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A protein encoded by the bovine herpesvirus 1 open reading frame E gene induces neurite-like morphological changes in mouse neuroblastoma cells and is expressed in trigeminal ganglionic neurons

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Bovine herpes virus 1 (BHV-1), like other α-herpesvirinae subfamily members, establishes latency in sensory neurons. Periodically BHV-1 reactivates from latency, resulting in virus shedding and spread to uninfected cattle. Although reactivation from latency does not usually lead to recurrent disease, the latency-reactivation cycle is crucial for virus transmission. The latency-related (LR) RNA is abundantly expressed during latency, and expression of a LR encoded protein is necessary for dexamethasone-induced reactivation from latency in cattle. Within LR promoter sequences, a small open reading frame (ORF) was identified (ORF-E) that is antisense to the LR-RNA, and downstream of the bICP0 gene. ORF-E transcription is consistently detected in trigeminal ganglia (TG) of latently infected calves, suggesting ORF-E expression plays a role in the latency-reactivation cycle. Polyclonal antiserum directed against an ORF-E peptide or the entire ORF-E protein specifically recognizes the nucleus of sensory neurons in TG of latently infected calves. The ORF-E peptide–specific antiserum also recognizes a protein when mouse neuroblastoma cells (neuro-2A) are transfected with an ORF-E expression construct. In contrast to the growth inhibiting properties of the LR gene, stably transfected ORF-E–expressing cells were obtained. Neuro-2A cells stably transfected with a plasmid expressing ORF-E induced morphological changes that resembled neurite-like projections. In contrast, neurite-like projections were not observed following transfection of neuro-2A cells with an empty vector. These studies suggest that a protein encoded by ORF-E has the potential to alter the physiology or metabolism of neuronal cell types, which may be important for long-term latency. *Journal of NeuroVirology* (2007) **13,** 139–149.

Keywords: bovine herpesvirus type 1; latency; neurite outgrowth; ORF-E

Introduction

Bovine herpes virus 1 (BHV-1) establishes lifelong latency in ganglionic neurons of the peripheral nervous system, primarily the trigeminal ganglia (TG), after initial replication in mucosal epithelium. Virus reactivation and spread to susceptible calves occur after natural or corticosteroid-induced stress (Rock *et al*, 1992; Sheffy and Davies, 1972). Infection can cause conjunctivitis, pneumonia, genital disorders, abortions, and an upper respiratory infection referred to as "shipping fever" (Tikoo *et al*, 1995). Because BHV-1–associated pathogenesis and shipping fever cost the U.S. cattle industry at least $500 million/year (Bowland and Shewen, 2000), developing better modified live vaccines is desirable.

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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 is translated into more than one protein (Devireddy and Jones, 1998; Hossain *et al*, 1995). LR gene products inhibit cell growth (Hossain *et al*, 1995; Jiang *et al*, 1998) and apoptosis (Ciacci-Zanella *et al*, 1999). A mutant BHV-1 strain that contains three stop codons near the beginning of the LR-RNA was constructed to test whether LR proteins play a role in virus growth in cattle (Inman *et al*, 2001). Calves infected with the LR mutant exhibit diminished clinical symptoms, and virus shedding in the eye (Inman *et al*, 2001), TG (Inman *et al*, 2002), or tonsils (Perez *et al*, 2005), compared to calves infected with wild-type (wt) BHV-1 or the LR rescued virus. wt BHV-1 or the LR rescued virus, but not the LR mutant virus, reactivate from latency following treatment with dexamethasone (DEX). 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 relative to calves infected with wt BHV-1 (Lovato *et al*, 2003). These studies indicate that wt expression of LR gene products is required for the latency-reactivation cycle in cattle.

A small open reading frame (ORF) within the LR promoter was identified, and designated ORF-E (Figure 1B and C) (Inman *et al*, 2004). ORF-E is antisense to the LR transcript, downstream of the bICP0 ORF, but does not overlap bICP0 (Figure 1B). A transcript encompassing ORF-E is expressed in 6/6 calves latently infected with BHV-1 (Inman *et al*, 2004). The LR promoter contains a neuronal-specific binding domain (NSB; Figure 1B) and has neuronal-specific transcriptional activity (Bratanich *et al*, 1992; Bratanich and Jones, 1992; Delhon and Jones, 1997; Jones *et al*, 1990). Sequences that activate expression of the LR-RNA during productive infection contain a long AT-rich motif (Figure 1B), suggesting these same promoter sequences also activate ORF-E RNA expression. ORF-E is 134 amino acids (aa) long and contains potential casein kinase 2 (ck2) as well as protein kinase C (pkc) phosphorylation sites (Figure 1C). A BLAST analysis did not reveal strong similarity to known cellular proteins.

In this study, we generated antiserum directed against the entire ORF-E or an ORF-E peptide. The ORF-E antiserum recognized a specific protein in transfected mouse neuroblastoma cells (neuro-2A). Unlike the LR gene, ORF-E does not induce cell cycle arrest, and consequently stably transfected cells were obtained. Neuro-2A cells stably transfected with ORF-E frequently have neurite-like projections, and are morphologically distinct compared to neuro-2A cells stably transfected with the empty vector. The ORF-E antiserum specifically reacted with TG neurons of infected calves, but when reactivation from latency was induced by addition of DEX, ORF-E protein expression was not readily detected. These studies suggest that the ORF-E protein is expressed in latently infected neurons, and that ORF-E alters the morphology and/or physiology of neuronal cell types.

Results

ORF-E protein expression in transfected neuro-2A cells

To test whether the ORF-E gene encodes a protein, it was necessary to generate specific antiserum directed against ORF-E. With this objective in mind, a peptide identified as the most immunogenic region of ORF-E was synthesized (Figure 1C), and injected into rabbits to generate an ORF-E specific antiserum (ORF-E peptide antiserum). In addition, the entire ORF-E was cloned into a baculovirus expression system, ORF-E was overexpressed (Figure 1D), purified, and the purified ORF-E protein used to generate rabbit polyclonal antiserum (ORF-E polyclonal antiserum).

The protein coding sequences of ORF-E (Figure 1C) were cloned upstream of the green fluorescent protein (GFP) coding sequences of phMGFP. The ORF-E/GFP plasmid or the blank GFP vector was transfected into mouse neuroblastoma cells (neuro-2A). As previously demonstrated (Inman *et al*, 2004), the ORF-E/GFP fusion protein was localized to the nucleus when expressed in neuro-2A cells (Figure 2A). In contrast, neuro-2A cells transfected with the GFP expression vector (phMGFP) contained GFP randomly distributed throughout the cell (Figure 2B). The ORF-E peptide antiserum specifically recognized a protein with an approximate molecular weight of 40 kDa at 24 h after transfection (Figure 2C). Based on the predicted size of ORF-E (approximately 15 kDa) and GFP (25 kDa), this was the expected size of the fusion protein. At 48 or 72 h after transfection, the ORF-E/GFP fusion protein was detectable, but at lower levels. As expected, the ORF-E peptide antiserum did not specifically recognize a 40 kDa protein when neuro-2A cells were transfected with the blank expression vector. When neuro-2A cells were transfected with an expression vector containing only ORF-E, the ORF-E peptide antiserum (Figure 2D) or the ORF-E polyclonal antiserum (data not shown) specifically detected a protein that migrates at approximately 14 kDa. Similar levels of protein were present in samples because when the blots were stripped and reprobed with a β-actin antibody similar levels of protein were detected (Figure 2C and D; β-actin panels).

ORF-E protein expression during productive infection

In contrast to the results in transiently transfected cells, the ORF-E polyclonal or peptide antiserum did not consistently detect ORF-E expression in productively infected Madin Darby bovine kidney (MDBK) cells. This suggested that ORF-E was not expressed

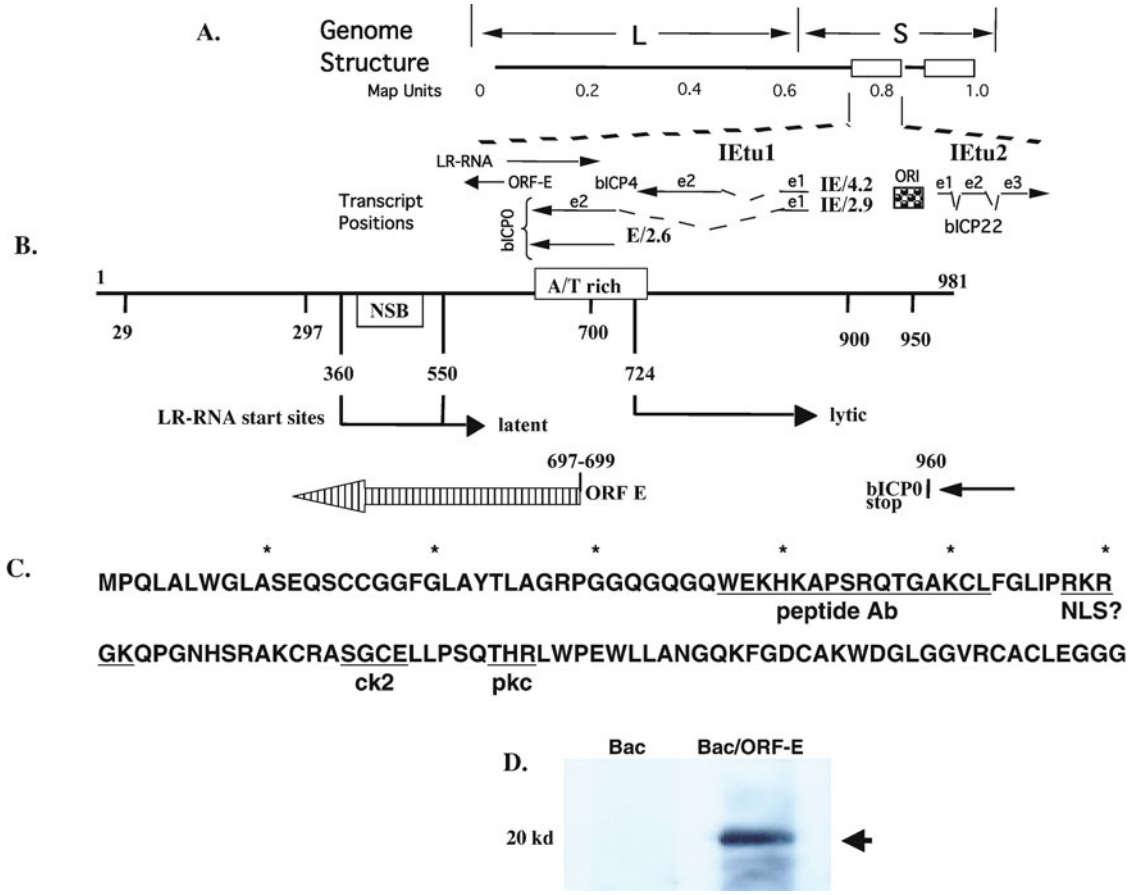

Figure 1 Location of the ORF-E gene within the BHV-1 genome. (**A**) Schematic representation of the BHV-1 genome. L and S indicate the unique long and short regions, respectively. The boxes represent the inverted and terminal repeats. The positions of the maps units are below the schematic of the genome. Positions of IE transcripts and LR transcript 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 activates expression of E2.6, which 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. Solid lines in the transcript position map represent exons (e1, e2, or e3). (**B**) Expanded map of the PstI fragment that contains the LR promoter, 5′ terminus of the LR transcript (Hossain *et al*, 1995), and ORF-E. The arrows denote start sites for LR transcription during latent or productive infection (Bratanich and Jones, 1992; Hossain *et al*, 1995). The boxes depict the neuronal specific binding (NSB) site, and A/T rich motif located within the LR promoter (Delhon and Jones, 1997). Also shown for reference is the termination site for bICP0 translation (bp 960) (Wirth *et al*, 1992). ORF-E is antisense with respect to the LR gene. (**C**) The ORF-E protein coding sequences are shown. The putative casein kinase 2 sites (ck2), protein kinase C sites (pkc), nuclear localization site (NLS), and the peptide used to generate a peptide specific polyclonal antibody are shown. (**D**) Western blot of ORF-E His-tagged fusion protein. Recombinant baculoviruses were prepared using Bac-N-Blue DNA (Invitrogen, Carlsbad, CA) and infectious baculovirus propagated in insect cells (SF9). The ORF-E fusion protein was partially purified using nickel affinity chromatography (Invitrogen). The arrow depicts a specific fusion-protein at a molecular weight of 20 kDa, which was detected using X-press antibody. The His tag is 6 kDa, and the predicted molecular weight of ORF-E is 14 kDa. Thus, 20 kDa was the expected size of the fusion protein. Cells infected with a baculovirus (Bac) only did not express a protein detected by the X-press antibody.

during productive infection or that only a subset of infected cells expressed low levels of ORF-E. Confocal microscopy was performed to discern between these possibilities. At 8 h after infection, we were unable to detect ORF-E expression, but we were able to readily detect bICP0 protein expression (data not shown). As judged by confocal microscopy, approximately 1 in 10 cells expressed low levels of ORF-E at 21 h after infection using the ORF-E peptide antibody (Figure 3A) or the ORF-E polyclonal antiserum (data not shown). ORF-E–positive cells were infected because they were also stained with an anti-BHV-1 antiserum. ORF-E protein expression was detected in the cytoplasm of a subset of infected cells, or dispersed throughout the cell, as judged by merging the image with 4′,6-Diamidino-2-phenylindole (DAPI)-stained cultures. It was also noted that staining was punctate in nature and not evenly dispersed throughout the cytoplasm. The nuclear structure was disrupted at late times after infection when DAPI staining of infected cultures was compared to infected cultures. As expected, the ORF-E peptide antiserum or the BHV-1 antiserum did not recognize mock-infected cells (Figure 3B). In another non-neural cell line (rabbit skin cells), the ORF-E–GFP fusion protein was primarily detected in the cytoplasm (Inman *et al*, 2004), suggesting that the ORF-E protein localizes to the cytoplasm of non-neural cells.

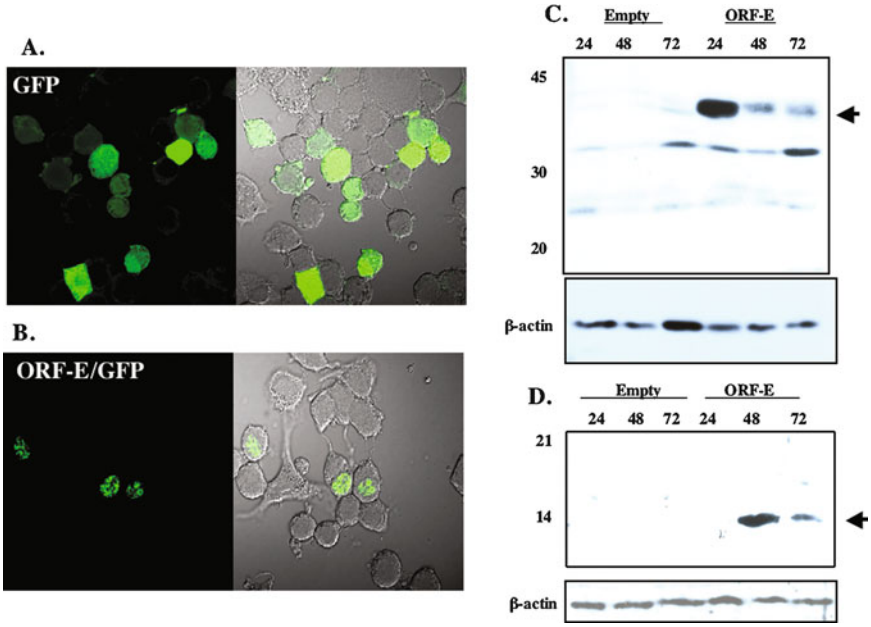

Figure 2 ORF-E expression in transfected mouse neuro-2A cells. Neuro-2A cells were transfected with the ORF-E/GFP plasmid (2 μg of plasmid; **A**) or the GFP blank expression vector (phMGFP, 2 μg; **B**). At 24 h after transfection, cells were viewed under a fluorescent microscope as described in Materials and Methods. The magnification for **A** and **B** is 100×. The image on the left is fluorescence only and the right image is the merge between fluorescence and phase contrast. These images are representative of several independent studies. (**C**) Neuro-2A cells were transfected with the ORF-E/GFP plasmid or the GFP empty expression vector (10 μg/100-mm dish). At the designated times after transfection, cells were processed for Western blotting. (**D**) Neuro-2A cells were transfected with an ORF-E expression plasmid, or an empty expression vector (pcDNA3.1–). For **C** and **D**, the ORF-E peptide antiserum was used to probe the Western blot. Approximately 50 μg protein was loaded per lane. The blot was stripped, and reprobed with an antibody directed against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

The ORF-E gene induces morphological changes in neuro-2A cells

The LR gene inhibits apoptosis and cell growth following transfection of certain cell types (Ciacci-Zanella *et al*, 1999; Geiser and Jones, 2005; Schang *et al*, 1996). Conversely, ORF-E overexpression did not have a dramatic effect on the frequency of apoptosis (induction or inhibition), nor did it inhibit cell growth. Following transfection of neuro-2A cells with an ORF-E expression plasmid containing a neomycin resistance gene (pcDNA3.1–), we were able to readily select stably transfected cells using G418. In these cultures, low levels of ORF-E protein expression were detected. When ORF-E stably transfected neuro-2A cells were subcultured, we consistently observed that these cells contained

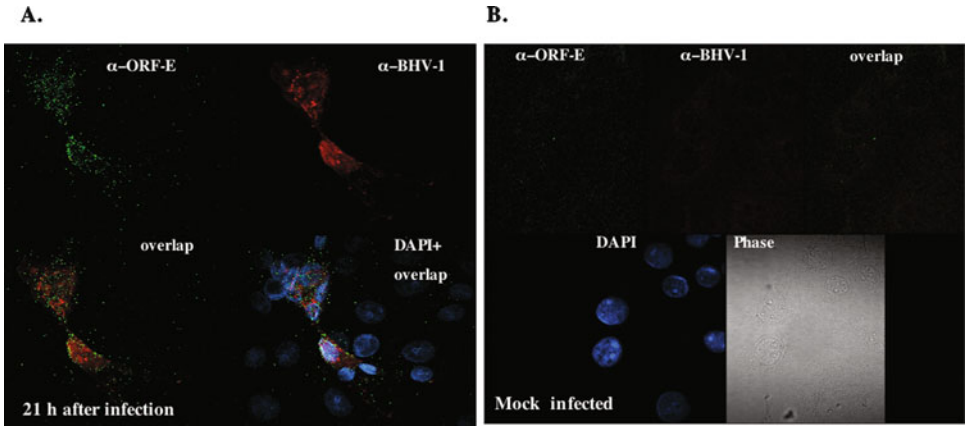

Figure 3 ORF-E protein expression in productively infected bovine kidney cells. Confocal microscopy was used to test whether ORF-E was expressed in productively infected bovine kidney (MDBK) cells. MDBK cells were infected with BHV-1 (MOI = 0.75 pfu/cell), and at 21 h after infection (**A**) cultures were fixed and confocal microscopy performed as described previously (Zhang and Jones, 2005). The ORF-E peptide antiserum or a BHV-1 antiserum was used to detect infected cells. Nuclei were detected by DAPI staining. As a control, mock-infected cells were used (**B**).

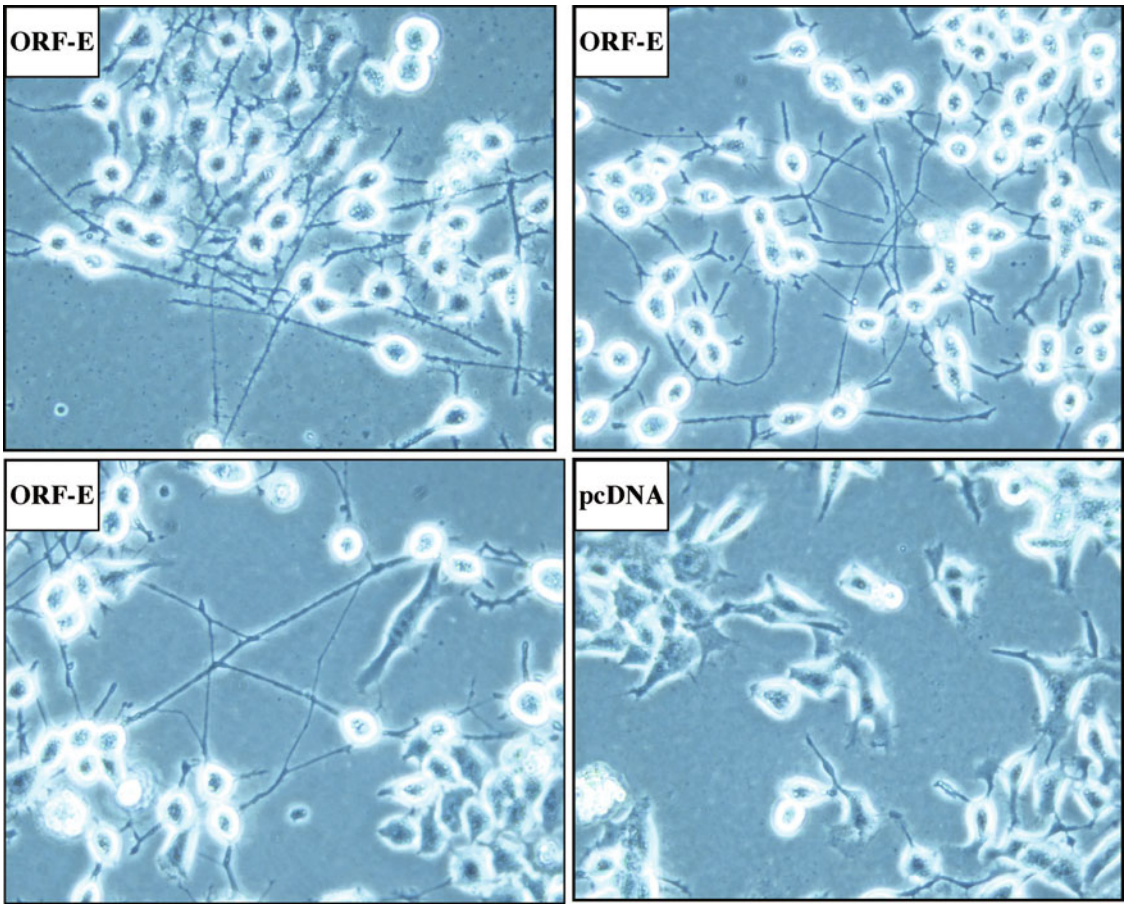

Figure 4 Stable transfection of neuro-2A cells with an ORF-E expression plasmid. A mammalian expression plasmid (pcDNA3.1–) containing ORF-E or the empty vector (pcDNA) was transfected into neuro-2A cells. At 24 h after transfection, stably transfected cells were incubated with the antibiotic G418 to select for transfected cells. Shown are representative fields (100× magnification) following 3 weeks of incubation with medium containing G418.

long extensions resembling neurites (Figure 4; ORF-E panels). In many cases, multiple extensions were observed, and many of these neurite-like extensions were quite long. Cells that contained neurite-like projections were viable as judged by trypan blue exclusion (data not shown), and they appeared to grow in culture. In sharp contrast to ORF-E, the LR gene inhibited growth of neuro-2A cells, but does not induce neurite-like growth (Geiser and Jones, 2005). When neuro-2A cells were transfected with a blank expression vector, neurite-like projections were not frequently observed (Figure 4; pcDNA). Furthermore, there was a morphological difference in cells stably transfected with ORF-E versus cells stably transfected with pcDNA3.1–. In contrast to the results obtained with neuro-2A cells, ORF-E did not alter the morphology of a bovine kidney or testicle cell line (unpublished data).

The ORF-E antiserum recognizes a protein in TG neurons of latently infected calves

Thin sections were prepared from TG of latently infected calves (60 days after infection), and the ability of the ORF-E antisera to specifically recognize these sections was tested. The ORF-E peptide antiserum (Figure 5A and B) or the ORF-E polyclonal antiserum (Figure 5C) specifically recognized a protein present in the nucleus of TG neurons. Neither ORF-E antiserum reacted with all neurons from latently infected calves. In contrast to the results obtained with TG sections prepared from latently infected calves, the ORF-E polyclonal antiserum (Figure 5D) as well as the ORF-E peptide antiserum (data not shown) did not react with the nucleus of sensory neurons from mock-infected calves. Even when the section cut through the nucleus of neurons from mock-infected calves, only the counter-staining of nucleoli was detected.

The TG used for this study were obtained from calves at 60 days after infection, and these calves were used in previously published studies. We know these calves are 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 are unable to detect a late transcript (gC) by reverse transcriptase–polymerase chain reaction (RT-PCR) using total RNA prepared

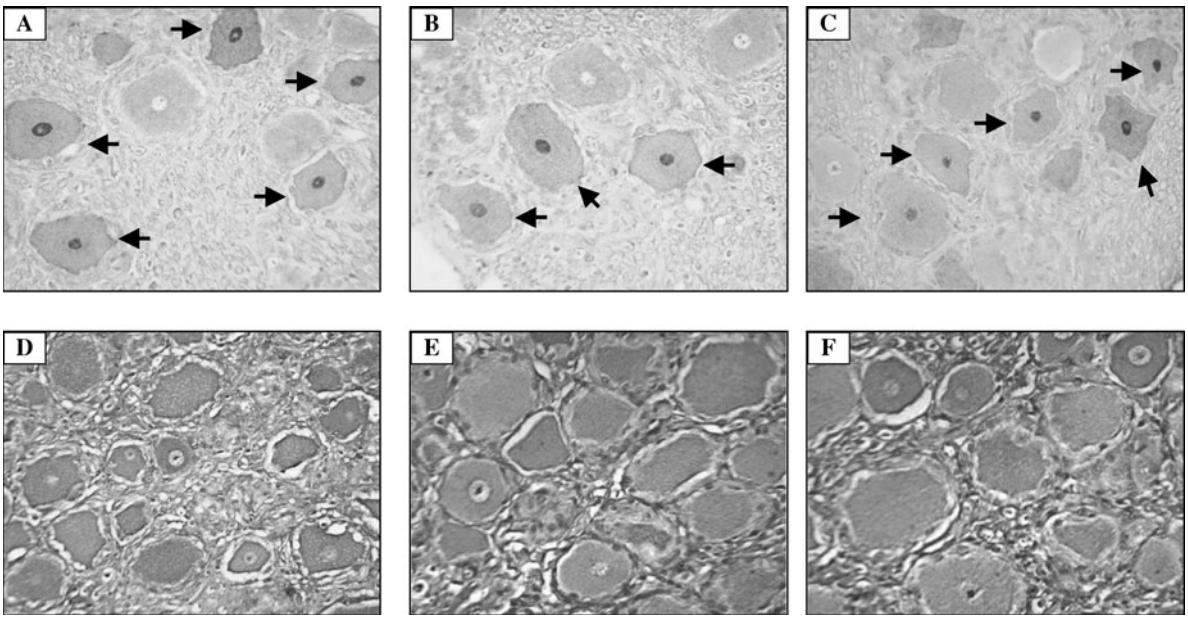

Figure 5 ORF-E is expressed in TG of latently infected calves. TG thin sections were prepared from two different latently infected calves (60 days after infection). (**A** and **B**) Sections were stained with the ORF-E peptide antiserum (1:1000 dilution). (**C**) Sections were stained with the ORF-E polyclonal antiserum (1:200 dilution). (**D**) TG sections from mock-infected calves were probed with the ORF-E polyclonal antiserum (1:100 dilution). (**E** and **F**) A bICP0-specific antibody (1:100 dilution) was incubated with TG thin sections prepared from latently infected calves. This antibody specifically reacts with the bICP0 protein (Inman *et al*, 2002; Zhang and Jones, 2001, 2005). Magnification was 20× for **A** to **D**, and 40× for **E** and **F**. Arrows denote neurons that were positively stained.

from TG of these calves at 60 days after infection (Inman *et al*, 2004). As another confirmatory assay to prove these calves were latently infected, TG sections were stained with an antibody that specifically recognizes bICP0 (Zhang and Jones, 2005). The bICP0 antiserum did not react with TG sections prepared from latently infected calves (Figure 5E and F), adding further support that these calves were latently infected. In summary, this study suggested that a protein encoded by ORF-E was expressed in TG neurons of calves latently infected with BHV-1.

ORF-E protein expression during acute infection and reactivation from latency

Additional studies were performed to test whether ORF-E was expressed in TG of acutely infected calves. Seven days after infection is the peak of acute infection in calves (Inman *et al*, 2001, 2002; Schang and Jones, 1997; Winkler *et al*, 2002), and thus this time was used for examining ORF-E expression in TG. For these studies, the ORF-E peptide antiserum was used. ORF-E protein expression was detected in a subset of neurons at 7 days after infection (Figure 6A and B). As was observed during latency (Figure 6D), nuclei of positive neurons were stained with the ORF-E peptide antiserum. If TG sections prepared from mock-infected calves were incubated with the ORF-E peptide antiserum, staining was not detected (Figure 6C).

Reactivation from latency can be initiated in latently infected calves or rabbits if a single injection of DEX is given (Ackermann *et al*, 1982; Inman *et al*, 2002; Jones *et al*, 2000; Rock *et al*, 1992; Sheffy and Davies, 1972). Because LR-RNA expression is dramatically reduced during reactivation from latency (Rock *et al*, 1992), we tested whether ORF-E protein expression was reduced in calves latently infected with BHV-1 following DEX treatment. At 24 h after DEX treatment, we detected ORF-E protein expression near the periphery of TG neurons using the ORF-E peptide antiserum (Figure 6E). It did not appear that the neuron itself was stained; rather satellite or infiltrating lymphocytes were apparently stained. At 48 h after DEX treatment, a few neurons were detected in which ORF-E was detected in the nucleus (Figure 6F).

Previous studies demonstrated that a subset of TG neurons express a protein recognized by a peptide antibody directed against the LR ORF-2 (Jiang *et al*, 1998). Studies were performed to compare the percentage of neurons that express ORF-E versus ORF-2. A polyclonal antibody directed against ORF-2 (Jiang *et al*, 2004) was used for these studies because this is the first ORF downstream of the LR-RNA, and ORF-2–specific antibodies specifically recognize a subset of neurons during latency (Jiang *et al*, 1998). These studies indicated that during acute infection (7 days after infection), approximately 6% of the total neurons were stained with the ORF-E peptide antiserum, but less than 1% of the total neurons were stained with the ORF-2 antiserum (Figure 7). During latency (60 days after infection), approximately 8% of the total neurons were stained with the ORF-E antiserum. In contrast, only 2% of the total neurons were ORF-2

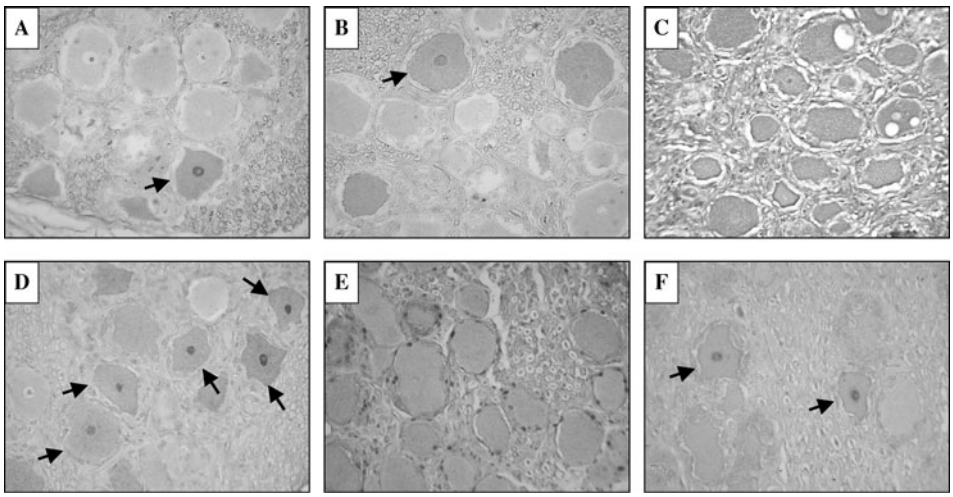

Figure 6 Analysis of ORF-E protein expression during acute infection, and reactivation from latency. TG thin sections were prepared from acutely infected calves (7 days after infection; **A**, **B**). (**C**) TG from mock-infected calf. (**D**) TG section from a latently infected calf (60 days after infection). (**E**) TG section from a latently infected calf (60 days after infection) that was treated with 100 mg DEX (IV injection) for 24 h to initiate reactivation from latency (Inman, 2002). (**F**) TG section from a latently infected calf (60 days after infection) that was treated with 100 mg DEX (IV injection) for 48 h to initiate reactivation from latency. TG sections were stained with the ORF-E peptide antibody (1:1000 dilution). Magnification for all sections was 20×. The images are representative of many slides that were obtained from at least two calves for each time point. Arrows denote cells positively stained by the antiserum.

positive. At 48 h after dexamethasone induced reactivation from latency, ORF-2 protein expression was not detected, which correlated with a dramatic reduction in the levels of LR-RNA (Rock *et al*, 1992). To avoid the uncertainty of whether ORF-E was detected in neurons or non-neuronal cells at 24 h DEX treatment, only those samples at 48 h after DEX treatment were used to count ORF-E positive neurons. Although the percentage of neurons that expressed ORF-E was reduced, we were able to detect ORF-E protein expression in TG neurons following DEX-induced reactivation from latency. In summary, these studies suggested that a higher percentage of TG neurons expressed detectable levels of ORF-E protein relative to ORF-2.

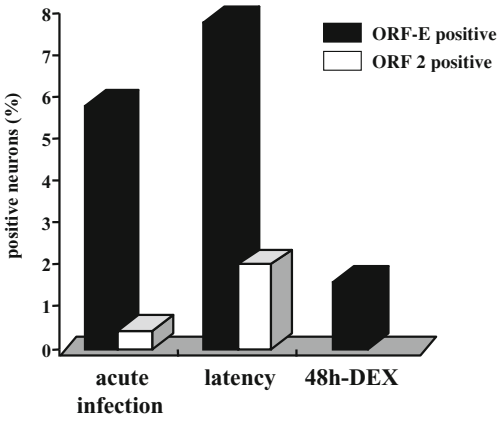

Figure 7 Estimation of the % of neurons that express ORF-E. The number of neurons expressing ORF-E or ORF-2 in four fields of three different TG sections was counted to estimate the % of neurons expressing each of the respective proteins. Approximately 1000 neurons were counted for each antibody. ORF-E–positive neurons are denoted by the black column, and ORF-2 by the white column. For these studies, TG from two different latently infected calves were used.

Discussion

Collectively, this study and other published studies (Jiang *et al*, 1998; Perez *et al*, 2006) suggested at least two viral proteins, ORF-E and proteins encoded by the LR gene, are expressed in a subset of TG neurons during BHV-1 latency. This is in sharp contrast to HSV-1 latency because the latency-associated transcript does not appear to encode a protein (Jones, 2003). We hypothesize that proteins encoded by ORF-E and the LR gene have the potential to be recognized by the immune system. For long-term latency to occur, a mechanism must exist that inhibits immune recognition of neurons expressing ORF-E or LR proteins. LR gene products, directly or indirectly, may be one factor that inhibits immune recognition in TG because higher inflammatory infiltrates are detected in TG of calves acutely infected with the LR mutant versus wt BHV-1 (Perez *et al*, 2006).

The ORF-E protein was primarily expressed in the nucleus of TG sensory neurons and transfected neuro-2A cells, whereas in non-neural cell types ORF-E was primarily in the cytoplasm. Most nuclear proteins contain a nuclear localization signal (NLS) that has one or two clusters of basic amino acids (K/R) (Kalderon *et al*, 1984; Lanford and Butel, 1984; Richter *et al*, 1985; Robbins *et al*, 1991). Although ORF-E contains one motif (RKRGK) that resembles a NLS (Figure 1C), it is not known if this

motif functions as a NLS or whether neuronal factors promote ORF-E nuclear localization. The presence of ORF-E in the nucleus of neuronal cells suggested ORF-E is a regulatory protein that may stimulate neurite-like growth in neuro-2A cells. However, ORF-E probably does not promote true neurite outgrowth because neurite formation is generally associated with terminal differentiation and cell cycle withdraw. Thus, ORF-E may cooperate with other cellular factors to promote neurite outgrowth following infection.

We assumed ORF-2 would be expressed in a higher percentage of TG neurons during latency because it is the first ORF within the LR gene. However, our studies suggested ORF-E was expressed in a higher percentage of neurons. Although one could argue that the ORF-E antibody had a higher titer than the ORF-2 antibody, this was not the case when Western blot studies were performed with ORF-E or ORF-2 fusion proteins overexpressed from a Baculovirus vector (data not shown). Unless the ORF-E antiserum works better with formalin-fixed and paraffin-embedded sections of TG, there is not a dramatic difference in the titers of the ORF-E or ORF-2 antiserum used for these studies.

The HSV-1 AL (antisense to LAT) gene is present in an analogous position on the HSV-1 genome as the ORF-E gene (Perng *et al*, 2002). An AL protein has been described (Perng *et al*, 2002); but there is virtually no similarity between ORF-E and the AL protein. To date, it is not known if the AL protein or RNA is expressed in latently infected TG, or if the AL gene stimulates neurite-like projections in neuro-2A cells.

Does ORF-E play a role in the latency-reactivation cycle of BHV-1? Although we believe that ORF-E plays a role in the latency-reactivation cycle, it is not essential because mutating the LR gene inhibits reactivation from latency (Inman *et al*, 2002), in part, by reducing the number of apoptotic neurons at the end of acute infection (Lovato *et al*, 2003). The LR mutant virus that was constructed contains stop codons at the 5′ terminus of the first LR ORF (Inman *et al*, 2001), and consequently the LR mutant virus can synthesize ORF-E transcripts. When latently infected calves or rabbits are treated with DEX for 24 h, extensive viral gene expression occurs in TG neurons, and then infectious virus is detected in nasal or ocular swabs (Rock *et al*, 1992; Winkler *et al*, 2000b, 2002). It is unlikely that ORF-E directly enhances reactivation from latency because the number of neurons expressing ORF-E decrease during the first 24 h after DEX treatment of latently infected calves. ORF-E also does not appear to directly inhibit apoptosis in infected neurons because ORF-E does not inhibit apoptosis in transient transfection assays (data not shown). Collectively, these studies suggest that ORF-E has an ancillary role in mediating certain aspects of the establishment and or maintenance of of long-term latency. We further predict that the ability of ORF-E to induce neurite-like growth in neurons plays a role in restoring mature neuronal functions after infection. To definitively prove what role ORF-E plays in the latency-reactivation cycle of BHV-1, it will be necessary to construct an ORF-E–null mutant BHV-1 strain, and an ORF-E/LR mutant virus. These respective mutant virus strains will then be compared to the phenotype of the LR mutant virus and wt BHV-1.

Materials and methods

Cells and virus

Bovine kidney cells (MDBK, ATCC CCL-22) were grown in Earl's modified Eagle's medium supplemented with 5% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 μg/ml). MDBK cells were split 1:6 every 2 to 3 days.

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

Neuro-2A cells (ATCC catalogue CCL131) are mouse neuroblastoma cells, which were grown in Earle's minimal essential medium supplemented with 10% FBS, penicillin (10 U/ml), and streptomycin (100 μg/ml).

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 1×10^7 pfu/ml of virus in each nostril and eye, dropwise without scarification, for a total of 4×10^7 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 these calves were not shedding infectious virus from the nasal cavity or the eye (Inman *et al*, 2002; Perez *et al*, 2005; Winkler *et al*, 2000a, 2000b, 2002). DEX was used to initiate reactivation from latency as described previously (Inman *et al*, 2002). TG were fixed in formalin, paraffin embedded, and thin sections cut for immunohistochemistry.

Cloning ORF-E into plasmid vectors

A plasmid containing the entire LR region (Figure 1) was digested to completion with PstI. The fragment containing ORF-E was agarose gel purified and used

for ligation. The correct orientation of the PstI insert was determined by restriction digest. To study cellular localization of ORF-E expression, ORF-E was cloned into the mammalian expression vector, Monster Green Fluorescent Protein (phMGFP Vector; Promega, catalogue number E6421) to generate an ORF-E–GFP fusion protein. PCR was performed on viral genomic DNA using primers that contained unique restriction sites for amplification of ORF-E as previously described (Inman *et al*, 2004).

Antibody production

To generate a recombinant Baculovirus that expresses ORF-E, a PCR-amplified product (Inman *et al*, 2004) was cloned into pBlueBacHis2 such that ORF-E was in frame with the histidine tag. A recombinant baculovirus was then constructed that expressed the ORF-E fusion protein. The recombinant baculovirus strain was grown in SF9 insect cells using procedures described by Invitrogen. Recombinant baculovirus ORF-E constructs were characterized by testing for ORF-E protein expression using the Express antibody that recognizes the X-press epitope at the 5′ terminus of LR protein sequences (R910-25; Invitrogen). Large-scale expression was carried out in 2-L flasks seeded with SF9 cells at a density of 2×10^6 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 ORF-E fusion protein was partially purified using nickel chromatography. The ORF-E fusion protein was further purified using preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the fusion protein band excised, eluted, and then injected into rabbits to generate a specific polyclonal antiserum (Animal Pharm Services; CA). This serum is referred to as the ORF-E polyclonal antiserum throughout the text. The antiserum directed against LR ORF-2 was previously described (Jiang *et al*, 2004).

A peptide corresponding to the immunogenic region of ORF-E was synthesized and a polyclonal antibody directed against this peptide generated in rabbits by Biosource (Camarillo, CA). The immunogenic region of ORF-E was predicted using programs on the ExPASy website (ProtScale) and the PredictProtein server (http://www.predictprotein.org). This serum is referred to as the ORF-E peptide antiserum throughout the text.

Confocal microscopy to examine subcellular localization of ORF-E

MDBK cells were cultured in 4-well Lab-Tek slides, and then infected for 21 h with wt BHV-1 (MOI = 0.75 pfu/cell). Cultures 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), slides were blocked in 3% bovine serum albumin (BSA) in TBS for 1 h and then incubated with the primary antibodies for 2 h at room temperature (RT). The primary antibody consisted of a mixture of anti-ORF-E peptide antibody (dilution 1:100) and anti-BHV-1 antibody (dilution 1:100) (American BioResearch Laboratories) in TBS 0.05% Tween 20 (TBS-T), 1% BSA. Three washes of 10 min each with TBS-T were followed by incubation with secondary antibody mix for 1 h at RT in the dark. The secondary antibody mixture consisted of donkey anti-rabbit immunoglobulin G (IgG) conjugated to Cy2 (dilution 1:50) and goat anti-donkey IgG conjugated to Cy5 (dilution 1:50). Three washes of 10 min each with TBS-T were followed by incubation with DAPI stain for 10 min. Slides were then mounted with 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.

Transient expression and Western blot analysis

Neuro-2A cells were transfected with 10 μg of the respective expression plasmids using TransIT transfection reagents (Mirus; Madison, WI) as described by the manufacturer. At the designated times after transfection, cells were collected, and lysed in 500 μl of 1 × SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β–mecaptoethanol). Cell extract was boiled for 5 minutes and the supernatant used for SDS-PAGE. Western blots were performed as described previously using the designated antibodies (Henderson *et al*, 2002; Jiang *et al*, 1998).

Immunohistochemistry

Tissue sections were deparaffinized, rehydrated in graded ethanol, and then treated with 3% hydrogen peroxide in phosphate-buffered saline (PBS) (pH 7.4) for 10 min to inactivate endogenous peroxidase. After washing in PBS, tissue sections were digested with proteinase K (DAKO) for 20 minutes at 37°C. Nonspecific binding was blocked by incubation with 5% normal goat serum, 0.25% BSA in PBS for 45 min at room temperature. Endogenous biotin was blocked by treatment of sections with avidin/biotin blocking reagents (Vector Labs). Tissue samples were incubated overnight at 4°C with the ORF-E polyclonal antiserum (1:100 or 1:200 dilution), or with the ORF-E peptide antiserum (1:1000 dilution). Slides were washed three times in PBS before addition of biotinylated secondary anti-rabbit immunoglobulin G (IgG) antibody (Vector Labs) for 30 min at room temperature. Slides were then incubated with freshly prepared substrate (Vector NovaRed substrate kit for peroxidase; Vector Labs), rinsed with distilled water, and counterstained in methyl green. Tissues from mock-infected calves, and incubation of infected tissue sections in the absence of primary or secondary antibodies, were used as controls to confirm the specificity of the test.

References

Ackermann M, Peterhans E, Wyler R (1982). DNA of bovine herpesvirus type 1 in the trigeminal ganglia of latently infected calves. *Am J Vet Res* **43:** 36–40.

Bowland SL, Shewen PE (2000). Bovine respiratory disease: commercial vaccines currently available in Canada. *Can Vet J* **41:** 33–48.

Bratanich AC, Hanson ND, Jones CJ (1992). The latency-related gene of bovine herpesvirus 1 inhibits the activity of immediate-early transcription unit 1. *Virology* **191:** 988–991.

Bratanich AC, Jones CJ (1992). Localization of cis-acting sequences in the latency-related promoter of bovine herpesvirus 1 which are regulated by neuronal cell type factors and immediate-early genes. *J Virol* **66:** 6099–6106.

Ciacci-Zanella J, Stone M, Henderson G, Jones C (1999). The latency-related gene of bovine herpesvirus 1 inhibits programmed cell death. *J Virol* **73:** 9734–9740.

Delhon G, Jones C (1997). Identification of DNA sequences in the latency related promoter of bovine herpes virus type 1 which are bound by neuronal specific factors. *Virus Res* **51:** 93–103.

Devireddy LR, Jones C (1998). Alternative splicing of the latency-related transcript of bovine herpesvirus 1 yields RNAs containing unique open reading frames. *J Virol* **72:** 7294–7301.

Geiser V, Jones C (2005). The latency related gene encoded by bovine herpesvirus 1 encodes a small regulatory RNA that inhibits cell growth. *J NeuroVirol* **11:** 563–570.

Henderson G, Peng W, Jin L, Perng G-C, Nesburn AB, Wechsler SL, Jones C. (2002). Regulation of caspase 8- and caspase 9-induced apoptosis by the herpes simplex virus latency-associated transcript. *J NeuroVirol* **8:** 103–111.

Hossain A, Schang LM, Jones C (1995). Identification of gene products encoded by the latency-related gene of bovine herpesvirus 1. *J Virol* **69:** 5345–5352.

Inman M, Lovato L, Doster A, Jones C (2001). A mutation in the latency-related gene of bovine herpesvirus 1 leads to impaired ocular shedding in acutely infected calves. *J Virol* **75:** 8507–8515.

Inman M, Lovato L, Doster A, Jones C (2002). A mutation in the latency related gene of bovine herpesvirus 1 interferes with the latency-reactivation cycle of latency in calves. *J Virol* **76:** 6771–6779.

Inman M, Zhou J, Webb H, Jones C (2004). Identification of a novel transcript containing a small open reading frame that is expressed during latency, and is antisense to the latency related gene of bovine herpes virus 1 (BHV-1). *J Virol* **78:** 5438–5447.

Jiang Y, Hossain A, Winkler MT, Holt T, Doster A, Jones C (1998). A protein encoded by the latency-related gene of bovine herpesvirus 1 is expressed in trigeminal ganglionic neurons of latently infected cattle and interacts with cyclin-dependent kinase 2 during productive infection. *J Virol* **72:** 8133–8142.

Jiang Y, Inman M, Zhang Y, Posadas NA, Jones C (2004). A mutation in the latency related gene of bovine herpesvirus 1 (BHV-1) inhibits protein expression of a protein from open reading frame 2 (ORF-2) and an adjacent reading frame during productive infection. *J Virol* **78:** 3184–3189.

Jones C (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Micro Rev* **16:** 79–95.

Jones C, Delhon G, Bratanich A, Kutish G, Rock D (1990). Analysis of the transcriptional promoter which regulates the latency-related transcript of bovine herpesvirus 1. *J Virol* **64:** 1164–1170.

Jones C, Newby TJ, Holt T, Doster A, Stone M, Ciacci-Zanella J, Webster CJ, Jackwood MW (2000). Analysis of latency in cattle after inoculation with a temperature sensitive mutant of bovine herpesvirus 1 (RLB106). *Vaccine* **18:** 3185–3195.

Kalderon D, Roberts BL, Richardson WD, Smith AE (1984). A short amino acid sequence able to specify nuclear location. *Cell* **39:** 499–509.

Kutish G, Mainprize T, Rock D (1990). Characterization of the latency-related transcriptionally active region of the bovine herpesvirus 1 genome. *J Virol* **64:** 5730–5737.

Lanford RE, Butel JS (1984). Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* **37:** 801–813.

Lovato L, Inman M, Henderson G, Doster A, Jones C (2003). Infection of cattle with a bovine herpesvirus 1 (BHV-1) strain that contains a mutation in the latency related gene leads to increased apoptosis in trigeminal ganglia during the transition from acute infection to latency. *J Virol* **77:** 4848–4857.

Perez S, Inman M, Doster A, Jones C (2005). Latency-realated gene encoded by bovine herpesvirus 1 promotes virus growth and reactivation from latency in tonsils of infected calves. *J Clin Micro* **43:** 393–401.

Perez, SL Lovato, Zhou J, Doster A, Jones C (2006). Comparison of inflammatory infiltrates in trigeminal ganglia of cattle infected with wild type BHV-1 versus a virus strain containing a mutaition in the LR (latency-related) gene. *J Neurovirol* **12:** 392–397.

Perng G-C, Maguen B, Jing L, Mott KR, Osorio N, Slanina SM, Yukht A, Ghiasi H, Nesburn AB, Inman M, Henderson G, Jones C, Wechsler SL (2002). A novel herpes simplex virus type 1 (HSV-1) transcript (AL-RNA) antisense to the 5′ end of LAT (latency associated transcript) produces a protein in infected rabbits. *J Virol* **76:** 8003–8010.

Richter JD, Young P, Jones NC, Krippl B, Rosenberg M, Ferguson B (1985). A first exon-encoded domain of E1A sufficient for posttranslational modification, nuclear localization, and induction of adenovirus E3 promoter expression in Xenopus oocytes. *Proc Natl Acad Sci USA* **82:** 8434–8438.

Robbins J, Dilworth SM, Laskey RA, Dingwall C (1991). Two independent basic domains in nucleoplasmin nuclear tageting sequences: identification of a class of bipartite nuclear targeting sequences. *Cell* **64:** 615–623.

Rock D, Lokensgard J, Lewis T, Kutish G (1992). Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. *J Virol* **66:** 2484–2490.

Rock DL, Beam SL, Mayfield JE (1987). Mapping bovine herpesvirus type 1 latency-related RNA in trigeminal ganglia of latently infected rabbits. *J Virol* **61:** 3827–3831.

Schang LM, Hossain A, Jones C (1996). The latency-related gene of bovine herpesvirus 1 encodes a product which inhibits cell cycle progression. *J Virol* **70:** 3807–3814.

Schang LM, Jones C (1997). Analysis of bovine herpesvirus 1 transcripts during a primary infection of trigeminal ganglia of cattle. *J Virol* **71:** 6786–6795.

Sheffy BE, Davies DH (1972). Reactivation of a bovine herpesvirus after corticosteroid treatment. *Proc Soc Exp Biol Med* **140:** 974–976.

Tikoo SK, Campos M, Babiuk LA (1995). Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. *Adv Virus Res* **45:** 191–223.

Winkler MT, Doster A, Sur JH, Jones C (2002). Analysis of bovine trigeminal ganglia following infection with bovine herpesvirus 1. *Vet Microbiol* **86:** 139–155.

Winkler MT, Schang LS, Doster A, Holt T, Jones C (2000a). Analysis of cyclins in trigeminal ganglia of calves infected with bovine herpesvirus-1. *J Gen Virol* **81:** 2993–2998.

Winkler MTC, Doster A, Jones C (2000b). Persistence and reactivation of bovine herpesvirus 1 in the tonsil of latently infected calves. *J Virol* **74:** 5337–5346.

Wirth UV, Fraefel C, Vogt B, Vlcek C, Paces V, Schwyzer M (1992). Immediate-early RNA 2.9 and early RNA 2.6 of bovine herpesvirus 1 are 3′ coterminal and encode a putative zinc finger transactivator protein. *J Virol* **66:** 2763–2772.

Zhang Y, Jones C (2001). The bovine herpesvirus 1 immediate-early protein (bICP0) associates with histone deacetylase 1 to activate transcription. *J Virol* **75:** 9571–9578.

Zhang Y, Jones C (2005). Identification of functional domains within the bICP0 protein encoded by BHV-1. *J Gen Virol* **86:** 879–886.