAT). In a rabbit ocular model of latency, CJLAT reactivated more efficiently than dLAT2903 and with similar or greater efficiency than wild-type (wt) HSV-1 McKrae strain (Perng *et al*, 2002). Compared to wt HSV-1 or dLAT2903, CJLAT killed mice with higher frequencies and caused more eye disease in latently infected rabbits. This study indicates that the LR gene enhances the pathogenic potential of the McKrae strain, and restores high levels of spontaneous reactivation to dLAT2903.

Plasmids containing various lengths of HSV-1 LAT (Ahmed *et al*, 2002; Inman *et al*, 2001b; Perng *et al*, 2000; Jin *et al*, 2003) or the BHV-1 LR gene (Ciacci-Zanella *et al*, 1999) enhance cell survival following apoptosis induction. These studies demonstrate that the antiapoptotic properties of LAT-expressing plasmids correlate with the ability of viruses expressing the corresponding LAT sequences to reactivate in the ocular rabbit model of HSV-1 latency. In the same rabbit ocular model of HSV-1 latency, a McKrae LAT deletion mutant (dLAT2903) had increased levels of apoptosis in rabbit TG (Perng *et al*, 2000). We hypothesized that the enhanced pathogenic properties of CJLAT may reflect novel antiapoptotic properties of the BHV-1 LR gene. Since caspases are cysteine proteases that stimulate apoptosis (Nicholson and Thornberry, 1997; Thornberry *et al*, 1997; White, 1996), we further hypothesized that the LR gene may have different effects on caspase activation when compared to LAT.

There are two major apoptotic pathways: the death receptor mediated pathway (Fas or tumor necrosis factor receptor for example) and the mitochondrial pathway (Kruegger, 2001; Schmitz *et al*, 2000; Wang, 2001). The death receptor mediated pathway activates caspase 8, leading to caspase 3 cleavage and activation. Activation of the mitochondrial pathway results in release of several proapoptotic molecules, cytochrome *c* and Smac/Diablo for example (Wang, 2001). Caspase 3 cleavage and activation leads to the morphological hallmarks of apoptosis.

Previous studies demonstrated that LAT inhibits apoptosis in neuro-2A cells (Inman *et al*, 2001b; Henderson *et al*, 2002; Jin *et al*, 2003). Neuro-2A cells are derived from the peripheral nervous system (Olmsted *et al*, 1970) and they express wt p53 (Devireddy, 1999), suggesting they are suitable to study the effects of LAT on apoptosis. When neuro-2A cultures were infected with wt McKrae, dLAT2903, dLAT2903R (marker rescued dLAT2903), or CJLAT (multiplicity of infection [moi] of 4), there was no dramatic difference in virus titers at 48 h after infection, which is consistent with other cell lines previously tested (Perng *et al*, 2002). However, cultures infected with dLAT2903 contained between 25% and 50% more apoptotic cells at 24 or 48 h after infection compared to cultures infected with HSV-1 or CJLAT (data not shown).

We initially tested whether the LR gene or LAT altered caspase 9 cleavage in neuro-2A cultures. The caspase 9 antibody used for this study detects the intact caspase 9 protein (denoted by the circle in Figure 1) and various cleavage products, the most prominent being the 37- and 39-kDa bands. The 39-kDa caspase 9 cleavage product is the result of caspase 3 cleaving caspase 9 at Asp368 (Li *et al*, 1997; Liu *et al*, 1996; Nicholson and Thornberry, 1997; Wolf and Green, 1999; Zou *et al*, 1999). The caspase 9 cleavage band at 37 kDa is the result of self-cleavage at Asp353. When neuro-2A cultures were treated with ultraviolet (UV) light for 30 s and then incubated for 4 h in medium, a prominent 37-kDa self-cleavage product was detected. Neuro-2A cells infected with dLAT2903 for 48 h or mock-infected cultures contained similar levels of the 37-kDa cleavage product (Figure 1). Neuro-2A cells exhibit increasing levels of apoptosis after plating because they deplete necessary growth factors. In contrast to mock- or dLAT2903-infected cultures, neuro-2A cultures infected with McKrae, dLAT2903R (marker rescued dLAT2903; data not shown), or CJLAT did not contain detectable levels of the 37-kDa cleavage product at 48 h after infection (Figure 1). The 39-kDa band and diffuse bands migrating below the 37-kDa band were also reduced in cultures infected with McKrae or CJLAT. The finding that wt McKrae reduced caspase 9 cleavage was not surprising because HSV-1 can inhibit apoptosis in certain cell types (Galvan and Roizman, 1998). No dramatic effects on caspase 8 cleavage were observed in neuro-2A cells following infection with any of the viruses (data not shown). In summary, these studies demonstrated that LAT and the LR gene inhibited self-cleavage of caspase 9 during productive infection of neuro-2A cells.

Caspase 3 is a key executioner of apoptosis, and can be cleaved by caspase 8 or 9 (Nicholson and Thornberry, 1997; Thornberry *et al*, 1997; Wang, 2001; Wolf and Green, 1999). Activation of caspase 3 requires proteolytic processing at Asp175 into a 19-kDa (prodomain + large fragment) and 17-kDa fragment (large fragment). An antibody that specifically recognizes the 17- and 19-kDa fragments of cleaved caspase 3 (cell signaling) was used for this study. High levels of the 17-kDa cleavage product of caspase 3 were detected in mock-infected neuro-2A cultures treated with UV light for 30 s and then incubated for 4 h (Figure 2A), confirming the antibody detects cleaved caspase 3. Increased levels of cleaved caspase 3 were also detected in mock-infected cells 48 h after infection. Compared to mock-infected cells, cultures infected with McKrae or dLAT2903 contained

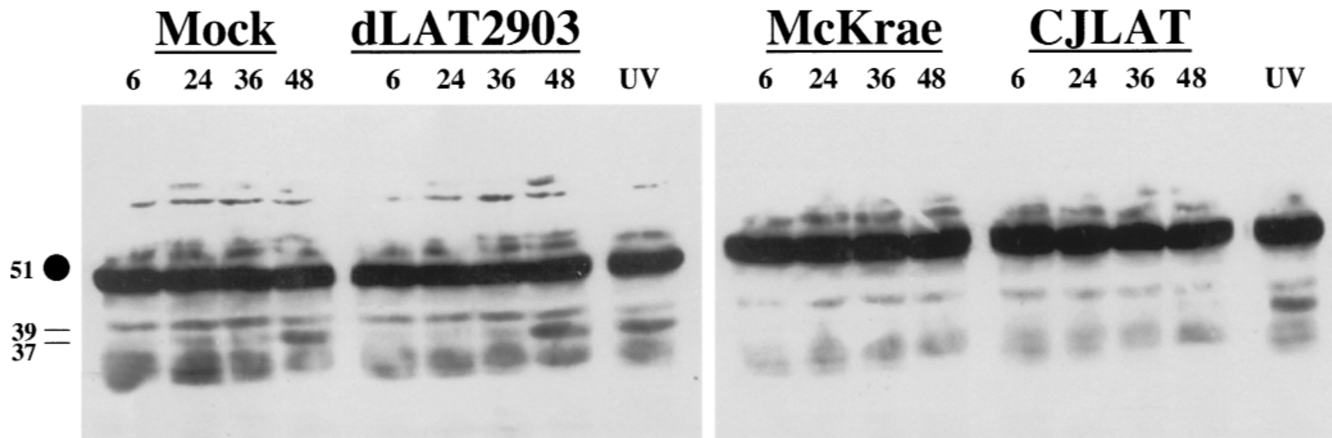

Figure 1 Analysis of caspase 9 cleavage in neuro-2A cells. Cultures of neuro-2A cells were infected with dLAT2903, McKrae, or CJLAT (moi = 4). At the designated times (hours) after infection, cell lysate was prepared as described by the supplier of the cleaved caspase 9 antibody (Cell Signaling) using their commercially available buffers. Mock-infected cells were used as a control. Cultures of neuro-2A cells in a 100-mm dish were exposed to UV light (312 nm) for 30 s. The medium was removed from the plate, the lid removed, and the dish was inverted onto a *trans*-illuminator (IBI; catalogue 46524). The same medium was added back to the culture, the cells were incubated at 37°C for 4 h, and then cell lysate prepared. Protein samples (100 μg protein) were loaded onto a 12% SDS-PAGE and Western blot analysis performed using an antibody that recognizes caspase 9 (Cell Signaling, catalogue no. 9504; Beverly, MA). The closed circle denotes the location of procaspase 9. The size of procaspase 9 and the major cleavage products are also indicated. These results are representative of four different experiments.

low levels of the 17-kDa cleavage product relative to the 19-kDa protein until 48 h after infection, suggesting that infection interfered with removal of the prodomain from the large subunit of caspase 3.

In contrast to cultures infected with dLAT2903 or McKrae, factors encoded or induced by CJLAT inhibited cleavage of procaspase 3 even at 48 h after infection (Figure 2A). Although we detected low levels of the 17-kDa cleavage fragment in cultures infected with dLAT2903 or wt McKrae until 48 h after infection, cultures infected with CJLAT clearly had lower levels of the 17-kDa cleaved fragment for at least 48 h after infection. Procaspase 3 levels were not increased dramatically by any of the viruses tested (Figure 2B).

The finding that LAT inhibited cleavage (activation) of caspase 9, but not caspase 3, was surprising because caspase 3 is down stream of caspase 9, and caspase 9 can activate caspase 3 (Nicholson and Thornberry, 1997; Thornberry *et al*, 1997; Wang, 2001; White, 1996). HSV-1 induces or inhibits apoptosis by cell type–dependent mechanisms (Galvan and Roizman, 1998) because it encodes several antiapoptotic factors (Blaho and Aubert, 2001). The HSV-1 antiapoptotic protein U_S3 blocks caspase 3 activation induced by two viral proteins ($U_S1.5$, and U_L13) (Hagglund *et al*, 2002). Based on these observations, we hypothesized that LAT does not play an easily detectable role in preventing caspase 3 activation in productively infected cells. However, we predicted that LAT inhibits caspase 3 activation in the absence of other viral genes.

As discussed in the preceding paragraph, several viral genes can regulate apoptosis, suggesting that LAT may have little or no detectable effect on caspase 3 cleavage in productively infected neuro-2A cells. To test whether LAT interfered with caspase 3 activation in the absence of the other viral genes that regulate apoptosis, we analyzed caspase 3 activation in neuro-2A cells cotransfected with a LAT expression plasmid (pLAT+2851), a cytomegalovirus (CMV) plasmid expressing Bax, and the pCaspase3-Sensor plasmid (Clontetech; Palo Alto, CA). Plasmid pCaspase3-Sensor encodes the enhanced yellow-green variant of the green fluorescent protein (GFP), which has three copies of the SV40 large T-antigen nuclear localization signal at the 3′ end of the GFP gene. At the 5′ terminus of the GFP gene, a sequence encoding the nuclear export signal (NES) of the mitogen-activated protein (MAP) kinase kinase is located. The NES is separated from GFP-coding sequences by a 36-bp cassette containing the region of poly (ADP-ribose) polymerase cleaved by caspase 3. As expected, GFP is primarily localized to the cytoplasm of healthy cells (Figure 3A and B). When neuro-2A cells were cotransfected with the CMV Bax expression plasmid and the pCaspase3-Sensor plasmid, caspase 3 was activated, NES was cleaved, and GFP localized to the nucleus (Figure 3C and D).

Approximately 3 times more cells contained GFP in the cytoplasm (nonapoptotic) when cultures were cotransfected with a LAT expression plasmid (LAT nucleotides −801 to +2851; pLAT+2851), the pCaspase3-Sensor plasmid, and the CMV Bax plasmid (Figure 3E). Because LAT has no effect on the CMV promoter (Inman *et al*, 2001b), this result was not due to LAT repressing Bax expression. Consistent

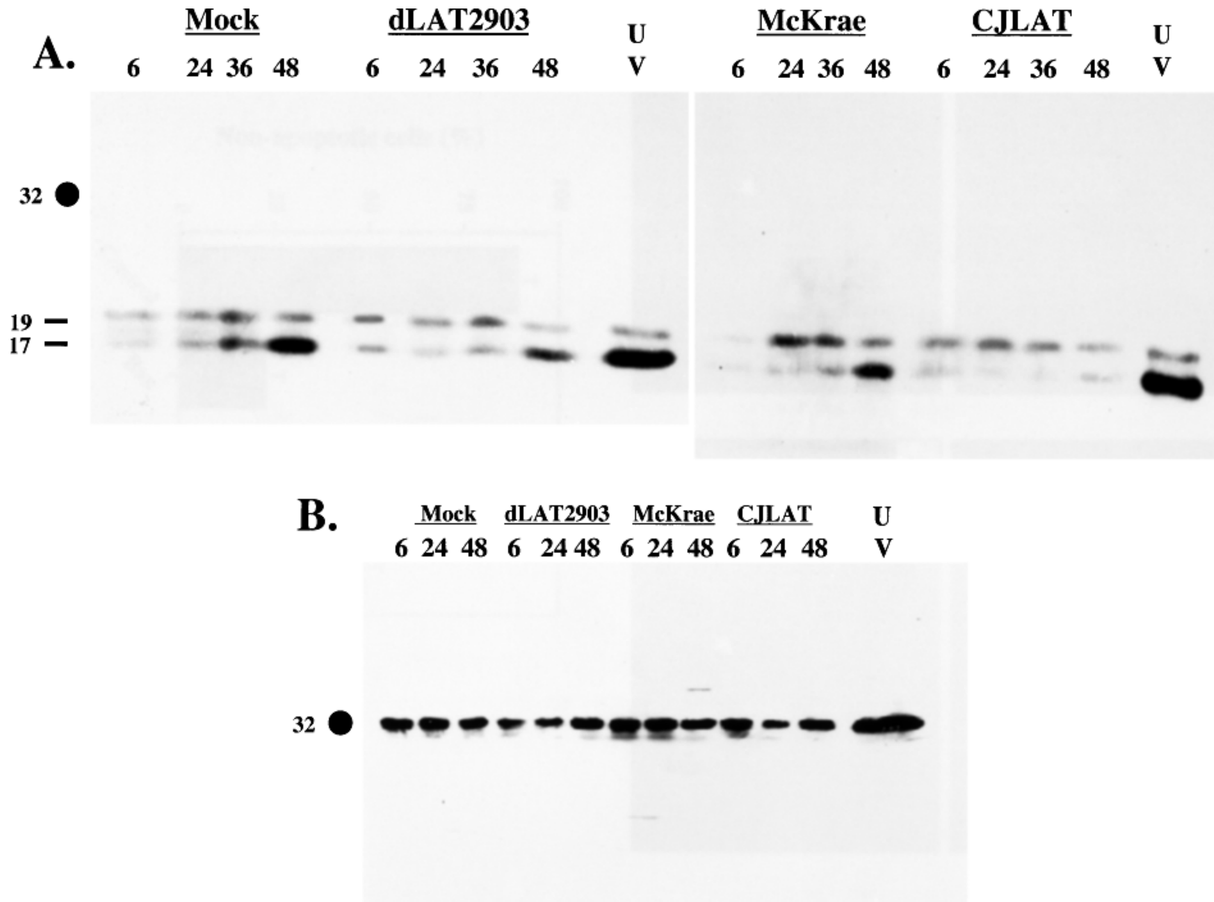

Figure 2 Analysis of caspase 3 cleavage in neuro-2A cells. Neuro-2A cultures were infected with dLAT2903, McKrae, or CJLAT (moi = 4). At the designated times after infection, cell lysate was prepared as described by the supplier using their cell lysis buffers. (**A**) Western blot analysis performed using an antibody that recognizes cleaved caspase 3 (Cell Signaling, catalogue no. 9661). The closed circle denotes the location of procaspase 3. The size of procaspase 3 and the major cleavage products are also indicated. These results are representative of three different experiments. (**B**) Western blot analysis performed using an antibody that recognizes caspase 3 (cleaved and uncleaved products) (Cell Signaling, catalogue no. 9662). At this exposure, the cleaved products were not detected. These results are representative of two different experiments.

with the finding that the LR gene inhibited caspase 3 cleavage in neuro-2A cells infected with CJLAT, we found that the LR gene inhibited caspase 3 activation in transiently transfected neuro-2A cells (Figure 3E). These results indicate that LAT and the LR gene can inhibit caspase 3 activation in transiently transfected neuro-2A cells.

Our studies indicated that the LR gene, but not LAT, has the potential to inhibit caspase 3 cleavage (activation) in productively infected Neuro-2A cells (Figure 2), suggesting that the LR gene has novel anti-apoptotic properties compared to LAT. It is also possible that LR gene products are expressed at higher levels in the context of CJLAT compared to LAT when cultures were infected with wt McKrae. The ability of the BHV-1 LR gene to inhibit procaspase 3 cleavage during productive infection correlates with the enhanced pathogenic properties of CJLAT (Perng *et al*, 2002). We hypothesize that CJLAT may survive more efficiently in certain cell types, allowing it to invade and spread to regions in the eye or nervous system that are usually not infected. Studies designed to test this hypothesis are currently underway.

When plasmids expressing LAT and the LR gene were cotransfected with Bax into neuro-2A cells, both inhibited caspase 3 (Figure 3), indicating that LAT can inhibit caspase 3 activation when other viral genes are not being expressed. Because HSV-1 has several apoptotic regulatory genes (Blaho and Aubert, 2002), the interaction between these genes and the functional redundancy of these genes can make it difficult to fully understand the function of a single gene during productive infection.

Because LAT and the LR gene inhibited caspase 9 cleavage during productive infection, it is logical to ask whether caspase 9 plays a role in neuronal apoptosis. Caspase 9 knockout mice are not viable because caspase 9 plays a crucial role in the developing mouse nervous system (Hakem *et al*, 1998; Kuida *et al*, 1998). Further support for the importance of caspase 9 in

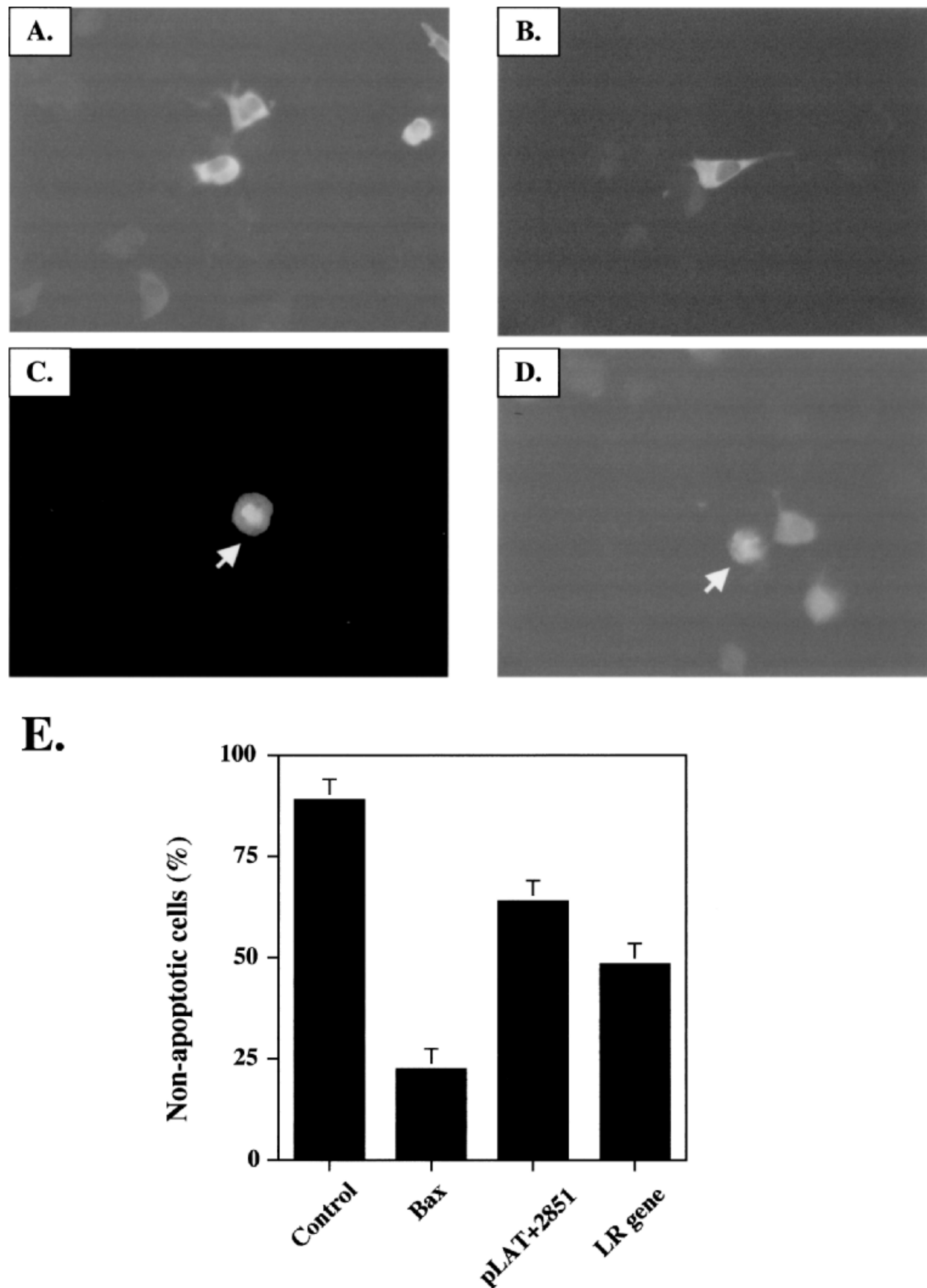

Figure 3 Inhibition of caspase 3 by LAT and the LR gene. (**A**) Neuro-2A cultures were cotransfected with the pCaspase3-Sensor plasmid (Clontech, Palo Alto, CA; 0.5 μg) and the following plasmids: pcDNA3.1− (Invitrogen, Carlsbad, CA; 4.0 μg) (**A** and **B**); pcDNA3.1− (3.25 μg) plus the CMV Bax expression vector (Upstate Biotechnology, Lake Placid, NY; 0.75 μg) (**C** and **D**). The cells in **A** to **D** were representative of cultures at 30 h after transfection. Arrows denote apoptotic cells (GFP primarily in nucleus). GFP+ cells were viewed with a Leica fluorescence microscope. (**E**) Neuro-2A cultures were cotransfected with the plasmids described below: Control: pCaspase3-Sensor plasmid (0.5 μg) and pcDNA3.1− (2.25 μg); Bax: pCaspase3-Sensor plasmid (0.5 μg), CMV Bax expression vector (0.75 μg), and pcDNA3.1− (1.5 μg); pLAT + 2851: pCaspase3-Sensor plasmid (0.5 μg), pLAT + 2851 (1.5 μg), and CMV Bax expression vector (0.75 μg); LR gene: pCaspase3-Sensor plasmid (0.5 μg), plasmid LRT^{wt} gene (1.5 μg), and the CMV Bax expression vector (0.75 μg). Transfection was carried out using TransIT transfection reagents (Mirus, Madison, WI) as described by the manufacturer. Five hours after the neuro-2A cultures were transfected, cultures were rinsed with serum-free medium (EMEM), new medium + 5% fetal calf serum + 2.5 mM sodium butyrate added, and cultures incubated overnight. The next morning, cultures were rinsed with medium containing 5% fetal calf serum, and fresh medium added. The percent of nonapoptotic cells (number of cells containing GFP in cytoplasm divided by the total number of GFP+ cells) was determined at 30 h after transfection by counting the number of GFP+ cells in a given area. The values in **E** are the average of three independent experiments. At least 300 GFP+ cells were counted for the respective samples. A CMV expression plasmid containing the intact BHV-1 LR gene (LRT^{wt}) was previously described (Ciacci-Zanella *et al*, 1999). Plasmid pLAT+2851 contains the LAT promoter and LAT coding sequences ($-801 \rightarrow +2851$) and was described previously (Henderson *et al*, 2002; Jin *et al*, 2003).

neuronal apoptosis comes from several other studies (Cowan *et al*, 2001; Keane *et al*, 2001; Kermer *et al*, 2000; Krajewski *et al*, 1999; Rigamonti *et al*, 2001). HSV induces DNA damage, even in the absence of productive infection (Chenet-Monte *et al*, 1986; Heilbronn and zur Hausen, 1989; Pilon *et al*, 1986; Schlehofer and Hausen, 1982; Hamper and Ellison, 1961), and caspase 9 promotes apoptosis after DNA damage (Soengas *et al*, 1999). Consequently, infection of sensory neurons by HSV-1 or BHV-1 may lead to DNA damage and caspase 9 activation. In conclusion, we suggest that LAT and the LR gene promote the latency-reactivation cycle by inhibiting the early stages of apoptosis.

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