

# Cloning and initial characterization of an alternatively spliced transcript encoded by the bovine herpes virus 1 latency-related gene

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**Bovine herpesvirus 1 (BHV-1) establishes latency in trigeminal ganglionic sensory neurons of infected cattle. The latency-related (LR) RNA is the only abundantly expressed viral transcript in sensory neurons of latently infected calves. Wild-type expression of LR gene products is required for the latency-reactivation cycle in calves. LR RNA is alternatively spliced in trigeminal ganglia (TG) after infection of calves, suggesting that these alternatively spliced transcripts encode novel factors that regulate specific steps during latency. To begin testing whether these alternatively spliced transcripts have novel functions, the authors cloned a full-length cDNA identified in TG of calves at 7 days post infection (dpi) and compared the functions of this cDNA to the intact LR gene. As a result of splicing, the 7 dpi cDNA contains a novel open reading (ORF) comprised of ORF-2 fused to ORF-1. Overexpression of the 7 dpi cDNA inhibited the BHV-1 immediate-early transcription unit 1 (IEtu1) promoter and the herpes simplex virus type 1 ICP0 promoter. Conversely, the 7 dpi cDNA stimulated the LR promoter in transiently transfected cells. A plasmid containing the LR gene had little effect on IEtu1 or LR promoter activity, indicating that the 7 dpi cDNA has novel functions. *Journal of NeuroVirology* (2003) 9, 612–622.**

**Keywords:** bovine herpes virus 1; latency-related gene; viral gene expression

## Introduction

Bovine herpesvirus 1 (BHV-1) belongs to the  $\alpha$ -herpesvirinae subfamily and shares a number of bi-

ological properties with herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) (Jones, 1998). Infection can cause conjunctivitis, pneumonia, genital disorders, abortions, and an upper respiratory infection referred to as “shipping fever” (Tikoo *et al*, 1995). Consequently, BHV-1 can induce immunosuppression culminating in secondary bacterial infections and pneumonia (Carter *et al*, 1989; Griebel *et al*, 1987a, 1987b, 1990). BHV-1 infection costs the US cattle industry at least \$500 million per year (Bowland and Shewen, 2000). Although modified live vaccines are available, they can cause disease in young calves or abortions.

The trigeminal ganglia (TG) are the major site of BHV-1 latency. The latency-related (LR) RNA is the only abundant viral transcript detected in latently infected sensory neurons (Kutish *et al*, 1990; Rock *et al*, 1987a, 1992). A fraction of LR RNA is polyadenylated and alternatively spliced in TG, suggesting this RNA is translated into more than one protein (Devireddy and Jones, 1998; Hossain *et al*, 1995). LR gene products inhibit S phase entry, and a LR protein is associated with cyclin-dependent kinase 2 (cdk2)/cyclin

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complexes (Hossain *et al*, 1995; Jiang *et al*, 1998). Furthermore, LR gene products inhibit apoptosis (Ciacci-Zanella *et al*, 1999), suggesting that one important function of the LR gene is to promote neuronal survival.

A mutant BHV-1 strain that contains three stop codons near the beginning of the LR RNA was constructed to test whether LR proteins play a role in virus growth in cattle (Inman *et al*, 2001). Calves infected with the LR mutant exhibit diminished clinical symptoms and ocular shedding of the virus relative to calves infected with wild-type (wt) or the LR rescued virus (Inman *et al*, 2001). Conversely, the LR mutant had similar growth properties in productively infected bovine kidney cells and the nasal cavity of calves during acute infection. Diminished levels of virus were also detected in TG of calves acutely infected with the LR mutant (Inman *et al*, 2002). The LR mutant virus does not reactivate from latency following treatment with dexamethasone (DEX). In contrast, all calves latently infected with wt virus or the LR rescued virus shed infectious virus following DEX treatment, which is indicative of reactivation from latency. During the transition from acute infection to latency (establishment of latency), higher levels of apoptosis occur in TG neurons of calves infected with the LR mutant when compared to calves infected with wild-type BHV-1 (Lovato *et al*, 2003). These studies indicate that wt expression of LR gene products is required for the latency-reactivation cycle in cattle.

BHV-1 gene expression during productive infection can be divided into three distinct classes: immediate-early (IE), early (E), and late (L) (Jones, 1998). IE transcription unit 1 (IEtu1) encodes proteins (bICP0 and bICP4) that are similar to HSV-1 ICP0 and ICP4, respectively. bICP0 is the major regulatory protein because it is expressed at high levels throughout infection (Wirth *et al*, 1992). A single transcript arises from IE transcription unit 2 (IEtu2) and this gene is similar to HSV ICP22 (Schwyzer *et al*, 1994). IE gene expression is stimulated by a virion component (bTIF), which interacts with a cellular transcription factor (Oct-1). The bTIF/Oct-1 protein complex binds TAATGARAT-like motifs in IE promoters and activates transcription (Misra *et al*, 1995, 1996).

Neuronal-specific transcription factors are believed to repress IE promoter activity and thus promote latency. Interference of VP16 and bTIF function by Oct-2 has also been suggested as another mechanism that prevents IE gene expression (Lillicrop *et al*, 1991, 1992, 1993). Oct-1, a class II POU (Pit-Oct-Unc) domain transcription factor, is bound by VP16 and the host cell factor (HCF). This complex specifically binds to a TAATGARAT motif in IE promoters and activates transcription (O'Hare and Goding, 1988, Preston *et al*, 1988). Oct-2 binds to the same sequence and inhibits IE promoter activity, presumably because it does not interact with VP16 (Lillicrop *et al*, 1991, 1992, 1993). The class IV POU domain transcription factor Brn-3b is expressed in sensory

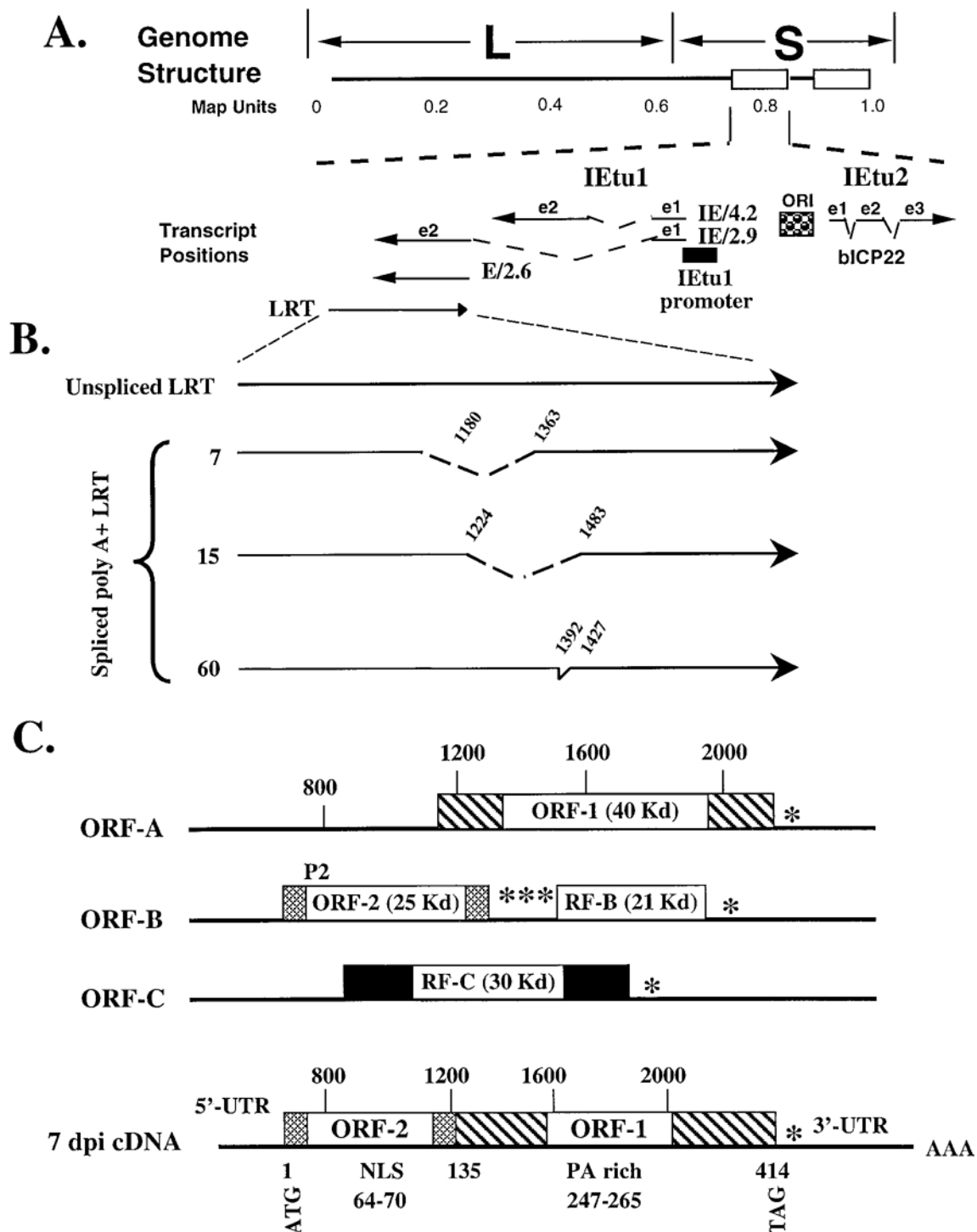
neurons and represses HSV-1 IE promoter activity (Lillicrop *et al*, 1992). Brn-3b belongs to a family of three closely related factors (Brn-3a, -3b, and -3c) (Gerrero *et al*, 1993, Turner *et al*, 1994) encoded by distinct genes (Theil *et al*, 1993). Brn-3a and -3c activate ICP0 and ICP4 promoter activity (Dawson *et al*, 1996, Lillicrop *et al*, 1995). Olf-1, a neuronal specific transcription factor that plays a role in retinal sensory neuronal development, activates ICP0 promoter activity in transiently transfected cells because a near-consensus Olf-1 binding site is present in the proximal promoter (Devireddy and Jones, 2000).

In this study, we cloned a full-length cDNA that was identified in bovine TG at 7 days post infection (dpi). The 7 dpi cDNA has the potential to encode a 65 kDa protein. Although this putative 65 kDa protein has 38% identity to the Brn-3a transcription factor, many gaps were introduced to make the best fit. Evidence is provided for expression of novel proteins by the cDNA following transient transfection of U2OS cells. The 7 dpi cDNA, but not the LR gene, reduced the activity of the BHV-1 IEtu1 promoter and a HSV-1 ICP0 promoter construct. Conversely, the 7 dpi cDNA, but not the LR gene, activated the LR promoter in transiently transfected neuroblastoma cells (neuro-2A). In summary, this study provides evidence that the 7 dpi cDNA has the potential to regulate gene expression.

## Results

### *Analysis and cloning of the 7 dpi cDNA*

The LR gene is adjacent to the inverted repeats in the BHV-1 genome (Figure 1A). Because the LR gene is not contained within the inverted repeats, it is a single-copy gene. The LR RNA partially overlaps and is antisense to an IE gene (IE/2.9), which encodes an ICP0-like protein (bICP0). RNA prepared from TG of calves infected with BHV-1 contains novel polyA+ alternatively spliced transcripts that arise from the LR gene (Devireddy and Jones, 1998) (Figure 1B). Productively infected bovine cells or transiently transfected cells do not contain the same spliced poly(A+) LR transcripts, suggesting that neuronal-specific splicing occurs in TG, and that these novel LR transcripts play specific roles during the latency-reactivation cycle. The LR gene contains 2 open reading frames (ORFs) that are designated ORF-1 and ORF-2 (Kutish *et al*, 1990) (Figure 1C). Two reading frames that lack an initiating ATG are present in the LR gene (RF-B and RF-C). A peptide antibody directed against the amino terminus of ORF-2 (P2) detects a 40-kDa protein in productively infected cells or in cells transfected with the LR gene (Ciacci-Zanella *et al*, 1999; Hossain *et al*, 1995; Jiang *et al*, 1998). The 40-kDa protein has been partially purified and is associated with cdk2 and cdc2 (cell division control 2). In contrast, we have been unable to detect ORF-1



**Figure 1** Schematic of BHV-1 genes in the repeats. (A) Positions of IE transcripts (Fraefel *et al*, 1994; Wirth *et al*, 1989, 1991, 1992) and the LR transcript (Rock *et al*, 1987a, 1987b) are presented. 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. The promoter that regulates expression of IETu1 is denoted by the black rectangle. E/2.6 is the early transcript that encodes bICP0 and an early promoter activates expression of this transcript. 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 a protein bICP22. Solid lines in the transcript position map represent exons (e1, e2, or e3). (B) Schematic of the major spliced and poly A+ LR transcripts identified in TG of acutely infected calves (Devireddy and Jones, 1998). The nucleotide number of the splice junction sites is given for each respective transcript. (C) The organization of open reading frames (ORFs) in the LR gene was previously described (Kutish *et al*, 1990). Numbers on top of reading frame A (ORF-A) indicate nucleotide positions and those on the bottom refer to amino acid positions. The hatched box denotes LR ORF-1. Reading frame B (*open box*; RF-B), which follows LR ORF-2 (*stippled box*), does not contain a methionine at its amino terminus. Reading frame C (*black box*; RF-C) also does not contain a methionine at its amino terminus. Asterisks indicate the position of in-frame stop codons. The 7 dpi cDNA splice junction site was previously reported (Devireddy and Jones, 1998). NLS refers to a putative nuclear localization sequence and PA to proline/alanine-rich sequences.

using a peptide antibody. Because the 7 dpi cDNA is not expressed during productive infection, we have no knowledge about the protein products that may be translated from this alternatively spliced transcript.

The major spliced polyA<sup>+</sup> LR transcript that was detected in TG of calves infected for 7 days contains an ORF comprised of the amino-terminal sequences of ORF-2 fused to the carboxyl-terminal sequences of ORF-1 (Figure 1B and C). This novel ORF contains proline/alanine (PA)-rich sequences located between amino acids 247 to 265 (Figure 1C). Similar sequences were present in p57<sup>kip2</sup>, a cdk inhibitor (Matsuoka *et al*, 1995), and the serotonin 1a gene, a neurotransmitter (Parks and Shenk, 1996). A putative nuclear localization signal (NLS) (aRRcRRcsgR; amino acids 64 to 70) was present in ORF-2, which was identical to the human transcription factor (Sp1) (Kadonaga *et al*, 1987), murine early B-cell factor (EBF) (Hagman *et al*, 1993), and rat AT-BP1 (Mitchelmore *et al*, 1991). We have detected the LR protein in the nucleus of neurons in latently infected calves and in nuclear fractions prepared from productively infected bovine cells (Jiang *et al*, 1998), suggesting that this putative nuclear localization signal is functional.

A GenBank search revealed that the largest ORF in the 7 dpi cDNA was 38% identical (146 out of 381 amino acids are identical in the intact ORF) and 46% similar (176 out of 381 amino acids) to Brn-3a. Brn-3a is a class IV POU domain transcription factor related to Brn-3b and -3c (Gerrero *et al*, 1993, Budhram-Mahadeo *et al*, 1994, 1995). The similarity to Brn-3a was higher in ORF-1 sequences compared to ORF-2. Virtually no amino acid identity was detected in the Brn-3a POU homeodomain and nine gaps greater than five amino acids were introduced to obtain the best fit. In addition, amino acids 101 to 421 of Brn-3a were not used for the comparison because the first 100 amino acids of Brn-3a has essentially no similarity to the 7 dpi cDNA. Although there was similarity to Brn-3a, numerous gaps were introduced into the 7 dpi cDNA to create the best fit.

RF-C was still intact in the alternatively spliced transcript, but as a result of splicing was fused to a portion of RF-B. The RF-C/RF-B fusion protein has the potential to encode a protein that is approximately 29 kDa. Following a Blast search, no dramatic similarities were detected between the RF-B/RF-C fusion protein and other proteins. In summary, the 7 dpi cDNA has the potential to encode two novel fusion proteins because of alternative splicing.

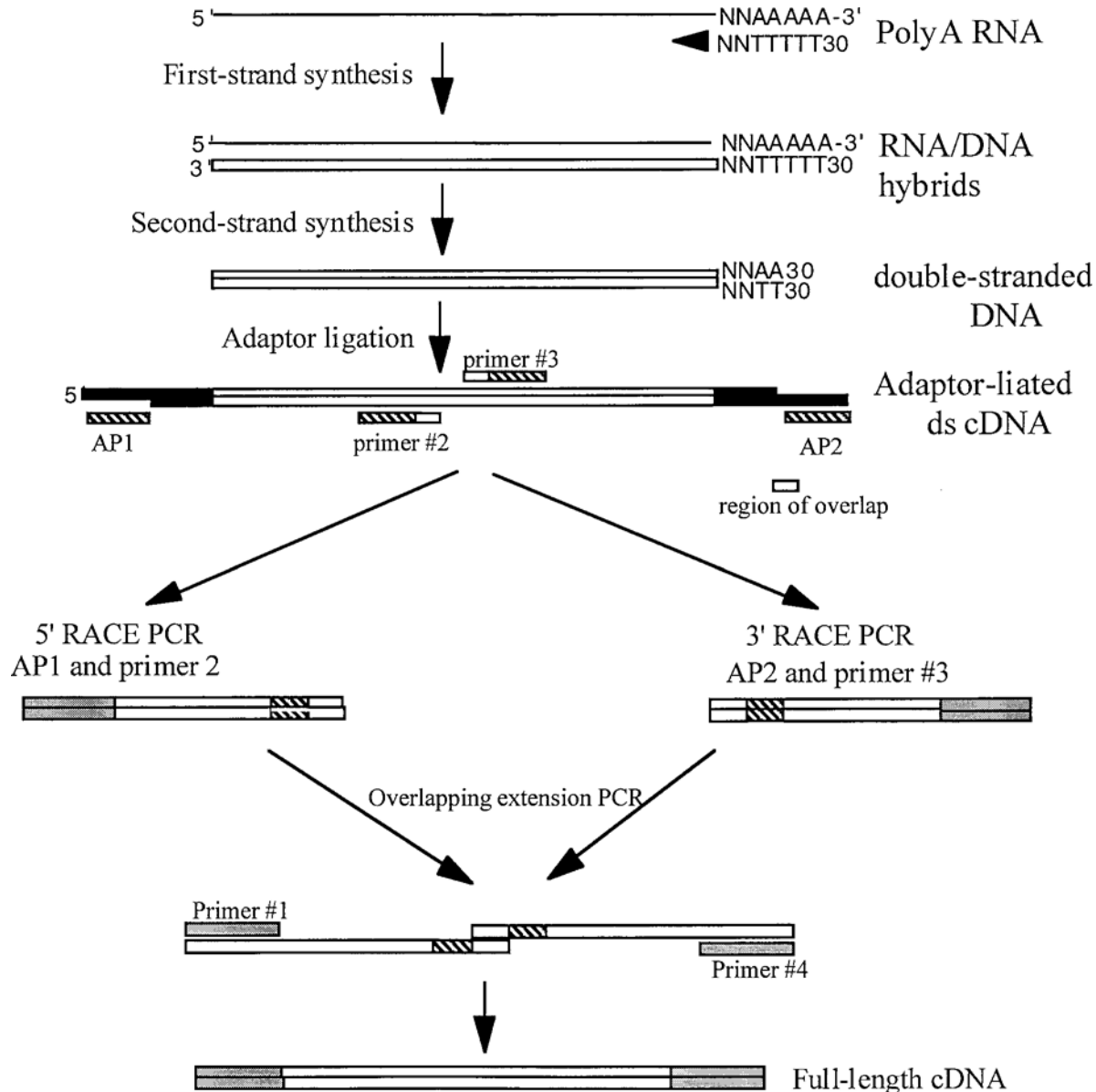
Traditional methods of screening a cDNA library were not feasible to clone the 7 dpi cDNA because we would have also cloned bICP0, unspliced LR RNA, or other spliced LR RNA variants. Although a portion of the cDNA was previously cloned (Devireddy and Jones, 1998), this only included the splice junction region, not the intact cDNA. A strategy was subsequently devised to clone the 7 dpi cDNA (outlined in Figure 2). Primers 2 and 3 were designed to amplify

the spliced poly(A<sup>+</sup>) LR RNA described in Figure 1C. Overlapping sequences in the 5' and 3' RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR) products allowed amplification of the full-length cDNA by a modified overlapping extension PCR (Nakshatri and Chambon, 1994). This process yielded bands with the expected sized PCR products (data not shown), and the full-length cDNA was cloned into a cytomegalovirus (CMV) expression vector (pcDNA3.1). The integrity of the clone was confirmed by sequencing the ends and the splice junction site.

#### *Detection of proteins expressed by the 7 dpi cDNA in transiently transfected U2OS cells*

To determine if the 7 dpi cDNA encoded a protein, a CMV expression plasmid (pcDNA3.1) containing the cDNA was transfected into U2OS cells and LR proteins were detected by Western blot analysis using the P2 antibody directed against LR ORF-2 (Hossain *et al*, 1995). U2OS cells were used for these studies because we have observed higher levels of LR protein expression in these cells compared to other cell lines. A Coomassie-stained gel of the respective extracts confirmed that similar amounts of protein were used for Western blot analysis (data not shown). U2OS cells transfected with the cDNA (Figure 3A, lane 2) contained several novel proteins recognized by the P2 antibody (denoted by asterisks) when compared to cells transfected with the blank expression plasmid (pcDNA3.1; lane 1) or the LR gene (pcDNA1/LRT; lane 3). Although we predicted that the 7 dpi cDNA would encode a 65 kDa protein, it was also possible that these smaller proteins arose because of degradation or perhaps because additional splicing occurred. Although Northern blots have revealed a single band using a probe directed against the LR gene, this band is diffuse and could contain more than one transcript because there are additional nonconsensus splice sites in the 7 dpi cDNA (data not shown). However, we cannot distinguish between the possibility that the 7 dpi cDNA undergoes additional splicing in transiently transfected cells or proteins that are expressed from the cDNA are prone to degradation. Cells transfected with plasmid pcDNA/1LRT and the 7 dpi cDNA contained a common 40 kDa protein recognized by the P2 antibody (Figure 3A, lanes 2 and 3, denoted by an arrow). This protein was not detected in cells transfected with pcDNA3.1.

To further examine the potential proteins that were expressed by the 7 dpi cDNA and LR gene, the 7 dpi cDNA and LR gene were cloned downstream of a Flag epitope using a mammalian expression vector (pCMV2C; Stratagene) such that all three potential reading frames could be expressed. In one reading frame, both the LR gene and 7 dpi cDNA expressed a Flag-tagged fusion protein that migrated with a molecular weight of approximately 30 kDa (Figure 3B). In cells transfected with the 7 dpi cDNA, a protein migrating with an approximate molecular

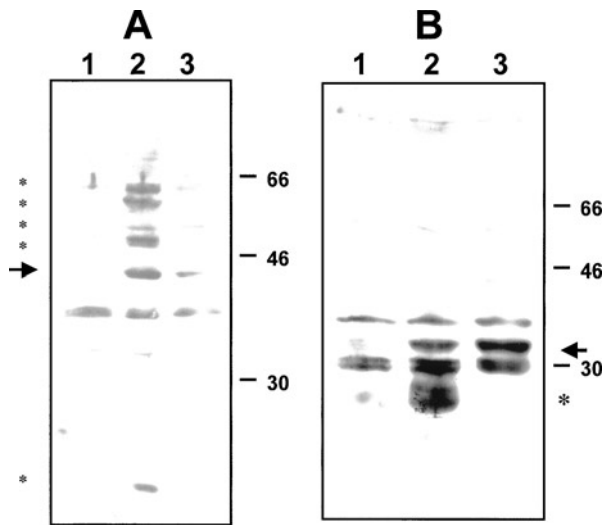


**Figure 2** Cloning strategy for 7 dpi cDNA. cDNA synthesis and adaptor ligation created a population of cDNAs. This population is a library of uncloned double-stranded cDNA from which the LR cDNA was amplified using primers 2 and 3 and adapter primers (supplied by the manufacturer). Utilization of primers 2 and 3 created overlapping PCR products, which were joined using primers 1 and 4. See Materials and methods for further details.

weight of 29-kDa was recognized by the Flag antibody. When either fragment was cloned such that the other reading frames were fused to the Flag epitope, no protein expression was detected (data not shown). The Flag fusion construct that expressed a Flag-tagged fusion protein in transiently transfected cells was in frame with RF-C (Figure 1). In summary, this study demonstrated that the 7 dpi cDNA expressed novel proteins that were recognized by the P2 antibody. Furthermore, this study suggested that RF-C might be expressed in transiently transfected cells when fused to an ATG. Additional experiments are necessary to determine if RF-C is really expressed in the context of the BHV-1 genome.

#### *Regulation of promoter activity by the 7 dpi cDNA and LR gene*

We predicted that the 7 dpi cDNA would have novel functions compared to the LR gene because alternative splicing yielded a transcript that has novel ORFs (Figure 1). Because the 7 dpi cDNA had certain domains that were similar to a known neuronal-specific transcription factor (Brn-3a), we also predicted that the 7 dpi cDNA might regulate gene expression in transient transfection assays. To test these predictions, we compared the ability of a plasmid containing the intact LR gene or the 7 dpi cDNA to regulate promoter activity in transient transfection assays. The BHV-1 IETu1 promoter was initially



**Figure 3** Western blot analysis of 7 dpi cDNA encoded protein. (A) Whole-cell lysate from U2OS cells transfected with pcDNA3.1 (lane 1), 7 dpi cDNA (lane 2), or LR transcript (lane 3) were subjected to Western blot analysis. Asterisks denote the presence of novel protein detected by the P2 antibody in cells transfected with the 7 dpi cDNA. The arrow indicates the position of 40-kDa protein detected by the P2 antibody. Molecular weight markers are indicated in kDa. (B) U2OS cells were transfected with Flag-tagged constructs containing the blank expression vector (pCMV-2B) (lane 1), 7 dpi cDNA (lane 2), or the LR gene (lane 3). At 48 h after transfection, cell lysate was prepared and Western blots performed using an antibody specific for the Flag epitope (Stratagene). For all lanes, 50  $\mu$ g protein was loaded.

examined because it regulates IE expression of bICP4 and bICP0 and thus is important for productive infection (Wirth *et al*, 1989, 1991, 1992). The IEtu1 promoter has low basal activity in transiently transfected cells but is strongly transactivated by bTIF or VP16 (Misra *et al*, 1995). Increasing concentrations of the 7 dpi cDNA inhibited transactivation of IEtu1 CAT by bTIF nearly fourfold (Figure 4A). In contrast, the LR gene was unable to reduce IEtu1 promoter activity (Figure 4B).

We also tested whether the 7 dpi cDNA would regulate HSV-1 IE promoter activity. The activity of pAB5 (ICP0 promoter construct  $-160$  to  $+150$ ) was reduced more than threefold by the 7 dpi cDNA (Figure 4A). pAB5 contains a single TAATGARAT motif, is responsive to VP16 (O'Hare and Goding, 1988), and has high basal activity in neuro-2A cells (Devireddy and Jones, 2000). The 7 dpi cDNA also reduced pAB2 promoter activity nearly fourfold. Plasmid pAB2 contains the ICP0 promoter ( $-130$  to  $+150$ ) but lacks the TAATGARAT motif in pAB5. In contrast to the ICP0 promoter, the 7 dpi cDNA had essentially no effect on ICP4 or CMV promoter activity (Figure 4A). The BHV-1 LR gene had no obvious effect on pAB5, pAB2, ICP4, or CMV promoter activity (Figure 4B).

A neuronal-specific promoter that is contained within a 980-bp PstI fragment regulates LR gene expression. This promoter has been cloned upstream of CAT (p0.95cat/1) and demonstrated to be functional

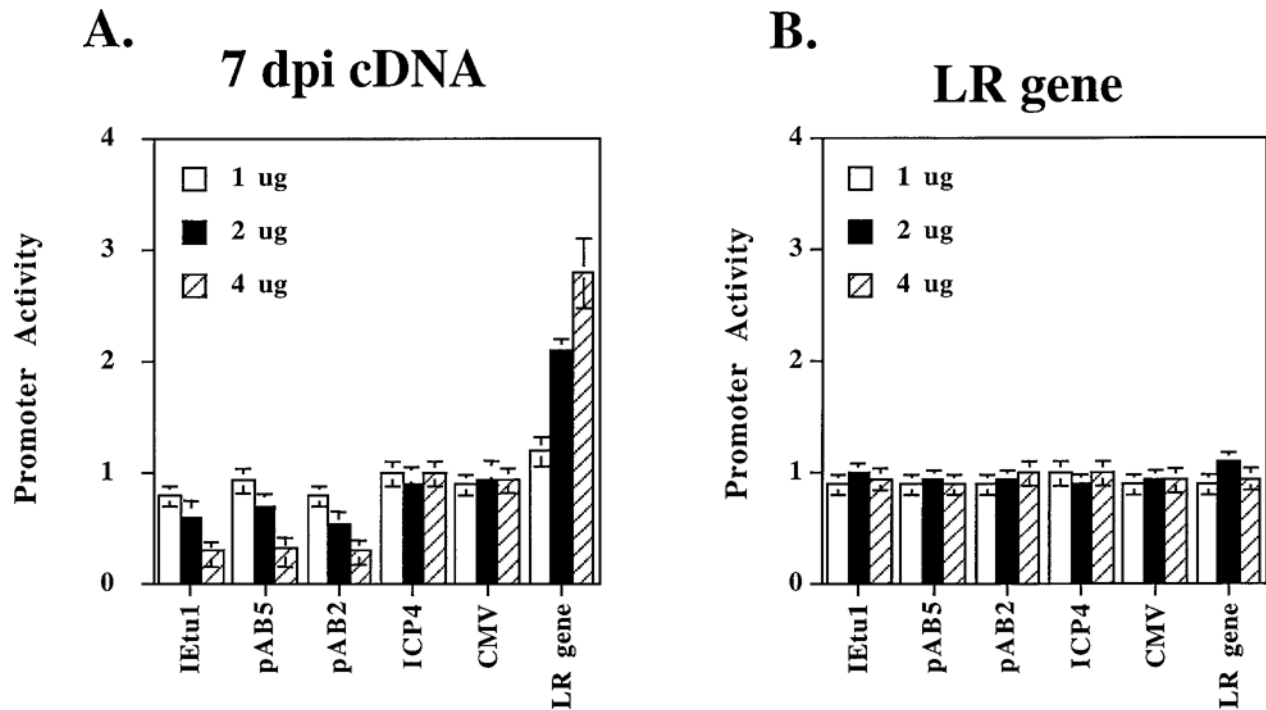
in transient transfection assays (Jones *et al*, 1990). The LR promoter displays neuronal-specific features in transient transfection assays and is comprised of multiple elements that regulate promoter activity. In cotransfection experiments, the 7 dpi cDNA, but not the LR gene, activated p0.95cat/1 promoter activity approximately threefold (Figure 4A and B). In summary, these studies indicated that the 7 dpi cDNA, but not the LR gene, had the potential to regulate certain promoters in transiently transfected neuro-2A cells.

## Discussion

A previous study demonstrated that alternative splicing of LR RNA occurred, suggesting these novel transcripts may have novel biological properties (Devireddy and Jones, 1998). To test this hypothesis, overlapping extension PCR (Ho, 1989; Nakshatri and Chambon, 1994) was used to specifically clone an alternatively spliced transcript that was detected in TG of infected calves at 7 dpi. An antibody directed against the amino terminus of ORF2 (P2) detected proteins in cells transfected with the 7 dpi cDNA that were not detected in cells transfected with the LR gene (Figure 3A). One of these proteins migrated between 60 and 70 kDa, which is approximately the predicted size of the largest ORF in the 7 dpi cDNA. The smaller proteins detected by the P2 antibody in cells transfected with the 7 dpi cDNA suggested the proteins were degraded or perhaps additional splicing occurred. When the 7 dpi cDNA was cloned downstream of the Flag tag sequences, we were unable to detect a 60 to 70-kDa protein, suggesting that LR protein expression was complicated and can be influenced by surrounding sequences. It is also possible that the Flag tag sequences were not readily exposed when fused to the large ORF in the 7 dpi cDNA.

Transient transfection assays demonstrated that the 7 dpi cDNA encodes a factor that reduces bTIF-mediated activation of the IEtu1 promoter (Figure 4A). It was necessary to use bTIF for these studies because basal levels of IEtu1 were low in neuro-2A cells. We hypothesize that the ability of the 7 dpi cDNA to inhibit IEtu1 promoter activity was not related to a reduction in bTIF levels because bTIF expression is regulated by the CMV IE promoter (Misra *et al*, 1995), which was not affected by the 7 dpi cDNA. The finding that ICP0, but not ICP4, promoter activity was reduced by the 7 dpi cDNA implied that *cis*-acting sequences present in certain promoters are targets for the 7 dpi cDNA. The TAATGARAT motif in pAB5 was not required for reducing ICP0 promoter activity. The inability of the LR gene to reduce IEtu1 or ICP0 promoter activity argues against nonspecific quenching of promoter activity.

Although it seems intuitive to predict that a protein encoded by the 7 dpi cDNA mediated repression of



**Figure 4** Regulation of promoter activity by the 7 dpi cDNA. Neuro-2A cells were cotransfected with 1  $\mu$ g of the designated promoter construct and 0, 1, 2, or 4  $\mu$ g of a plasmid containing the 7-dpi cDNA (**A**) or the LR gene (**B**). Because IEtu1 CAT promoter activity is nearly undetectable in the absence of bTIF, 4  $\mu$ g of bTIF were also included in those samples containing IEtu1 CAT. CAT activity was measured 48 h after transfection. The percent acetylated chloramphenicol (%Ac-CM) values were determined using a PhosphorImager. The amount of promoter activity in the presence of the 7 dpi cDNA, LR gene, or blank expression vector was measured. The relative promoter activity was determined by comparing the values obtained when the designated promoter was cotransfected with the LR gene or 7 dpi cDNA divided by the values obtained when the promoter was cotransfected with the same concentration of the blank expression vector. Thus, a value of 1 means that the LR gene or the 7 dpi cDNA had no effect on that particular promoter when compared to the blank expression vector. The values are the means of five independent studies, and the bars indicate standard errors of the means.

transcription, it was conceivable that the RNA itself inhibited transcription. For example, a non-protein-coding RNA is a steroid receptor coactivator that is required for transcriptional activation (Lanz *et al*, 1999). Antisense repression did not appear to be a factor in repression of IEtu1 and ICP0 promoter activities because the coding sequences of the 7 dpi cDNA do not overlap the IEtu1 promoter and there is little sequence similarity to the ICP0 promoter. Regardless of the mechanism of action, this study demonstrates that the 7 dpi cDNA encodes a novel factor that regulates transcription.

Brn-3a to -3c are class IV POU domain transcription factors that are encoded by different genes (Gerrero *et al*, 1993; He *et al*, 1989; Lillycrop *et al*, 1992; Ninkina *et al*, 1993; Xiang *et al*, 1993, 1995). Brn-3 family members are expressed in distinct but overlapping populations of neurons (Gerrero *et al*, 1993; He *et al*, 1989; Ryan and Rosenfeld, 1997) and these genes regulate development of the nervous system (Theil *et al*, 1993). Brn-3a levels are low in proliferating cells but high in differentiated neurons (Lillycrop *et al*, 1992). Conversely, Brn-3b levels are high in proliferating cells but reduced in differentiated neurons (Budhram-Mahadeo *et al*, 1994, 1995; Lillycrop *et al*, 1992; Xiang *et al*, 1993, 1995). In sensory gan-

glia, a primary site of BHV-1 latency, Brn-3a, but not Brn-3b, is expressed at high levels. Brn-3a and Brn-3c activate ICP0 and ICP4 promoter activity two- to fourfold (Dawson *et al*, 1996; Lillycrop *et al*, 1993, 1995). Brn-3b represses ICP4 and ICP0 promoter activities (Lillycrop and Latchman, 1995). Spacing of two TAATGARAT motifs in the ICP4 promoter plays a role in activation by Brn-3a and repression by Brn-3b. Although there is scattered amino acid similarity between the 7 dpi ORF and Brn-3, the 7 dpi cDNA has distinct functional properties. For example, the TAATGARAT motif in pAB5 (ICP0 promoter) played no role in repression, the ICP4 promoter activity was not repressed, and there is no obvious POU domain in the 7 dpi cDNA.

In terms of biological relevance, one could ask whether the 7 dpi cDNA would be present at high enough concentrations to inhibit productive infection in infected neurons. Our previous studies demonstrated that this alternatively spliced product was the major spliced poly(A<sup>+</sup>) LR RNA at 7 dpi in TG of infected calves (Devireddy and Jones, 1998), suggesting that it may have an impact on IEtu1 promoter activity. We speculate that in conjunction with neuronal-specific factors, the 7 dpi cDNA plays a role in reducing IE gene expression

during the transition from acute infection to establishment of latency and may play a role in maintaining latency. In latently infected B cells, Epstein-Barr virus (EBV) encodes gene products that repress expression of viral promoters required for productive infection. For example, EBNA3C is a potent repressor of transcription when tethered to DNA (Bain *et al*, 1996). Furthermore, EBNA2C activates a cellular protein (BATF) that is a member of the AP-1 family of transcription factors that functions as a negative regulator of AP-1 activity and as an antagonist of cell growth (Johansen *et al*, 2003). BATF inhibits expression of the EBV BZLF1 promoter, which is required for activation of the EBV lytic transcription program. Thus, it appears that herpesviruses, including BHV-1, have evolved to promote viral latency, but inhibit lytic-cycle entry in cells that are sites for latency.

## Materials and methods

### Cells

Mouse neuroblastoma (neuro-2A) and human osteosarcoma cells (U2OS) (American Type Culture Collection, Rockville, MD) were grown in Earle's minimum essential medium supplemented with 10% fetal calf serum.

### Plasmids

IEt1 chloramphenicol acetyl transferase (CAT) and the LR promoter linked to CAT (p0.95cat/1) were previously described (Bratanich *et al*, 1992; Bratanich and Jones, 1992; Jones *et al*, 1990). pSV2 CAT (SV40 enhancer-promoter linked to the CAT gene) was obtained from Bruce Howard, NIH. ICP4 CAT contains DNA sequences from the HSV-1 ICP4 promoter (-331 to +25) linked to CAT (Liu *et al*, 1990). A HSV-1 ICP0 promoter construct (-160 to +150) is designated pAB5 and contains one TAATGARAT motif (O'Hare and Goding, 1988). Plasmid pAB2 contains the ICP0 promoter (-130 to +150) but lacks the TAATGARAT motif in pAB5. The bTIF expression plasmid contains the BHV-1 VP16 homologue cloned into a CMV expression vector (pcDNA-bTIF). pcDNA3.1 and pcDNA3.1 CAT were purchased from Invitrogen. pcDNA1/LRT was described previously (Hossain *et al*, 1995).

The LR gene (SphI fragment) and 7 dpi cDNA (XhoI-SalI fragment) were inserted into Flag-tagged expression vectors (Stratagene; La Jolla, California USA) such that all three reading frames could be expressed with a Flag-tagged epitope at the amino terminus of the protein.

Cells were transfected by calcium phosphate precipitation as described previously (Devireddy and Jones, 1999) or by Superfect transfection reagent (Qiagen) following the manufacturers instructions. The amount of DNA transfected was kept constant by adding pUC19 carrier DNA.

### Preparation of poly(A+) RNA

Poly(A+) RNA was prepared using the mRNA purification kit from Pharmacia according to the manufacturer's instructions and measured spectrophotometrically at 260 nm (Devireddy and Jones, 1998).

### cDNA synthesis and construction of full-length 7 dpi cDNA

Priming poly(A+) RNA with an oligo (dT) primer using the Marathon cDNA amplification kit from Clontech according to the manufacturer's instructions generated the cDNA library. The resulting cDNA library was used to amplify the 7 dpi cDNA as described in Figure 2. Primer 2 is antisense to LR transcripts and spans nucleotide 1151 to 1180 (5'-CTCGGCGCCAGCGAGCGCCATGGCGCGGGCGCA CCTGG-3'). Primer 3 is the same sense as LR transcript and spans nucleotide 1363 to 1393 (5'-CGCTCGCTGGCGCCGAGCTCCGAGCGACGGAAG GTGCC-3'). The numbering system of Kutish *et al* (1990) was used to describe these primers. These primers were designed based on the splice junction sites of poly(A+) LR transcript at 7 dpi (Devireddy and Jones, 1998). Primers 2 and 3 have 9-bp overlapping sequences that are underlined (also indicated as small open boxes in Figure 2). The reason for this overlap is the final PCR products will have overlapping sequences when amplified with these primers. The 5' and 3' RACE PCRs were performed using the Marathon cDNA amplification kit (Clontech) using primers 2 and 3. These RACE PCR products were cloned into pCR-Script vector (Stratagene) and PCR products sequenced using a kit from Oncor.

For construction of a full-length cDNA, the overlapping extension PCR method was modified (Nakshatri and Chambon, 1994). Primers 1 and 4 were designed for this purpose. Primer 1 spans nucleotides 776 to 808, has Kozak consensus sequences for efficient translation (underlined) (Kozak, 1995) and contains an EcoR I site (italicized) (5'-CGGAATTCGCCACCATGCGCGACCTGGGCCATA AA-3'). Primer 4 is antisense to LR transcript, spans nucleotides 2202 to 2173, and has a XhoI site (underlined) (5'-CGGCCGCTCGAGTCAGCAGTCTG CGCCTCCTGGCGCCGTGG-3'). Primers 1 and 4 are designed based on the previous analysis of the organization of open reading frames within a spliced poly(A+) LR RNA detected at 7 dpi (Devireddy and Jones, 1998). The method of Nakshatri and Chambon (1994) was modified to include a mixture of Deep Vent DNA polymerase (New England Biolabs) and *Taq* polymerase (1.25 U of Deep Vent polymerase for every 100 U of *Taq* polymerase) to provide proof reading activity in the reaction. This polymerase mixture worked better than other combinations that were tested. One-tenth of the 5' and 3' RACE PCR products were mixed and amplified. The 50- $\mu$ l PCR reaction contained 1 $\times$  buffer (10 $\times$  buffer supplied with the Deep Vent DNA polymerase), primer 1



(200 ng), primer 4 (200 ng), dNTPs (400  $\mu$ M, Perkin Elmer Cetus), 10% glycerol, 2 mM MgSO<sub>4</sub>, and 0.5  $\mu$ l of each polymerase. The assembly PCR reaction had three steps. Step 1 had five cycles of PCR (94°C [1 min], 37°C [2 min], and 72°C [2 min]). Step 2 had five cycles of PCR (94°C [1 min], 45°C [2 min], and 72°C [2 min]). Step 3 had 40 cycles of PCR (94°C [1 min], 55°C [2 min], and 72°C [2 min]). Final extension was conducted at 72°C for 7 min. The resulting full-length cDNA was cloned into pcDNA3.1 (Invitrogen) using the unique EcoRI and XhoI sites.

#### *Chloramphenicol acetyl transferase assays*

Neuro-2A cells were transfected with the indicated plasmids, cell lysates harvested 48 h after transfection, and CAT activity measured (Devireddy and Jones, 1999). Quantification was performed using a Phosphorimager from Molecular Dynamics. As an internal control, pSV2  $\beta$ -galactosidase ( $\beta$ -gal) (Clontech) was cotransfected with the test

plasmids. The  $\beta$ -gal activity was assayed by a colorimetric method using *ortho*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG, Sigma) (Nakshatri and Chambon, 1994). The amount of extract used to measure CAT activity was normalized based on  $\beta$ -gal activity.

#### *Western blot analysis*

Whole cell extract was prepared from transfected and untransfected cells according to the method described previously (Harlow, 1988). Extracts were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to an Immobilon (Millipore) membrane following the manufacturer instructions, and probed with the P2 antibody. The P2 antibody is directed against the amino terminus of ORF-2 (Hossain *et al*, 1995). Western blots were developed using an enhanced chemiluminescence (ECL plus) kit (Amersham) following the manufacturer's instructions.

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