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Short communication

Identification of herpes simplex virus type 1 proteins encoded within the first 1.5 kb of the latency-associated transcript

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Expression of the first 1.5 kb of the latency-associated transcript (LAT) that is encoded by herpes simplex virus type 1 (HSV-1) is sufficient for wild-type (wt) levels of reactivation from latency in small animal models. Peptide-specific immunoglobulin G (IgG) was generated against open reading frames (ORFs) that are located within the first 1.5 kb of LAT coding sequences. Cells stably transfected with LAT or trigeminal ganglionic neurons of mice infected with a LAT expressing virus appeared to express the L2 or L8 ORF. Only L2 ORF expression was readily detected in trigeminal ganglionic neurons of latently infected mice. *Journal of NeuroVirology* (2009) **15**, 439–448.

Keywords: herpes simplex virus type 1; latency-associated transcript

Most adults in the United Staes are infected with herpes simplex virus type 1 (HSV-1) (Nahmias and Roizman, 1973; Whitley, 1997). Recurrent ocular HSV-1 is the leading cause of infectious corneal blindness in industrialized nations (Whitley, 1997). Acute infection is typically initiated in the epithelium, which culminates in HSV-1 establishing latency in sensory neurons located in trigeminal ganglia (TG) or sacral dorsal root ganglia (Jones, 1998; Wagner and Bloom, 1997). Periodically, HSV-1 reactivates from latency, resulting in virus shedding and recurrent disease.

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The latency-associated transcript (LAT) is abundantly transcribed in latently infected neurons of mice, rabbits, or humans (Croen *et al*, 1987; Deatly *et al*, 1987, 1988; Krause *et al*, 1988; Mitchell *et al*, 1990; Rock *et al*, 1987; Stevens *et al*, 1987; Wagner *et al*, 1988a, b). Splicing of the primary 8.3-kb transcript yields a stable 2-kb LAT and an unstable 6.3-kb LAT (Deatly *et al*, 1988; Rock *et al*, 1987; Zwaagstra *et al*, 1990) (for schematic of the 8.3-kb LAT and genes in this regions, see Figure 1A). The 2-kb LAT can be further spliced in infected neurons (Mador *et al*, 1995). LAT enhances the latency-reactivation cycle in small animal models (reviewed in Jones, 1998, 2003; Wagner and Bloom, 1997). For example, the HSV-1 McKrae strain is frequently shed in tears of infected rabbits as a result of spontaneous reactivation (Perng *et al*, 1994a, 1996a, 1996b, 1996c, 1999). In contrast, a LAT deletion mutant (dLAT2903) does not induce high levels of spontaneous reactivation in rabbits (Perng *et al*, 1994a, 1996a) or high levels of induced reactivation in mice (Perng *et al*, 2001). dLAT2903 contains a deletion from −161 to +1667 relative to the start of the primary 8.3-kb LAT, and thus does not express detectable levels of LAT (Perng *et al*, 1994a, 1996a). The spontaneous reactivation

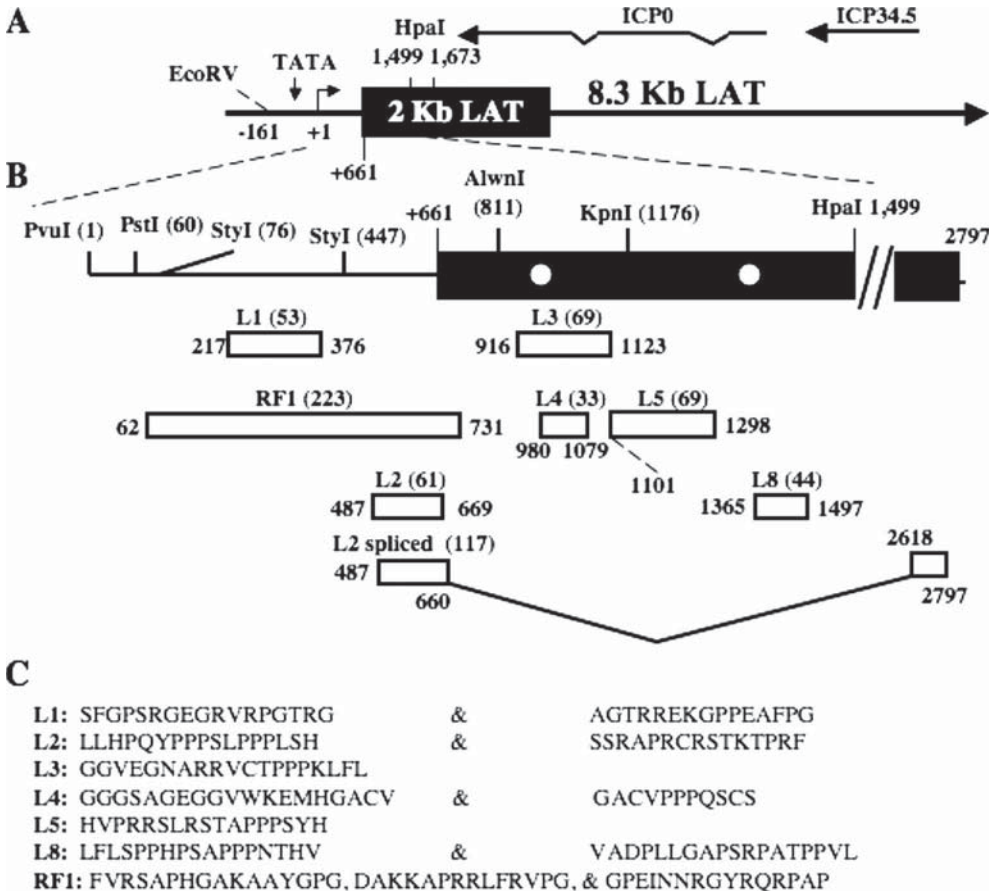


Figure 1 Schematic of viruses used in this study and the organization of the gene encoding LAT. (**A**) Schematic of genes within the long repeats that contain the LAT locus. The large arrow indicates the primary LAT transcript. The solid rectangle represents the very stable 2-kb LAT intron. The LAT TATA box is indicated by TATA. The start of LAT transcription is indicated by the arrow at +1 (genomic nucleotide 118801). Several restriction enzyme sites and the relative locations of the ICP0 and ICP34.5 transcripts are shown for reference. (**B**) Partial restriction map of LAT and position of LAT open reading frames (L1 to L8) within the first 1.5 kb of strain McKrae LAT coding sequences, which were based on previous studies (Drolet *et al*, 1998). The numbering system of the ORFs was consistent with a previous study (Drolet *et al*, 1998). Only the ORFs with at least 30 amino acids are shown (the number of amino acids in each ORF is denoted by the numbers in parenthesis). A reading frame that lacks an ATG (RF1) is also shown. The L2-spliced ORF exists because the C-terminus of L2 is at the splice junction of the stable 2-kb LAT. Open circles denote the position of two LAT small RNAs that are encoded within the first 1.5 kb of LAT coding sequences (Peng *et al*, 2008). (**C**) Antigenic peptides within the respective ORFs that were used to generate peptide-specific antibodies. The peptides and peptide-specific antiserum were prepared by ABR Affinity Bioreagents (Golden CO). For most LAT ORFs, two peptides were synthesized. The ORF-specific serum from two rabbits was pooled and peptide-specific IgG was affinity purified.

phenotype of dLAT2903 is restored to wt levels when the first 1.5 kb of LAT coding sequences (LAT nucleotides 1 to 1499; Figure 1B), driven by the LAT promoter, is inserted into an ectopic location in the virus (Jin *et al*, 2003; Perng *et al*, 1996a).

The first 1.5 kb of LAT has antiapoptosis functions (Ahmed *et al*, 2002; Inman *et al*, 2001; Jin *et al*, 2003; Peng *et al*, 2000, 2003). Plasmids expressing LAT products inhibit caspase 8– and caspase 9–induced apoptosis (Henderson *et al*, 2002; Jin *et al*, 2003), the major apoptosis pathways in mammals. LAT also reduces apoptosis of productively infected tissue culture cells (Jin *et al*, 2004), and promotes neuronal survival in TG of infected rabbits (Perng *et al*, 2000) and mice (Ahmed *et al*, 2002; Branco and Fraser, 2005). Inhibiting apoptosis appears to be the most important function of LAT because three antiapoptosis genes restore wild-type (wt) levels of spontaneous reactivation to a LAT null mutant (Jin *et al*, 2005, 2008; Mott *et al*, 2003; Perng *et al*, 2002).

It is not clear whether LAT encodes a protein that regulates the latency-reactivation cycle. However, several studies have suggested that a protein encoded within sequences encompassing LAT is expressed (Doerig *et al*, 1991; Lagunoff and Roizman, 1994; Lock *et al*, 2001; Randall *et al*, 1997; Thomas *et al*, 1999, 2002). Putative LAT proteins were suggested to substitute for ICP0 functions (Thomas *et al*, 1999, 2002), interfere with binding of ICP4 to DNA (Randall *et al*, 1997), or their functions were not described. In addition, these proposed LAT proteins map downstream of the critical first 1.5 kb of the primary LAT transcript, a region that appears both

sufficient and necessary for LAT's antiapoptosis activity and its ability to support a wild-type spontaneous reactivation phenotype (Perng *et al*, 1996a, 2001). Other studies suggested LAT does not encode a protein (Drolet *et al*, 1998). None of the published studies prepared antiserum directed against potential open reading frames (ORFs) located within the first 1.5 kb of LAT coding sequences.

Within the first 1.5 kb of LAT, eight ORFs exist, and six of these are greater than 30 amino acids (aa) (Drolet *et al*, 1998) (Figure 1B). A large reading frame that lacks an initiating ATG is also present (RF1). One or more of these ORFs may play a role in inhibiting apoptosis because when all of the initiating ATGs for the respective ORFs are mutated to TTG, the first 1.5 kb of LAT no longer inhibits apoptosis (Carpenter *et al*, 2008). Antigenic peptides directed against the six ORFs within the first 1.5 kb of LAT that were larger than 30 aa, and against RF1, were synthesized (Figure 1C), and then were used to generate peptide-specific antiserum that was affinity purified. For most ORFs or RF1, more than one antigenic peptide was identified. The peptides for each ORF were coinjected into the same rabbit to enhance the immune response to each respective ORF.

Initially, LAT protein expression was analyzed in C1300 (mouse neuroblastoma) cells that stably express a NotI fragment containing LAT coding sequences (DC-LAT6 cells) (Figure 2A). DC-LAT6 cells are resistant to cold-shock–induced apoptosis (Carpenter *et al*, 2007), indicating LAT is functional in these cells. A previous study demonstrated that abundant levels of the stable 2-kb LAT were expressed in DC-LAT6 cells (Carpenter *et al*, 2007). The △PstI LAT cell lines contain the same NotI fragment, but lacks the TATA box and the major start site for LAT transcription. The △PstI LAT cell lines express little or no stable 2-kb LAT, and are not resistant to apoptosis (Carpenter *et al*, 2007). To test whether low levels of LAT expression occurred in △PstI LAT cells, primers were designed that are downstream of the LAT promoter and upstream of the stable 2-kb LAT intron sequences (Figure 2A, arrowheads). As expected, a LAT-specific product was detected in DC-LAT6 cells (Figure 2B, lane +). Low levels of a similar sized cDNA product were detected in the two △PstI LAT–transfected cell lines (Figure 2B, lanes △P), but not parental C1300 cells. Inclusion of reverse transcriptase to the cDNA synthesis reaction was required for amplification of these LAT-specific bands (−RT lanes). Subsequent studies were performed to examine LAT expression in productively infected cells using these primers. As expected, the LAT-specific cDNA product was expressed in neuro-2A cells infected with dLAT2903R (wild-type marker rescue of dLAT2903) (Figure 2C, 2903R lanes), but not in mock-infected cells or cells infected with a LAT null mutant (dLAT2903; Figure 2C, 2903 lanes) that lacks the first 1.5 kb of LAT coding sequences. Low levels of LAT may have been expressed in the △PstI cell lines because of a proposed second LAT promoter upstream of the stable 2-kb LAT sequences that begins at +661 (Chen *et al*, 1995; Goins *et al*, 1994), a region downstream of the start site of LAT transcription that is necessary for long-term expression during latency (Lokensgard *et al*, 1997), and/or integration of LAT sequences in these cell lines may be downstream of cellular promoter elements.

The L2 peptide–specific immunoglobulin G (IgG) recognized a protein that migrated at approximately 12 kDa in DC-LAT6 cells, but not parental C1300 cells (Figure 2D, Panel L2,+lane). One of the △PstI cell lines also expressed low levels of a 12-kDa protein that was recognized by the L2 peptide–specific IgG. A protein migrating at approximately 13 kDa was detected by the L8-specific IgG in DC-LAT6 cells (panel L8,+lane) and the two △PstI cell lines, but not in the parental C1300 cell line. Similar levels of protein were loaded in each lane, as judged by β-actin levels.

LAT-specific proteins were not detected by peptide-specific IgG directed against L1, L3, L4, or L5 (Figure 2D). The RF1-specific peptide IgG consistently cross-reacted with several cellular proteins when Western Blots were performed using cell lysate from C1300 cells, which made it difficult to conclude whether RF1 was expressed. In summary, these studies suggested that L2 and L8 were expressed in DC-LAT-6 cells. During productive infection of cultured cells, we have also seen low levels of L2 expression in some experiments (data not shown). The putative L2 protein may not be readily detected during productive infection because the LAT promoter is repressed by ICP4 (Batchelor and O'Hare, 1990), the transcript that is translated into the L2 ORF is not abundantly expressed during productive infection, or the L2 protein is not stably expressed in productively infected cells.

Immunohistochemistry (IHC) was performed on formalin-fixed and paraffin-embedded TG sections using the respective peptide-specific IgG. Swiss Webster mice were infected with a LAT+ virus (dLAT2903R) or a LAT null mutant (dLAT2903) (Perng *et al*, 1996d) using 1×10^5 plaque-forming units (PFU)/eye as described previously (Jones *et al*, 2005; Peng *et al*, 2005). The L2 peptide–specific IgG was initially used to test whether the L2 protein was expressed in TG during latency (30 days after infection). At 30 days after infection with dLAT2903R, TG neurons were stained with the L2-specific IgG (Figure 3A, 2903R panel; arrows denote positive neurons). In general, when L2 was detected in TG neurons, there was a tendency to observe a cluster of TG neurons that was stained by the L2-specific IgG. Most neurons stained by the L2-specific IgG were stained in the nucleus as well as weak cytoplasmic staining. L2-positive neurons

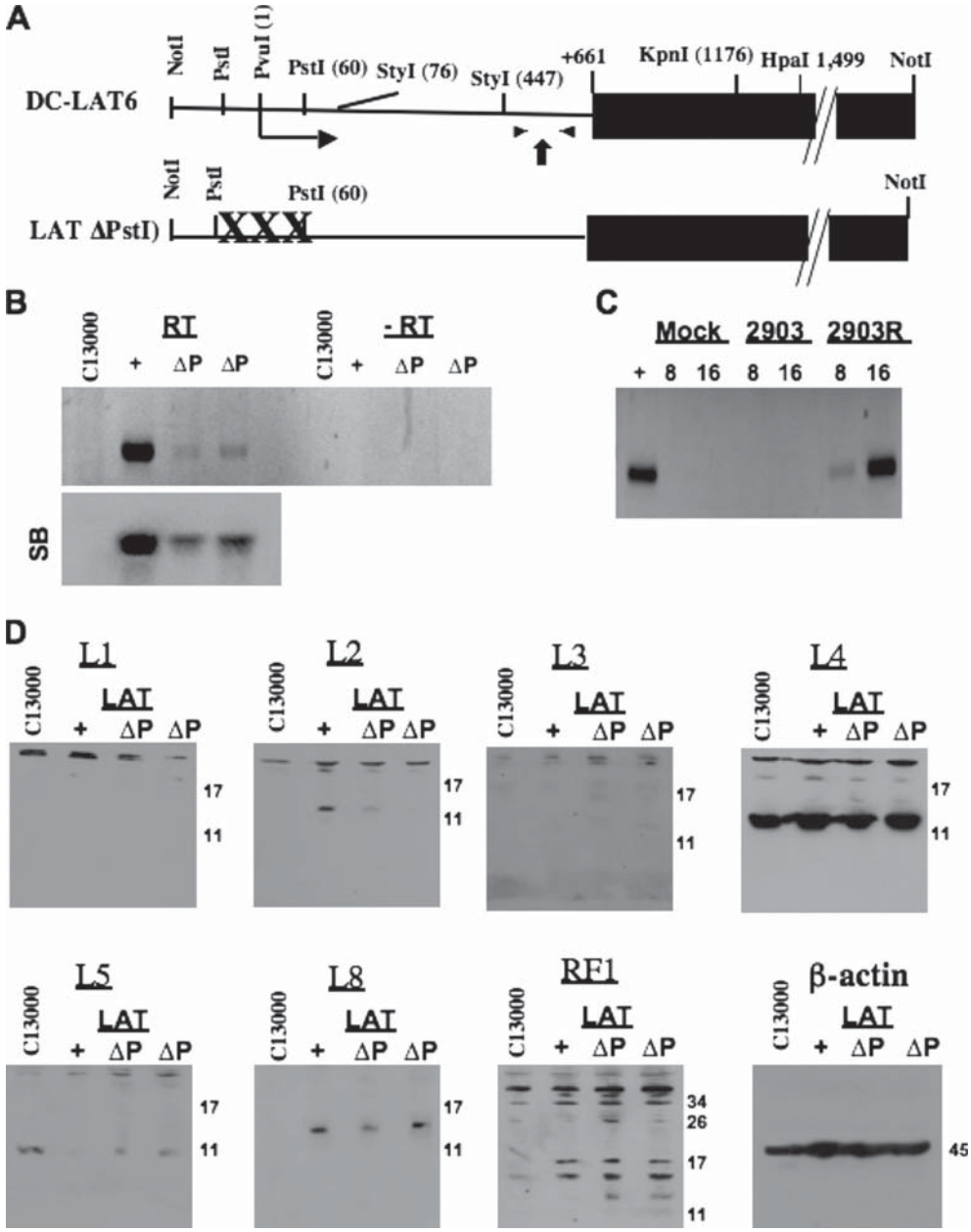


Figure 2 (*Continued*)

were not frequently detected at 30 days after infection with dLAT2903 (2903 panel). Approximately 19% of TG neurons were stained by the L2-specific IgG 30 days after infection with dLAT2903R, whereas only 2% of TG neurons were stained when mice were infected with dLAT2903 (Figure 4C). When the L2 peptides used to generate the L2-specific antiserum were included in the IHC reaction mixture, the numbers of neurons that were positively stained was reduced to background levels (data not shown), suggesting that binding of L2-specific IgG to neurons was not random. Because it has been estimated that up to 30% of murine sensory neurons in TG are latently infected (Sawtell, 1997), it seemed possible that 19% of the neurons could be L2 positive.

Additional studies were performed to assess L2 protein expression in TG of acutely infected mice. Three days after infection with dLAT2903R, 36% of TG neurons were stained by the L2-specific IgG (Figure 3B, 2903R 3 d panel, and Figure 4C). Less than 1% of TG neurons were stained with the L2-specific IgG at 3 days after infection with dLAT2903 (Figure 4C), which was similar to that observed in mock-infected mice stained by the L2-specific IgG (Figure 3B; Mock panel). By 6 days after infection with dLAT2903R, 26% of TG neurons were stained

with the L2-specific IgG (Figure 3B, 2903R 6 d panel, and Figure 4C). When mice were infected with dLAT2903 for 6 days (Figure 3B, 2903 6 d panel), 4% of TG neurons were stained by the L2-specific IgG (Figure 4C).

When mice were infected with dLAT2903R, L8-specific IgG recognized approximately 16% of TG neurons at 3 days after infection (Figure 4A; 2903 3d panel, and Figure 4C). By 6 days after infection (2903R 6 d panel), 12% of TG neurons were stained with the L8-specific IgG. During latency (30 days after infection) 4% of TG neurons were stained by the L8-specific IgG when mice were infected with dLAT2903R, whereas 2% of TG neurons were stained if mice were infected with dLAT2903 (Figure 4C). Three days after mice were infected with dLAT2903, 4% of TG neurons were stained with the L8-specific IgG (Figure 4A, 2903 panels, and Figure 4C). In general, the intensity of neuronal staining by the L8-specific IgG was less than that observed with the L2 IgG. Although the L1-specific IgG detected 6% of TG neurons at 3 days after infection with dLAT2903R, at 6 or 30 days after infection, the L1-specific IgG did not detect more TG neurons in mice infected with dLAT2903R than in mice infected with dLAT2903 (Figure 4B and C). The RF1-specific IgG cross-reacted with TG neurons prepared from mock-infected mice as well as infected mice (data not shown) and thus no conclusions regarding potential RF1 expression could be reached. The results of the RF1-specific IgG in TG of mock-infected mice were consistent with the results shown in Figure 2D.

Although LAT is considered to be a non–protein-encoding transcript, evidence is presented in this study suggesting that the L2 and L8 ORFs were expressed in DC-LAT6 cells and in TG neurons of infected mice. The L8 ORF, unlike L2, was not detected in TG neurons of latently infected neurons. This suggested that L8 was preferentially expressed during the establishment of latency. The L8 ORF is only 44 aa long, but the protein recognized in Western Blots migrated approximately as a 13-kDa protein, suggesting the protein may be modified or the protein migrated aberrantly on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The L2 ORF is 61 aa and is predicted to migrate as a 6.4-kDa protein (Figure 1B). Because the C-terminus of L2 is at the 2-kb LAT exon-intron border, L2 can be fused in frame with an additional 59 aa, and this ORF (L2 spliced, Figure 1B) would encode a protein with a predicted molecular weight of 13.2 kDa. The L2 protein expressed in DC-LAT6 cells migrated with an apparent molecular weight of approximately 12 kDa, suggesting this band represented the L2-spliced ORF or that the L2 ORF was post-translationally modified. We suggest L2 may play a role in the latency-reactivation cycle because L2 appeared to be expressed in TG neurons of latently infected mice, and mutagenesis of the initiating methionine residues of all ORFs in the first 1.5 kb of LAT coding sequences reduced the anti-apoptosis functions of this fragment to background levels (Carpenter *et al*, 2008). If proper splicing of the LAT intron is important for the latency-reactivation cycle and inhibiting apoptosis (Kang *et al*, 2003), it would further suggest that the spliced L2 ORF may be important for some aspect of the latency-reactivation cycle. Furthermore, the L2 ORF encoded by strain 17syn+ and McKrae have only two aa

Figure 2 Analysis of LAT RNA in cells stably expressing LAT or productively infected neuroblastoma cells. (**A**) Schematic of LAT sequences used to stably transfect C1300 mouse neuroblastoma cells. Partial restriction map of the NotI-NotI fragment that contains the LAT promoter and coding sequences cloned into pUC19. A PstI deletion construct (△PstI) was also used to stably transfect C1300 cells, and these cells were designated DC-△LAT35 and DC-△LAT311 (Carpenter *et al*, 2007). Position of primers used to detect LAT transcription is also shown (*black arrowhead*). The position of the probe used for Southern blotting after RT-PCR (*vertical black arrow*). The sequence of the primers used are positive primer: CCACAACGGCCCGGCGCATGCGCTGTGGTT, negative primer: CTTTGTTGAACGA-CACCGGGGCGCCCTCGA, and the probe used for Southern blotting: TCTCCCCCCCCCCTTCTTCACCCCCAGTAC. (**B**) Total RNA was extracted from C1300 cells or the stably transfected cell lines. DC-LAT6 cells are denoted by the +. The △P lanes were total RNA extracted from C1300 cells stably transfected with the △PstI NotI LAT fragment DC-△LAT35 and DC-△LAT311 (Carpenter, 2007). The top panel was the EtBr-stained gel, and the bottom was the Southern Blot of the same gel using the oligonucleotide probe described above. (**C**) Neuro-2A cells were infected with dLAT2903 (LAT null mutant; 2903 lanes) or dLAT2903R (LAT+ rescued virus; 2903R lanes) for the designated times (hours). Total RNA was then extracted. For **B** and **C**, RT-PCR was performed using the negative primer to prime cDNA synthesis. For cDNA synthesis, 2 μg total RNA was used. PCR was performed as described previously (Peng *et al*., 2005). **(D)** Total cell lysate was prepared from DC-LAT6 cells (+ lanes) or the LAT △PstI cell lines, DC-△LAT35 and DC-△LAT311 (△P lanes). Cells were scraped into PBS, pelleted at 1000 rpm, and then lysed by adding fresh Chaps Cell Extract Buffer 9852 (Cell Signaling) to the cell pellet. Freeze thawing was performed 3 times (−70°C to 37°C) to ensure complete lysis. Cell debris was removed by centrifuging for 14,0000 rpm for 20 min in a microcentrifuge. Five hundred micrograms protein of the cell lysate was loaded per lane of a 15% SDS PAGE gel. Proteins were transferred onto Immobilon-P transfer membrane (IPVH00010) using semidry gel electrophoresis in transfer buffer (25 mM Tris base, 0.2 M glycine, 20% methanol, pH 8.5). After transfer, the membrane was washed for 5 min in 1×TBS (from 10× stock of 24.2 g Tris base, 80 g NaCl/1 L water, pH 7.6) at room temperature (RT). The membrane was incubated in 25 ml blocking buffer (1× TBS, 0.1% Tween-20 with 5% nonfat dry milk [Cell Signaling catalog no. 9999]) for 3 h at RT. The membrane was then washed 3× for 15 min in 1× TBS containing 0.1% Tween-20 (TBS/T) at RT. The primary antibody was diluted from 10 to 1 mg/ml in double-distilled water and then diluted to a final concentration of 1:2000 in 10 ml blocking buffer. The membrane and primary antibody were incubated with gentle agitation overnight at 4°C. The membrane was then washed 3 times for 15 min in 1× TBS/T at RT, and then incubated with HRP-conjugated secondary anti-rabbit antibody (1:2000) (Amersham NA 934) with gentle agitation for 1 h at room temperature. The membrane was finally washed 3 times for 15 min in 1× TBS/T, and antigen-antibody complexes detected using the ECL Detection Kit from Amersham (RPN 2106). Peptides used to generate LAT ORF–specific IgG are summarized in Figure 1D. The β-actin antibody was purchased from Santa Cruz. Each lane was loaded with 100 μg protein. The numbers to the right of each panel denote the position of molecular weight markers.

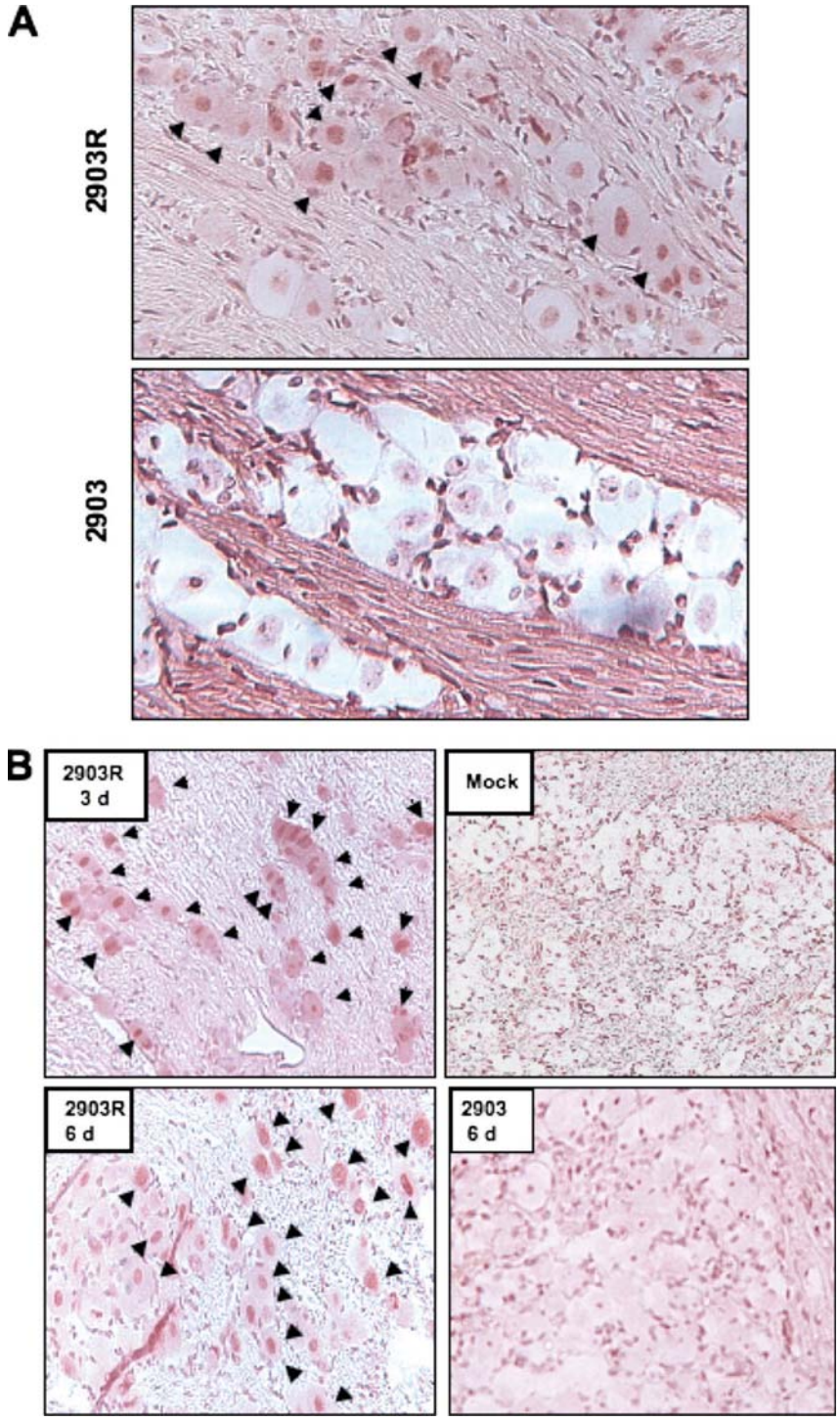
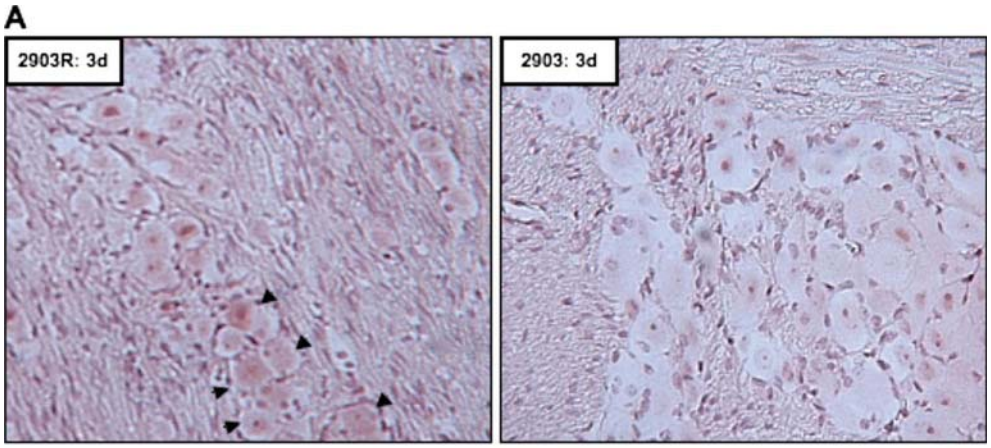


Figure 3 Analysis of L2 protein expression in TG of infected mice. Eight- to 10-week-old Swiss-Webster female mice (Jackson Labs) were infected with 2×10^5 PFU/eye of dLAT2903 (2903 panels) or dLAT2903R (2903R panels). Viral infections were done without scarification as previously described (Jones, 2005; Perng *et al*, 1994b, 1996e, 2001). TG were removed, formalin fixed, embedded in paraffin, and thin sections cut. Latent TG was prepared at 30 days after infection (**A**) or 3 or 6 days after infection (acute infection, **B**). IHC (immunohistochemistry) was performed using the L2-specific IgG. Arrows denote L2+ neurons. L2-specific IgG was diluted 1:5000 for IHC studies. IHC was performed as previously described (Perez *et al*, 2006, 2007; Winkler *et al*, 2000a, 2000b, 2002).

differences (Drolet *et al*, 1998), adding further support to the concept that the L2 ORF may be important. It is difficult to predict what the function of L2 or the L2-spliced ORF are because a BLAST analysis revealed only scattered similarity to known proteins. Consequently, it will be necessary to generate a



C

	L2 (2903R)	L2 (2903)	L8 (2903R)	L8 (2903)	L1 (2903R)	L1 (2903)
3d	36% (88/247)	0.1% (2/313)	16% (71/446)	4% (18/473)	6% (16/249)	0 (0/315)
6d	26% (95/271)	4% (10/264)	12% (54/258)	1% (4/440)	1% (2/196)	2% (6/300)
Latency	19% (48/255)	2% (7/367)	4% (12/314)	2% (9/416)	0% (0/350)	0% (0/249)

Figure 4 Analysis of L8 and L1 protein expression in TG of infected mice. (**A**) The L8-specific IgG was used to test for L8 protein expression in TG of mice infected with dLAT2903 or dLAT2903R as described in Figure 3. L8-specific IgG was diluted 1:5000 for IHC studies. (**B**) Thin sections were stained with the L1-specific IgG. L1-specific IgG was diluted 1:5000 for IHC studies. Arrows denote neurons that were positively stained by the designated antibody. (**C**) The number of neurons stained by the specific IgG was counted at the designated time after infection. The number of positive neurons versus the total number of neurons counted is in parenthesis. For these studies, TG from at least eight mice was examined. Only those sections in which folding of the TG was not observed after paraffin embedding were used for counting positive neurons because folding of TG tissue results in high levels of background staining.

mutant virus that does not express the L2 protein and then test whether expression of L2 influences reactivation from latency or neurovirulence.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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