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# Identification of a novel protein encoded by the latency-related gene of bovine herpesvirus 1

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**The latency-related (LR) RNA encoded by bovine herpesvirus 1 (BHV-1) is abundantly expressed and alternatively spliced in trigeminal ganglia. A mutant BHV-1 strain that contains three stop codons at the beginning of LR open reading frame (ORF)-2 (LR mutant virus) does not express ORF-2 or an adjacent reading frame that lacks an initiating ATG (RF-C). Calves latently infected with wild-type (wt) BHV-1, but not with the LR mutant virus, reactivate from latency, indicating that proteins encoded by the LR gene regulate the latency-reactivation cycle. The LR gene also contains another large ORF (ORF-1) that is approximately 200 bp downstream of stop codons inserted at the N-terminus of ORF-2. To test whether the LR mutant virus can expresses ORF-1, the authors developed antiserum directed against ORF-1. The ORF-1 antiserum recognizes specific proteins in bovine cells productively infected with wt BHV-1. ORF-1 protein expression is reduced, but not blocked, when bovine cells are infected with the LR mutant virus. Confocal microscopy demonstrated ORF-1 is present in the cytoplasm and nucleus of productively infected cells, whereas RF-C or a fusion protein containing RF-C localizes to the cytoplasm. Trigeminal ganglia from calves latently infected with wt BHV-1 contain neurons specifically stained with the ORF-1 antiserum. These studies suggest ORF-1 expression may be important for the BHV-1 latency-reactivation cycle.** *Journal of NeuroVirology* (2007) **13,** 569–578.

**Keywords:** bovine herpesvirus type 1; latency-related gene

## Introduction

Bovine herpesvirus 1 (BHV-1) belongs to the $\alpha$-herpesvirinae subfamily and shares a number of biological properties with herpes simplex virus (HSV) types 1 and 2 (HSV-1 and HSV-2) (Jones, 1998). Like other $\alpha$-herpesvirinae subfamily members, BHV-1 establishes lifelong latency in ganglionic neurons of the peripheral nervous system after initial replication in mucosal epithelium. Virus reactivation and spread to susceptible animals occur after natural or corticosteriod-induced stress (Rock *et al*, 1992; Sheffy and Davies, 1972). Although the primary site of BHV-1 latency is sensory neurons within trigeminal ganglia (TG), long-term persistence and reactivation occur within germinal centers of pharyngeal tonsil (Winkler *et al*, 2000b).

BHV-1 infection can cause conjunctivitis, pneumonia, genital disorders, abortions, and an upper respiratory infection referred to as "shipping fever," which costs the U.S. cattle industry at least $3 billion/year (Bowland and Shewen, 2000; Ishmael, 2001; Kapil and Basaraba 1997; Powell, 2005; Tikoo *et al*, 1995). BHV-1 infection leads to transient immunosuppression, thus promoting secondary bacterial infections and pneumonia (Carter *et al*, 1989; Griebel *et al*, 1987a, 1987b, 1990). The UL49.5 gene encoded by BHV-1 blocks CD8+ T-cell recognition of infected

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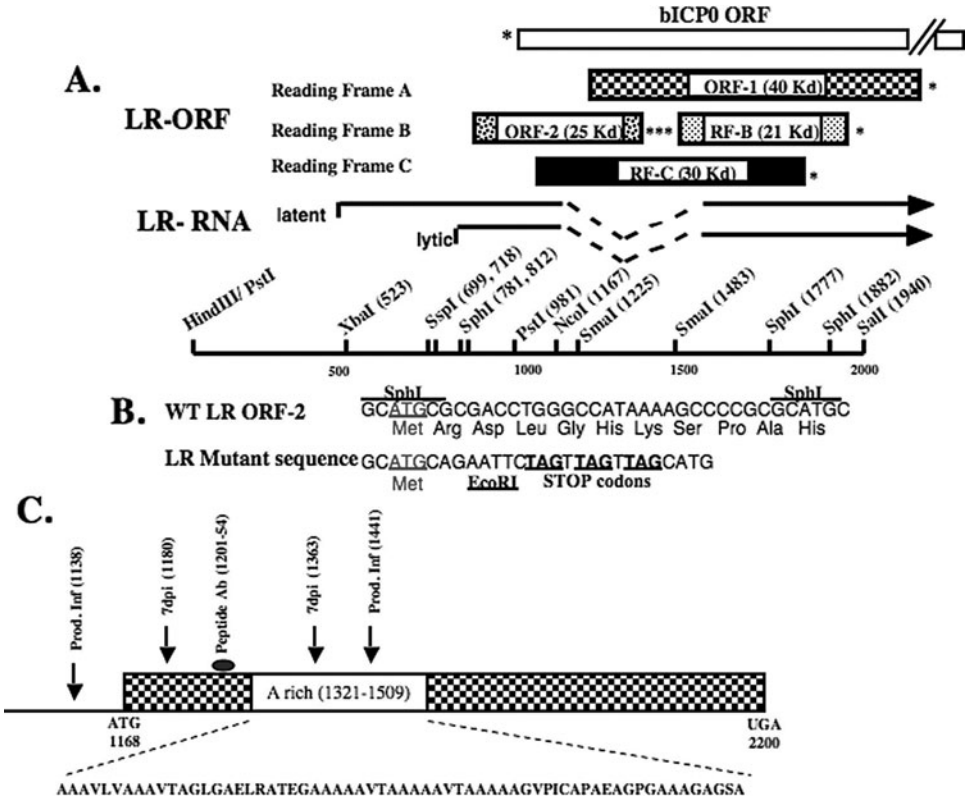


**Figure 1** Schematic of the LR gene and the targeted site for mutagenesis. (**A**) Partial restriction map, location of LR-RNA, organization of LR ORF, and 3′ terminus of the bICP0 ORF. The start sites for LR transcription during latency and productive infection were previously described (Devireddy and Jones, 1998; Hossain *et al*, 1995). Reading frames B and C (RF-B and RF-C) each contain an open reading frame that lacks an initiating Met. The asterisk (*) denotes the position of stop codons that are in frame with the respective ORF. (**B**) DNA sequence of the SphI-SphI fragment (781 to 812) and the mutant oligonucleotide. The first ATG in the wt sequence is the first in frame ATG for ORF2 and this Met is underlined. Stop codons in the mutant oligonucleotide are in all three reading frames (bold and underlined). The EcoRI restriction enzyme site (GAATTC) was incorporated into the mutant oligonucleotide to facilitate screening. (**C**) Schematic diagram of ORF-1. The numbers below ORF-1 indicate the nucleotide numbers in the LR gene. The alanine-rich domain in ORF-1 (A-rich) spans nucleotides 1321 to 1509. The position of the ORF-1 peptide antibody that was used in a previous study is denoted by an oval. The 5′ and 3′ splice sites that were detected during productive infection (Prod. Inf) or at 7 days after infection in TG (7 dpi) are denoted by the arrows. Both splice variants yield a transcript in which a portion of ORF-2 is fused to ORF-1.

cells by repressing expression of major histocompatibility complex class I and the transporter associated with antigen presentation (Koppers-Lalic *et al*, 2005). CD4+ T-cell function is impaired during acute infection because BHV-1 infects CD4+ T cells and induces apoptosis (Winkler *et al*, 1999).

The latency-related (LR) RNA is abundantly expressed in latently infected neurons (Kutish *et al*, 1990; Rock *et al*, 1987, 1992). A fraction of LR-RNA is polyadenylated and alternatively spliced in TG, suggesting this RNA encodes a family of proteins (Devireddy and Jones, 1998; Hossain *et al*, 1995). The LR gene contains two open reading frames (ORF-1 and ORF-2) and two reading frames that lack initiating ATGs (reading frame [RF]-B and RF-C) (see Figure 1A) (Kutish *et al*, 1990). LR gene products inhibit S phase entry, and an LR protein associates with cyclin-dependent kinase 2 (cdk2) or cdc2/cyclin complexes (Schang *et al*, 1996; Jiang *et al*, 1998). The LR gene inhibits apoptosis (Ciacci-Zanella *et al*, 1999), in part because a novel protein encoded by an alternatively spliced LR-RNA interacts with two proteins (Bid and Cdc42) that are proapoptotic (Meyer *et al*, 2007).

A mutant BHV-1 strain that contains three stop codons at the N-terminus of ORF-2 has been partially characterized. Two specific proteins are recognized by polyclonal antiserum directed against (1) a fusion protein containing a portion of ORF-2 and ORF-1 (ORF-2 fusion antiserum), or (2) a fusion protein that contains a portion of RF-C and RF-B (RF-C fusion antiserum) (Jiang *et al*, 2004). In contrast, these proteins are not detected when bovine cells are infected with the LR mutant virus. The LR mutant virus does not reactivate from latency following treatment with dexamethasone (Inman *et al*, 2002), whereas all calves latently infected with wild-type (wt) BHV-1 or the LR rescued virus shed infectious virus following dexamethasone treatment. During the transition from acute infection to latency (establishment of latency), higher levels of apoptosis occur in TG neurons of calves infected with the LR mutant virus

when compared to calves infected with wt BHV-1 (Lovato *et al*, 2003). Calves infected with the LR mutant virus exhibit diminished clinical symptoms and have reduced virus shedding in certain tissues (eyes, TG, and tonsils) relative to calves infected with wt BHV-1 or the LR rescued virus (Inman *et al*, 2001, 2002; Perez *et al*, 2005). Collectively, these studies suggest that expression of fusion proteins containing ORF-2 and/or RF-C regulate crucial steps during the latency-reactivation cycle.

In this study, we developed polyclonal antiserum directed against ORF-1, and then tested whether ORF-1 is expressed following infection of bovine kidney cells or in TG neurons during latency. Our studies indicate that ORF-1 is expressed in productively infected bovine cells and TG neurons of calves latently infected with wt BHV-1. Following infection of bovine kidney cells with the LR mutant virus, ORF-1 expression is reduced. Confocal microscopy revealed ORF-1 is localized to the nucleus and/or cytoplasm during productive infection, whereas antiserum that recognizes the ORF-2 fusion protein detects a nuclear protein (Jiang *et al*, 1998). The RF-C fusion antiserum recognizes a viral specific protein that localizes to the cytoplasm in productively infected cells. Because the ATG of ORF-1 is approximately 200 bases downstream from stop codons at the 5′ terminus of ORF-2 in the LR mutant virus, we suggest that these stop codons do not completely block ORF-1 expression.

## Results

*Preparation of ORF-1 polyclonal antiserum*

The LR gene has two open reading frames, ORF-1 and ORF-2, and two reading frames that lack an initiating ATG (RF-B and RF-C) (Figure 1A). The LR mutant virus contains three stop codons adjacent to the first in frame ATG of ORF-2 (Inman *et al*, 2001) (Figure 1B). Consequently, the LR mutant virus does not express detectable levels of ORF-2, RF-C, or their respective fusion proteins that would normally be expressed as a result of alternative splicing (Jiang *et al*, 2004). Antiserum directed against a peptide near the N-terminus of ORF-1 (Figure 1C) did not reproducibly recognize a viral-specific protein in infected or transfected cells (Hossain *et al*, 1995). The major spliced LR-RNA detected during productive infection or in TG at 7 days after infection lacks sequences encompassing the N-terminus of ORF-1 because of splicing (Devireddy *et al*, 2003) (Figure 1C). These LR transcripts can be translated into a protein with ORF-2 fused to ORF-1 (ORF-2/ORF-1 fusion protein) (Devireddy *et al*, 2003; Devireddy and Jones, 1998), suggesting that a portion of ORF-1 is expressed after splicing of LR-RNA. Because LR-RNA splicing is complicated and there may be more spliced transcripts, generating additional peptide antibodies or monoclonal antibodies directed against ORF-1 was not a logical approach. Consequently, we chose to generate polyclonal antiserum directed against most of the ORF-1 protein coding sequences.

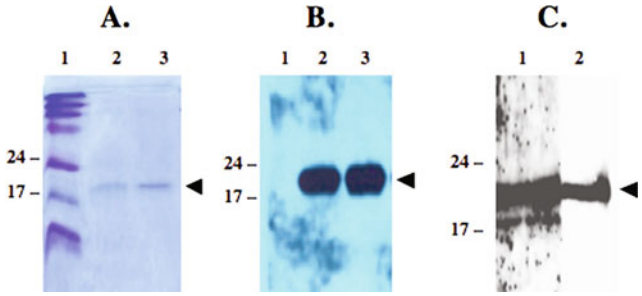


**Figure 2** Analysis of the ORF-1 protein overexpressed in baculovirus. (**A**) A Coomassie blue–stained gel of two fractions that contain the partially purified His- and Xpress-tagged-ORF-1 fusion protein prepared from baculovirus-infected SF9 cells (lane 2). Lane 1 shows molecular weight markers. (**B**) A Western blot of a similar gel as in **A** using the Xpress antibody (lanes 2 and 3). (**C**) The partially purified His- and Xpress-tagged ORF-1 protein was used for a Western blot. The rabbit ORF-1 polyclonal antiserum was used to probe lane 1, and the X-press antibody was used to probe lane 2. Molecular weight markers appear in kDa.

To develop polyclonal antiserum that recognized the majority of ORF-1 coding sequences, an NcoI-SalI fragment (nucleotides 1167 to 1940 in the LR gene; Figure 1A) was cloned into a baculovirus expression vector. A recombinant baculovirus was prepared using Bac-N-Blue DNA (Invitrogen, Carlsbad, CA) and infectious baculovirus propagated in insect cells (SF9). The His- and Xpress-tagged ORF-1 protein was partially purified using nickel affinity chromatography (Invitrogen) (Figure 2A). The anti-Xpress antibody specifically recognized a protein migrating with an approximate molecular weight of ~24 kDa (Figure 2B). The His-tagged ORF-1 protein was further purified using sodium dodecyl sulfate–polyacrylamide gel electrophorsis (SDS-PAGE), the fusion protein band excised, eluted, and then injected into rabbits to generate polyclonal antibodies. As expected, ORF-1 antiserum recognized the His- and Xpress-tagged ORF-1 protein expressed from the baculovirus vector (Figure 2C, lane 1).

*The ORF-1 antiserum recognizes viral-specific proteins during productive infection*

To test whether ORF-1 was synthesized during productive infection, we used the ORF-1 antiserum to immunoprecipitate cell lysate prepared from infected MDBK cells. MDBK cells were infected with wt BHV-1 or the LR mutant virus for a total of 20 or 24 h, and cultures metabolically labeled as described in Materials and Methods. Whole-cell lysate was incubated with ORF-1 antiserum overnight to immunoprecipitate ORF-1–specific proteins. Two proteins migrating at near 35 kDa were immunoprecipitated by the ORF-1 antiserum following infection of MDBK cells with wt virus for 20 or 24 h (Figure 3, lanes 2 and 3). In addition, two prominent bands migrating at 28–30 kDa were consistently detected at 20 or 24 h after infection. MDBK cells infected with the LR

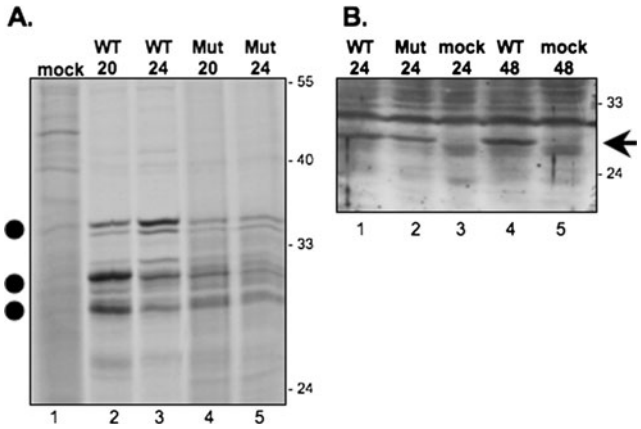


**Figure 3** ORF-1 expression during acute infection. (**A**) MDBK cells were infected with wt BHV-1 or the LR mutant virus (MOI of 2) and labeled with $^{35}$S-methionine for 3 h before immunoprecipitating samples with the ORF-1 polyclonal antiserum as described in Materials and Methods. Autoradiography of the dried gel shows immunoprecipitated proteins at 20 or 24 h of infection with each virus (wt or mut; lanes 2 to 5). As a negative control, cell lysate from mock-infected cells was used (lane 1). The amount of cell lysate immunoprecipitated was approximately $1.5 \times 10^7$ acid-precipitable counts. Numbers to the right denote the location of molecular weight standards in kDa. Closed circles denote virus-specific proteins immunoprecipitated by the ORF-1 antiserum. (**B**) MDBK cells were infected as described in **A**, but with an MOI of 4. Two hundred micrograms of whole-cell lysate prepared from wt, LR mutant virus (mut), or mock-infected cells were resolved in a 12% polyacrylamide gel and Western blot analysis performed with the ORF-1 antiserum that was diluted 1:500. The arrow denotes a viral specific protein recognized by the antiserum. Numbers to the right indicate the position of molecular weight markers in kDa.

mutant virus contained lower levels of the same viral specific bands detected in cells infected with wt BHV-1 (Figure 3, lanes 4 and 5). The immunoprecipitated viral-specific proteins varied in intensity as a function of time following infection of MDBK cells with wt BHV-1. In contrast to the bands near 35 kDa, which increased as a function of time after infection, the bands migrating at 28–30 kDa generally decreased in intensity at 24 h after infection, suggesting post-translational processing occurred. In contrast, low levels of the viral-specific proteins were detected following infection with the LR mutant virus. Viral-specific proteins were not readily detected in cell lysate prepared from mock-infected MDBK cultures (Figure 3A, lane 1).

An ORF-1–specific protein was also detected by Western blot analysis (Figure 3B). Both wt and the LR mutant virus expressed a viral specific protein of about 28 kDa in MDBK cells. Although the background for the ORF-1 antiserum was low in immunoprecipitation studies (Figure 3) or confocal microscopy (Figure 4), the ORF-1 antiserum cross-reacted with denatured cellular proteins in Western Blots, making it difficult to determine whether additional bands were present. In conclusion, these studies demonstrated that the ORF-1 antiserum recognized viral-specific proteins during productive infection.

*Localization of LR proteins during productive infection by confocal microscopy*

Confocal microscopy was used to localize ORF-1 within infected MDBK cells. Cells were infected for 21 h with wt BHV-1, and probed with a mixture of anti-ORF-1 and anti-BHV-1 antibodies (see Materials and Methods). The purpose of this dual staining was to prove that ORF-1–positive cells were infected. ORF-1 exhibited a punctate staining pattern (Figure 4, ORF-1 panel) that was only detected in infected cells (red cells, BHV-1 panel). Although greater than 75% of the total cells were infected at 21 h after infection, only one out of two infected cells expressed detectable levels of the ORF-1 protein. Although ORF-1 was present in the cytoplasm and nucleus (Figure 4, merge + DAPI panel), the staining was punctate, suggesting ORF-1 was not randomly distributed throughout the cell. As expected, the ORF-1 antiserum did not cross-react with cells that were mock infected (Figure 4, Mock infected panels).

We previously prepared polyclonal antiserum directed against a fusion protein containing ORF-2 and ORF-1 (ORF-2 fusion antiserum) or RF-C and RF-B (RF-C fusion antiserum) (Jiang *et al*, 2004). These respective antisera were used to compare the subcellular localization of ORF-1 to RF-C and ORF-2. At 16 h after infection, the RF-C fusion antiserum recognized a viral specific protein that was localized to the cytoplasm, whereas the ORF-2 fusion antiserum recognized a protein that was primarily localized to the nucleus (Figure 5). As expected, the RF-C fusion antiserum (Figure 5) or ORF-2 fusion antiserum (data not shown) did not recognize proteins in mock-infected cells. In summary, the localization of ORF-1 was distinct compared to ORF-2 and RF-C or fusion proteins containing these ORFs.

*Detection of ORF-1 in TG of calves latently infected with wt BHV-1*

The TG used for this study were obtained from calves at 60 days after infection. These calves were latently infected because infectious virus is not detected in nasal or ocular swabs (Inman *et al*, 2002; Perez *et al*, 2005; Winkler *et al*, 2000a, 2000b, 2002). Furthermore, we could not detect a late transcript (gC) by reverse transcriptase–polymerase chain reaction (RT-PCR) using total RNA prepared from TG of these calves at 60 days after infection (Inman *et al*, 2004). The ORF-1 polyclonal antiserum yielded slightly higher background than the RF-C fusion antiserum when TG from two mock-infected calves were stained (Figure 6). However, it was clear that certain TG neurons from two calves latently infected with wt BHV-1 were stained by the ORF-1 antiserum. Most of the ORF-1 positive neurons from TG of these latently infected calves contained nuclear staining, but there were also neurons that had strong cytoplasmic staining. Two calves latently infected with the LR mutant virus did not contain ORF-1 positive TG neurons

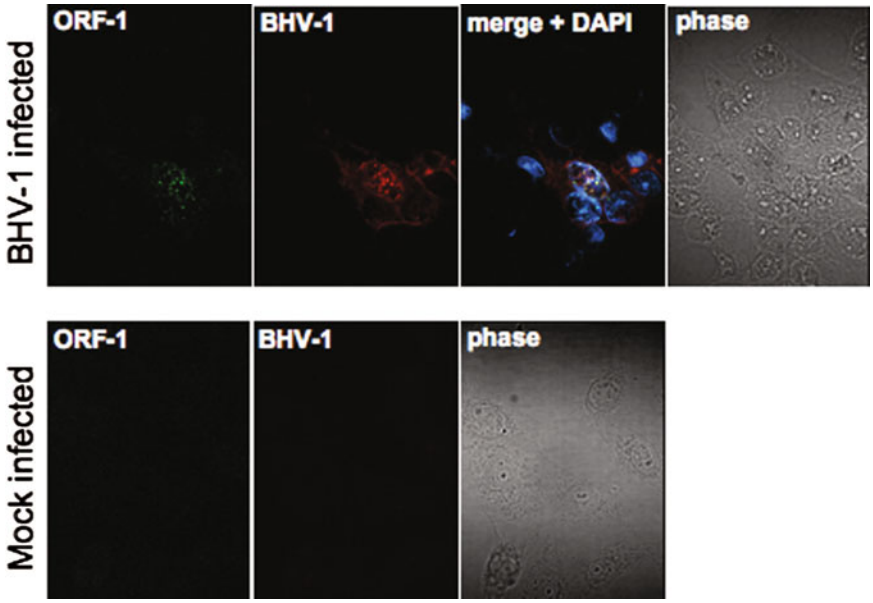


**Figure 4** Localization of ORF-1 by confocal microscopy. MDBK cells were infected with wt BHV-1 at an MOI of 0.5 for 21 h (*upper panels*) and prepared for confocal microscopy as described in Materials and Methods. As a negative control, MDBK cultures were mock infected (*lower panels*). A combination of ORF-1 antiserum (*green*) and anti-BHV-1 antiserum (*red*) was used to stain cells. Individual staining of cells with the respective antiserum, a merge of ORF-1, and BHV-1, and DAPI (to show nuclear staining), and the phase-contrast image are shown. For mock-infected cultures, it was only necessary to show that BHV-1 proteins or ORF-1 was not detected. Magnification of all images is 1000×.

using the same procedures described for calves latently infected with wt BHV-1 (data not shown).

In contrast to the cytoplasmic localization of RF-C during productive infection, the RF-C fusion antiserum recognized a protein in the nucleus of neurons prepared from TG of latently infected calves (Figure 6). The background of the RF-C fusion antiserum was low, and we did not detect staining in the nucleus of neurons obtained from TG of mock-infected calves. In summary, this study suggested that a subset of neurons express ORF-1 and/or RF-C during latency.

## Discussion

For this study, we designed a polyclonal antiserum directed against the majority of ORF-1 coding sequences. Although a previous study did not detect ORF-1 using a peptide antibody, the ORF-1 peptide antibody was directed to sequences near the N-terminus (Hossain *et al*, 1995). Because LR-RNA undergoes splicing at or near this region (Devireddy and Jones, 1998) (Figure 1C), the peptide antiserum could not have detected an ORF-1 protein that was translated from a spliced transcript. Furthermore, it

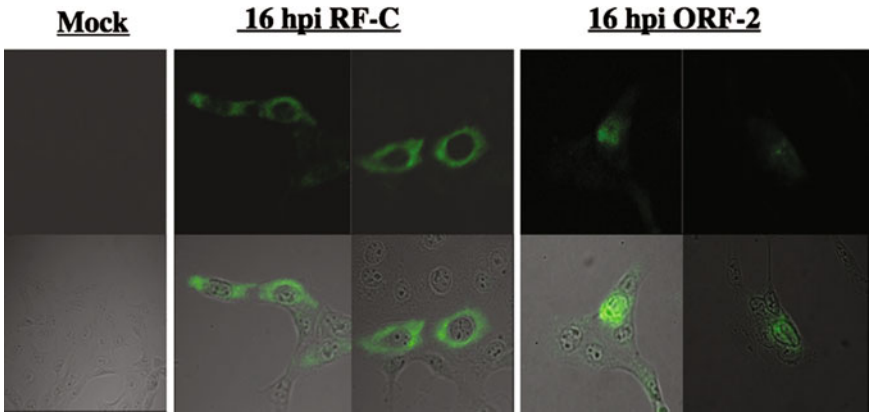


**Figure 5** Localization of ORF-2 and RF-C by confocal microscopy. MDBK cells were infected for 16 h (MOI = 0.5) and confocal microscopy performed. The antiserum directed against ORF-2 or RF-C was previously described (Jiang *et al*, 2004). Greater than 75% of cells at 16 h after infection are infected as judged by morphological changes and staining with the anti-BHV-1 antibody (data not shown).

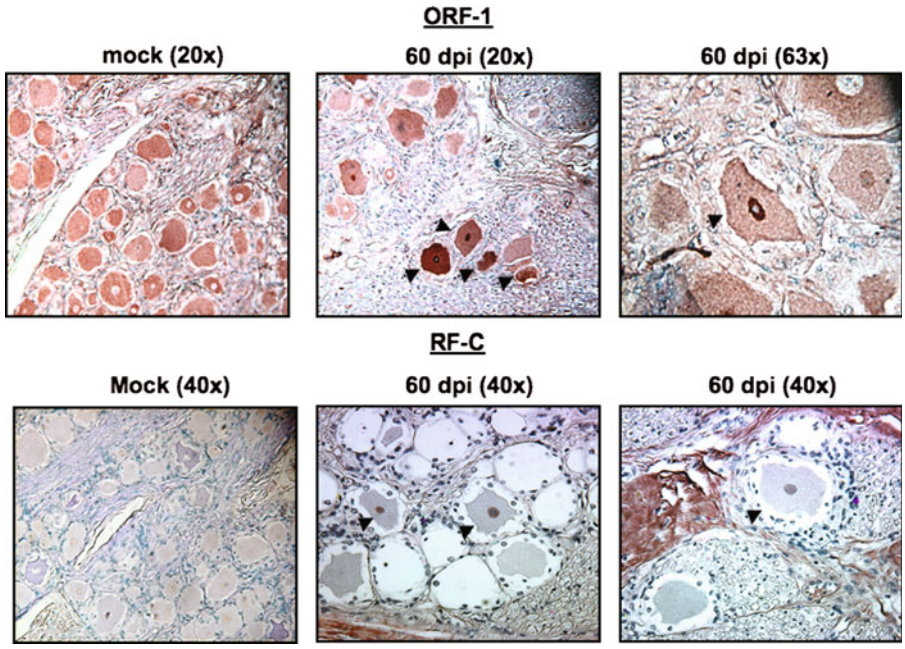


**Figure 6** Detection of ORF-1 and RF-C in TG of calves latently infected with wt BHV-1. Thin sections from TG were prepared from two different calves latently infected with wt BHV-1 (60 days after infection). Immunohistochemistry was performed using the ORF-1 or RF-C antiserum as described previously (Jiang*et al*, 1998; Perez, 2005, 2006). The magnification for each panel is denoted in the figure. Arrows denote positively stained neurons. The results shown are from one calf, and are representative of the results obtained from both calves. The ORF-1 antiserum did not specifically stain TG from two calves that were latently infected with the LR mutant virus (data not shown).

does not appear that the intact ORF-1 was expressed during productive infection because the ORF-1 antiserum recognized proteins migrating between 28 and 30 kDa and 20 and near 35 kDa (Figure 2), whereas the intact ORF-1 has a predicted molecular weight of 40 kDa (Figure 1A). The presence of several ORF-1–specific protein bands suggested that (1) post-translational modification of ORF-1 occurs; (2) more than one spliced variant of the LR transcript contains a portion of ORF-1; or (3) the ORF-1 protein was prone to degradation or proteolytic processing. Interestingly, a spliced polyA− LR transcript is expressed during productive infection that contains a portion of ORF-2 fused to ORF-1 (Devireddy and Jones, 1998), suggesting ORF-1 is expressed as a fusion protein during productive infection. In summary, our studies indicated that tissue-specific alternative splicing of LR-RNA expands the coding potential of the LR gene.

Calves latently infected with wt BHV-1 contained TG neurons that were recognized by the ORF-1 antiserum (Figure 6). ORF-1 was not detected in TG of calves latently infected with the LR mutant virus (data not shown), suggesting that ORF-1 was not expressed. However, it is important to note that reduced levels of viral DNA are present in TG of calves latently infected with the LR mutant virus versus calves latently infected with wt BHV-1 (Inman *et al*, 2002). Thus, fewer neurons are latently infected with the LR mutant virus, or each latently infected neuron contains fewer copies of viral DNA. If one correlates ORF-1 expression in TG neurons to that observed during productive infection, ORF-1 may be expressed at low levels in TG neurons of calves latently infected with the LR mutant virus.

A BLAST analysis revealed that the alanine-rich domain in ORF-1 (Figure 1C) contains regions of identity to four cellular transcription factors: (1) histone demethylase 1 (61% identical, 21/34 amino acids); (2) the human headcase protein homolog (52% identical, 35/67 amino acids); (3) Brn-3a, a neuronal specific transcription factor (51% identical, 28/54 amino acids) (Devireddy *et al*, 2003); and (4) the homeobox protein Hox-A13 (45% identical, 34/76 amino acids). The lysine-specific histone demethylase 1 is required for cell proliferation (Scoumanne and Chen, 2007). The headcase gene, initially identified in *Drosophila melanogastor*, mediates development of the adult head structure (Weaver and White, 1995). The Hox-A13 protein regulates growth and patterning of digits and interdigital tissues, in part, by interacting with tissue-specific gene regulatory factors (Knosp *et al*, 2004). The alanine-rich domain within ORF-1 was not the only region that contained similarity to cellular proteins because the C-terminus of ORF-1 has 50% identity (25/50 amino acids) to the chromatin-helicase DNA-binding protein 1 (Delmas *et al*, 1993). This analysis suggested that the alanine-rich domain in ORF-1 might have functional significance.

Does ORF-1 play a role in the BHV-1 latency-reactivation cycle? We predict that if the intact ORF-1

is expressed, it plays an important role in this process because the LR mutant virus does not express detectable levels of ORF-1 during latency. Even if the intact ORF-1 protein is not expressed, ORF-1 protein coding domains can be fused with ORF-2 to yield a protein that regulates gene expression and inhibits apoptosis (Devireddy *et al*, 2003; Devireddy and Jones, 1998; Meyer *et al*, 2007). Furthermore, genetic evidence clearly demonstrates that expression of ORF-2 or a fusion protein containing a portion of ORF-2 with ORF-1 is required for the latency reactivation cycle in cattle (Inman *et al*, 2002), in part because the LR mutant virus induces higher levels of apoptosis in TG neurons (Lovato *et al*, 2003). Regardless of whether ORF-1 is expressed as an intact protein, certain ORF-1 protein coding domains are likely to be important because they can be fused with ORF-2 as a result of alternative splicing of LR-RNA.

## Materials and methods

*Virus and cells*

Madin Bovine kidney cells (MDBK; ATCC CCL-22) were maintained in minimal essential medium (EMEM). The medium was supplemented with 5% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 $\mu$g/ml).

The BHV-1 Cooper strain (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa. The LR mutant virus was constructed by replacing wt LR gene sequences (25 bp) with an oligonucleotide that contains a unique EcoR1 restriction site and three stop codons (see Figure 1 for a diagram of the LR mutant). The three stop codons were designed to prevent protein expression from all 3 reading frames. A complete description of the LR mutant virus has been previously reported (Inman *et al*, 2001). Viral stocks were prepared by infecting MBDK cells with a multiplicity of infection (MOI) of .001 from a plaque-purified virus. Virus was titrated on MDBK cells by using 10-fold dilutions and determining the tissue culture infectious dose (TCID) 50% or plaque forming units (PFU).

*Generation of polyclonal antiserum that detects ORF-1*

To generate a recombinant baculovirus that expresses ORF-1, a NcoI-SalI fragment from the LR gene (Figure 1A) was cloned into pBlueBacHis2 such that ORF-1 was in frame with the histidine (His) and Xpress tag. A recombinant baculovirus was then constructed that expresses the His- and Xpress-tagged ORF-1 protein. The recombinant baculovirus strain was grown in SF9 insect cells using procedures described by Invitrogen. Recombinant baculovirus that contained ORF-1 sequences were characterized by testing for ORF-1 protein expression using the Xpress antibody that recognizes the Xpress epitope at the 5′ terminus of ORF-1 protein sequences (R910-25; Invitrogen). Large-scale expression was carried out in 2-L flasks seeded with SF9 cells at a density of $2 \times 10^6$ cells/ml in a total volume of 1000 ml. At 4 days after infection, cells were pelleted by centrifuging for 30 min at 8000 rpm (Beckman J2-21 centrifuge, JA-10 rotor), and suspended in 20 ml of Guanidinium Lysis Buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8). Suspended cells were then passed through an 18-gauge needle four times to lyse cells and shear cellular DNA. The His- and Xpress-tagged ORF-1 protein was partially purified using nickel chromatography. The His- and Xpress-tagged ORF-1 protein was further purified using preparative SDS-PAGE, the ORF-1 protein band excised, eluted, and then injected into rabbits to generate ORF-1–specific polyclonal antiserum (Animal Pharm Services, CA). The polyclonal anti-serum directed against ORF-1 is referred to as ORF-1 antiserum throughout the text. The antisera directed against LR ORF-2 fusion proteins of ORF-1/ORF-2 (ORF-2 fusion antiserum) or RF-C/RF-B (RF-C fusion antiserum) were previously described (Jiang *et al*, 2004).

*Western blot*

To detect ORF-1 in MDBK cells, cultures were infected with wt BHV-1 or the LR mutant virus at an MOI of 4. As a negative control, MDBK cells were mock-infected. At the designated times after infection, cells were harvested and lysed by incubating with NP40 lysis buffer (1% Nonidet P-40 [NP40], 50 mM Tris-HCl pH8, 150 mM NaCl, plus 1 tablet of protease inhibitor cocktail [Roche, no. 11 8361 53001] for every 10 ml). Cell lysate (200 $\mu$g) was loaded on a 12% SDS-PAGE gel. The details of performing Western blots were previously described (Inman *et al*, 2002; Lovato *et al*, 2993; Meyer *et al*, 2007). In brief, proteins were transferred to PVDF (Polyvinylidene Fluoride) membrane (Millipore), blocked for 1 h with 5% nonfat dry milk (NFDM) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), and incubated with anti-ORF-1 antiserum (1:500 dilution in 5% NFDM in PBS-T) overnight at 4°C. A 30 minute washing step with PBS-T was followed by incubation of donkey anti-rabbit horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (Amersham Biosciences) diluted 1:1000 in 1% NFDM PBS-T.

To detect partially purified ORF-1 protein from baculovirus-infected cells, approximately 1 $\mu$g of the partially purified protein (Figure 2B) or the purified protein (Figure 2C) were probed with the anti-Xpress antibody (1:5000 dilution) or with the ORF-1 polyclonal antiserum (1:500 dilution). An anti-mouse or ant-rabbit HRP-conjugated IgG secondary antibody was then added.

For all Western blots, membranes were washed for 30 min with PBS-T, developed with Amersham enhanced chemiluminescence (ECL) reagents, and then exposed to Kodak x-ray film.

*Measuring acid precipitable counts after $^{35}S$ metabolic labeling*

Five microliters of precleared whole-cell lysate was incubated in 10 ml of 1 M HCl, 0.1% sodium pyrophosphate, for 10 min at 4°C. The mixture was passed through a glass microfibre filter GF/C (Whatman) using vacuum, and then washed twice with 1 M HCl, 0.1% sodium pyrophosphate, and once with 100% ethanol. The acid-precipitable counts on dried filters were determined using a liquid scintillation counter.

*Metabolic labeling of cells with $^{35}S$-methionine and immunoprecipitation*

MDBK cells were infected with BHV-1 at an MOI of 2 and cultured in regular EMEM + 5% fetal calf serum (FCS) for 15 or 19 h at 37°C in a 5% $CO_2$ chamber. The medium was removed, and cells washed once with warm PBS. EMEM + 2.5% dialyzed FCS that lacked methionine and cystein (Sigma) was added to cultures, and incubated for 2 h. $^{35}S$-labeled methionine, 150 $\mu$Ci, in 1 to 2 ml of EMEM lacking methionine and cysteine was added to each 60-mm dish and incubated for 3 additional hours. Cells were then harvested and lysed in RIPA buffer (1% Nonidet P-40, 0.1% SDS, 0.5% deoxicholate in PBS). Cell lysate was precleared with 10 ug of normal rabbit serum for 30 minutes at room temperature. Acid precipitable counts were measured using 1% of the cell lysate. IgG was captured by adding 50 $\mu$l of a slurry of protein A magnetic beads (NEBiolabs) for 30 min at room temperature. Approximately $1.5 \times 10^7$ acid-precipitable counts of each sample were incubated 16 h with 300 $\mu$l of anti-ORF-1 antiserum. The next morning, antibodies were precipitated by adding 50 $\mu$l of protein A magnetic beads slurry to each sample for 30 min at room temperature. Magnetic beads were washed three times with RIPA buffer and once with 20 mM Tris-HCl pH 6.8. The beads were boiled for 5 min in SDS reducing sample buffer and the supernatant electrophoresed on a 12 % SDS-PAGE gel. The gel was then dried and exposed to Kodak x-ray film.

*Confocal microscopy to examine localization of LR proteins*

MDBK cells were grown in 4-well Lab-Tek 4 culture slides and infected for 21 h with wt BHV-1 or the LR mutant virus at a MOI of 0.75 PFU/cell. Cells were washed twice with EMEM (without serum) and fixed in cold 100% ethanol at −20°C for 5 min. After a brief wash with Tris-buffered saline (TBS) (137 mM NaCl, 2.68 mM KCl, 24.8 mM Tris base, pH 7.4), samples were incubated with 3% bovine serum albumin (BSA) in TBS for 1 h to reduce nonspecific binding. Samples were then incubated with primary antiserum for 2 hours at room temperature. The primary antiserum consisted of a mixture of ORF-1 antiserum (dilution 1:75; preabsorbed overnight on ethanol-fixed uninfected MDBK cells) and anti-BHV-1 antiserum (dilution 1:100; American BioResearch Laboratories) in TBS 0.05%Tween 20 (TBS-T), 1% BSA. Three 10-min washes with TBS-T were followed by incubation with the secondary antibody mix for 1 h at room temperature in the dark. The secondary antibody mixture consisted of donkey anti-rabbit IgG conjugated to Cy2 (dilution 1:50) and Goat anti-donkey IgG conjugated to Cy5 (dilution 1:50). Three washes with TBS-T (10 min each) were followed by incubation with DAPI (4′,6-diamidino-2-phenylindole) stain for 10 min. Slides were then mounted with mounting gel (Sigma) and a coverslip. Images were collected using a Bio-Rad confocal laser-scanning microscope (MRC-1024ES) with excitation/emission at 488/520 nm.

Similar procedures as described above were used to detect the ORF-2 or RF-C fusion proteins. The ORF-2 fusion antiserum or RF-C fusion antiserum were used at a 1:200 dilution.

*Animal experiments*

BHV-1-free crossbred calves (~250 kg) were randomly assigned and housed in isolation rooms to prevent cross contamination. Calves were sedated with xylazine (approximately 50 mg/50 kg body weight; Bayer, Shawnee Mission, KS.). Calves were then inoculated with 1 ml of a solution containing $1 \times 10^7$ PFU/ml of virus in each nostril and eye, dropwise without scarification, for a total of $4 \times 10^7$ PFU per animal as described previously (Inman *et al*, 2001, 2002; Schang and Jones, 1997; Winkler *et al*, 2002). Experiments using animals were performed in accordance with the American Association of Laboratory Animal Care guidelines. Calves were housed under strict isolation containment and given antibiotics before and after BHV-1 infection to prevent secondary bacterial infection. All calves designated as being latently infected were euthanized at 60 days after infection, and were not shedding infectious virus from the nasal cavity or eye (Inman *et al*, 2002; Perez *et al*, 2005; Winkler *et al*, 2000a, 2000b, 2002).

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