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A Mutation in the Latency-Related Gene of Bovine Herpesvirus 1 Leads to Impaired Ocular Shedding in Acutely Infected Calves

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Bovine herpesvirus 1 (BHV-1) is an important pathogen of cattle, and infection is usually initiated in the ocular or nasal cavity. Like other alphaherpesviruses, BHV-1 establishes latency in sensory neurons but has the potential of reactivating from latency and spreading. The only abundant viral transcript expressed during latency is the latency-related (LR) RNA, which is alternatively spliced in trigeminal ganglia during acute infection (L. R. Devireddy and C. Jones, J. Virol. 72:7294–7301, 1998). LR gene products inhibit cell cycle progression (Y. Jiang, A. Hossain, M. T. Winkler, T. Holt, A. Doster, and C. Jones, J. Virol. 72:8133–8142, 1998) and chemically induced apoptosis (J. Ciacci-Zannela, M. Stone, G. Henderson, and C. Jones. J. Virol. 73:9734–9740, 1999). Although these studies suggest that LR gene products play an important role in the latency/pathogenesis of BHV-1, construction of a mutant is necessary to test this hypothesis. Because the bICP0 gene overlaps and is antisense to the LR gene, it was necessary to mutate the LR gene without altering bICP0 expression. This was accomplished by inserting three stop codons near the beginning of the LR RNA, thus interfering with expression of proteins expressed by the LR RNA. The LR mutant virus grew with wild-type (WT) efficiency in bovine kidney (MDBK) cells and expressed bICP0 at least as efficiently as WT BHV-1 or the LR rescued virus. When calves were infected with the LR mutant, we observed a dramatic decrease (3 to 4 log units) in ocular shedding during acute infection relative to WT or the LR rescued virus. In contrast, shedding of the LR mutant from the nasal cavity was not significantly different from that of the WT or the LR rescued virus. Calves infected with the LR mutant exhibited mild clinical symptoms, but they seroconverted. Neutralizing antibody titers were lower in calves infected with the LR mutant, confirming reduced growth. In summary, this study suggests that an LR protein promotes ocular shedding during acute infection of calves.

Bovine herpesvirus 1 (BHV-1) is an important viral pathogen of cattle that can cause severe respiratory infection, conjunctivitis, abortions, vulvovaginitis, balanopostitis, and generalized systemic infection in neonate calves (40). BHV-1-induced immunosuppression frequently leads to secondary bacterial infections, resulting in bronchopneumonia and occasionally death. Increased susceptibility to secondary infection correlates with depressed cell-mediated immunity after infection (2, 8–10). $CD8^+$-T-cell recognition of infected cells is impaired by down regulation of major histocompatibility complex class I expression and the transporter associated with antigen presentation (11, 12, 22). $CD4^+$-T-cell function is impaired during acute infection of calves because BHV-1 has the ability to infect $CD4^+$ T cells and induce apoptosis (34).

BHV-1 belongs to the subfamily *Alphaherpesvirinae* and shares a number of biological properties with herpes simplex virus type 1 (HSV-1) and HSV-2 (16). BHV-1 establishes lifelong latency in ganglionic neurons of the peripheral nervous system after initial replication in the mucosal epithelium. Virus reactivation and spread to other susceptible animals occur after natural or corticosteroid-induced stress (26, 32). Although the primary site of BHV-1 latency is sensory neurons, there is evidence that long-term persistence and reactivation also occur within germinal centers of the pharyngeal tonsil (36).

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In contrast to the 70 to 80 viral genes expressed during productive infection, LR RNA is the only abundant viral transcript detected in latently infected neurons. A small fraction of LR RNA is polyadenylated and alternatively spliced in trigeminal ganglia, suggesting this RNA is translated into an LR protein (5, 13). LR gene products inhibit S-phase entry, and LR protein is associated with cyclin-dependent kinase 2 (Cdk2)-cyclin complexes (13, 15). LR gene products also promote cell survival following induction of apoptosis in transiently transfected cells (4). Although these studies imply that the LR gene plays a role in latency and/or pathogenesis, the effects of LR gene products on growth of the virus in cultured cells or in cattle has not been studied.

In this study, we constructed an LR mutant virus that contains three stop codons near the beginning of the LR RNA. The LR mutant had growth properties similar to those of the WT in productively infected bovine kidney (MDBK) cells. Since HSV-1 latency-associated transcript (LAT) null mutants have growth properties in tissue culture cells and infected rabbits or mice similar to those of wild-type (WT) virus (reviewed in references 16 and 33), this result was expected. Surprisingly, calves infected with the LR mutant consistently exhibited diminished clinical symptoms and ocular shedding. However, similar levels of the LR mutant, WT BHV-1, and the LR rescued virus were shed from the nasal cavities of calves during acute infection. Taken together, these results suggested that LR gene products promote virus growth in certain cell types in the eye or optic nerve during acute infection of cattle.

MATERIALS AND METHODS

Virus and cells. The designated cells were plated at a density of 5×10^5 per 100-mm^2 plastic dish in Earle's modified medium supplemented with 5 to 10% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 μg/ml). Bovine kidney (MDBK) cells (CCL-22; American Type Culture Collection [ATCC]) were grown in 5% FBS, split 1:6 every 4 to 5 days, and used to propagate BHV-1. Primary bovine epidermal cells were grown in 10% FBS and were used to generate the mutant and rescued viruses because they can be transfected with high efficiency. These cells are immortalized with the simian virus 40 large T antigen and have fibroblastlike characteristics (11a).

The Cooper strain of BHV-1 (WT virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa. Viral stocks were prepared by infecting MBDK cells at a multiplicity of infection (MOI) of 0.001 from a plaque-purified virus and were subsequently titrated on MDBK cells.

Animal experiments. BHV-1-free crossbred calves (≈250 kg) were randomly assigned and housed in isolation rooms to prevent cross contamination. The calves were anesthetized with Rompun (approximately 50 mg/50 kg of body weight; Bayer Corp., Shawnee Mission, Kans.). The calves were then inoculated in each nostril and eye with 1 ml of a solution containing 1×10^7 PFU of the indicated virus/ml, without scarification, for a total of 4×10^7 PFU per animal, as described previously (30, 34–36). Experiments using animals were performed in accordance with the American Association of Laboratory Animal Care guidelines. Calves were housed under strict isolation containment and were given antibiotics before and after BHV-1 infection to prevent secondary bacterial infection. Nasal swabs, ocular swabs, and serum samples were taken at the designated times.

Plasmids. The plasmid used for generating the LR mutant (pBlueL/mLAT) was constructed as follows: 825 bp of the *Hin*dIII L fragment that is directly upstream of the LR promoter (D fragment) was cloned into pBlueBacHisA (Invitrogen, Carlsbad, Calif.). pBR322-*Hin*dIII L fragment contains the *Hin*dIII L fragment of BHV-1 (Cooper strain), and this plasmid was digested with *Nhe*I. The resulting products were treated with mung bean exonuclease (New England BioLabs) to blunt the ends for ligation of *Bam*HI linkers. After phenol-chloroform extraction, the DNA was digested with *Hin*dIII and then *Bam*HI. The products were electrophoresed on an agarose gel, and the 825-bp product was isolated. The 825-bp product, containing a 5′ *Bam*HI site and a 3′ *Hin*dIII site, was ligated into the pBlueBacHis vector digested with *Bam*HI plus *Hin*dIII, and the resulting plasmid was designated pBlueL. A fragment containing the entire LR promoter and coding region (1,940 bp) was cloned into the *Hin*dIII and *Sal*I sites of pBlueL, and the resulting plasmid was designated pBlueL/LAT. The *Pst*I fragment (1 to 981 nucleotides [nt]) was excised from pBlueL/LAT and cloned into the pBlueBacHis vector. This subcloning was performed because there are three *Sph*I sites in the coding region of the LR gene (781, 812, and 1,777 nt). The *Sph*I fragment (781 to 812 nt) was excised, and the mutant oligonucleotide was inserted (Fig. 1C). The mutated *Pst*I fragment was then cloned back into the original *Pst*I-digested pBlueL/LAT, and the resulting construct was designated pBlueL/mLAT. Restriction enzyme mapping and DNA sequencing determined the proper orientation of the *Pst*I fragment. All cloning procedures (restriction digests, ligations, calf intestinal phosphatase treatment, etc.) were performed by standard procedures described previously (4, 13, 29).

The HSV-1 ICP0 (infected cell protein 0)-expressing plasmid was a gift from S. Silverstein (Columbia University, Columbia, N.Y.).

Extraction of viral DNA. Isolation of intact BHV-1 viral DNA has been previously described (1). Briefly, MDBK cells were infected with either BHV-1 Cooper or the LR mutant at an MOI of approximately 10. The clarified lysate was pelleted using a 30% sucrose–Tris-EDTA cushion (25,000 rpm for 2 h in a Beckman Lt-65 using an SW28 rotor at 4°C). Virions were disrupted with sodium dodecyl sulfate (SDS) and RNase treatment, followed by proteinase K treatment and extraction with phenol-chloroform-isoamyl alcohol (50:48:2). The integrity and quantity of viral DNA were determined by 1% agarose gel electrophoresis.

Transfection and identification of the LR mutant. Bovine epidermal cells were cotransfected with 6 μg of pBlueL/mLAT, 2 μg of a plasmid encoding HSV-1 ICP0, and 2 μg of viral DNA (Cooper or LR mutant) by using Superfect (Qiagen) as previously described (14).

Sixteen hours after transfection, the cells were split 1:2, incubated for 16 additional hours, and then overlaid with 0.7% SeaPlaque agarose. When visible plaques appeared (3 to 4 days postinfection [p.i.]), each plaque was isolated, propagated in MDBK cells, and screened by PCR for the mutant oligonucleotide insert. PCR was performed on the extracted DNA using the p4 (nt 873; 5′CGT GTATTTGCGACCCCCAGCCT3′) and p5 (nt 596; 5′GCCAGACCAAACCC CCCGCA3′) primers (Fig. 1). After a hot start, each cycle consisted of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min (30 cycles total). To ensure complete elongation of the amplified products, the reaction mixture was incubated at 72°C for an additional 10 min. The products were digested with *Eco*RI and electrophoresed on a 2% agarose gel, and the DNA was visualized by staining it with ethidium bromide.

Growth characteristics of the LR mutant, detection of virus shedding, and virus-specific neutralizing antibodies. MDBK cells were infected with various MOIs of BHV-1 for 1 h at 37°C. The monolayers were then rinsed two times with phosphate-buffered saline containing 0.5× trypsin to inactivate any surface-bound virus. Complete medium was then added to the cultures to inactivate the trypsin. At various times, total cell lysate or the supernatant from infected cultures was subjected to three freeze-thaw cycles, clarified, and titrated on MDBK cells.

Nasal and ocular swabs were stored at −80°C in 2 ml of tissue culture medium supplemented with 10 μg of amphotericin B (Fungizone)/ml and 45 μg of gentamicin/ml. Samples were thawed quickly in a 37°C water bath, vortexed, and centrifuged ($1{,}500 \times g$ for 10 min). All titrations were performed using 10-fold serial dilution and were plated in quadruplicate.

The Veterinary Diagnostic Service, University of Nebraska, Lincoln, performed neutralizing-antibody titrations utilizing the Cooper strain as the stock virus.

Western blot analysis of bICP0. After infection of the cells with the different viruses, whole-cell lysate was collected at various times (13). Proteins (50 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and then transferred to Immobilon-P membranes (Millipore, Bedford, Mass.). The membranes were rinsed for 5 min in TBS (0.02 M Tris base, 0.13 M NaCl, pH 7.6) and then blocked in a buffer (TBS, 0.1% Tween 20, 5% nonfat dry milk) for 1 h at room temperature. The membrane was then incubated with rabbit anti-bICP0 (M. Schwyzer, Zurich, Switzerland) that was diluted 1:1,000 in primary antibody buffer (TBS, 0.1% Tween 20, 5% bovine serum albumin) for 16 h at 4°C. The membrane was washed three times for 5 min each time with TBS–0.1% Tween 20. Detection of bound primary antibody was performed using the ECL detection system (Amersham Pharmacia, Piscataway, N.J.) (using goat anti-rabbit antibody) as previously described (13). The only change made to this protocol was to use the blocking buffer mentioned above as the secondary antibody dilution buffer. For loading controls, the membrane was stripped as previously described (ECL Western blotting Protocols Manual; Amersham Pharmacia) and reprobed with goat anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) as the primary antibody. A horse anti-goat peroxidase-conjugated antibody (Santa Cruz Biotechnology) was used for detection as described above.

RESULTS

Construction of a BHV-1 LR mutant virus. Our previous studies have focused on performing functional studies of the LR gene and putative proteins encoded by this gene. These studies have demonstrated that LR gene products interfere with cell cycle progression (29) and chemical induction of apoptosis (4). To test whether BHV-1 LR gene products play a role in virus growth and/or latency, we constructed a BHV-1 LR mutant virus that contains stop codons near the 5′ terminus of the LR transcript and tested this mutant in cultured cells or calves.

The LR gene is transcribed antisense with respect to the immediate-early (IE) and early (E) gene transcript (IE/2.9 and E/2.6) that encodes bICP0 (Fig. 1A and B). The lytic start site for the LR RNA is at nt 724 (1, 13), and the first in-frame ATG for LR open reading frame 2 (ORF2) is at nt 783 to 785 (Fig. 1C), whereas the stop site for bICP0 is at nt 956 (LR numbers) (7, 37–39), which complicates construction of an LR mutant virus. The *cis*-acting sequences that regulate poly(A) addition for the transcript that encodes bICP0 are also near sequences that contain the LR gene TATA box. This prevented insertion of a reporter gene near the start site of LR gene expression or an extensive deletion of LR gene sequences. Consequently, we inserted three stop codons that should prevent LR protein

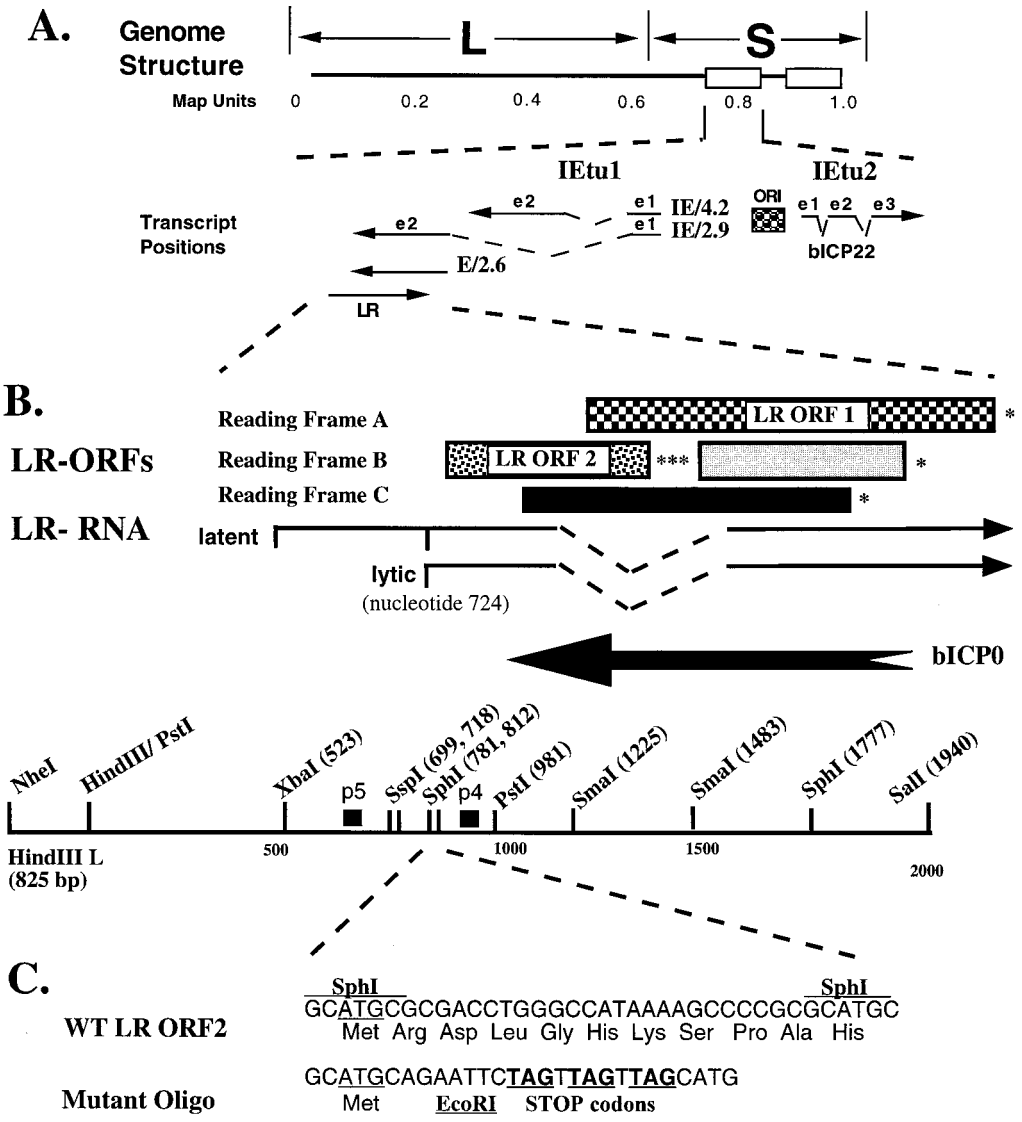

FIG. 1. Schematic of the LR gene and the targeted site for mutagenesis. (A) Positions of IE transcripts (7, 37–39) and the LR transcript (27, 28) are presented. IE/4.2 is the IE transcript that encodes bICP4. IE/2.9 is the IE transcript that encodes bICP0. One IE promoter activates expression of IE/4.2 and IE/2.9, and this IE transcription unit is designated IEtu1. E/2.6 is the early transcript that encodes bICP0. Exon 2 (e2) of bICP0 contains all of the protein coding sequences of bICP0. The origin of replication (ORI) separates IEtu1 from IEtu2. IEtu2 encodes a protein, bICP22. The solid lines in the transcript position map represent exons (e1, e2, and e3). The arrows indicate the direction of the respective transcripts. (B) Partial restriction map, location of LR RNA, organization of LR ORF, and 3′ terminus of bICP0. The start sites for LR transcription during latency and productive infection were previously described (5, 13). Reading frame C contains an ORF but lacks an initiating Met. The asterisks denote the positions of stop codons that are in frame with the respective ORFs. A region of the *Hin*dIII L fragment was cloned upstream of the LR gene, as described in Materials and Methods, to facilitate homologous recombination. The positions of the primers that were used to amplify the mutated region of the LR gene were designated p4 and p5. The approximate location of the 3′ end of bICP22 is shown by the arrow. (C) DNA sequence of the *Sph*I fragment and the mutant oligonucleotide (oligo). The first ATG in the WT sequence is the first in-frame ATG for ORF2 and is underlined. Stop codons in the mutant oligonucleotide are in all three reading frames (boldface and underlined). The *Eco*RI restriction enzyme site (GAATTC) was incorporated into the mutant oligonucleotide to facilitate screening.

expression in all three reading frames (Fig. 1C). This mutation was also designed to allow for WT levels of bICP0 expression. The entire promoter and coding region of the LR gene was cloned into the pBlueBacHisA vector as described in Materials and Methods. A total of 825 bases from the adjacent *Hin*dIII L fragment (18) were cloned upstream of the LR promoter to ensure that efficient homologous recombination occurred. The LR sequences between the two *Sph*I sites (nt 781 to 812) were replaced with the mutant oligonucleotide (Fig. 1C). The mutant oligonucleotide contains the first in-frame ATG of ORF2, a unique *Eco*RI restriction site to facilitate screening, and three stop codons that are in each reading frame. In transiently transfected cells, this LR mutant gene construct expresses the LR RNA, but the protein detected by a peptide antibody directed against the N terminus of LR ORF2 (P2) is not detected (4, 13). Since alternative splicing of LR RNA occurs in

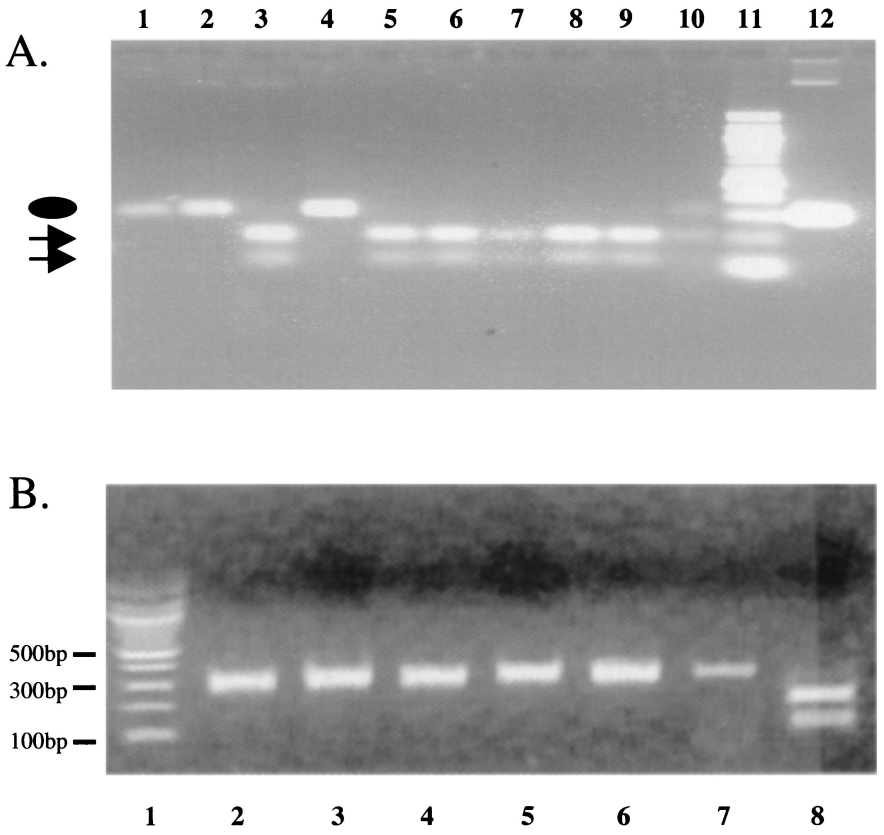

FIG. 2. PCR of plaque-purified recombinant viruses. (A) Bovine epidermal cells were cotransfected with a plasmid encoding HSV-1 ICP0 (2 μg), BHV-1 DNA (2 μg), and pBlueL/mLAT (6 μg). Plaques were isolated, and PCR was performed on extracted viral DNA using the p4 and p5 primers (see Fig. 1C and Materials and Methods for the locations and sequences of these primers). Amplified products were digested with *Eco*RI and visualized by ethidium bromide staining on 2% agarose gel electrophoresis. WT virus yields a single band migrating at 298 bp (oval), while mutant oligonucleotide insertion yields two bands migrating at 105 and 193 bp (arrows). Lanes 1 and 2, WT virus plaques. Lane 3 pBlueL/mLAT plasmid DNA. Lane 4, pBlueL/LAT plasmid DNA. Lanes 5 to 9, viral DNA extracted from single plaques after the third round of plaque purification of the LR mutant virus. Lane 10, example of a mixed-population virus stock. Lane 11, 100-bp ladder (New England BioLabs). Lane 12, PCR positive control (WT Cooper strain viral DNA). (B) Bovine epidermal cells were cotransfected with a plasmid encoding HSV-1 ICP0 (2 μg), LR mutant viral DNA (2 μg), and pBlueL/LAT (6 μg). Viral DNA was prepared from individual plaques, and PCR was performed using the p4 and p5 primers. Lane 1, 100-bp ladder. Lanes 2 to 6, individual plaques from the third round of plaque purification of the LR rescued virus. Lane 7, WT virus DNA. Lane 8, LR mutant DNA, which served as a PCR control.

trigeminal ganglia of calves during acute infection (5), it is possible that a protein encoded by the LR gene could be expressed, even if this mutation is present.

BHV-1 DNA was extracted from infected cells, and its integrity was examined by agarose gel electrophoresis. BHV-1 DNA is not very infectious when transfected into cultured bovine epithelial cells. Efficient plaque formation was not observed at 14 days posttransfection, a time when cells were lifting off the plates. When BHV-1 DNA and plasmids encoding bICP0 (14) or HSV-1 ICP0 (data not shown) were cotransfected into bovine cells, efficient plaque formation was consistently observed 48 h after transfection. A plasmid expressing HSV-1 ICP0 was used for these studies because bICP0 sequences overlapped the LR mutant region, and thus we were concerned this might reduce the efficiency of homologous recombination.

The viral genome was cotransfected into bovine epithelial cells with HSV-1 ICP0 and the plasmid containing the mutant oligonucleotide (pBlueL/mLAT). Plaques were isolated and screened for insertion of the mutant oligonucleotide sequence by PCR using the p4 and p5 primers (Fig. 1C). The amplified products were then digested with *Eco*RI. If homologous recombination between the LR mutant plasmid and the viral genome occurred, two bands (105 and 193 bp) would be observed following digestion with *Eco*RI (Fig. 2A lane 3). WT virus yielded a single band (298 bp) as expected (Fig. 2A, lanes 1, 2, and 4). After the potential LR mutants were subjected to three rounds of plaque purification, the same banding pattern was observed, demonstrating that the mutant virus from a plaque was not contaminated with WT virus and was stable. (Fig. 2A, lanes 5 to 9). Occasionally, the WT band was detected in some samples (Fig. 2A, lane 10, for example), indicating that the *Eco*RI digestion was not complete or there was slight contamination with WT virus. Several plaques containing the mutant were selected and plaque purified two more times to ensure they were not contaminated with WT virus.

To ensure that a resulting phenotype was not due to secondary site mutations, a rescued virus was constructed (LR rescued virus). The LR mutant viral genome was cotransfected with the WT LR gene cloned into pBlueBacHisA (pBlueL/

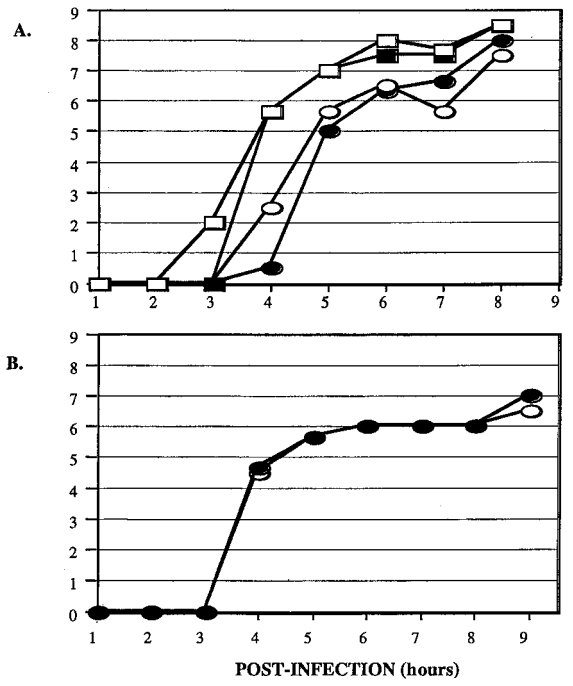

FIG. 3. Growth properties of WT and LR mutant viruses in MDBK cells. Growth curves were performed as described in Materials and Methods. (A) Cells were infected at an MOI of 1. LR mutant virus is denoted by open symbols, and WT virus is denoted by solid symbols. Released virus is denoted by ovals, and cell-associated virus is denoted by rectangles. There were no significant differences between the growth curves or final titers of WT and the LR mutant virus. (B) Cells were infected at an MOI of 5. Solid ovals denote WT virus titers, and open ovals denote LR mutant virus titers. The results are representative of four different experiments.

LAT) and a plasmid encoding HSV-1 ICP0 into bovine cells. The p4 and p5 primers were used to identify amplified products that were not digested by *Eco*RI, which was indicative of the LR WT gene. Figure 2B (lanes 2 to 6) shows five individual plaques that were rescued back to the WT sequence. Viral sequences encompassing the manipulated regions of the LR gene in the LR mutant and LR rescued virus were sequenced and contained the expected sequences (data not shown).

Analysis of the BHV-1 LR mutant virus in MDBK cells. Infection of MDBK cells with BHV-1 Cooper strain produces visible cytopathic effects by 7 to 10 h p.i. followed by efficient plaque formation. IE gene expression can be detected within 1 to 2 h p.i. (6, 14, 30, 38, 39). Although not statistically significant, growth curves suggested that the mutant released virus slightly faster from MDBK cells early in infection at an MOI of 1 (Fig. 3A). However, the end point titers were consistently the same. At an MOI of 5, there were no differences in the titers of cell-associated (data not shown) and released (Fig. 3B) virus. The differences in the virus titers between Fig. 3A and B were a result of the cells used to titer the virus in Fig. 3B being more confluent. We have consistently observed that resting cells or cells that are too confluent do not yield as much virus as actively growing cells. However, this difference did not alter our conclusion that the LR mutant and WT virus had similar growth properties in MDBK cells. LR mutant, LR rescued, and WT viruses also had similar growth properties in rabbit epidermal (CCL-68; ATCC), rabbit lung (CCL-193; ATCC), rabbit skin fibroblasts (CRL-1414; ATCC), and bovine epidermal cells (data not shown).

The LR RNA is antisense to the IE and E transcript that is translated into bICP0 (20), suggesting bICP0 expression could be altered by the mutation within the LR gene. Since bICP0 is essential for productive viral replication (7, 14, 19), we compared expression of bICP0 in the WT, LR rescued, and LR mutant viruses following infection of MDBK cells. These studies demonstrated that expression of bICP0 in MDBK cells infected with the LR mutant was at least as high as in those infected with the WT or the LR rescued virus (Fig. 4). In several experiments, it appeared that the LR mutant expressed slightly higher levels of bICP0.

Analysis of the LR mutant in calves. Calves were infected with a total of 4×10^7 PFU of the WT, LR rescued, or LR mutant virus/ml via the intranasal and intraocular routes as described previously (30, 34, 36). Acute BHV-1 infection in cattle lasts approximately 10 days. Significant amounts of ocular and nasal discharge were readily observed in all calves infected with the WT or LR rescued virus (Table 1). Inflammation, herpetic lesions in the nostrils, and severe conjunctivitis were routinely detected on days 4 to 8 p.i. As a result of these clinical symptoms, the calves go off feed for several days and are listless (depressed) (Table 1). In contrast, the calves infected with the LR mutant virus showed little discharge from the nose or eyes and consequently did not exhibit severe clinical symptoms (Table 1).

Infectious virus was collected from ocular and nasal swabs in 2 ml of medium (Becton Dickinson, Franklin Lakes, N.J.). Samples were subjected to two freeze-thaw cycles and clarified by centrifugation, and then titers were determined on MDBK cells. WT and LR rescued virus groups are considered one group because there were no differences in the titers. At 1 day p.i., similar titers of virus were detected in ocular swabs of calves infected with the LR mutant or WT-LR rescued virus group (Fig. 5). At all other time points, calves infected with the LR mutant had 2 to 4 log units less virus in ocular swabs than calves infected with the WT-LR rescued virus. At 6 days p.i., only 2 out of 10 calves infected with the LR mutant were shedding measurable virus in ocular swabs. In contrast, the WT-LR rescued virus group shed an average of 10^5 50% tissue culture infective doses of virus at 6 days p.i.

From nasal swabs, the highest titer was obtained at 2 days p.i. for all groups (9.0 tissue culture infective doses/ml) (Fig. 6). Although we consistently detected 0.5- to 1-log-unit-lower titers in nasal swabs obtained from the LR mutant group, the differences in titers between the WT-LR rescued virus group and the LR mutant group were not significant. Considering there was reduced nasal discharge from the LR mutant group, it was somewhat surprising to find that viral titers in nasal swabs collected from these calves were similar to those in swabs from the WT-rescued virus group. Necropsy of the calves infected with WT showed herpetic lesions and mucous secretions in the turbinate at 6 and 10 days p.i. However, the LR mutant group exhibited reduced lesions and secretions (Table 1). In summary, these results demonstrated that the LR mutant virus was not shed efficiently from the eyes of infected calves but was shed efficiently from the nose.

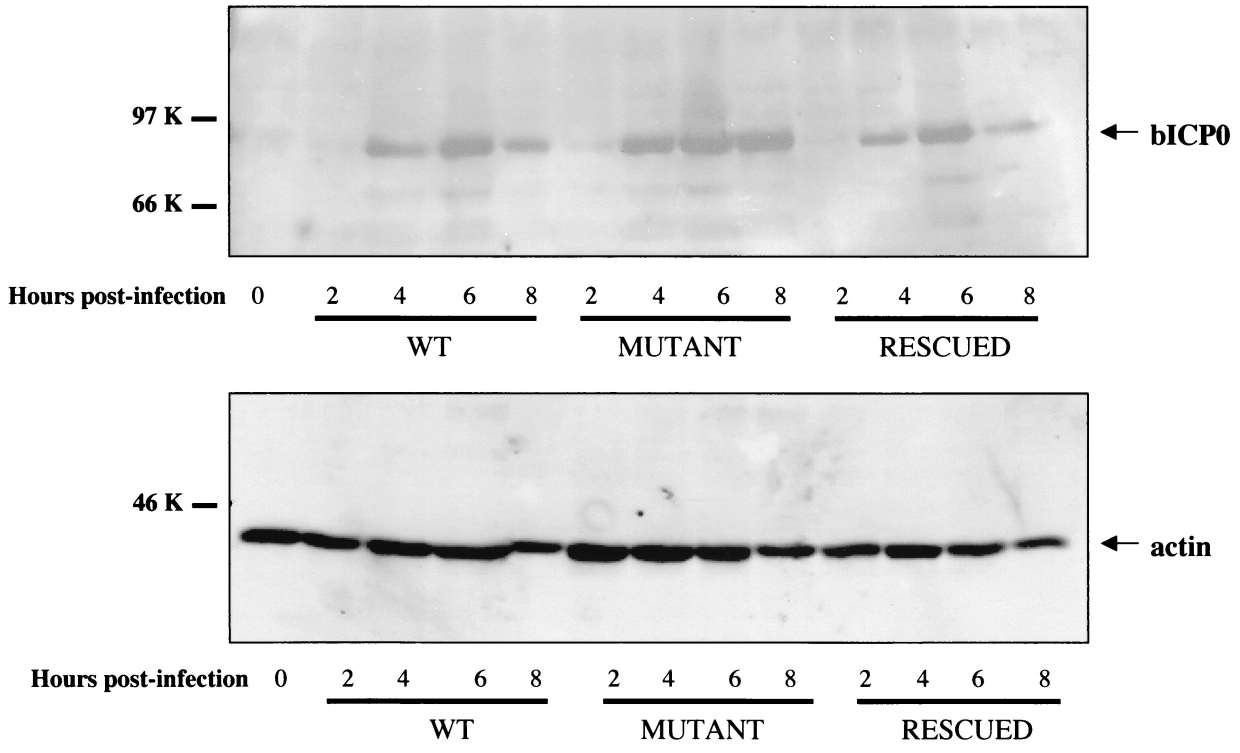

FIG. 4. Western blot of bICP0 expression of WT, LR rescued, and LR mutant viruses during infection of MDBK cells. MDBK cells were infected with the indicated viruses (MOI, 5), and whole-cell lysates were assayed for bICP0 expression at the indicated times. The membrane was probed with a polyclonal rabbit serum directed against bICP0 (1:1,000 dilution), and bICP0 was detected with the ECL detection kit. The predicted molecular mass of bICP0 is 97 kDa (7). We find that on an SDS–10% polyacrylamide gel electrophoresis gel, bICP0 routinely runs just below the 97-kDa protein marker. The membrane was stripped and reprobed for β-actin expression as a loading control.

To confirm that the LR mutant was secreted from infected calves, DNA was extracted from ocular swabs prepared from LR mutant- or WT virus-infected calves. Using primers p4 and p5, PCR was performed, and the resulting products were digested with *Eco*RI. Prior to infection (0 days p.i.), viral DNA was not detected for either group (Fig. 7). Virus was detected in ocular swabs from 1 through 4 days p.i. from animals infected with the WT or LR mutant virus. After 4 days p.i., viral DNA was not consistently detected in calves infected with the LR mutant. In contrast, viral DNA was consistently detected at days 6, 8, and 14 p.i. in calves infected with the WT virus. Although data for only one animal are shown in Fig. 7, these results are representative of several animals from each of the groups.

Neutralizing-antibody titers are used to determine if animals were vaccinated or previously infected with BHV-1 (17). This suggests that an increase in the amount of virus replication and shedding correlated with an increase of neutralizing-antibody titers. At 10 and 14 days p.i., calves infected with the WT-LR rescued virus produced higher titers of neutralizing antibodies than calves infected with the LR mutant (Fig. 8). At 14 days p.i., the WT-LR rescued group had an average titer of 78, but

TABLE 1. Clinical data obtained from calves infected with WT, rescued, or LR mutant viruses

Group	Clinical symptom					
	Nasal shedding[a]	Ocular shedding[a]	Respiratory distress[b]	Off feed[c]	Conjunctivitis[d]	Secretion or lesions in turbinate[e]
WT	5–6 d (9.0 logunits)	5–6 d (7.2 logunits)	3–4 d	3 d	++++	++++
LR rescued	5–6 d (9.0 logunits)	5–6 d (7.0 logunits)	3–4 d	3 d	++++	ND
LR mutant	2–3 d (8.0 logunits)	0–1 d (5.5 logunits)	0–1 d	1.5 d	−−−	+

[a] The values indicate the length of time during the acute infection that nasal and ocular secretions were observed on average for each group. d, days. The numbers in parentheses indicate the highest titer obtained in each group during the time of shedding.

[b] Respiratory distress was deemed to be present when animals presented signs such as shallow quick breaths, hanging head, and locked front legs. The values represent the number of days (d) on average that each group showed the above-mentioned symptoms.

[c] Calves were considered off feed if food remained 12 to 16 h after feeding. The value represents the number of days on average that each group showed this symptom.

[d] A typical clinical sign of BHV-1 infection is conjunctivitis. Severe conjunctivitis (++++) was observed during the acute infection. No conjunctivitis (−−−) was seen in calves infected with the LR mutant virus.

[e] Calves were necropsied on days 0, 2, 4, 6, 10, and 14 p.i. A major amount of mucus (++++) was observed in the turbinates of calves infected with the WT virus, whereas low levels of mucus (+) were observed in the LR mutant-infected group. Typical herpetic lesions were observed in the turbinate in the WT group (days 10 to 14 p.i.), whereas few lesions were observed in the LR-mutant group. ND, not done.

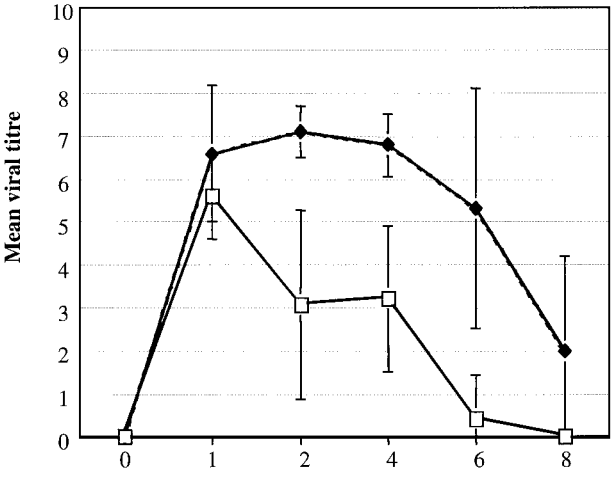

FIG. 5. Titer of ocular virus shedding. Ocular swabs were obtained at the indicated times p.i. and stored at −80°C. Titers of the clarified lysate were determined on MDBK cells in quadruplicate. The cells were stained with formalin and crystal violet to determine the 50% endpoint. The solid symbols represent calves infected with WT or LR rescued virus, and the open symbols represent calves infected with LR mutant virus. Shown are the average means from each group at each time indicated. $n = 10$ for the LR mutant, and $n = 12$ for the WT-LR rescued group. Calves infected with WT and LR rescued viruses showed no observable differences, either in virus shedding or clinical signs. From this point on, calves in the WT and LR rescued groups were considered one group. Note that at 6 days p.i., only two calves infected with the LR mutant shed virus, and at very low titers. Days 2, 4, 6, and 8 p.i. are statistically significant: $P < 0.005$. Statistical analysis was performed using Microsoft Excel's descriptive statistics. P values represent the probability that the result occurred by chance, using 95% confidence (a P value of <0.05 is statistically significant). The error bars represent the standard errors of the mean.

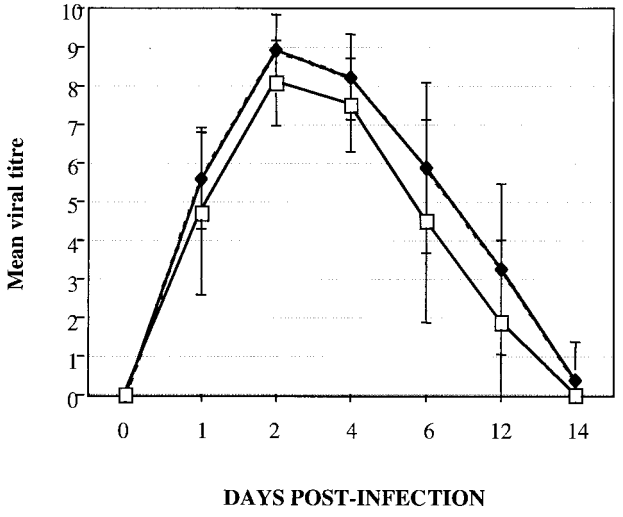

FIG. 6. Titer of nasal virus shedding. Nasal swabs were obtained at the indicated times p.i. and were stored at −80°C. Titers of the clarified lysate were determined on MDBK cells in quadruplicate. The cells were stained with formalin and crystal violet to determine the 50% endpoint. The solid symbols represent calves infected with the Cooper virus strain, and the open symbols represent calves infected with the LR mutant virus. Shown are the average means from each group at each time indicated ($n = 10$ for the LR mutant, and $n = 12$ for the Cooper-rescued group). All time points are not statistically significant: $P > 0.05$. See the legend to Fig. 5 for an explanation of the statistical methods used for this study.

that of the LR mutant group was 20. In summary, this study demonstrated that there was a correlation between reduced ocular shedding of the LR mutant virus and neutralizing-antibody titers.

DISCUSSION

This report describes the construction, growth properties, and gross pathogenesis of a BHV-1 LR mutant that contains stop codons near the start site of LR transcription. This mutation should interfere with expression of proteins encoded by the LR gene (Fig. 1) (13, 20). An earlier study demonstrated that a peptide antibody directed against the amino terminus of LR ORF2 recognized a 35- to 40-kDa protein in transiently transfected cells, but insertion of this mutant oligonucleotide interfered with its expression (4). The LR RNA has the potential to produce a family of proteins that may include functionally distinct proteins because alternative splicing occurs after infection of calves or cultured cells (5, 13). Because of the complicated nature of LR ORF organization and splicing of the LR transcript, it is conceivable that the mutation we generated may not block expression of all proteins encoded by the LR gene. We are currently developing additional antibodies that will recognize these putative proteins to identify which ones are expressed following infection of calves and how this mutation disrupts protein expression.

Shedding of the LR mutant from the eye was reduced 3 to 4 log units compared to that of WT or rescued virus (Fig. 5), suggesting virus replication in the eye or optic nerve was inhibited. Curiously, the LR mutant virus produced slightly larger plaques that lacked a distinct border compared to the Cooper (WT) virus or the LR rescued virus in MDBK cells (data not shown). However, the LR mutation had little effect on virus growth (Fig. 3) following infection of MDBK cells. Furthermore, similar levels of virus shedding were detected in nasal swabs of calves infected with the different viruses (Fig. 6). We have previously demonstrated that LR gene products interfere with cell cycle progression (29) and apoptosis in transiently transfected cells (4). It will be interesting to determine if these activities are necessary for reduced virus shedding in the eyes of infected calves. It is also possible that the LR mutant does not play a direct role in virus replication. In productively infected MDBK cells, the LR mutant virus appeared to be released slightly faster than WT or rescued virus when the cells were infected at an MOI of 1 (Fig. 3A). If premature shedding occurred in ocular tissue, the released virus would likely be an easier target for immune recognition and thus viral titers would be lower.

This study suggested that a mutation designed to interfere with expression of LR proteins mediated the phenotype of the LR mutant. The LR mutant appeared to produce slightly higher levels of bICP0 expression in productively infected cells (Fig. 4). If higher levels of bICP0 were expressed in certain cell types during acute infection of calves, this could also have an effect on virus growth because bICP0 is toxic to cells and

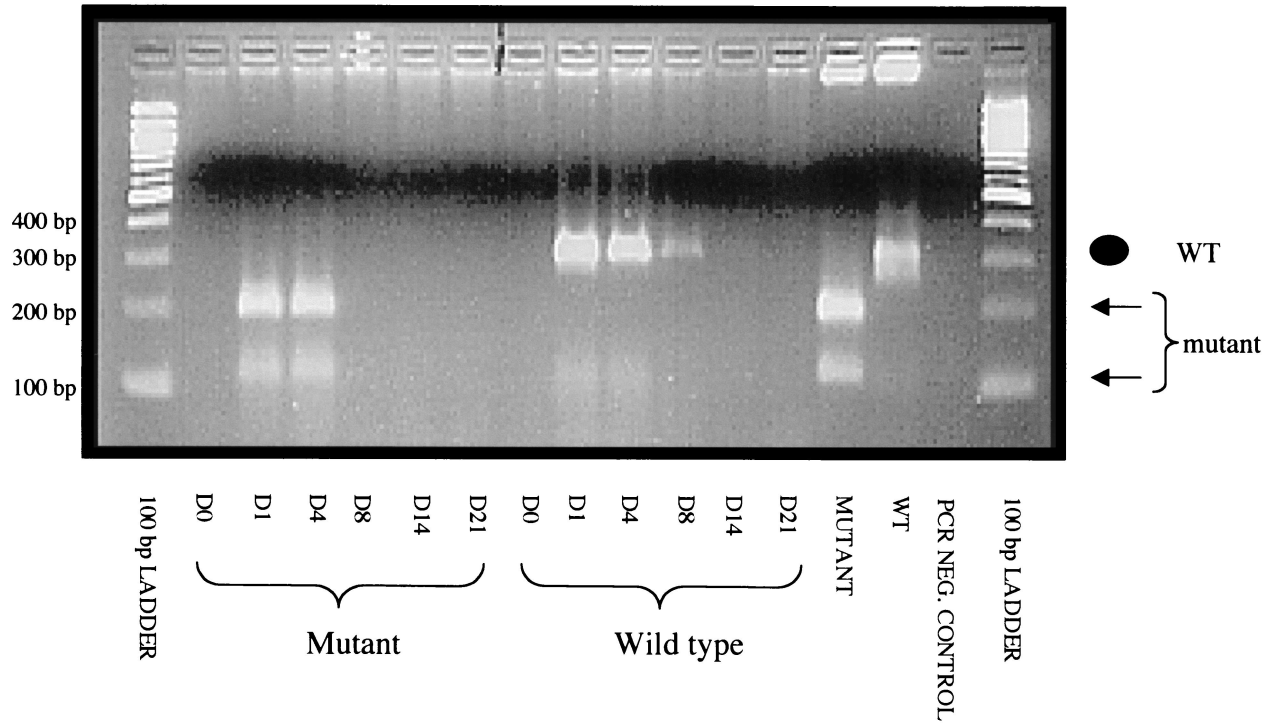

FIG. 7. PCR of total DNA prepared from MDBK cells infected with ocular swabs. Clarified lysate from ocular swabs was obtained at the indicated times p.i. and then used to infect MDBK cells. DNA was extracted, and PCR was performed using the primers p4 and p5 (described in Materials and Methods). The amplified products were digested with *Eco*RI and visualized by ethidium bromide staining on a 2% agarose gel. The positions of the WT sequence (not digested by *Eco*RI) and the LR mutant sequence (digested by *Eco*RI) are shown. Calves infected with the LR mutant virus only shed virus on days 1 and 4 p.i. Calves infected with the WT virus shed only the WT virus, which was detected at 1, 4, 8, and 14 days p.i. The mutant and WT lanes contained plaque-purified viruses prior to infection of the calves. the molecular size ladder was the 100-bp ladder from New England BioLabs.

activates productive infection (14). Finally, it is possible that this small mutation has subtle effects on LR RNA expression in certain cell types. Although the data presented in this study strongly suggested that LR protein expression plays an important role in virus shedding in the eye, we cannot rule out the possibility that increased bICP0 expression in certain cell types contributed to the observed phenotype of the LR mutant.

The BHV-1 LR gene is considered by some to be a functional homologue of the HSV-1 gene encoding LAT. This analogy can be made because LR RNA and LAT are abundantly expressed during latency, their RNA is localized in the nuclei of latently infected neurons, and the respective RNAs overlap and are antisense to a potent transcriptional activator (bICP0 or ICP0) (16, 33). A number of studies have described the growth properties of HSV-1 LAT mutants during infection of rabbits or mice (16, 33). None of the published HSV-1 LAT mutants exhibit reduced growth in the eyes of acutely infected animals, suggesting that the LR gene has novel functions or this phenotype is only observed in the natural host. In addition to reduced ocular shedding, we predict that the LR mutant will have reduced establishment and reactivation from latency because LAT sequences regulate establishment of latency (24) and spontaneous reactivation (23) in rabbits. Studies designed to address this hypothesis are in progress.

BHV-1 lacks several genes contained in the HSV-1 genome which mediate pathogenesis and/or latency, for example, 34.5

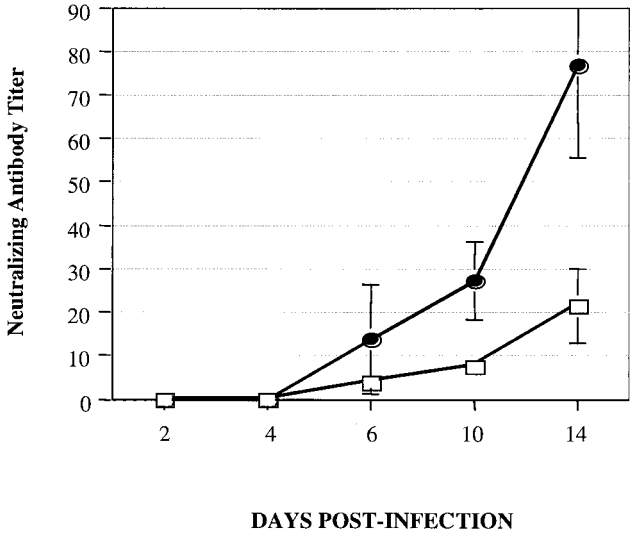

FIG. 8. BHV-1 neutralizing-antibody titers. Serum was collected from calves at the indicated times and stored at −20°C until it was tested. Standard testing was performed using constant amounts of virus (Cooper strain) and twofold dilutions of the serum. The Veterinary Diagnostic Services, University of Nebraska, Lincoln, performed the assay. Solid symbols represent calves infected with WT or LR rescued virus. Open symbols represent calves infected with LR mutant virus. Each time point represents at least 10 calves for each virus. Days 10 and 14 p.i. are statistically different ($P < 0.005$). See the legend to Fig. 5 for an explanation of the statistical methods used for this study.

(31). The 34.5 gene plays a crucial role in neurovirulence by inhibiting antiviral functions of the interferon-inducible double-stranded-RNA-dependent protein kinase R (PKR) (3, 21). 34.5 null mutants have reduced pathogenesis in rabbits and mice, in large part because of poor growth properties in the eyes and trigeminal ganglia (25). Although there does not appear to be a high amino acid similarity between 34.5 and the LR ORFs, it is tempting to speculate that the LR gene contains certain LAT-like and 34.5-like functions. A better understanding of LR gene function will help to clarify its role in latency or pathogenesis in cattle and may help us understand the differences in the genomes of alphaherpesviruses.

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