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Identification of Gene Products Encoded by the Latency-Related Gene of Bovine Herpesvirus 1

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Bovine herpesvirus 1 (BHV-1) establishes a latent infection in sensory ganglionic neurons of infected animals. Expression of latency-related (LR) gene products is controlled by a 980-bp fragment (LR promoter). DNA sequence analysis revealed that two major open reading frames (ORFs) are in the LR gene. Antibodies directed against both ORFs were generated in rabbits by using synthetic peptides. Antibody P2, which is directed to sequences near the amino terminus of ORF 2, recognized a 41-kDa protein in lytically infected cells, suggesting that ORF 2 encodes a protein. When the LR gene was inserted into a mammalian expression vector and subsequently transfected into COS-7 cells, a 41-kDa protein was detected by use of silver-stained sodium dodecyl sulfate-polyacrylamide gels and by the P2 antibody. In contrast, this protein was not detected in mock-transfected cells. Deletion of DNA sequences containing ORF 2 blocked synthesis of the 41-kDa protein in COS-7 cells. Reverse transcriptase-mediated PCRs indicated that splicing occurs near the C terminus of ORF 2. Further studies indicated that LR RNA was alternatively spliced in latently infected cattle and that a fraction of LR RNA was poly(A)$^+$. Taken together, these studies suggested that a spliced LR transcript has the potential to encode a 41-kDa protein.

Bovine herpesvirus 1 (BHV-1) is a significant viral pathogen of cattle which can induce respiratory disease, abortion, or occasionally encephalitis (reviewed in reference 28). Furthermore, BHV-1 is believed to be a causative agent of shipping fever, or bovine respiratory complex. As a consequence of the pathogenic potential of BHV-1, the cattle industry suffers losses of millions of dollars per year. Like all members of the alphaherpesvirus family, BHV-1 establishes a latent infection in sensory ganglionic neurons of an infected host (15, 20–22). The virus can persist in a latent state for the lifetime of the infected host or can periodically reactivate. During reactivation, virus particles are synthesized and subsequently passed to other susceptible cattle. In essence, latently infected animals serve as a reservoir for the virus and are primarily responsible for transmission of the virus.

During a primary infection of cattle, BHV-1 infects epithelial cells of the upper respiratory tract or, in some instances, the genital tract. Subsequently, virus enters the peripheral and central nervous systems, where it establishes a latent infection in sensory neurons (1, 20). A latent infection of BHV-1 can be divided into at least three stages: (i) establishment of a latent infection in sensory neurons, (ii) maintenance of the latent state, and (iii) reactivation of latent virus. In contrast to the 70 to 80 viral genes expressed during a lytic infection of bovine cells, it is clear that viral gene expression is severely impaired during a latent infection (20–22). One small region of the genome is transcriptionally active in latently infected neurons, and this region is designated the latency-related gene (LRG). The results of previous studies revealed that expression of the LRG is controlled by a 980-nucleotide (980-nt) fragment (3, 4, 16). During a productive infection in bovine cells, the transcriptional start site for LR RNA is downstream of two TATA elements (3). DNA sequence analysis revealed that two major open reading frames (ORFs) exist within the LRG (18).

Although the LR promoter has been analyzed in considerable detail (3, 4, 16), it is not known whether a protein is synthesized from the LRG. The aim of this work was to determine if the LRG can encode a protein. For this purpose, we inserted the LRG into mammalian expression vectors. Transfection of these constructs into COS-7 cells resulted in expression of a 41-kDa protein which could be detected by an antipeptide antibody and by silver staining of protein gels. However, the protein was not detected in a construct containing a deletion at the 5′ end of the LRG. Synthesis of a similar-size protein was also detected during a lytic infection. Reverse transcriptase-mediated PCR (RT-PCR) analysis indicated that the LR RNA is spliced near the C terminus of ORF 2 and has novel 5′ termini in latency. Furthermore, a fraction of LR RNA is poly(A)$^+$. Taken together, these results suggest that a spliced LR RNA is translated into a 41-kDa protein encoded by ORF 2.

MATERIALS AND METHODS

Virus and cells. COS-7 cells (American Type Culture Collection, Rockville, Md.) were grown in Earle's modified Eagle's medium supplemented with 5% fetal bovine serum. Bovine turbinate (BT) cells were maintained as described previously (16). The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa (16). BT cell monolayers were infected with the Cooper strain of BHV-1 (multiplicity of infection, 5 PFU per cell) 24 h prior to preparation of cell lysates or at the indicated time before RNA extraction.

Virus infection and preparation of cell lysates. After infection, BT cells were washed once with phosphate-buffered saline (PBS) and cells were collected in PBS with a disposable cell scraper. The cells were then centrifuged in a microcentrifuge (16,000 × *g*, 5 min, 4°C) and suspended in lysis buffer (10 mM Tris-HCl buffer [pH 7.2], 50 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors (10 μg of aprotinin per ml, 10 μg of soybean trypsin inhibitor per ml, 100 μg of leupeptin per ml, 10 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM iodoacetamide, 20 mM sodium P_i, 50 mM sodium fluoride, and 1 mM sodium *o*-vanadate; Sigma, St. Louis, Mo.). The lysis buffer was prepared from stock solutions immediately before use. An equal volume of hot 5% sodium dodecyl sulfate (SDS) solution (100°C) was added to the cell suspension, which was then vortexed briefly and boiled for 5 min. The cell lysates

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were then centrifuged for 5 min (16,000 × *g*), and the supernatant was collected and stored in small aliquots at −20°C.

Plasmids. A *Sal*I site was first introduced into pcDNAI/Amp (Invitrogen, San Diego, Calif.) by inserting a *Sal*I linker at the *Xba*I site, and this plasmid was designated pCDNAI/Amp-SalI. The 2.0-kb *Hin*dIII-*Sal*I fragment containing the entire LR promoter and gene was purified on a 5% polyacrylamide gel and cloned into the unique *Hin*dIII-*Sal*I site of pCDNAI/Amp1-SalI (23). This construct was termed pcDNA/LR-1. pcDNA/LR-1Δ SphI was constructed by deleting a 1.0-kb *Sph*I fragment from pcDNA/LR-1.

The *Hin*dIII-*Sal*I fragment has three *Sph*I sites, at nt 774, 805, and 1773 (see Fig. 1 and 4). To delete the 31-bp fragment between the first two *Sph*I sites and introduce a *Sph*I stop linker, the *Hin*dIII-*Sal*I fragment was digested with *Xba*I and *Pst*I. The 457-bp *Xba*I-*Pst*I fragment (nt 524 to 981) was purified and cloned into phagemid Bluescript II SK$^+$ (Stratagene, La Jolla, Calif.). The resulting construct was designated SK/LR-1. A *Sph*I stop linker (Sigma) was introduced into SK/LR-1 to create SK/LR-3. The *Hin*dIII-*Sal*I fragment was purified from pcDNA/LR-1 and digested with *Pst*I-*Sal*I, and the 1,019-bp *Pst*I-*Sal*I fragment was cloned into SK/LR-3 to generate SK/LR-4, which contains the 1,544-bp *Xba*I-*Sal*I fragment of the original 2.0-kb *Hin*dIII-*Sal*I fragment in which a *Sph*I stop linker has been introduced. The *Xba*I-*Sal*I fragment was purified from SK/LR-4 and cloned into pcDNA/LR-1, which was digested with *Xba*I-*Sal*I to release its *Xba*I-*Sal*I fragment to create pcDNA/LR-1 Stop. pcDNA/LR-1 Stop thus contains the *Hin*dIII-*Sal*I fragment of the LR promoter and gene from which the 31-bp fragment between the first two *Sph*I sites (nt 778 and 801) was deleted and into which a *Sph*I stop linker was inserted. For a diagram of the respective LR constructs, see Fig. 4A.

Transfection. COS-7 cells were transfected with the different LR constructs by calcium phosphate precipitation (13). Eighteen hours prior to transfection, cells were plated at a density of 3×10^5/100-mm-diameter dish. Generally, 10 to 15 μg of plasmid DNA was used for each 100-mm-diameter dish. At 24 and 48 h posttransfection, cell monolayers were washed with PBS and cell lysates were prepared as described above.

Antibodies, immunoprecipitation, and Western immunoblotting. For immunodetection of proteins, rabbit antisera raised against synthetic peptides (18 amino acids long; Chiron Corporation, Clayton, Australia) corresponding to sequences near the N terminus and C terminus of LR ORF 2 were used. In addition, a synthetic peptide derived from the sequences near the N terminus of LR ORF 1 was used. These peptides were conjugated to diphtheroid toxoid and injected into rabbits for production of antisera according to standard procedure. The protein concentrations of cell lysates were determined (2), and samples (50 μg) were electrophoresed by SDS–10% polyacrylamide gel electrophoresis (PAGE) and electroblotted onto Immobilon P membranes (Millipore, Bedford, Mass.) by using a Trans-Blot semi-dry transfer cell (Bio-Rad, Richmond, Calif.) for 1 h at 20 V. The filter was blocked for 2 h at 4°C in PBS (0.5×) containing 5% powdered milk, 1.0% bovine serum albumin (Sigma), 1.0% fetal calf serum, 7.5% glycine (Sigma), and 0.1% Tween 20. The filter was then washed (30-min wash, four times) in PBS (0.5×) containing 1.0% fetal calf serum and 0.1% Tween 20. Primary antibody was used at a 1:5,000 dilution in wash buffer (0.5× PBS containing 1.0% fetal calf serum and 0.1% Tween 20), and the blot was incubated overnight at 4°C. The blot was incubated with wash buffer (30-min wash, six times) at room temperature. Horseradish peroxidase-conjugated anti-rabbit whole antibody raised in donkeys (Amersham Corp., Arlington Heights, Ill.) was used as a secondary antibody at a 1:1,000 dilution in wash buffer, and the blot was incubated at 4°C for 2 h. After four washes at room temperature (30 min each wash), the blot was developed with the ECL chemiluminescence system (Amersham) and then exposed to XAR5 film (Kodak, Rochester, N.Y.).

For preparation of cell lysates to be used in immunoprecipitations, infected BT cells or transfected COS-7 cells were washed once with PBS, 1 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 125 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 2 mM Na-orthovanadate, 10 mM PMSF, 10 mM $NaPP_i$, and 10 μg each of soybean trypsin inhibitor, leupeptin, and aprotinin per ml) was added per 100-mm-diameter dish, and cells were collected with a cell scraper. The cell suspension was shaken for 30 min at 4°C and then centrifuged for 5 min (16,000 × *g*) at 4°C. The supernatant was collected and stored in small aliquots at −20°C. Cell lysates (200 μg) precleared with normal rabbit serum were incubated with P2 antibody (1:1,000 dilution in PBS) for 3 h at 4°C. Immune complexes were collected by adsorption with 25 μl of protein A-Sepharose (Pharmacia, Piscataway, N.J.). The beads were sedimented and washed three times with 10 mM Tris-HCl buffer containing 50 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40. The proteins were separated by SDS-PAGE, and Western blots were incubated with a 1:2,500 dilution of primary antibody as described above. In blocking experiments, P2 antibody was preincubated with P2 peptide (10 to 40 nmol) for 1 h at 37°C and the immunoprecipitation reaction was carried out as described above.

Isolation of RNA. RNA from trigeminal ganglia was prepared as described by Chomczynski and Sacchi (5), with slight modifications. Trigeminal ganglia from latently infected or uninfected cattle (a generous gift from F. Osorio) were frozen at −70°C. The infection of cattle and extraction of trigeminal ganglia have been described elsewhere (11). Frozen tissue (1 to 2 g) was minced. Subsequently, the tissue was placed in a guanidine-isothiocyanate solution (4 M guanidine thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, and 14 mM β-mercaptoethanol) and homogenized for 10 to 15 s with a Brinkman Polytron tissue grinder. The tissue grinder probe had previously been incubated in 1 M KOH overnight and rinsed extensively with sterile diethyl pyrocarbonate-treated water (to eliminate RNase activity). Grinding was repeated until the tissue was completely suspended (once or twice). Two phenol extractions were performed to ensure that the RNA was free of protein. RNA concentrations were determined spectrophotometrically (260 nm). From 1 or 2 g of tissue, approximately 1 to 2 mg of RNA was obtained. Aliquots of RNA (50 μg) were suspended in 1 volume of Tris-EDTA containing 0.3 M Na acetate, 2.5 volumes of ethanol were added, and the samples were stored at −120°C. RNA extraction from tissue culture was performed essentially as described by Chomczynski and Sacchi (5).

Identification of splicing in LR RNA. RNA samples were treated with 2 U of DNase I (RNase free) per μg of RNA for 20 min at 20°C and then ether extracted and ethanol precipitated. This treatment eliminates contaminating DNA but does not interfere with RT activity (24). Five micrograms of total RNA and the proper amount of primer (0.75 μM for sequence-specific primer or 2.5 μM for $T_{(12-18)}$; both final concentrations), in 4 μl of Tris-EDTA, were incubated at 65°C for 5 min and chilled on ice (denaturation). Sixteen microliters of ice-cold RT mix (20 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 1 mM dithiothreitol, 0.5 mM each deoxynucleoside triphosphate, 10 U of RNasin [Promega, Madison, Wis.], and 100 U of RNase H$^-$ RT [Stratagene]) was added. The reaction mixture was incubated at 20°C for 10 min and then at 45°C for 60 min. Reverse transcription was terminated by incubation at 95°C for 5 min. An aliquot (2.5 μl) of the RT reaction mixture was used for PCR. PCRs were carried out with 50 μl of 1× commercial PCR buffer–1.0 mM $MgCl_2$–10% glycerol–200 μM each deoxynucleoside triphosphate–1 μM each primer–1 U of *Taq* polymerase. The locations of primers used for amplification are shown in Fig. 6. Amplification was carried out for 40 cycles by denaturing at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 3 min in the first cycle, increasing 2 s per cycle. Upon completion of the last cycle, the reaction mixtures were further incubated at 72°C for 7 min in order to ensure complete extension of the amplified products. All PCRs were hot started (components were mixed at 4°C, and the reaction mixture was placed in a preheated 95°C heat block) after 3 min of denaturation at 95°C. The use of RNase H$^-$ RT and a high temperature for reverse transcription as well as 10% glycerol and Hot start in the PCR mixtures allows amplification through RNA regions of complex secondary structure. Amplified products were resolved by agarose gel electrophoresis, blotted onto a nylon membrane, and hybridized to probe 1. After autoradiography, the membranes were stripped and rehybridized with probe 2.

The primers used to identify the LR splice site within ORF 2 are as follows. Primer 1815 (5′-GACGAGACCCCCGATTGCCG-3′) is antisense to LR RNA and was used to prime total LR RNA cDNA synthesis. Primer 1629 (5′-GGC CCGCCGGAGAAGAAGGACAGAGT-3′) is antisense to LR RNA and was used for PCR amplification. Primer 872 (5′-AGGCTGGGGGTCGCAAATAC ACGGC-3′) has the same sense as LR RNA and was used for PCR amplification. To prime poly(A)$^+$ RNA, a commercial poly(dT) primer which is 12 to 18 nt long (Invitrogen) was used. The hybridization probes used for identifying spliced LR RNA were probe 1, from nt 890 to 920 (5′-GGTGCGGTGTGGT GGCCTGCGGG-3′), and probe 2, from nt 1606 to 1585 (5′-AGTTAGAC CGCGCCCGGACCCC-3′).

Mapping the 5′ end of LR RNA. Total RNA extracted from infected BT cells (2 μg) or latently infected trigeminal ganglia from cattle (5 μg) was reverse transcribed and amplified with a RACE (rapid amplification of cDNA ends)-PCR kit according to the instructions of the manufacturer (Clontech, Palo Alto, Calif.). The primers used for RACE-PCR are as follows. Primer RI was used for cDNA synthesis, is antisense to LR RNA, and is located between nt 913 and 942 (5′-TCCCCGCCCACCCACTCCGCGACCCGCAGC-3′) (upstream primer: the complement to the anchor oligonucleotide provided by the Amplifinder RACE kit [Clontech]). Primer RII is antisense to LR RNA and is located between positions 894 and 913 (5′-CCCACCACACCGCACCGCCG-3′). Amplified products were hybridized with an oligonucleotide complementary to positions 811 to 841 (5′-GCGAGCAGTTACTTTCGGTTTGGGGATGACA-3′), designated probe RIII.

Mapping the 3′ end of LR RNA in BHV-1. Total RNA extracted from either lytically infected BT cells (2 μg) or latently infected trigeminal ganglia from cattle (5 μg) was reverse transcribed and PCR amplified. cDNA synthesis was primed with 1 μg of $T_{(12-18)}$. The primers used to map the 3′ terminus of LR RNA were as follows: L3A primer pair, upstream sense primer from nt 1672 to 1693 (5′-CGCTCCCCTTCGTCCCTCCTCA-3′) and downstream antisense primer complementary to nt 1835 to 1815 (5′-GACGAGACCCCCGATTGC CG-3′); L3B primer pair, upstream sense primer from nt 1755 to 1775 (5′-TTCTCTGGGCTCGGGGCTGC-3′) and downstream antisense primer complementary to nt 1924 to 1947 (5′-AGAGGTCGACAAACACCCGCGGT-3′); and L3C primer pair, upstream sense primer from nt 1856 to 1875 (5′-TGG GGGGCGCGGAAACTG-3′) and downstream antisense primer complementary to nt 2003 to 2021 (5′-AGTCGAGGCGCACGCGGG-3′). These primers also amplify IE 2.9/E 2.6, which overlaps (in the opposite sense) the bulk of LR RNA (see Fig. 6). PCR conditions were as follows: denaturing at 95°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 3 min in the first cycle and increasing 2 s per cycle. All PCRs were hot started after 3 min of denaturation at 95°C. Glycerol (10%) was added to the buffer to disrupt secondary structure. The $MgCl_2$ concentrations were 1.5 mM for primer pair L3C and 1.0 mM for

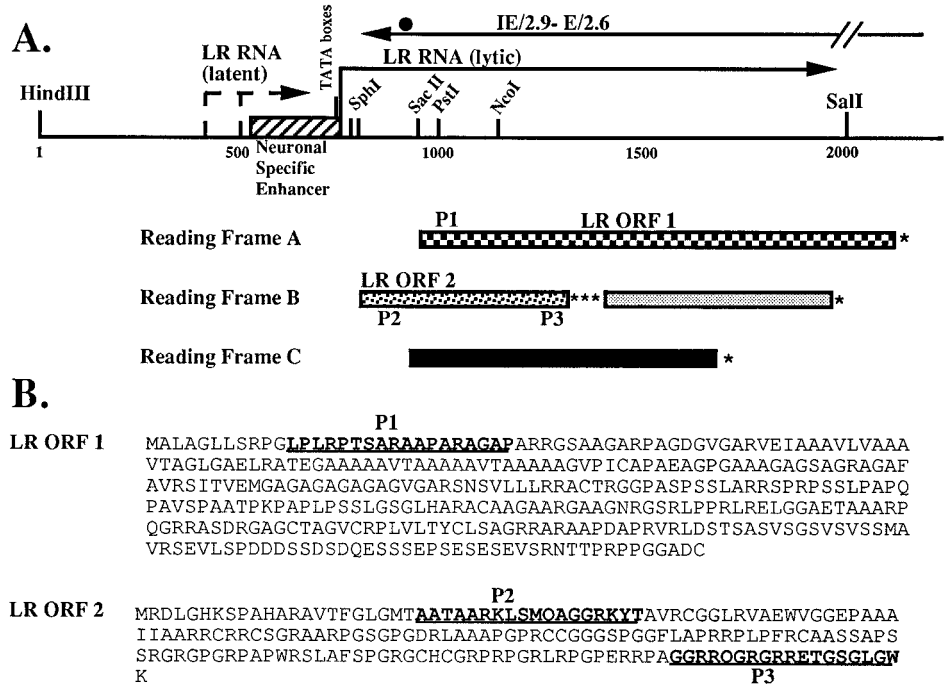

FIG. 1. Organization of the LRG and protein coding regions. (A) Diagram of LRG plus partial restriction enzyme map. Positions of immediate-early transcripts and LR transcript are shown (arrows). IE 2.9 is the 2.9-kb immediate-early transcript derived from IEtu1, and E 2.6 is the 2.6-kb early transcript (26, 27). IE 2.9 and E 2.6 are translated into identical proteins which have transcriptional transacting activity; the position of the stop codon (●) is indicated. The location of the two overlapping TATA boxes, start site of LR transcription, and neuron-specific enhancer in the LR promoter were described previously (3, 4, 16). Translation of LR DNA sequences was conducted by using the Wisconsin Genetics Computer Group sequencing package. The two major ORFs which were previously described (18) are shown. The other regions which have the potential to encode a protein (▨ and ■) are in reading frames B and C, respectively, but do not have methionine residues at their amino termini. Stop codons following the reading frames are indicated (★). (B) Predicted amino acid sequence of LR ORF 1 and LR ORF 2. Oligopeptides P1 to P3 derived from LR ORF 1 and LR ORF 2 (boldface and underlined) were synthesized, conjugated to the diphtheroid toxoid, and injected into rabbits to generate specific antiserum.

primers pairs L3A and L3B. Amplifications were carried out for 40 cycles. Upon completion of the last cycle, the reaction mixtures were further incubated at 72°C for 7 min to ensure complete extension of amplified products. Amplified products were electrophoresed in a 2% agarose gel.

RESULTS

Immunodetection of 41-kDa protein in BHV-1-infected BT cells. When DNA sequences comprising the LRG were analyzed for protein coding regions, two major ORFs were identified (LR ORF 1 and LR ORF 2) (18) (Fig. 1). The first in-frame ATG of LR ORF 2 is 51 nt from the start site of transcription, and the ATG of LR ORF 1 is 444 nt from the start site of transcription. After the three stop codons at the 3′ terminus of LR ORF 2, there is not another stop codon until DNA sequences near the *Sal*I site. In reading frame C, there is a long ORF but no initiating methionine at its amino terminus. Since LR ORF 2 is the first ORF after the start site of transcription, and since its initiating methionine is in proper context for translation (17), it appears to have a higher probability of being expressed relative to LR ORF 1. Two peptides from LR ORF 2 and one from LR ORF 1 were synthesized and designated P1 through P3 (Fig. 1B). To enhance immunogenicity of the respective peptides, each 18-amino-acid peptide was conjugated to the diphtheroid toxoid. The conjugated peptides were injected into rabbits, and subsequently several booster injections were given to induce antibodies against the peptides. Each antibody had a titer of >10,000 for its respective peptide as determined by enzyme-linked immunosorbent assay (data not shown).

Antibodies directed against P1, P2, or P3 were used to determine if proteins were synthesized from the LRG during a productive infection. BT cells were infected with BHV-1 (5 PFU per cell), and whole-cell lysates were prepared at 24 and 48 h postinfection. At 48 h postinfection, the P2 antibody recognized a 41-kDa protein (Fig. 2, panel P2, lane 3). In addition, several smaller proteins were recognized by the P2

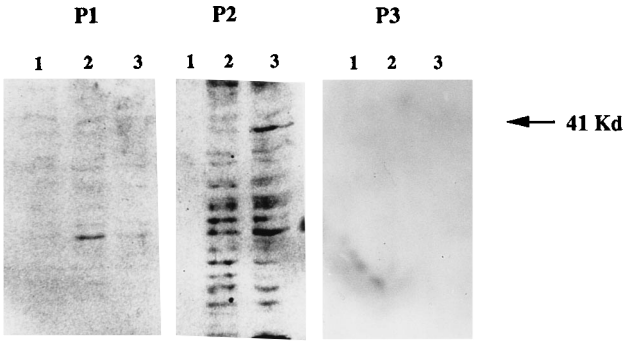

FIG. 2. Western blot analysis of BHV-1-infected BT cell lysates with P1, P2, and P3 antibodies. BT cell monolayers were infected with the Cooper strain of BHV-1 (multiplicity of infection, 5). At 0, 24, and 48 h postinfection (lanes 1 to 3, respectively), cell lysates (50 μg) were electrophoresed by SDS–10% PAGE, electroblotted onto Immobilon P membranes, and analyzed by immunoblotting with antibodies generated in rabbit against the synthetic ORF peptides. Horseradish peroxidase-conjugated anti-rabbit whole antibody raised in donkeys (Amersham Corp.) was used as a secondary antibody, and the blot was developed by using the ECL chemiluminescence system (Amersham) according to the instructions of the manufacturer.

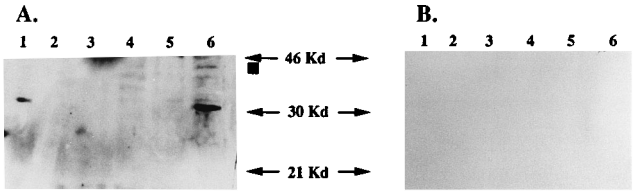

FIG. 3. Western analysis of BHV-1-infected BT cell lysates immunoprecipitated with P2 antibody (1:1,000 dilution) or P2 antibody preincubated with P2 peptide (10 nmol) for 1 h at 37°C prior to use (A) or immunoprecipitated with normal rabbit serum (B). After immunoprecipitation, proteins were separated by SDS-PAGE and immunodetection was performed with a 1:2,500 dilution of primary antibody as described in the legend to Fig. 2. Lanes 1, mock-infected BT cell lysate; lanes 2 to 6, BT cell lysates after 1 h of incubation with BHV-1 (lanes 2); at 36 h postinfection, with or without P2 antibody preincubated with peptide antigen conjugated to diphtheroid toxoid (lanes 3 and 4, respectively); and at 24 h postinfection, with or without P2 antibody preincubated as for lane 3 but with peptide antigen (lanes 5 and 6, respectively).

antibody. At 24 h postinfection, the 41-kDa protein was nearly undetectable but the smaller virus-specific proteins were present. In several independent experiments, the P3 or P1 antibody did not react with any viral or virus-induced protein (Fig. 2, panels P1 and P3). The small protein in panel P1, lane 2, was not routinely observed, suggesting that this was the result of a cross-reaction with a viral or cellular protein.

To examine the specificity of the P2 antibody, blocking experiments were performed with peptide P2. After the various cell lysates were immunoprecipitated with the P2 antibody, samples were electrophoresed by SDS-PAGE, proteins were transferred to nitrocellulose, and the Western blot was probed with P2 antibody. Addition of the P2 peptide to immunoprecipitation reaction mixtures prevented immunoprecipitation of the 41-kDa protein at 24 or 36 h postinfection (Fig. 3A, lanes 3 and 5). In contrast, the 41-kDa protein was readily detected if the peptide was not included in the immunoprecipitation assay (Fig. 3A, lane 6). Although the 41-kDa protein has been consistently detected during a productive infection of bovine cells, smaller proteins were also detected. The number, size, or intensity of these smaller proteins differed from experiment to experiment, suggesting that they are proteolytic degradation products of the 41-kDa protein. If a cocktail of protease and phosphatase inhibitors (10 μg of aprotinin per ml, 10 μg of soybean trypsin inhibitor per ml, 100 μg of leupeptin per ml, 10 mM PMSF, 10 mM iodoacetamide, 20 mM sodium P_i, 50 mM sodium fluoride, and 1 mM sodium *o*-vanadate) was not included in the lysis buffer, the relative intensity of the 41-kDa protein decreased and the intensity of the smaller proteins increased. As expected, normal serum did not recognize the 41-kDa protein in mock-infected or infected BT cells (Fig. 3B). In summary, these findings suggested that either (i) LR ORF 2 encodes a protein during a productive infection, (ii) the P2 antibody cross-reacted with another viral protein, or (iii) the P2 antibody reacted with a cellular protein induced by viral infection.

Immunodetection of 41-kDa protein in transfected cells. To determine if the LRG encodes the 41 Kd protein, restriction fragments containing LRG sequences were inserted into a mammalian expression vector (pcDNAI/Amp; Fig. 4A). Since COS-7 cells express the simian virus 40 large T antigen, plasmids containing the simian virus 40 origin of replication should replicate in COS-7 cells (12), thus allowing for efficient expression of LRG products. A novel 41-kDa protein was detected by silver staining an SDS-polyacrylamide gel which contained COS-7 cell extracts transfected with pcDNA/LR-1 (Fig. 4B, lane 4). To further demonstrate that the 41-kDa protein was encoded by ORF 2, a 1.0-kb *Sph*I fragment of the LRG was deleted from pcDNA/LR-1. In effect, this deletion eliminated LR ORF 2 but retained the LR promoter. The 41-kDa protein was not present in cell lysates prepared from cells transfected with pcDNA/LR-1Δ SphI (Fig. 4B, lane 3). A similar-size protein was also present in COS-7 cell lysates transfected with constructs containing the *Xba*I-*Sal*I fragment of the LRG (data not shown).

Forty-eight hours after COS-7 cells were transfected with pcDNA/LR-1, a 41-kDa protein was detected by the P2 antibody (Fig. 4C, lanes 1 and 3). In addition, several smaller proteins migrating between 21 and 35 kDa were detected. A 41-kDa protein was not detected when cells were transfected with a construct that has a mutation at the amino terminus of LR ORF 2 (pcDNA/LR-1 Stop; Fig. 4C, lane 2). Addition of the P2 antibody to the reaction mixture inhibited immunoprecipitation of the 41-kDa protein (Fig. 4C, lanes 4 and 5), suggesting that binding of the antibody to the 41-kDa protein was specific. The P1 or P3 antibody did not react with the 41-kDa protein in cells transfected with pcDNA/LR-1 (data not shown). The simplest interpretation of these experiments is that the P2 antibody recognized a 41-kDa protein encoded by LR ORF 2.

Structure of LR RNA in latently infected cattle and productively infected bovine cells. To better understand LR RNA structure, total RNA was prepared from BT cells infected with 5 PFU of BHV-1 per cell (5 or 7.5 h postinfection) or from trigeminal ganglia of calves latently infected with BHV-1, and LR RNA was detected by RT-PCR. As controls, RNAs were prepared from mock-infected BT cells and trigeminal ganglia of an uninfected calf.

The 5′ termini of LR RNAs in latently infected cattle were localized by RACE-PCR (Invitrogen). During a latent infection, two RACE-PCR products (approximately 380 and 610 bp) were detected (Fig. 5A). Thus, two unique 5′ termini were located near nt 524 and near nt 310. In contrast, two smaller bands (approximately 100 and 200 bp) were observed during a productive infection (Fig. 5A), which is in agreement with previous results obtained using primer extension (3). This result suggested that transcription of LR RNA was regulated by neuron-specific transcriptional regulatory elements residing upstream of the promoter utilized during a lytic infection.

The studies presented in previous sections indicated that a protein with an apparent molecular mass of 41 kDa was synthesized from LR ORF 2 (Fig. 2 to 4). Inspection of LR ORF 2 revealed that it is 181 amino acids long and thus should encode a protein with a molecular mass of 22.8 kDa (Fig. 1). Although posttranslational modification of proteins can lead to slower mobility on SDS-polyacrylamide gels, it is not likely that the apparent molecular mass would be increased 1.7 times. All three LR reading frames contain potential protein coding domains, without initiating methionines, which could be utilized to produce a larger protein if splicing occurred near the carboxy terminus of LR ORF 2 (Fig. 1). Finally, analysis of LRG DNA sequences indicates that several potential splice acceptor donor sites are 3′ and 5′ to the three stop codons at the carboxy terminus of LR ORF 2 (data not shown). To test whether the RNA was spliced, a primer located near P2 (primer 872) and one downstream of the three stop codons at the end of ORF 2 were chosen to amplify LR RNA (see Fig. 6 for locations of primers). The expected size of the amplified product was 757 bp. When cDNA synthesis was primed with an LR RNA-specific primer (primer 1815), several bands smaller than 757 bp were detected after PCR, suggesting that the LR RNA was spliced (Fig. 5B). Since the amplified products hybridized to probes 1 and 2 (Fig. 6 and data not shown), they were not the result of mispriming. The distributions of these bands were

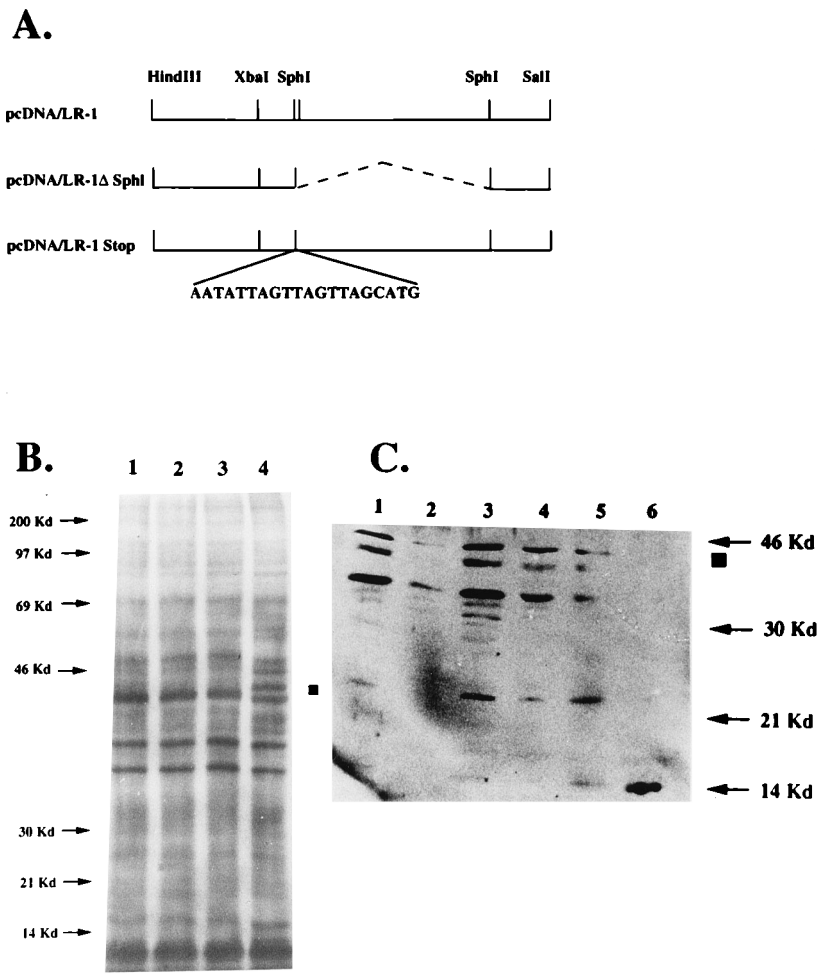

FIG. 4. Detection of 41-kDa protein in COS-7 cells transfected with plasmids containing LRG. (A) LRG constructs used in this study. pcDNA/LR-1 contains the full-length 2.0-kb LR promoter and gene; pcDNA/LR-1Δ SphI was constructed by deleting a 1.0-kb *Sph*I fragment from pcDNA/LR-1; and pcDNA/LR-1 Stop was constructed by introducing a *Sph*I stop linker at the first *Sph*I site (nt 778). See Fig. 1 for a map of LRG and Materials and Methods for details of plasmid constructs. (B) Silver staining of an SDS-polyacrylamide gel to detect protein encoded by ORF 2. COS-7 cells were transfected with the respective LR constructs, cell lysates were prepared 48 h posttransfection, proteins were separated by SDS-PAGE as described in the legend to Fig. 2, and the gel was silver stained. Lane 1, mock-transfected-cell proteins; lane 2, pcDNAI/Amp1; lane 3, pcDNA/LR-1Δ SphI; lane 4, pcDNA/LR-1. The position of the 41-kDa protein is indicated (square). (C) Immunoprecipitation and Western analysis of COS-7 cell lysates with P2 antibody transfected with different LR constructs. P2 antibody (1:1,000 dilution) or P2 antibody preincubated with P2 peptide was used in immunoprecipitation reactions. Immune complexes were collected by adsorption to protein A-Sepharose and analyzed by SDS-PAGE and Western blotting as described in Materials and Methods. Lanes 1 and 3, COS-7 cell lysate transfected with pcDNA/LR-1; lane 2, pcDNA/LR-1 Stop; lane 4, pcDNA/LR-1, with P2 antibody preincubated with P2 peptide antigen (10 nmol); lane 5, same as for lane 4 but with 40 nmol of antigen used; lane 6, pcDNAI/Amp (blank expression vector). The position of the 41-kDa band is indicated (square).

different in lytic and latent infections. In contrast, when cDNA synthesis was primed with a poly(dT) oligonucleotide and then LR cDNA was amplified with primers 872 and 1629, one prominent band smaller than 757 bp was detected in a latent or lytic infection (Fig. 5C). In summary, these studies suggested that LR RNA was spliced near the C terminus of LR ORF 2, that a fraction of LR RNA was poly(A)$^+$, and that the splicing of poly(A)$^+$ and poly(A)$^-$ LR RNAs was different.

To localize the 3′ terminus of LR RNA, three sets of primers located near the putative end of the LR RNA were designed and used for RT-PCR (for locations of primers, see Fig. 6). Previous studies relied on in situ hybridization to map the 3′ terminus of the LR RNA (20, 21). These studies did not take into account whether LR RNA was poly(A)$^+$ or poly(A)$^-$, nor were their methods as sensitive as RT-PCR. Since the three sets of primers used for these studies would also amplify IE 2.9/E 2.6, which is antisense to LR RNA (18, 27) (Fig. 1 and 6), RNA extracted from latently infected cattle was used. As expected, IE 2.9/E 2.6 RNA was not detected in latently infected cattle when primers which amplify IE 2.9/E 2.6 were used (Fig. 7, primer L3C). For all of these experiments, cDNA synthesis was done with a $T_{(12-18)}$ primer. LR RNA was amplified when L3A or L3B primers were used (Fig. 7). In contrast, L3C primers did not amplify RNA prepared from latently infected cattle. As expected, all three primers detected RNA prepared from productively infected BT cells. Southern blotting also confirmed that the amplified products were derived from the LR region, and samples lacking RT were consistently negative (data not shown). In summary, these studies demonstrated that the 3′ terminus of LR RNA was located between positions 1948 and 2018.

DISCUSSION

In this study, experiments to identify and characterize LRG products were conducted. A small fraction of LR RNA was

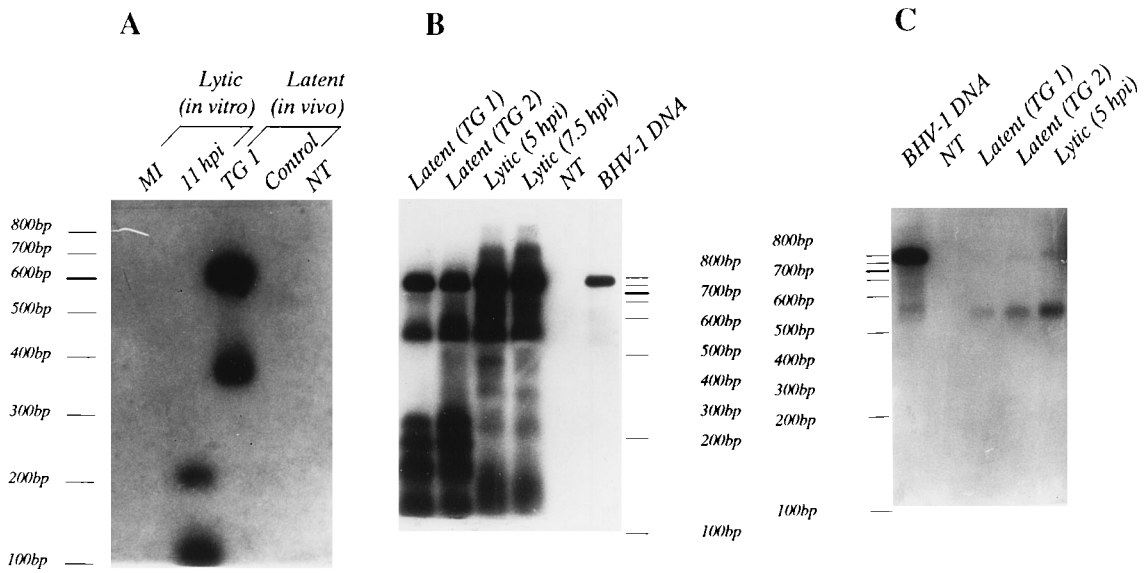

FIG. 5. Comparison of LR RNA in latently infected cattle and productively infected bovine cells. Samples were electrophoresed in 2% agarose gels, and the DNA was transferred to nylon. The Southern blots were probed with probe RIII (A), probe 1 (C), or probe 2 (23a). Radioactive probes were prepared by incubating the respective oligonucleotides with [γ-^{32}P]ATP and polynucleotide kinase. Details of the respective probes are given in the text. (A) Mapping of the 5′ end of LR transcript in a latent infection by RACE-PCR. Lanes: MI, amplification of RNA extracted from mock-infected BT cells; 11 hpi, amplification of RNA extracted from lytically infected BT cells at 11 h postinfection; TG1, amplification of RNA extracted from trigeminal ganglia of a cow latently infected with BHV-1; Control, amplification of RNA extracted from trigeminal ganglia of an uninfected cow; NT, no-template control. (B) Amplification of total LR RNA by RT-PCR. Lanes: Latent (TG1) and Latent (TG2), amplification of RNAs extracted from the trigeminal ganglia of two cows latently infected with BHV-1; Lytic (5 hpi), amplification of RNA extracted from lytically infected BT cells at 5 h postinfection; Lytic (7.5 hpi), amplification of RNA extracted from lytically infected BT cells at 7.5 h postinfection; NT, no-template control; BHV-1 DNA, positive control showing the expected full-length (757-bp) amplified product. (C) Amplification of polyadenylated LR RNA by RT-PCR. Lanes are as described for panel B.

poly(A)$^+$, and this RNA was spliced. Poly(A)$^-$ RNA was alternatively spliced, and distinct patterns of splicing were evident when RNA prepared from trigeminal ganglia of latently infected cattle was compared with RNA from productively infected bovine cells. Peptide antibodies directed against sequences near the amino terminus of LR ORF 2 detected a 41-kDa protein during the late stages of a productive infection, suggesting that LR RNA is translated into a protein. The same antibody detected a protein in COS-7 cells transfected with a plasmid containing the LRG. Since LR RNA is the only viral gene expressed during a latent infection, it is hypothesized that the LR protein plays a role during some phase of a latent infection.

The finding that antibodies directed against P2 but not P3 detected the 41-kDa protein (Fig. 2) suggested that amino acids constituting P3 were not present in the protein or that this region of ORF 2 was not immunogenic. RT-PCR analysis demonstrated that splicing occurs near the C terminus of LR ORF 2 (Fig. 5B and C), supporting the hypothesis that P3 was not present in the 41-kDa protein. Since all three reading

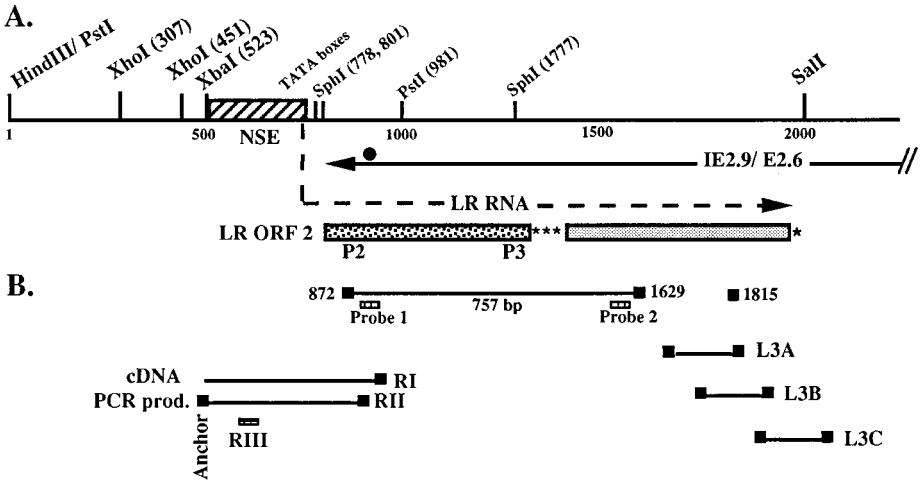

FIG. 6. Location of LR primers used to analyze LR RNA structure. (A) Restriction map of the LR promoter region, start site of LR transcription, termination of IE 2.9/E 2.6, and position of neuron-specific enhancer (NSE) (3, 4, 16). The DNA sequences which encompass the amino acids used to synthesize P2 are from nt 841 to 895. The DNA sequences which encompass P3 are from nt 1261 to 1315. The first termination codon for LR ORF 2 is located at nt 1319 to 1321. The position of the termination codon for IE 2.9/E 2.6 is indicated (solid circle). (B) Location of primers used for analyzing LR RNA. The primers are described in Materials and Methods.

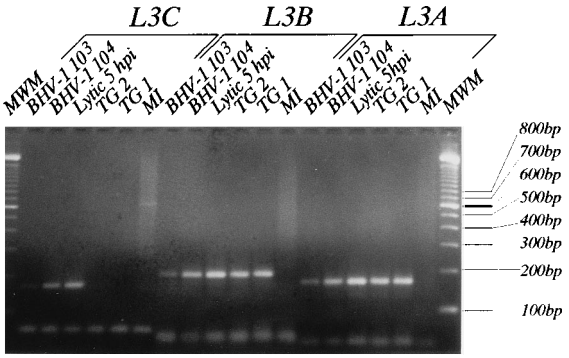

FIG. 7. Mapping of the 3′ end of LR RNA. The primer pairs used for this study (L3C, L3B, and L3A) are presented in Fig. 6. Lanes: BHV-1 10^3 and BHV-1 10^4, DNA extracted from BHV-1 (10^3 and 10^4 genome copies, respectively); Lytic 5 hpi, RNA extracted from BT cells at 5 h postinfection; TG 2 and TG 1, RNAs extracted from trigeminal ganglia of two cows latently infected with BHV-1; MI, RNA extracted from mock-infected BT cells; MWM, 100-bp ladder (GIBCO-BRL).

frames could be utilized after splicing occurs near the 3′ terminus of LR ORF 2, protein isoforms with the same N terminus but different C termini could be generated by alternative splicing. It is unlikely that the C terminus of LR ORF 1 is utilized, because LR RNA terminates between positions 1948 and 2018 (Fig. 7) and LR ORF 1 terminates at position 2200 (18) (Fig. 1). Several putative splice acceptor donor sites exist near the C terminus of LR ORF 2, lending support to the hypothesis that a spliced RNA encodes the 41-kDa protein. These studies also suggested that LR ORF 1 was not expressed during a lytic infection, because the P1 antibody did not recognize a virus-specific protein (Fig. 2). The fact that the initiating methionine of ORF 1 is 444 nt 3′ from the initiating methionine of ORF 2 would also favor expression of ORF 2. Furthermore, the initiating methionine of ORF 2 appears to be in proper context to be translated (17). In summary, several lines of evidence suggested that LR ORF 2 was expressed during a productive infection.

In addition to the P2 antibody recognizing a 41-kDa protein in infected or transfected cells, the antibody also recognized several other, smaller proteins (Fig. 2 to 4). Peptide blocking studies suggested that the antibody specifically recognized some of the smaller proteins (Fig. 3 and 4). These small proteins and the 41-kDa protein were not detected when LR ORF 2 was disrupted (Fig. 4), arguing that the antibody was not cross-reacting with other viral proteins. Addition of protease inhibitors reduced the number and intensity of the smaller bands, suggesting that these smaller proteins were the result of proteolysis. Since the P2 antibody was directed against an 18-amino-acid peptide, this antibody may also cross-react weakly with cellular proteins. On the basis of these observations, it is hypothesized that the 41-kDa protein encoded by LR ORF 2 is labile because it is susceptible to proteolytic degradation or undergoes processing. Experiments designed to distinguish between these two possibilities are being pursued.

There are two major differences in the structure of LR RNAs in latently infected cattle versus productively infected bovine cells: (i) alternative splicing of poly(A)$^-$ LR RNA occurs, and (ii) the 5′ termini of the LR RNAs were different. Recent studies demonstrated that novel promoters in the LRG are active in latently infected cattle and that these promoters, in part, direct tissue-specific expression of LR RNA (7). These novel promoter elements are 5′ from the initiation sites detected in latently infected cattle and thus are activated by neuron-specific transcription factors. Numerous studies indicated that LR RNA is predominantly localized to the nucleus during a latent infection (20, 21). It is likely that the majority of poly(A)$^-$ RNA remains in the nucleus, where it is alternatively spliced. Tissue-specific splicing factors exist (6, 14, 19), suggesting that neuron-specific splicing of LR RNA occurs during a latent infection. Neuron-specific splicing of neurexins, cell surface proteins, is known to occur (25). α-Neurexin contains five canonical sites of alternative splicing. Although each splice site normally contains only two variants, some of the splice sites have more than 10 variants. As a result, the total number of neurexins in the brain exceeds 1,000 forms. Another study has demonstrated that RT-PCR of the splice junction sites of plasma membrane calcium ATPases, an alternatively spliced mRNA, leads to additional spurious bands (29). Thus, it is not clear if each band in Fig. 5B is due to an independent splicing event. These studies clearly indicated that a small fraction of LR RNA is poly(A)$^+$ in latently infected cattle and productively infected bovine cells, suggesting that this RNA was translated (Fig. 5 and 7). Although poly(A)$^+$ forms of LR RNA appear to be spliced in similar fashions during latency and productive infection, it will be necessary to sequence splice junction sites to confirm this hypothesis and determine if the poly(A)$^+$ RNA is a template for translation. Efforts to clone the cDNA products of LR RNA have not been successful because this region was not stably maintained in bacteria. This instability could be due to secondary structure of the LR RNA, as suggested for herpes simplex virus type 1 (HSV-1) (9), or the result of deleterious effects of LR protein expression in bacteria.

Earlier studies suggested that HSV-1 encodes a latency-associated antigen (LAA) (8). A role for LAA during latency has not been reported. A mutant which contains stop codons in the major ORF of the latency-associated transcript (LAT) can establish, maintain, and reactivate from a latent infection (10), suggesting that LAA is not required for a latent infection. A comparison of ORF 2 of BHV-1 and the amino acid sequence of the major ORF of HSV-1 LAT has not revealed extensive amino acid similarities (data not shown). However, until a function is assigned to this protein and functional domains are identified, it may be difficult to compare the two ORFs.

The capacity of BHV-1 to remain latent in sensory neurons of cattle and periodically reactivate is one of its most intriguing biological properties. This complex virus-host interaction facilitates the spread of virus and complicates antiviral therapeutic strategies. Since LR transcripts are the only viral RNAs expressed during a latent infection (18, 20, 21), they may regulate some aspect of latency. Preliminary studies have not conclusively demonstrated whether ORF 2 is expressed during a latent infection (data not shown). Since only 1 to 10% of sensory neurons are believed to be latently infected (20) and since sensory neurons are a minor cell population in ganglia, LR protein expression may be difficult to detect during latency. It is also possible that only a subset of neurons which are latently infected expresses the 41-kDa protein. Experiments designed to determine a functional role for the 41-kDa protein encoded by LR ORF 2 and whether it plays a role during latent infection are under way.

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