Small Noncoding RNAs Encoded within the Bovine Herpesvirus 1 Latency-Related Gene Can Reduce Steady-State Levels of Infected Cell Protein 0 (bICP0)[▽]

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Following acute infection in mucosal epithelium, bovine herpes virus 1 (BHV-1) establishes lifelong latency in sensory neurons within trigeminal ganglia. The latency-related RNA (LR-RNA) is abundantly expressed in sensory neurons of latently infected calves. Expression of LR proteins is necessary for the latency reactivation cycle because a mutant virus that does not express LR proteins is unable to reactivate from latency after dexamethasone treatment. LR-RNA sequences also inhibit bICP0 expression, productive infection, and cell growth. However, it is unclear how LR-RNA mediates these functions. In this study, we identified a 463-bp region within the LR gene (the XbaI-PstI [XP] fragment) that inhibited bICP0 protein and RNA expression in transiently transfected mouse neuroblastoma cells. Small noncoding RNAs (sncRNAs) encoded within the XP fragment (20 to 90 nucleotides in length) were detected in transiently transfected mouse neuroblastoma cells. Two families of sncRNAs were cloned from this region, and each family was predicted to contain a mature microRNA (miRNA). Both miRNAs were predicted to base pair with bICP0 mRNA sequences, suggesting that they reduce bICP0 levels. To test this prediction, sequences encompassing the respective sncRNAs and mature miRNAs were synthesized and cloned into a small interfering RNA expression vector. Both sncRNA families and their respective miRNAs inhibited bICP0 protein expression in mouse neuroblastoma cells and productive infection in bovine cells. In trigeminal ganglia of latently infected calves, an sncRNA that migrated between nucleotides 20 and 25 hybridized to the XP fragment. During dexamethasone-induced reactivation from latency, XP-specific sncRNA levels were reduced, suggesting that these sncRNAs support the establishment and maintenance of lifelong latency in cattle.

Bovine herpes virus 1 (BHV-1) infection leads to respiratory and genital disorders, abortion, conjunctivitis, and/or multisystemic infection in small calves (19–21, 23). Consequently, BHV-1 infections are a significant economic loss to the cattle industry. As with other *Alphaherpesvirinae* subfamily members, the primary site for a BHV-1 latent infection is sensory ganglionic neurons (19, 20, 23). Virus reactivation from latency can occur after stress, suggesting that corticosteroids play a role in this process.

During latency, viral gene expression is restricted to the latency-related (LR) gene and open reading frame E (ORF-E) (13, 23, 35, 36). The LR gene contains two open reading frames (ORF1 and ORF2) and two reading frames (RF-B and RF-C) (24). A fraction of LR-RNA is polyadenylated and alternatively spliced in trigeminal ganglia (TG), suggesting that more than one protein is expressed (4, 5, 12). A peptide antibody directed against ORF2 recognizes a protein encoded by the LR gene (12, 17, 18). LR protein expression is necessary for the latency reactivation cycle because a mutant BHV-1 strain with three stop codons at the N terminus of ORF2 does not reactivate from latency (14, 33). Furthermore, the LR

mutant virus has diminished clinical symptoms and reduced shedding of infectious virus from the eye, TG, and tonsil (14, 15, 33). Finally, the LR mutant virus induces higher levels of apoptosis in TG neurons, in part because a protein encoded by the LR gene (ORF2) inhibits apoptosis (3, 14, 15, 26, 40). Three LR proteins, including ORF2, have reduced or no expression in cells infected with the LR mutant virus (18, 27).

Although proteins encoded by the LR gene are necessary for the latency reactivation cycle, non-protein coding functions within LR-RNA have also been identified. For example, the intact LR gene inhibits the ability of bICP0 to stimulate productive infection in a dose-dependent manner (1, 9). Insertion of three in-frame stop codons at the amino terminus of the first ORF within the LR gene (ORF2) inhibited bICP0 repression with an efficiency similar to that of the wild-type (wt) LR gene, suggesting that expression of an LR protein is not required (9). Since the LR gene is antisense to bICP0 coding sequences, we assumed that LR-RNA hybridized to bICP0 RNA sequences and interfered with bICP0 expression. However, we were unable to obtain data suggesting that antisense repression was the major reason why the LR gene inhibited bICP0 expression. LR gene products also inhibit mammalian cell growth (8, 38), and the cell growth-inhibitory function of the LR gene maps to a 463-bp XbaI-PstI (XP) fragment (8). Sequences within the XP region have the potential to form stem-loop secondary structures, suggesting that there are small noncoding RNAs (sncRNAs) expressed from the XP region.

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In this study, we demonstrated that the XP fragment efficiently inhibits bICP0 protein levels and, to a lesser extent, bICP0 RNA levels. Northern blot analysis using the XP fragment as a probe detected sncRNAs migrating between 20 and 90 nucleotides (nt). Two families of sncRNAs with the same 5' terminus but different 3' termini were cloned from this region. Members of these two families of sncRNAs inhibited bICP0 expression with an efficiency similar to that of the XP fragment. Each family of sncRNAs has the potential to generate a mature microRNA (miRNA). Sequences encompassing the mature miRNA also inhibited bICP0 expression in transiently transfected cells. Although the miRNA sequences have the potential to base pair with bICP0 mRNA, the miRNA sequences do not overlap bICP0 RNA sequences. Finally, LR-specific sncRNAs and miRNAs inhibited productive infection approximately 2-fold, suggesting that LR-specific sncRNAs support the establishment and maintenance of lifelong latency in cattle.

MATERIALS AND METHODS

Cell lines and viruses. Neuro-2A (mouse neuroblastoma) cells were plated in 100-mm or 60-mm plastic dishes in Earl's modified Eagle's medium supplemented with 10% fetal bovine serum. All medium contained penicillin (10 U/ml) and streptomycin (100 µg/ml).

The Cooper strain of BHV-1 (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, IA. Stock cultures of BHV-1 were prepared in CRIB cells. A BHV-1 mutant containing the β -galactosidase (β -Gal) gene in place of the viral glycoprotein C (gC) gene was obtained from S. Chowdhury (Baton Rouge, LA) (gCblue virus). The gCblue virus grows to similar titers as the wild-type parent virus and expresses the β -Gal gene as a true late gene.

Preparation of RNA and RT-PCR. Total RNA was prepared using Trizol reagent (Life Technologies) as described by the manufacturer. RNA was treated with amplification grade DNase I (Invitrogen). Reverse transcription (RT) was performed using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's directions. RNA was reverse transcribed using oligo(dT) primers (Invitrogen). Five percent of the resulting cDNA was used as a template for PCR using specific primers for bICP0. PCR was performed using GoTaq DNA polymerase (Promega) and initiated at 90°C for 10 min. This was followed by 30 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 45 s. Extension was at 72°C for 10 min. PCR products were analyzed on a 1.5% agarose gel. The following primer sequences were used: bICP0 forward primer, 5'-CGTCAGGTCTATCACTGTGGAAT-3', and bICP0 reverse primer, 5'-GCGGAAAGACAGTATGTCAGCACT-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for equivalent sample loading (forward primer, 5'-CCATGGAGAAGGCTGGGG-3'; reverse primer, 5'-CAAAGTGTGTCATGGATGACC-3').

Plasmids. Several LR gene fragments were cloned into cytomegalovirus (CMV) expression vectors, and these were described previously (8, 12, 38). The XP expression plasmid contains an XbaI-PstI fragment (523 to 986 bp). The XSpH expression plasmid contains an XbaI-SphI fragment (523 to 778 bp). The SphP expression plasmid contains an SphI-PstI fragment (809 to 986 bp). The SspP expression plasmid contains an SspI-PstI fragment (718 to 986 bp). The XS2 plasmid contains an Xba-SphI fragment (523 to 809 bp). PCR was used to amplify an XbaI-SphI fragment (nucleotide position 523 to 809) from the XP construct using a forward primer that contains the XbaI restriction site sequence (5'-ATTCTAGACGGGGCTTTGTG-3') and reverse primer that contains the HindIII restriction site sequence (5'-TCAAGCTTCTGCTCGCGCAT-3'). The PCR product was digested with XbaI and HindIII and cloned into the pcDNA3.1(-) expression vector (Invitrogen). A schematic of the respective LR plasmids is shown in Fig. 1A.

Two sncRNA families (sncRNA1 and sncRNA2) were identified from the LR gene because they have the potential to form stem-loop structures. Putative stem-loop structures were identified using a Global MicroRNA Amplification Kit (System Biosciences). The most abundant clone derived from sncRNA1 and sncRNA2 was synthesized into double-stranded DNA (Integrated DNA Technology, IA). The synthesized sncRNAs contain BamHI and HindIII restriction enzyme sites at their 5' or 3' termini, respectively. The respective sncRNAs were cloned between the unique BamHI and HindIII sites of pSilencer 2.1-U6 neo

(Ambion). The construct contains sequences from nt 525 to nt 604 according to the LR numerical system (24) and is referred to as LR sncRNA1; LR sncRNA2 contains sequence from nt 591 to nt 684. The predicted mature miRNAs within LR sncRNA1 and LR sncRNA2 were synthesized and cloned in the pSilencer 2.1-U6 neo as described above. The construct containing sequences from nt 521 to nt 546 is designated LR miRNA1. The construct designated LR miRNA2 contains sequence from nt 588 to nt 613. The control construct contains LR sequences from nt 642 to nt 809 and was used as a negative control because it does not alter bICP0 protein levels.

A herpes simplex virus type 1 (HSV-1) ICP0 expression plasmid was obtained from S. Silverstein (Columbia University) and was used as a control for certain studies.

Small RNA isolation and Northern blot analysis. Cultured cells or TG were lysed using lysis/binding solution (Ambion). For TG samples, the tissue sample and lysis/binding solution were added, and the tissue was solubilized using a Polytron tissue grinder (Kinematica). Small RNA was prepared using a mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. RNA samples were precipitated and suspended in 25 µl of formamide. Northern blot analysis was performed as described previously (30). Briefly, samples were resolved in 12 to 15% polyacrylamide-7 M Urea in 20 mM morpholinepropane-sulfonic acid (MOPS)-0.1 mM EDTA, pH 7.0. Gels were electrophoretically transferred to Hybond-NX membrane (Amersham BioScience). Membranes were placed on a sheet of 3 MM Whatman filter paper completely saturated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) prepared in 0.13 M 1-methylimidazole (Sigma) at pH 8.0. The gel was wrapped in Saran wrap and incubated at 55°C for 2 h (30). Blots were hybridized with ³²P-labeled DNA probes at 40°C for 48 h using a High Efficiency Hybridization System-Super Hyb (Molecular Research Center). Probes were prepared using an NEBlot kit (New England BioLabs) as described by the manufacturer. Radioactive probe bound to the membrane was removed by incubating the filter in a solution containing boiling 0.1% sodium dodecyl sulfate (SDS) for 15 min, and this step was then repeated.

Western blot analysis. Total cell lysate was prepared from the designated cell lines. Cells were lysed using buffer A (1% Triton X-100, 1% sodium deoxycholate, 25 mM Tris, pH 8.0, 50 mM NaCl). Equal amounts of each sample (200 µg) were loaded onto a 9% SDS-PAGE gel. Proteins were transferred onto Immobilon-P transfer membrane (Millipore) using semidry gel electrophoresis. The membrane was incubated with a bICP0 peptide-specific primary antibody (200 µg/ml) overnight at 4°C and then incubated with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (1:2,000) (Amersham NA 934) with gentle agitation for 1 h at room temperature. Antigen-antibody complexes were detected using an ECL Detection Kit from Amersham. A β -actin antibody (Santa Cruz) was used to determine if similar levels of protein were loaded in each lane. Antiserum directed against HSV-1 ICP0 (H1A027-100) was purchased from Virusys Corp.

bICP0 peptide-specific antiserum was prepared by Affinity Bioreagents (Golden, CO). The four best antigenic peptides within bICP0 were coinjected into rabbits and boosted three times. The four peptides used were RAGRGADAAQEFIDRVARG (amino acids [aa] 152 to 170), GFDDGLADAMEP (aa 226 to 238), LIGEDDAPAFVRS (aa 239 to 251), and LTANAPARPA DPAP (aa 492 to 505). The total serum was affinity purified using the four peptides. The specificity of this peptide-specific antiserum was previously demonstrated (37).

Cloning of small RNAs. One hundred nanograms of the total small RNA fraction was used for cloning. Amplification of cDNAs was performed with a Global MicroRNA Amplification Kit (System Biosciences [SBI]) according to the manufacturer's protocol. PCR products were cloned into a vector supplied with the TOPO cloning kit (Invitrogen) and transformed into *Escherichia coli* strain DH5 α (Invitrogen). The individual bacterial colonies were grown in 5 ml of LB broth, purified with a DNA purification kit (SBI), digested with restriction enzymes (BamHI and HindIII), and then loaded onto a 2.5% agarose gel. Digested plasmid DNA was transferred to a positively charged nylon membrane (Hybond N plus; Amersham Biosciences) using semidry gel electrophoresis for 3 h. To fix DNA to the membrane, the membrane was UV cross-linked. Southern blotting was performed to detect LR-positive clones. Blots were hybridized with a ³²P-labeled DNA probe at 42°C for 16 h using a High Efficiency Hybridization System-Super Hyb (Molecular Research Center). Inserts from clones that gave a positive hybridization signal were sequenced. BLASTnt in NCBI was performed with the insert sequences to identify the source of the cloned RNAs. Radioactive probes were prepared using an NEBlot kit (New England BioLabs) as described by manufacturer.

Prediction of mature miRNAs within LR small RNAs. The BayesMIRNA gene prediction program (version 1.3) (<http://wotan.wistar.upenn.edu/micro-RNA>) was used to predict mature miRNAs within the LR sncRNAs (50).

FIG. 1. The XP fragment inhibits bICP0 protein expression. (A) Schematic of the LR gene and plasmid constructs used in this study. Partial restriction map of the LR gene is shown. The XP plasmid contains LR gene sequence from nt 523 to 986 (XbaI-PstI). The numbering system of the LR gene was derived from a previous study (24). Numbers in parenthesis are the BHV-1 genomic locations (accession number AJ004801). The XSphI plasmid contains sequences from nt 523 to 778 (XbaI-SphI). The SphP plasmid contains sequences from nt 809 to 986 (SphI-PstI). The XS2 plasmid contains sequences from nt 523 to 809 (XbaI-SphI) and was constructed as described in Materials and Methods. The constructs were prepared in a human CMV expression plasmid (pcDNA3.1). (B) The XP expression construct reduced bICP0 protein levels. As described in Materials and Methods, Neuro-2A cells were cotransfected with 1 μ g of a plasmid expressing bICP0 and 5 μ g of the designated LR gene construct shown in panel A. Lane M was transfected with 5 μ g of the empty vector (pcDNA3.1+). The empty (E) lanes are Neuro-2A cells cotransfected with 1 μ g of bICP0 and 5 μ g of the empty vector (pcDNA3.1+). The XP lane was cotransfected with the XP expression plasmid and the bICP0 expression plasmid. The XS2 lane was cotransfected with a plasmid containing the XS2 expression plasmid and the bICP0 construct. The XPrev lane was cotransfected with a plasmid containing the XP fragment in the reverse orientation and bICP0. The XSphI lane was cotransfected with the XSphI plasmid and the bICP0 expression construct. The SphP lane was cotransfected with the SphP plasmid and the bICP0 expression plasmid. Cells were lysed 24 h after transfection, and a cell lysate was prepared as described in Materials and Methods. A total of 200 μ g of protein from the cell lysate was loaded in each lane of a 9% SDS-PAGE gel. Proteins in the gel were electrophoretically transferred and immunoblotted using peptide-specific IgG directed against bICP0. β -Actin protein levels were analyzed in the respective samples as a loading control. (C) Localization of sequences within the XP fragment that reduces bICP0 protein levels. As described in panel B, the designated LR plasmids were cotransfected with the bICP0 expression plasmid, and their effects on bICP0 protein levels were analyzed. The numbers to the left of the blots in panels B and C are the positions of molecular weight markers. (D) Summary of results shown in panels B and C. The average of three independent experiments is shown. The error bars indicate standard deviations.

Analysis of productive infection following transfection of cells with genomic BHV-1. Stock cultures of the gCblue virus were grown in bovine kidney cells (CRIB). Procedures for preparing BHV-1 genomic DNA were described previously (6, 7, 9, 15). Rabbit skin (RS) cells grown in 60-mm dishes were transfected with 1 μ g of sncRNA constructs, blank pcDNA3.1, or a control sequence of LR-RNA that does not inhibit bICP0 expression by using a Transil-LTR kit according to the manufacturer's specifications (MIR2305; Mirus). Cells were incubated for 24 h and then transfected with 1 μ g of purified gCblue viral genomic DNA using Lipofectamine 2000 according to the manufacturer's instructions (11668-019; Invitrogen). Because of the size difference of the plasmid and the BHV-1 genome, there is an approximate 64:1 molar ratio between the sncRNA plasmid and BHV-1 DNA. At 24 h after transfection of the BHV-1 genome, cells were fixed and stained for β -Gal expression. The number of β -Gal-positive (β -Gal⁺) cells was counted as described previously (6, 7, 9). The number of blue cells in cultures expressing the blank vector was set to 100%. To calculate the relative level of

productive infection, the number of blue cells in cultures transfected with sncRNA constructs was divided by the number of blue cells in cultures transfected with the blank vector. Cotransfection with a green fluorescent protein (GFP) expression plasmid confirmed that similar levels of plasmids were transfected into the respective cultures (data not shown). The results are the average of three independent experiments.

Animals. BHV-1-free crossbred calves (~200 kg) were used for this study. Calves were inoculated with 10⁷ PFU of wild-type BHV-1 into each nostril and eye, for a total of 4 $\times$ 10⁷ PFU/animal, as described previously (14, 15, 26, 31–33). Calves were housed under strict isolation and given antibiotics before and after BHV-1 infection to prevent secondary bacterial infection. Experiments were performed in accordance with the American Association of Laboratory Animal Care guidelines and the University of Nebraska IACUC committee. At 60 days after infection, calves were injected intravenously with 100 mg of dexamethasone (DEX) to induce reactivation from latency. TG tissue was derived from calves used in previous studies (14, 15, 26, 31–33, 45).

FIG. 2. The XP expression plasmid reduces bICP0 RNA steady-state levels. (A) As described in the legend of Fig. 1, Neuro-2A cells were cotransfected with the designated LR plasmids (5 μ g) and the bICP0 expression construct (1 μ g). At 24 h after transfection, total RNA was prepared, and bICP0 RNA levels were examined by a semiquantitative assay, as described in Materials and Methods. Analysis of GAPDH was used to confirm that similar levels of RNA were used for each lane. (B) Summary of three independent experiments. The error bars indicate standard deviations. (C) Neuro-2A cells were transfected with the HSV-1 ICP0 expression plasmid (1 μ g) and the XP expression plasmid (5 μ g) or the SphP plasmid (5 μ g). The empty lane was cotransfected with the HSV-1 ICP0 expression plasmid (1 μ g) and the empty expression plasmid pcDNA3.1 (5 μ g). At 48 h after transfection, cell lysate was collected, and 600 μ g of protein was loaded in each lane. Expression of ICP0 was monitored using an ICP0-specific antiserum. As a loading control, β -actin levels were analyzed by Western blot analysis.

RESULTS

The XP fragment inhibits bICP0 protein expression. A previous study demonstrated that the LR gene (schematic shown in Fig. 1A) inhibited productive infection and bICP0 mRNA steady-state levels (1, 9). RNA sequences encoded within the XbaI-PstI (XP) fragment of the LR gene inhibit cell growth (8), suggesting that the XP fragment encodes an sncRNA that influences bICP0 levels.

To test whether the XP fragment inhibited bICP0 protein levels, Neuro-2A cells (mouse neuroblastoma) were cotransfected with a bICP0 expression vector and plasmids that express XP or smaller fragments derived from XP (Fig. 1A). Twenty-four hours after transfection, cells were lysed, and Western blot analysis was performed. bICP0 protein levels were reduced approximately 10-fold when bICP0 was cotransfected with plasmids expressing XP, XS2, or XSphI (Fig. 1B to D). The bICP0 peptide-specific antiserum used for these studies nonspecifically reacts with a denatured protein migrating at approximately 75 kDa. Although the levels of bICP0 were reduced when cells were cotransfected with the XP expression plasmid, levels of the 75-kDa protein remained the same. Conversely, a CMV plasmid expressing the SphP fragment consistently increased bICP0 protein levels slightly (Fig. 1B and D). The SspP fragment reduced bICP0 protein levels approximately 40% (Fig. 1D), which may be because the SspP sequences overlap bICP0 coding sequences. The effect of SspP on bICP0 protein levels was consistently less than 2-fold, whereas the effect of XP on bICP0 protein levels was 10-fold.

If the XP expression plasmid encoded an LR-specific

sncRNA that inhibited bICP0 protein levels, expression of an LR-specific transcript from XP should be important. To test this prediction, the bICP0 expression vector was cotransfected with a plasmid containing the XP fragment in the reverse orientation (XPrev). When the XPrev expression plasmid was cotransfected with the bICP0 expression plasmid, XPrev did not reduce bICP0 protein levels (Fig. 1B and D). In summary, these results suggested that the first 255 bp of the XP fragment encoded an sncRNA that inhibited bICP0 protein expression.

The XP fragment reduces steady-state levels of bICP0 RNA but does not inhibit ICP0 protein levels. To test whether the XP expression construct or smaller fragments derived from XP also inhibited bICP0 RNA expression, Neuro-2A cells were cotransfected with the bICP0 expression plasmid and one of the LR constructs (XP, XSphI, SphP, or SspP). Twenty-four hours after transfection, cells were lysed, and total RNA was prepared. RT-PCR suggested that bICP0 mRNA levels were reduced slightly following transfection of Neuro-2A cells with XP (Fig. 2A and B). In contrast, the levels of bICP0 RNA detected when cells were transfected with XSphI (Fig. 2) or XS2 (data not shown) were similar to those in cells transfected with the empty vector. The SphP or SspP constructs did not dramatically reduce bICP0 RNA levels (Fig. 2A and B).

To begin to understand whether the effect of XP on bICP0 protein expression was specific for bICP0, we tested whether XP would reduce HSV-1 ICP0 protein levels in Neuro-2A cells. Although ICP0 and bICP0 are considered to be functional homologues, they do not have a great deal of amino acid or nucleic acid similarity, apart from the zinc RING finger

FIG. 3. Identification of small RNA in Neuro-2A cells transfected with plasmids expressing XP or fragments within XP. (A) Approximately 1×10^6 Neuro-2A cells in a 100-mm dish were transfected with 5 μ g of the XP plasmid as described in Materials and Methods. At 24 h after transfection, total small RNA was prepared using a mirVana kit as described in Materials and Methods. Either 14 or 40 μ g of total small RNA was loaded onto a denaturing polyacrylamide gel. As a negative control, 14 μ g of total small RNA from mock-transfected cells was electrophoresed on a denaturing gel. Two micrograms of total small RNA was loaded on a 1.5% agarose gel, and this gel was stained with ethidium bromide to confirm that similar levels of total small RNA were loaded in each lane. The panel to the right of panel A shows an overexposure of the gel in the range of 9 to 30 nucleotides. (B) The designated plasmids were transfected into Neuro-2A cells, and a small RNA fraction was obtained as described for panel A. Fourteen micrograms of total small RNA was loaded in each lane. (C) The gel from panel B was overexposed to visualize faint bands.

located at the N terminus of the respective proteins (21). Thus, one would not expect XP to have a dramatic effect on HSV-1 ICP0 protein levels unless XP had a general effect on reducing protein levels expressed from a plasmid. Neuro-2A cells were cotransfected with a plasmid that expresses HSV-1 ICP0 and either XP, SphP, or a blank expression plasmid (pcDNA3.1). The XP expression plasmid was not able to dramatically reduce ICP0 protein levels in Neuro-2A cells relative to the blank expression vector or the SphP expression plasmid (Fig. 2C). Although this study does not rule out the possibility that XP can reduce the levels of other BHV-1 or cellular proteins, it suggests that XP does not randomly reduce protein levels expressed from a plasmid in transient assays.

The XP fragment encodes small RNAs in transfected cells.

To begin to understand whether the XP fragment encodes sncRNAs, Neuro-2A cells were transfected with either the XP expression plasmid or an empty vector (pcDNA3.1+). Twenty-four hours after transfection, cells were lysed, and a small RNA fraction was prepared using a mirVana kit. Northern blot analysis was then performed using the XP fragment as a probe. Two prominent RNA bands that migrated between nucleotides 70 and 90 were detected in cells transfected with the XP expression plasmid but not with the empty vector (Fig. 3A). In some experiments, it appeared that a band migrating between nucleotides 20 and 30 hybridized to the XP fragment. However, when 40 μ g of total small RNA was analyzed, a band migrating between nucleotides 20 and 30 was not detected. Regardless of whether 14 or 40 μ g of total small RNA was loaded in each lane, it was evident that a distribution of small RNAs, not a specific band migrating between nucleotides 20 and 30, hybridized to the XP fragment. Longer exposure of the Northern blot confirmed this observation. Relative to the XP plasmid construct, levels of small RNAs expressed from the XSphI or XS2 plasmid constructs were not as abundant (Fig. 3B). After prolonged exposure of the Northern blot (Fig. 3C),

small RNA species were detected, and in general, the pattern was similar to that of the XP fragment.

Cloning small RNAs encoded within the XP fragment. To expand the Northern blot results, the small RNA fraction was cloned using a Global MicroRNA Amplification Kit (SBI) following transfection of Neuro-2A cells with the XP plasmid construct. Potential plasmids containing small RNA clones with XP-specific inserts were initially identified by Southern blot analysis using the XP fragment as a probe. From this analysis, 98 clones out of 400 were identified, and the inserts were sequenced. Using NCBI BLASTnt, 19 out of 98 clones contained an insert that matched sequences located within the XbaI-PstI fragment. Following alignment of these clones, two families of sncRNAs were identified (Fig. 4 shows the location of the LR sncRNA1 family and LR sncRNA2 family). The LR sncRNA1 family contained six clones that had a 5' terminus beginning at position 525 but contained different 3' termini. The LR sncRNA2 family contained 13 clones. The 5' terminus of the LR sncRNA2 family began at position 591 of the LR gene sequence. As with the LR sncRNA1 family, the 3' termini mapped to different locations.

LR-specific sncRNA inhibits bICP0 expression. To test whether LR sncRNAs inhibited bICP0 protein expression, the most abundant clone from the LR sncRNA1 family (Fig. 4; nucleotides 525 to 623) plus the most abundant clone from the LR sncRNA2 family (nt 591 to 684) was synthesized and then cloned into a small interfering RNA (siRNA) expression vector (pSilencer 2.0-U6; Ambion). The pSilencer 2.0-U6 plasmid contains a human U6 promoter that drives short hairpin RNA expression. Cotransfection of a plasmid expressing the LR sncRNA1 family member (psncRNA1) (Fig. 5A and B, lanes 1) or a plasmid expressing the LR sncRNA2 family (psncRNA2) (lanes 2) with the bICP0 expression vector into Neuro-2A cells reduced bICP0 protein expression compared to expression in cells cotransfected with bICP0 and a plasmid

FIG. 4. Cloning of sncRNAs encoded by the XP fragment in transiently transfected Neuro-2A cells. The start sites for LR transcription during latency and lytic infection were previously described (1, 4, 5, 12). Selected restriction enzyme sites within the LR gene are shown. The BHV-1 genomic nucleotide positions are the numbers in parenthesis. The numbers in the parenthesis are consistent with the LR numbering system (24). As described in Materials and Methods, small RNA was prepared from Neuro-2A cells transfected with the XP plasmid, and the total small RNA fraction was cloned. One hundred nanograms of small RNA was used for cloning. Amplification of cDNAs was performed with a Global MicroRNA Amplification Kit (SBI) (see Materials and Methods for details). Using NCBI BLASTnt, 19 clones out of 98 (19.4%) were identified that corresponded to sequences within the XbaI-PstI fragment. Two families of small RNAs were identified. The first small RNA family (LR sncRNA1) was represented by six clones. All of the sncRNA1 clones began at position 525 of the LR gene sequence. The second small RNA family (LR sncRNA2) was represented by 13 clones. The 5' terminus of the sncRNA2 family began at position 591 of the LR gene sequence. The numbers in brackets represent the number of identical clones that were identified. The positions at which the putative LR miRNA1 and miRNA2 base pair with bICP0 mRNA are indicated by the filled circles. The numbers in parentheses are the genomic positions of the 5' nucleotides of bICP0 mRNA where potential base pairing occurs with the two LR miRNAs. Details of LR miRNAs are shown in Fig. 6A to C.

expressing LR sequences between nucleotides 642 and 809 (lanes C). The control plasmid was designed by cloning nt 642 to nt 809 from the LR region, which was not expected to reduce bICP0 expression, into pSilencer 2.0-U6 (Fig. 4 shows the location of this region). As expected, the XP expression plasmid inhibited bICP0 expression, but the plasmid SspP did not (Fig. 5B). LR sncRNA1 or sncRNA2 consistently inhibited bICP0 protein levels as efficiently as XP in three independent studies (Fig. 5C).

The BayesMiRNA gene prediction program (version 1.3) (50) was used to predict whether a mature miRNA was present within the LR sncRNA1 family or LR sncRNA2 family. This analysis suggested that both sncRNA families could be processed into a mature miRNA (Fig. 6A and B). Although the sequence of the putative LR miRNAs does not overlap bICP0 coding sequences, both miRNAs have the potential to base pair with bICP0 RNA sequences (Fig. 6C and D). Interestingly, LR miRNA1 interacts with a different region of the bICP0 mRNA than LR miRNA2 (Fig. 4 shows the location of base pairing between bICP0 and LR miRNAs). The predicted mature miRNA sequences (LR miRNA1 and LR miRNA2) were synthesized and cloned into the pSilencer expression vector.

To test whether the predicted mature miRNAs inhibited bICP0 protein expression, increasing levels of a plasmid expressing LR miRNA1 or LR miRNA2 were cotransfected with a bICP0 expression vector into Neuro-2A cell lines. Reduced levels of bICP0 protein expression were detected following cotransfection of Neuro-2A cells with LR miRNA1 or LR miRNA2 relative to cells cotransfected with bICP0 and in-

creasing concentrations of the control plasmid (Fig. 6C). Relative to the XP expression plasmid, plasmids expressing the putative LR-specific miRNAs inhibited bICP0 expression to similar levels (Fig. 6E). Plasmids expressing LR sncRNA1, LR sncRNA2, LR miRNA1, or LR miRNA2 had only a marginal effect on steady-state levels of bICP0 RNA (data not shown), suggesting that they primarily inhibited translation of bICP0 mRNA.

LR sncRNAs and miRNA sequences repress productive infection. To test whether LR sncRNAs or miRNAs have an effect on productive infection, plasmids expressing the respective small RNAs were cotransfected with BHV-1 genomic DNA into rabbit skin (RS) cells, and their effects on productive infection were measured. For this study, we used the gCblue virus, which contains a β -Gal insert downstream of the gC promoter. At 24 h after transfection, cells were fixed, and β -Gal⁺ cells were identified. This time point was used to minimize the number of virus-positive cells that result from virus spread. Furthermore, at later times, many β -Gal⁺ cells lift off the dish, making it difficult to count virus-positive cells (9, 16). The number of β -Gal⁺ cells directly correlates with the number of plaques produced following transfection with the gCblue virus (7, 9, 16). In three independent studies, LR miRNA1 and LR miRNA2 inhibited the number of β -Gal⁺ cells 2- to 3-fold relative to cells transfected with genomic DNA (Fig. 7). The XP plasmid, sncRNA1, or sncRNA2 reduced the number of β -Gal⁺ cells approximately 2-fold relative to cells transfected with just genomic DNA or cells cotransfected with genomic DNA plus control sequences within the LR gene. In summary,

FIG. 5. LR sncRNAs inhibit bICP0 protein expression. The LR sncRNA1 family member that was cloned twice (Fig. 4) was synthesized and cloned into an siRNA expression vector (pSilencer 2.0-U6). The most abundant member of LR sncRNA family 2 (Fig. 4) was synthesized and cloned into pSilencer 2.1-U6 neo. The control oligonucleotide containing LR sequences from nt 642 to 809 was synthesized, cloned into pSilencer 2.1-U6 neo, and used as a negative control (lanes C). (A) Neuro-2A cells were cotransfected with 1 μ g of the bICP0 expression plasmid and 5 μ g of a plasmid that expresses LR sncRNA1 (lane 1), LR sncRNA2 (lane 2), XP, or the LR control oligonucleotide (lane C). The mock lane was Neuro-2A cells transfected with 5 μ g of only the empty vector (pSilencer 2.1-U6 neo). (B) As described in panel A, Neuro-2 cells were cotransfected with a plasmid expressing bICP0 and the designated LR constructs. Lane SspP was cotransfected with bICP0 and the plasmid expressing the SspP fragment. For panels A and B, Neuro-2A cells were lysed 24 h after transfection. A total of 200 μ g of protein/lane was loaded onto a 9% SDS-PAGE gel. Proteins in the gel were electrophoretically transferred and immunoblotted using a rabbit bICP0 peptide antiserum. β -Actin was used as a control for equivalent sample loading. (C) The average of three independent experiments is shown. The error bars indicate standard deviations. Column E represents the value obtained from cells cotransfected with the bICP0 expression plasmid and the empty siRNA expression vector (pSilencer 2.1-U6 neo). The value for E was arbitrarily set at 100%.

this study indicated that the LR-specific sncRNAs or miRNA sequences reduced or delayed productive infection.

Identification of small RNAs in TG of infected calves. To test whether LR-specific small RNAs were expressed during latency, a small RNA fraction was prepared from TG of three latently infected calves. Northern blot analysis was then performed using the XbaI-PstI fragment as a probe. A prominent small RNA band migrating at approximately 20 nucleotides was detected in all three latently infected samples but not in small RNA prepared from tissue from a mock-infected calf (Fig. 8A). In addition, several bands migrating between nucleotides 70 and 90 hybridized to the XP fragment, suggesting that these bands were precursors of a mature miRNA. Bands that specifically hybridized to the XP probe were also detected between nucleotides 45 and 50 (Fig. 8, filled circles). In some Northern blots, the 45- to 50-nucleotide bands were more intense.

Dexamethasone induced reactivation from latency reduces steady-state levels of LR-RNA (34), suggesting that LR sncRNAs may decrease following treatment of latently infected calves with dexamethasone. To test this prediction, levels of LR-specific RNAs in latently infected calves treated with dexamethasone were compared to levels in latently infected

calves. The 20-nucleotide band that hybridized to the XP probe was not readily detected in two latently infected calves treated with dexamethasone for 6 h (Fig. 8B). The bands that migrated between nucleotides 70 and 90 were also not readily detected in one calf treated with dexamethasone for 6 h. At 48 h after dexamethasone treatment, the fragment at nucleotide 20 and a subset of the bands migrating between nucleotides 70 and 90 were detected. At 48 h after dexamethasone treatment, LR-RNA levels were restored to levels similar to those during latency (34).

DISCUSSION

A recent study identified herpes simplex virus type 1 (HSV-1) miRNA in TG of latently infected humans (44). Surprisingly, miRNA from varicella-zoster virus (VZV) was not detected in the same samples even though these individuals were latently infected with VZV. Since BHV-1 is considered to be a *Varicellovirus* and since a protein encoded by the LR gene is necessary for the latency reactivation cycle in calves (14), we wondered whether the LR gene encoded sncRNAs and whether these sncRNAs were expressed during latency. In this study, two families of sncRNAs were identified within the XP

[illegible]

FIG. 6. LR-specific miRNAs inhibit bICP0 protein levels. (A and B) The BayesMiRNA gene prediction program (version 1.3) was used to predict the mature miRNA within LR snRNA family 1 and 2. The predicted mature miRNA sequences (LR miRNA1 and LR miRNA2) are in bold and underlined. The LR sequence of miRNA1 is comprised of nt 521 to 546, and the genomic sequence spans nt 100234 to 100255. LR sequence spanning 100234 to 100259 was synthesized and cloned into the siRNA expression vector (pSilencer 2.1-U6 neo). The LR sequence of miRNA2 spans nt 588 to 613, and the genomic sequence spans nt 100300 to 100323. LR sequence spanning 100301 to 100326 was synthesized and cloned into the siRNA expression vector. (C) The potential of LR miRNA1 to hybridize to bICP0 was analyzed by RNAhybrid, version 2.2. The potential for miRNA1 (top strand) to hybridize with bICP0 mRNA is shown (bottom strand). The 5' nucleotide is genomic position 101081 (accession number AJ00481) or bICP0 nucleotide position 1956 (accession number M84465). (D) The potential of LR miRNA2 to hybridize to bICP0 was analyzed by RNAhybrid, version 2.2. The potential for miRNA2 (top strand) to hybridize to bICP0 mRNA is shown (bottom strand). The 5' nucleotide is genomic position 101698 (accession number AJ004801) or position 1339 for the bICP0 sequence (accession number M84465). The approximate location of base pairing between bICP0 mRNA and LR miRNA1 or miRNA2 is shown in Fig. 4. (E) LR miRNA1 and LR miRNA2 sequences were synthesized as double-stranded DNA and then cloned into pSilencer 2.1-U6 neo. Neuro-2A cells were cotransfected with 1 μ g of the bICP0 expression plasmid and increasing amounts of plasmids expressing LR miRNA1, LR miRNA2, or the control oligonucleotide. The mock lane (M) was transfected with just 5 μ g of the empty vector (pSilencer 2.1-U6 neo). As a positive control, Neuro-2A cells were cotransfected with the bICP0 expression plasmid (1 μ g) and the XP plasmid (5 μ g). Cells were lysed at 24 h after transfection, and 200 μ g of protein/lane was loaded onto a 9% SDS-PAGE gel. Proteins in the gel were electrophoretically transferred and immunoblotted using the bICP0 peptide antiserum. β -Actin was used as a control for equivalent sample loading.

fragment of the LR gene. The XP fragment also detected virus-specific sncRNAs that appeared to be the same size as mature miRNAs in TG of latently infected calves. The two families of sncRNAs each contained a putative viral miRNA that reduced bICP0 protein levels, but not steady-state levels of bICP0 RNA, in transfected Neuro-2A cells. Both families of LR-specific sncRNAs were adjacent to the 3' terminus of the bICP0 mRNA sequences (46–48) (Fig. 4 and 6), but they did not overlap bICP0 coding sequences. However, LR miRNA1 and LR miRNA2 have the potential to base pair with bICP0 mRNA sequences, as judged by analysis using the RNAhybrid program (Fig. 6C and D), which suggests that this interaction is important for reducing bICP0 protein levels. LR sncRNAs and putative miRNAs inhibited productive infection 2- to

3-fold in transient transfection assays. This effect may have been underestimated because of endogenous levels of LR-specific sncRNAs expressed during productive infection. Since wt bICP0 protein expression is necessary for reactivation from latency in calves (37), the ability of LR-RNA to reduce bICP0 protein levels may block productive infection when bICP0 protein levels are limiting. We suggest that bICP0 is a specific target for sncRNAs encoded within the XP fragment because the XP expression vector did not have a dramatic effect on HSV-1 ICP0 protein levels.

In TG of latently infected calves, but not Neuro-2A cells transfected with the XP plasmid, a distinct band that hybridized to the XP probe was detected at nucleotides 20 to 25. Expression plasmids containing the LR snRNA1 or snRNA2

FIG. 7. LR sncRNAs inhibit productive infection. RS cells were transfected with 1 μ g of blank expression vector (pcDNA3.1), a plasmid expressing the control oligonucleotide that does not inhibit bICP0 expression, or the designated LR sncRNA constructs. Twenty-four hours later, cells were transfected with 1 μ g of the gCblue virus genome as described in Materials and Methods. The ratio between plasmid and viral genome is approximately 64:1. At 24 h after transfection with the viral genome, cells were fixed and stained for β -Gal expression, and β -Gal⁺ cells were counted. The number of β -Gal-positive cells in cultures cotransfected with the blank vector and the gCblue virus was set to 100%. The number of blue cells in cultures transfected with the blank vector was used to calculate the relative levels of productive infection in cultures transfected with plasmids expressing the designated LR sequences. The results are an average of three independent experiments.

expressed an LR-specific band migrating between nucleotides 20 and 25 in some experiments (data not shown), suggesting that LR miRNAs were efficiently processed or stabilized by neuronal-specific factors abundantly expressed during latency. During productive infection, the 5' terminus of LR-RNA begins at position 724, whereas in TG the 5' terminus of LR-RNA is localized to positions 360 and 550 (5, 12) (Fig. 4). This further suggested that neuronal factors are necessary for regulating expression of the upstream region of LR-RNA that contains the sncRNAs. The 20- to 25-nucleotide fragment that hybridized to the XP fragment was not readily detected during productive infection (data not shown), which also implied that neuronal factors stimulate processing of sncRNAs in TG. A recent study identified BHV-1 miRNA during productive infection (10), but LR miRNA1 and LR miRNA2 were not detected. Collectively, these observations implied that neuronal-specific processing led to abundant levels of LR miRNAs in TG of latently infected calves.

In the context of the lifelong latency reactivation cycle in cattle, we suggest that LR-specific sncRNAs and the putative miRNAs support this process by inhibiting bICP0 protein levels. It is unlikely that LR-specific sncRNAs and putative miRNAs are required for the latency reactivation cycle because genetic studies demonstrated that LR protein expression is necessary for dexamethasone-induced reactivation from latency (14, 33). The antiapoptosis functions of ORF2 (3, 11, 26, 40) correlate with production of infectious virus during dexamethasone-induced reactivation from latency. We further predict that ORF2 or LR sncRNAs do not directly promote reactivation from latency because LR RNA levels (34), LR

FIG. 8. Identification of small RNAs in TG of BHV-1 latently infected calves. Total small RNA was prepared from TG of three calves (animals 37, 43, and 47) that were latently infected with BHV-1 (60 days after infection). These TG were obtained during the course of other published studies (14, 15, 26, 31–33, 45). (A) Total small RNA from the respective samples (15 μ g of RNA/lane) was separated on a denaturing polyacrylamide gel, the RNA was transferred to a membrane, and the gel was probed with a radioactive XP fragment. The nucleotide position of markers is shown on the left of the gel. The bracket denotes XP-specific bands that migrated between nt 70 and 90, and the arrow indicates the approximate position of where mature miRNAs would migrate (nt 20 to 25). The closed circles indicate the XP-specific bands migrating between nucleotides 45 and 50. The bottom panel was total small RNA loaded onto a 1.5% agarose gel as a loading control (1 μ g RNA/lane). (B) Small RNA from TG of a latently infected calf (lane L) or TG of latently infected calves that were injected with 100 mg of dexamethasone, as described in Materials and Methods, to stimulate reactivation from latency. At 6 h (lane 6) or 48 h (lane 48) after dexamethasone treatment, TG were obtained, and total small RNA was prepared. Northern blot analysis was performed using the XP fragment as a probe. The arrow denotes the position of a potential mature miRNA that was recognized by the XP probe. Total small RNA is shown as a loading control. The amount of total small RNA was calculated by measuring the optical density at 260 nm of the respective sample. In addition, samples of total small RNA (approximately 2 μ g) were electrophoresed on a 1.5% agarose gel, and the levels of small RNA were visualized using a Molecular Imager FX instrument (Bio-Rad) after staining with ethidium bromide.

sncRNAs (Fig. 8), and presumably ORF2 levels were reduced during dexamethasone-induced reactivation from latency. Dexamethasone appears to stimulate reactivation from latency by several distinct pathways. For example, dexamethasone reduces LR promoter activity (22) and LR RNA expression (34) but stimulates cellular gene expression (28) and bICP0 early promoter activity (49). Since bICP0 transcription is readily detected throughout dexamethasone-induced reactivation from latency (49), reduced levels of LR-specific sncRNAs during the early stages of DEX-induced reactivation from latency appear to be biologically relevant. These observations have led us to propose that LR gene products (ORF2, sncRNAs, and perhaps additional unknown factors) promote the establishment and maintenance of latency. The ability of ORF2 to inhibit apoptosis and, to a lesser extent, the ability of sncRNAs or miRNAs to inhibit bICP0 protein levels and productive infection are predicted to increase the pool of latently infected neurons that can support reactivation for latency.

The prediction that LR-specific sncRNAs and miRNAs support the establishment and maintenance of alphaherpesvirus latency is consistent with several other studies. For example, HSV-1 and HSV-2 encode sncRNAs and miRNAs within the latency-associated transcript (LAT), and these small RNAs can reduce the levels of viral regulatory proteins (ICP0 and ICP4) (39, 41–44). A chicken alphaherpesvirus, Marek's disease virus (MDV), that causes a lymphoproliferative disorder and induces tumors, also encodes miRNAs that map to LAT coding sequences (2). The miRNAs encoded from the MDV LAT cluster are well conserved and are consistently expressed in tumors derived from infected chickens (29). Although our studies clearly demonstrated that LR-specific sncRNAs and miRNAs reduced bICP0 protein levels, they may also influence expression of cellular genes, promote neuronal differentiation as seen with other sncRNAs (25), or inhibit expression of other viral genes. Studies designed to further characterize the function of these small RNAs are under way.

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