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Short Communication

Localization of sequences within the latency-related gene of bovine herpesvirus 1 that inhibit mammalian cell growth

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The latency-related (LR) RNA of bovine herpes virus 1 (BHV-1) is abundantly expressed in sensory neurons of latently infected cattle. Wild-type expression of LR gene products is required for the latency-reactivation cycle. LR gene products inhibit apoptosis, bICP0 expression, and mammalian cell growth. The cell growth inhibitory function of the LR gene maps to a 463-bp XbaI-PstI fragment. Introduction of stop codons into the XbaI-PstI fragment had no effect on inhibiting growth. Expression of a LR strand-specific transcript correlates with growth inhibition in bovine fibroblasts and mouse neuroblastoma cells. *Journal of NeuroVirology* (2005) **11,** 563–570.

Keywords: bovine herpesvirus 1; cell growth; latency-related gene

Bovine herpesvirus 1 (BHV-1) is an α-*Herpesvirinae* subfamily member that establishes lifelong latency in ganglionic neurons of the peripheral nervous system after initial replication in mucosal epithelium (Jones, 1998). Long-term persistence also occurs within germinal centers of pharyngeal tonsil (Winkler *et al*, 2000). Infection can lead to conjunctivitis, pneumonia, genital disorders, abortions, and an upper respiratory infection referred to as "shipping fever" (Tikoo *et al*, 1995). BHV-1 initiates shipping fever by suppressing cell-mediated immunity during acute infection, thus allowing secondary bacterial infections to occur (Carter *et al*, 1989; Griebel *et al*, 1990, 1987a, 1987b; Hariharan *et al*, 1993; Hinkley *et al*, 1998; Nataraj *et al*, 1997; Winkler *et al*, 1999). Shipping fever cost the US cattle industry approximately $500 million/year (Bowland and Shewen, 2000; NASS, 1996).

The latency-related (LR) gene is abundantly transcribed in trigeminal ganglia (TG) of latently infected calves (Kutish *et al*, 1990; Rock *et al*, 1987; 1992), and is antisense to bICP0 transcripts (Figure 1A). The LR gene has two start sites for transcription (Delhon and Jones, 1997; Devireddy and Jones, 1998; Hossain *et al*, 1995), two open reading frames (ORF-1 and ORF-2), and two reading frames that lack an initiating ATG (RF-B and RF-C) (Figure 1B). A mutant BHV-1 virus with three stop codons at the beginning of ORF-2 (Figure 1C) was constructed to test whether LR protein expression regulates the latency-reactivation cycle in cattle (Inman *et al*, 2001). Antibodies directed against ORF-2 and RF-B recognize proteins expressed in bovine cells infected with wild-type (wt) or the LR rescued virus, but not cells infected with the LR mutant (Hossain *et al*, 1995; Jiang *et al*, 1998, 2004). Calves infected with the LR mutant exhibit diminished clinical symptoms and reduced shedding of infectious virus (Inman *et al*, 2001, 2002; Perez *et al*, 2005). The LR mutant virus does not reactivate from latency following dexamethasone (DEX) treatment (Inman *et al*, 2002), suggesting that LR protein expression is required for the latency-reactivation cycle. LR gene products inhibit mammalian cell growth by blocking S-phase entry (Schang *et al*, 1996), bICP0 expression (Bratanich *et al*, 1992; Geiser *et al*, 2002;

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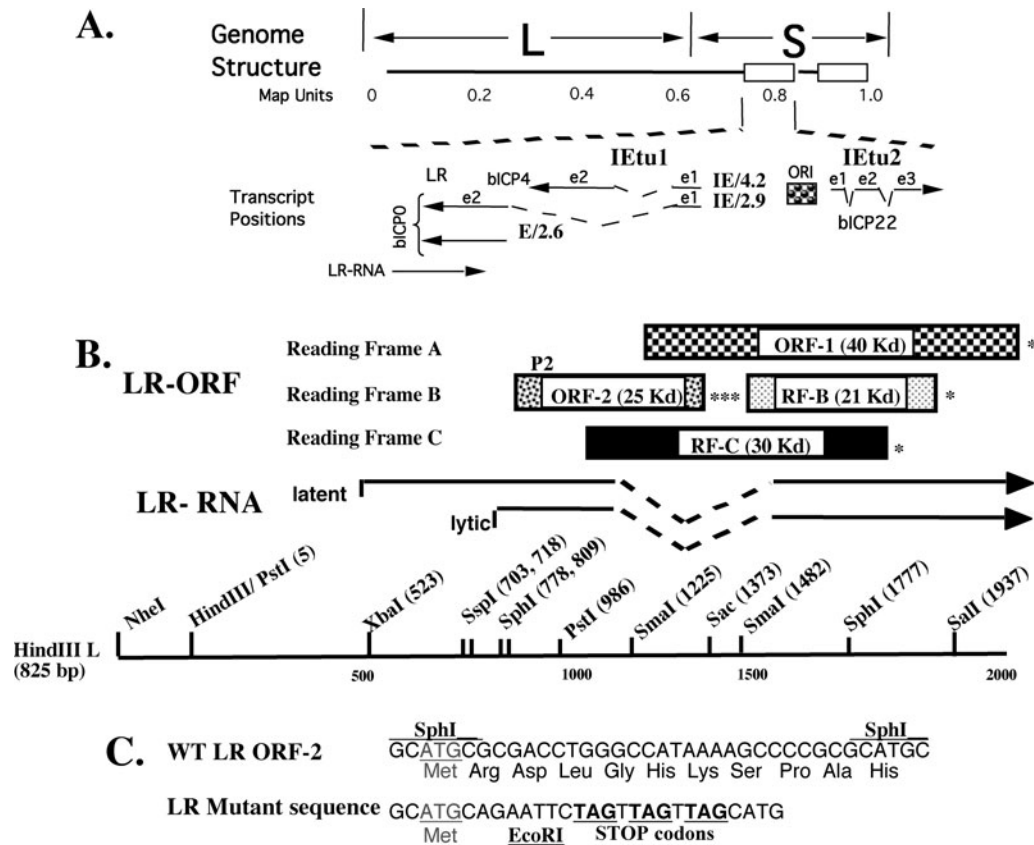

Figure 1 Schematic of the BHV-1 LR gene. (**A**) Schematic representation of the BHV-1 genome. L and S indicate the unique long and short regions, respectively. The boxes represent the inverted or terminal repeats, and positions of the map units are below the schematic of the genome. IE/4.2 is the IE transcript that encodes bICP4. IE/2.9 is the IE transcript that encodes bICP0. One IE promoter activates expression of IE/4.2 and IE/2.9 and this IE transcription unit is designated IEtu1. E/2.6 is the early transcript that encodes bICP0. Exon 2 (e2) of bICP0 contains all of the protein coding sequences of bICP0. The origin of replication (ORI) separates IEtu1 from IEtu2. IEtu2 encodes the bICP22 protein. Solid lines in the transcript position map represent exons (e1, e2, or e3), and dashed lines are introns. (**B**) Partial restriction map, location of LR-RNA, and organization of LR open reading frames (ORFs). The start sites for LR transcription during latency and productive infection were previously described (Devireddy *et al*, and Jones, 1998; Hossain *et al*, 1995). The dashed lines denote the regions of the LR-RNA that undergo alternative splicing (Devireddy 2003; Hossain *et al*, 1995). Reading frame B (RF-B) and reading frame C (RF-C) contain an open reading frame, but lack an initiating Met. The asterisk (*) denotes the position of stop codons that are in frame with the respective ORF. The numbering system of Kutish *et al.* (1990) was used for denoting the cut sites of the respective restriction sites. (**C**) DNA sequence of the SphI fragment and the mutant oligonucleotide. The first ATG in the wild-type sequence is the first in frame ATG for ORF2 and is underlined. Stop codons in the mutant oligonucleotide are in all three reading frames (bold and underlined). The EcoRI restriction enzyme site (GAATTC) was incorporated into the mutant oligonucleotide to facilitate screening.

Schang *et al*, 1996), and apoptosis (Ciacci-Zanella *et al*, 1999; Henderson *et al*, 2004), suggesting these functions regulate the latency-reactivation cycle. LR protein expression is necessary for inhibiting apoptosis, but it is not clear whether LR protein expression is necessary for inhibiting cell growth.

To test whether LR protein expression was necessary for inhibiting cell growth, we compared the growth inhibitory properties of a wt LR gene construct (LRT^{wt}) to a LR gene construct that contained three stop codons adjacent to the initiating methionine of ORF-2 (LRTstop; Figure 1C). Bovine fibroblasts (9.1.3) or mouse neuroblastoma cells (neuro-2A) were cotransfected with LRTstop, LRT^{wt}, a vector control (pcDNA3.1(−) or pcDNA1; Invitrogen, Carlsbad, CA), and a puromycin resistance plasmid (pPur; BD Biosciences, Clontech, San Diego, CA). Cultures were transfected at a ratio of 1:10 (pPur:LR plasmid) to ensure that puromycin-resistant cells contained the gene of interest. At 24 h after transfection, medium with puromycin (2.5 μg/m—for neuro-2A cells or 4 μg/ml for 9.1.3 cells) was added to cultures. The number of colonies present after antibiotic selection correlates with the ability of transfected cells to progress through the cell cycle, proliferate, and develop a colony (Schang *et al*, 1996). A cluster of puromycin-resistant cells that contained at least 10 cells was considered to be a colony (for example, see Figure 2A). In general, neuro-2A or 9.1.3 cells transfected with LRTstop or LRT^{wt} survived indefinitely, but were unable to proliferate and form large colonies (Figure 2A), which was consistent with a previous study (Schang *et al*, 1996). The number of colonies in cultures transfected with the vector control was set at 100% for each experiment. Mouse neuroblastoma (neuro-2A) or bovine fibroblasts (9.1.3)

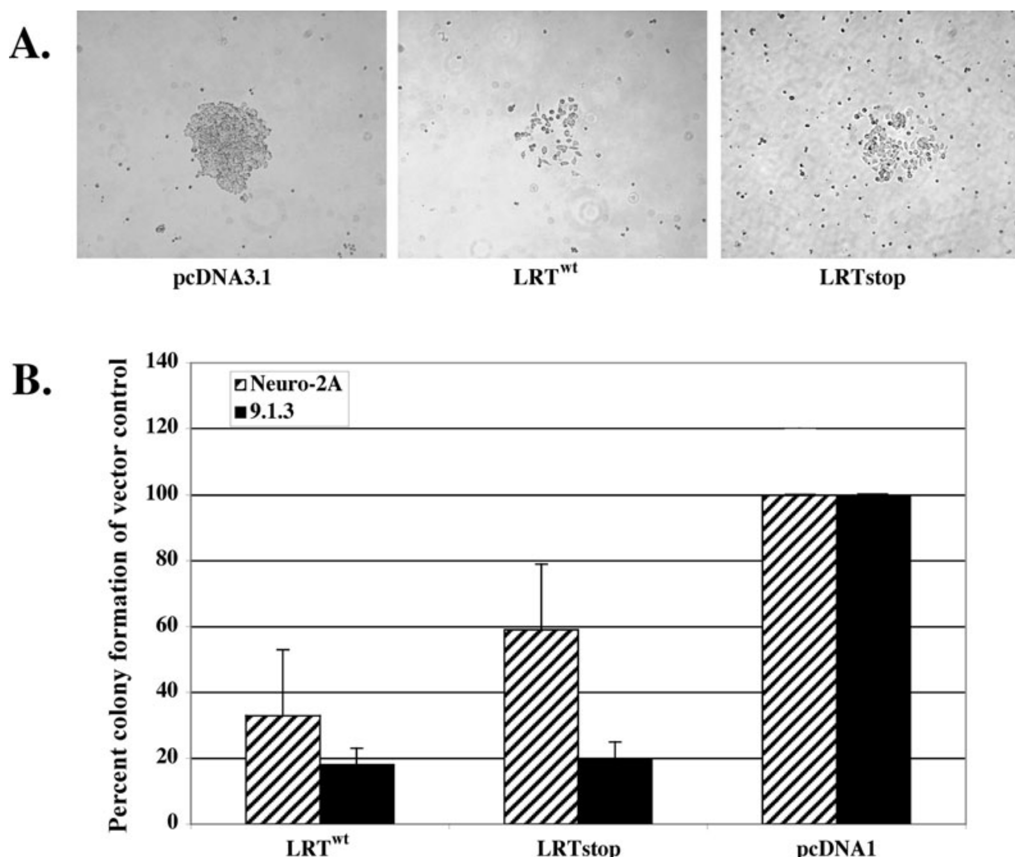

Figure 2 Expression of a protein encoded by the BHV-1 LR gene is not required for inhibiting cell growth. (**A**) Bovine fibroblast 9.1.3 cells or neuro-2A cells (1×10^5 cells in a 60-mm dish) were transfected using Superfect (Qiagen) for 9.1.3 cells or TransIT LT-1 (Mirus) for neuro-2A cells with 2.7 μg of the indicated plasmid and 0.3 μg of pPur. Cells were passaged at 24 post transfection into medium containing puromycin (2.5 μg/ml for neuro-2A cells or 4 μg/ml for 9.1.3 cells). Neuro-2A cells that comprised representative colonies were photographed at 10 × magnification using a Leica DM IRB microscope (Leica Microsystems, Bannockbum, IL). Images were captured with Leica DC viewer 3.2.0.0 software (Leica Microsystems). Robust growing colonies were not routinely observed when neuro-2A cells were transfected with LRT^{wt} or LRTstop. (**B**) The number of colonies present in the vector control after antibiotic selection was set at 100% for each experiment (*black bar*). This minimized the differences in cell confluence, Superfect lot variation, and transfection efficiency. The values are the means of three independent studies for 9.1.3 cells, and five experiments for neuro-2A cells. Bars indicate the standard errors of the means.

cells transfected with LRT^{wt} contained only 33% or 17% of the colonies, respectively, compared to the vector control (Figure 2B), which is consistent with previous studies (Schang *et al*, 1996). LRTstop contained only 59% or 21% of the puromycin resistant colonies relative to the vector control in neuro-2A or 9.1.3 cells, respectively (Figure 2B), suggesting that LR protein expression was not required for inhibiting cell growth. A Tukey-Kramer multiple comparisons post-ANOVA test demonstrated that the difference between LRT^{wt} and LRTstop was significantly different compared to cells transfected with the blank vector, pcDNA1 ($P < .004$). Although LRT^{wt} appeared to inhibit cell growth more efficiently than LRTstop in neuro-2A cells, there was not a significant difference ($P > .05$).

Additional LR gene deletion constructs were prepared to localize sequences that inhibit cell growth (Figure 3A). Cell growth was inhibited when DNA sequences that encompass the LR splicing region (LRTΔ*Sma*I), or the first 523 bp of the LR promoter (LRTΔHX) were deleted (Figure 3B). A construct that contained 986 bp of the LR gene (LRTΔ*Pst*I) or just the 463 bp XbaI-PstI fragment (LRT-XP) inhibited cell growth with similar efficiency as LRT^{wt} or LRTstop (Figure 3B). A LR plasmid with a deletion between the *Sph*I restriction sites (LRTΔ*Sph*I) (Schang *et al*, 1996) and a construct containing 564 bp of the 3′ terminus of LR-coding RNA sequences (LRT *Sac*I) were not able to inhibit cell growth (Figure 3B). These studies demonstrated that LRT-XP, which contained the XbaI-PstI restriction fragment (nucleotides 523 to 986), inhibited cell growth efficiently.

The XbaI-PstI fragment was digested with SphI and the respective fragments cloned into pcDNA3.1−(LRT-XSph and LRT-SphP; Figure 3A) to localize sequences within the XbaI-PstI fragment that inhibited cell growth. The LRT-SphP construct inhibited cell growth with approximately 1/2 the efficiency as LRT-XP (Figure 3B). In contrast, plasmid LRT-XSphλ had no effect on cell growth relative to blank expression vectors (pcDNA3.1 or

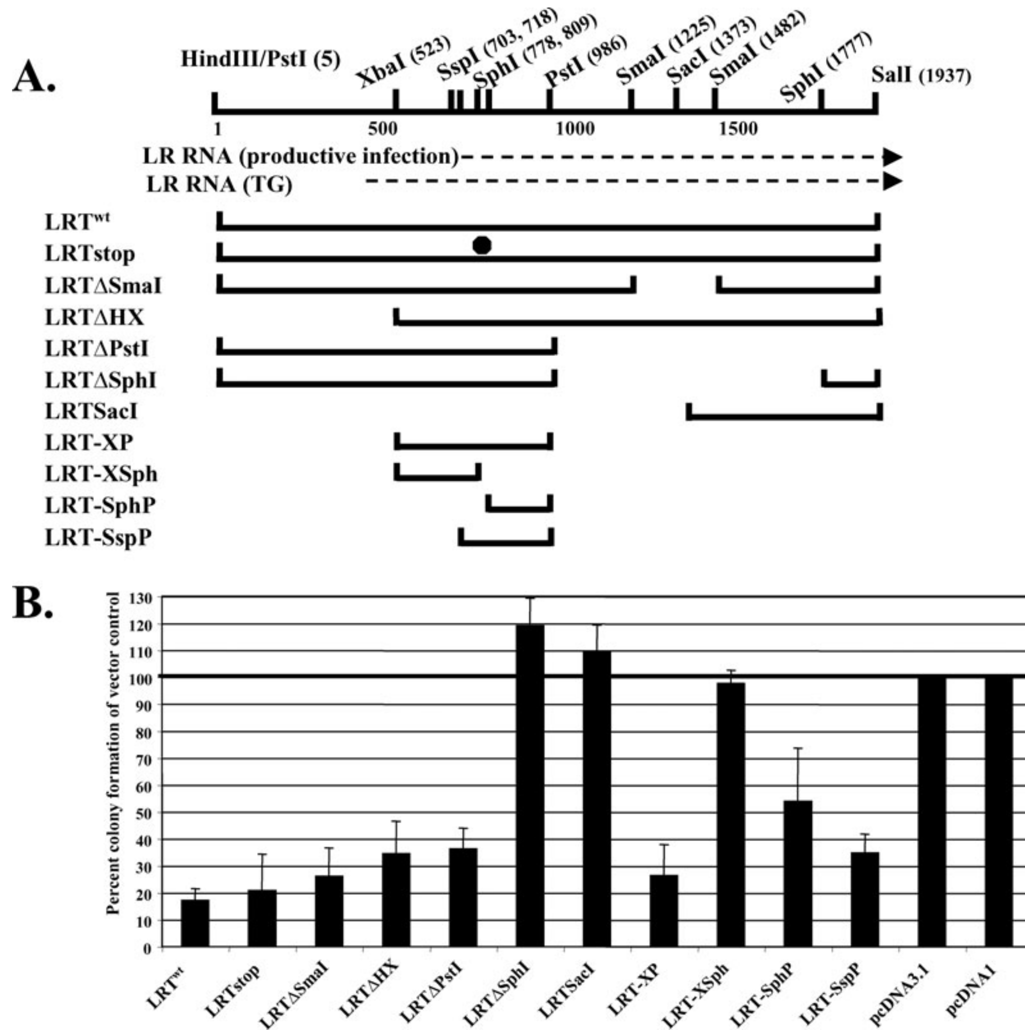

Figure 3 Location of LR gene sequences that inhibit cell growth. (**A**) Partial restriction map of the 2-kb LR gene. The approximate position of the LR RNA that is transcribed during productive and latent infection is included as a reference point (Bratanich *et al*, 1992; Delhon and Jones, 1997; Schang *et al*, 1996). LRTstop is a plasmid that has a deletion between two SphI sites (778 to 809 bp), an oligonucleotide containing three stop codons, and a unique EcoRI site inserted between the two SphI sites (the position of the stop codons are denoted by the hexagon). LRTΔSmaI has a deletion in the SmaI region that encompasses splicing sites within the LR gene. LRTΔHX has a deletion of the first 523 bp of the LR promoter. LRTΔPstI is a LR promoter construct that contains approximately 200 nt of LR-coding RNA sequences. LRTΔSphI has a deletion of the SphI fragment (781 to 1777 bp). LRTSacI contains 564 bp of the 3′ terminus of LR-coding RNA sequences. Construction of these respective plasmids was previously described (Ciacci-Zanella *et al*, 1999; Geiser *et al*, 2002; Inman *et al*, 2002; Jones *et al*, 1990; Schang *et al*, 1996). The XbaI to PstI LR fragment (523 to 986 bp) was cloned into the corresponding sites of the pUC19 (pUC19LRT-XP). The XbaI to PstI fragment of the LR gene was then excised by digestion with HindIII and XbaI, and this fragment was cloned into pcDNA3.1− (Invitrogen) digested with HindIII and XbaI (LRT-XP). The XbaI to SphI LR gene fragment (523 to 778 bp) was cloned into pUC19 (pUC19LRT-XSph). The XbaI to SphI LR gene fragment of pUC19-LRT-XSph was excised by digestion with HindIII and XbaI, and then cloned into pcDNA3.1− digested with HindIII and XbaI (LRT-XSph). The SphI to PstI LR gene fragment (809 to 981 bp) from pUC19LRT-XP was cloned into pUC19 (pUC19LRT-SphP). The SphI to PstI LR gene fragment was excised from pUC19LRT-SphP by digestion with HindIII and XbaI, and then cloned into pcDNA3.1+ digested with HindIII and XbaI (LRT-SphP). The SspI to PstI LR gene fragment (718 to 986 bp) from pUC19LRT-XP was cloned into the SmaI to PstI sites of pUC19 (pUC19LRT-SspP). The SspI to PstI LR gene fragment was released from pUC19LRT-SspP by digestion with HindIII and EcoRI, and this fragment cloned into pcDNA3.1− digested with HindIII and EcoRI (LRT-SspP). Following lysis of bacteria, all plasmids were purified by two CsCl gradients. (**B**) Neuro-2A or bovine fibroblast 9.1.3 cells (1×10^5 cells) were transfected using Superfect (Qiagen) with 2.7 μg of the indicated plasmid and 0.3 μg of pPur. Cells were passaged at 24 h post transfection into medium containing puromycin (4 μg/ml). The number of colonies present in the vector control after antibiotic selection was set at 100% for each experiment (*black bar*). This minimized the differences in cell confluence, Superfect lot variation, and transfection efficiency. The values are the means of at least three independent studies for 9.1.3 cells or five experiments for neuro-2A cells, and the bars indicate the standard errors of the means.

pcDNA1). The growth inhibitory effect of LRT-XP relative to LRT-SphP and LRT-XSph was significantly different ($P = .003$). A construct containing the SspI-PstI fragment (LRT-SspP) had 35% growth inhibition, which was not significantly different from the results obtained from LRT-SphP ($P = .097$). Although further deletion of the XbaI-PstI fragment decreased the growth inhibitory functions of the LR gene, it was clear this function mapped to sequences spanning the SspI-PstI fragment (nucleotides 718 to 986), and the 5′ region of LR-RNA coding sequences.

We predicted that the XbaI-PstI fragment encoded a transcript that was necessary for inhibiting cell growth. If this prediction were true, orientation of the insert would be important. Conversely, if LR DNA sequences within the XbaI-PstI fragment were responsible for inhibiting cell growth, this would not be the case. To test these possibilities, a plasmid was constructed that contained the XbaI-PstI fragment in the reverse orientation (LRT-XPrev) (Figure 4A). The LRT-XP construct, but not LRT-XPrev, inhibited cell growth in 9.1.3 or neuro-2A cells (Figure 4B).

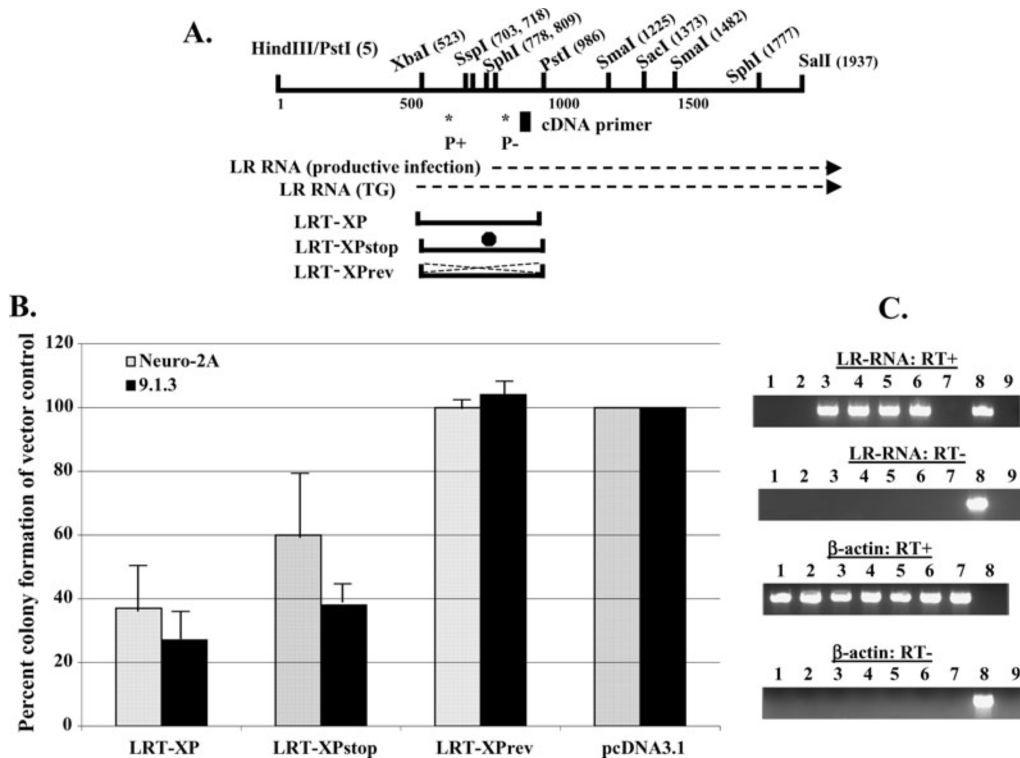

Figure 4 Analysis of the XbaI-PstI fragment for growth inhibitory functions. (**A**) Partial restriction map of the 2-kb LR gene. Strand-specific priming of LR cDNA was performed using an antisense primer to LR-RNA that spans nucleotides 874 to 851 of the LR gene (5′-CTGCATACTTAACTTTCGAGCCGC-3′), and is designated the cDNA primer. PCR amplification of LR-RNA was performed using the P+ primer (nucleotides 582 to 605; 5′-CGGCTAAAGTATAGGCCAGACCAA-3′) and the P-primer (nucleotides 834 to 811; 5′-CCCAAACCGAAAGTAACTGCTCGC-3′). LRT-XP was constructed as described in Figure 3. The XbaI to PstI fragment from the LRTstop plasmid was cloned into pUC19 (pUC19LRT-XPstop). The XbaI to PstI LR gene fragment of pUC19LRT-XPstop was released by digestion with HindIII and XbaI, and then cloned into pcDNA3.1− digested with HindIII and XbaI (LRT-XPstop). The XbaI to PstI LR gene fragment from pUC19LRT-XP was released by digestion with HindIII and XbaI, and this fragment was cloned into pcDNA3.1+ that was digested with HindIII and XbaI (LRT-XPrev). (B) The ability of the designated plasmids to regulate neuro-2A or 9.1.3 cell growth was measured as described in Figures 2 and 3. (C) 9.1.3 cells (6×10^5 cells) were transfected using Superfect (Qiagen) with 16.2 μg of the indicated plasmid and 1.8 μg of pPur. At 48 h post transfection, RNA was prepared using Trizol reagent and the RNA was subsequently treated with RNase-free DNase I for 30 min at 37°C. First-strand LR cDNA was synthesized using 1 μg of total RNA as a template, 0.5 μg of the cDNA primer, and a SuperScript preamplification kit (Invitrogen, Carlsbad, CA). Oligo(dT) priming was performed to detect β-actin mRNA. For each sample, 1/10 of the cDNA reaction mixture was used with the PCR primers. The primers used to detect β-actin were described previously (Inman *et al*, 2001). PCR reactions were carried out with 5 μl of 10 × commercial PCR buffer, 2 μl of 25 mM $MgCl_2$, 10 μl of GCMelt (BD, Palo Alto, CA), 1 μl of 40 mM deoxynucleoside triphosphates, 1 μM each primer, and 1 U of *Taq* polymerase for each 50 μl reaction. After hot start for 1 min, each cycle consisted of 95°C for 30 s, 45 s at the annealing temperature (57°C for LR-RNA, 55°C for β-actin), and 72°C for 1 min. PCR was carried out for 25 cycles (LR-RNA) or 35 cycles (β-actin). To ensure complete elongation of the amplified products, the reaction mixture was incubated at 72°C for an additional 10 min. The products were electrophoresed on a 2% agarose gel, and the DNA was visualized by staining with ethidium bromide. For all of the panels, lanes 1 to 7 contained amplified products from cells transfected with mock transfected (1), pcDNA3.1 (2), LRT^{wt} (3), LRTstop (4), LRT-XP (5), LRT-XPstop (6), LRT-XPrev (7). For the LR-RNA samples, lane 8 contained LRT^{wt} plasmid DNA, and lane 9 was the no template control. In the β-actin: RT+ panel, lane 8 was the no template control. For the β-actin: RT-panel, lane 8 contained total cellular DNA, and lane 9 was the no template control. Omitting reverse transcriptase from the reaction eliminated amplification of the specific bands (RT− reactions).

Plasmid LRT-XPstop, which contained the same stop codons as LRTstop, inhibited cell growth with similar efficiency as LRT-XP in 9.1.3 cells, adding further support that LR protein expression was not required for inhibiting cell growth. Although LRT-XPstop exhibited more variability with respect to LRT-XP inhibiting neuro-2A cell growth, the difference between the two plasmids was not significant ($P > .05$).

RT-PCR was performed to test whether a LR-specific transcript was expressed from the XbaI-PstI fragment in transfected 9.1.3 cells (Figure 4C). An oligonucleotide that primes LR-specific cDNA was used (cDNA primer), and cDNA products were amplified with primers (P+ and P−) located between the XbaI-PstI restriction sites (Figure 4A). A LR-specific transcript was readily detected in 9.1.3 cells transfected with LRT^{wt}, LRTstop, LRT-XP, or LRT-XPstop (Figure 4C; LR-RNA:RT+, lanes 3 to 6, respectively). In contrast, the amplified cDNA product was not detected when cells were transfected with LRT-XPrev (lane 7) and pcDNA3.1 (lane 2), or mock-transfected cells (lane 1). As expected, the cDNA-amplified product was not detected when reverse transcriptase was omitted from the cDNA reaction (LR-RNA: RT−). Similar levels of RNA were used for cDNA amplification, as judged by the levels of β-actin cDNA in the respective samples (β-actin RT+). In summary, these studies demonstrated that expression of a LR-specific transcript containing the XbaI-PstI fragment correlated with growth inhibition.

LR protein expression is necessary for the BHV-1 latency-reactivation cycle (Inman *et al*, 2002), and inhibiting apoptosis (Ciacci-Zanella *et al*, 1999), because addition of stop codons adjacent to the beginning of ORF-2 blocks these functions. Non–protein-coding LR-RNAs that can inhibit bICP0 expression (Geiser *et al*, 2002) and cell growth may also play a supportive role in the latency-reactivation cycle. For example, inhibiting bICP0 expression would promote establishment or maintenance of latency by inhibiting productive infection, thus enhancing survival of infected neurons. Secondly, inhibiting cell growth may enhance neuronal survival because cyclin expression is induced in trigeminal ganglia neurons during acute infection or reactivation from latency (Schang *et al*, 1996;Winkler *et al*, 2000), and cell cycle regulatory proteins can initiate neuronal apoptosis (Freeman *et al*, 1994; Gill and Windebank, 1998; Herrup and Busser, 1995; Levkau *et al*, 1998; Meikrantz *et al*, 1994; Meikrantz and Schlegel, 1996, Park *et al*, 1996, 1997a, 1997b; Shirvan *et al*, 1997a, 1997b, 1998). Collectively, these LR gene functions would enhance neuronal survival. Because the LR gene enhances growth in the tonsil (Perez *et al*, 2005) and eye (Inman *et al*, 2001), the ability of the LR gene to inhibit cell growth may also regulate these cell type–specific virus host interactions.

Surprisingly, small RNAs that do not express proteins can regulate gene expression (Dykxhoorn *et al*, 2003; Hanon, 2002), neuronal differentiation (Kuwabera *et al*, 2004), cell death (Iseni *et al*, 2002; Schors *et al*, 2002; Xu *et al*, 2004), interferon-induced apoptosis (Nanbo *et al*, 2002), or cell growth (Rastinejad *et al*, 1993). Non–protein-coding RNAs that regulate certain aspects of the latency-reactivation cycle (maintenance for example) may have a selective advantage because these latently infected neurons would escape immune surveillance. Recent studies have indicated that herpesviruses encode microRNAs (Pfeffer *et al*, 2004, 2005). Sequences within the XbaI-PstI fragment have the potential to form stem-loop secondary structures that are present in microRNAs, suggesting that the LR gene encodes a microRNA. Future studies will test whether these LR sequences encode a microRNA or if a larger RNA is encoded that plays a role in inhibiting cell growth.

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