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A Protein Encoded by the Bovine Herpesvirus 1 Latency-Related Gene Interacts with Specific Cellular Regulatory Proteins, Including CCAAT Enhancer Binding Protein Alpha

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Following acute infection, bovine herpesvirus 1 establishes latency in sensory neurons of trigeminal ganglia (TG). Reactivation from latency occurs periodically, resulting in the shedding of infectious virus. The latency-related (LR) RNA is abundantly expressed in TG of latently infected calves, and the expression of LR proteins is necessary for dexamethasone-induced reactivation from latency. Previously published studies also identified an alternatively spliced LR transcript which is abundantly expressed in TG at 7 days after infection and has the potential to encode a novel LR fusion protein. Seven days after infection is when excessive viral gene expression is extinguished in TG and latency is established, suggesting that LR gene products influence the establishment of latency. In this study, we used a bacterial two-hybrid assay to identify cellular proteins that interact with the novel LR fusion protein. The LR fusion protein interacts with two proteins that can induce apoptosis (Bid and Cdc42) and with CCAAT enhancer binding protein alpha (C/EBP-α). Additional studies confirmed that the LR fusion protein interacts with human or insect C/EBP-α. C/EBP-α protein expression is induced in TG neurons of infected calves and after dexamethasone-induced reactivation from latency. Wild-type C/EBP-α, but not a DNA binding mutant of C/EBP-α, enhances plaque formation in bovine cells. We hypothesize that interactions between the LR fusion protein and C/EBP-α promote the establishment of latency.

Bovine herpesvirus-1 (BHV-1) is an Alphaherpesvirinae subfamily member (35) that establishes lifelong latency in ganglionic neurons of the peripheral nervous system following acute replication in the mucosal epithelium. Infection of permissive cells (13) or calves (65) leads to rapid cell death, in part due to apoptosis. Infection can also cause conjunctivitis, pneumonia, genital disorders, abortions, and a complex upper respiratory tract infection referred to as shipping fever (bovine respiratory complex) (59). BHV-1 transiently depresses cell-mediated immunity (8, 21–23), resulting in secondary bacterial infections and pneumonia (shipping fever). BHV-1 infection and shipping fever cost the United States cattle industry at least $500 million/year (5, 46). Although modified live vaccines are available, they can cause disease in young calves or abortion in pregnant cows.

Viral gene expression is regulated in three distinct phases: immediate early (IE), early (E), or late (L). IE gene expression is stimulated by a virus component, alpha trans-inducing factor (44), and bICP0. The bICP0 protein is constitutively expressed throughout productive infection because the gene has an IE and E promoter. The bICP0 protein trans-activates all viral promoters and thus promotes efficient productive infection (reviewed in references 36 and 38). IE proteins activate E gene expression, and viral DNA replication ensues. L gene expression then occurs, culminating in virion production.

The BHV-1 latency-related (LR) RNA is abundantly transcribed in latently infected neurons (40, 54, 55) and is antisense to the bICP0 gene. 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) (Fig. 1, panel II). A fraction of LR RNA is polyadenylated and alternatively spliced in trigeminal ganglia (TG), suggesting that different LR proteins are expressed at specific times during the latency-reactivation cycle (13, 27) (Fig. 1, panel I). One such transcript is expressed in TG at 7 days after infection (7d cDNA) and has the potential to encode an ORF-2/RF-C fusion protein (Fig. 1, panel III, LR fusion protein) (11, 12). When the 7d cDNA is expressed in insect cells from a recombinant baculovirus, a 40-kDa protein is expressed (33).

A mutant BHV-1 strain with three stop codons at the N terminus of ORF-2 (LR mutant virus) was constructed (30). ORF-2 and RF-C are expressed in bovine cells infected with wild-type (wt) or the LR-rescued virus but not in cells infected with the LR mutant virus (33). Calves infected with the LR mutant exhibit diminished clinical symptoms and reduced shedding of infectious virus from the eye, TG, or tonsil (30, 31, 51). The LR mutant virus does not reactivate from latency following treatment with dexamethasone (DEX) (31), whereas all calves latently infected with wt virus or the LR-rescued virus shed infectious virus following DEX treatment (31). Thus, disruption of LR protein expression is necessary for DEX-induced reactivation from latency in calves. LR gene products inhibit cell proliferation, bICP0 RNA expression (6, 18, 56), and apoptosis (10). LR protein expression is necessary for inhibiting apoptosis but not for cell growth or bICP0 expression (6, 16, 18, 56). It is not clear whether LR protein expres-
sion enhances the establishment or maintenance of latency and/or reactivation from latency.

In this study, we performed a two-hybrid screening to identify cellular proteins that interact with the LR fusion protein. Several such cellular proteins were identified. Two of these proteins, Bid and Cdc42, can induce apoptosis. Since the LR gene inhibits apoptosis (10, 43), the ability of an LR protein to interact with proapoptotic cellular proteins was expected. The LR fusion protein also interacts with CCAAT enhancer binding protein alpha (C/EBP-H9004) in the two-hybrid assay. Independent studies confirmed that the LR fusion protein interacts with insect or human C/EBP-H9004.

MATERIALS AND METHODS

Cells and virus. Bovine kidney (MDBK) cells were grown in Earle’s modified Eagle’s medium supplemented with 5% fetal calf serum (FCS). Human neuroblastoma cells (SK-N-SH) and bovine testicle cells (9.1.3) were grown in Earle’s modified Eagle’s medium supplemented with 10% FCS. Spodoptera frugiperda (SF9) insect cells were maintained in Grace’s supplemented insect medium (Gibco) with 10% FCS. All media contained penicillin (10 U/ml) and streptomycin (100 μg/ml).

The wt Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services (Ames, IA). The BHV-1 gC-blue virus contains the β-galactosidase gene inserted into the glycoprotein C gene, which was provided by S. Chowdhury (Kansas State University). A recombinant baculovirus expressing the 7d cDNA sequence (His66) was described previously (33), and His66 was used to infect SF9 insect cells.

Plasmids. pBT7day was generated by cloning the SphI fragment of the 7d cDNA (33) into the bait plasmid pBT. The BHV-1 gC-blue virus contains the β-galactosidase gene inserted into the glycoprotein C gene, which was provided by S. Chowdhury (Kansas State University). A recombinant baculovirus expressing the 7d cDNA sequence (His66) was described previously (33), and His66 was used to infect SF9 insect cells.

Plasmid pC/EBP-α 290nts contains a mutation that produces a single amino acid change (R290A) in C/EBP-α, which results in loss of DNA binding activity (61). This mutant is referred to as C/EBP-αDB in this study.
The hICP0 plasmid encodes hICP0 coding sequences cloned into a human cytomegalovirus expression vector.

**Bacterial two-hybrid assay.** All procedures were performed according to recommendations of the manufacturers of the BacterioMatch II two-hybrid system (Stratagene). An E. coli reporter strain was co-transformed with pBT7day and pTRG containing the rat brain cDNA library. Cotransformants were screened by using medium lacking histidine and containing 5-aminotriazole, as recommended by the manufacturer. Positive interactions were further confirmed by activation of the streptomycin resistance gene on selective plates. The pTRG plasmid derived from positive interactions was isolated by standard methods. DNA sequence reactions of the respective inserts in pTRG were performed at the University of Nebraska DNA sequencing core facility. A BLAST search at the NCBI website was performed to identify proteins encoded by the respective cDNAs.

**Binding assays.** The His66 recombinant baculovirus, containing the 7d cDNA expressed as a histidine-tagged protein (33), was used to infect SF9 cells. Cells were collected when the cytopathic effect was 70 to 80%, lysed, and then loaded on a 2-mI N\textsuperscript{7} column under (i) native or (ii) denaturing conditions, according to the manufacturer’s recommendations (Probebound, K850-01; Invitrogen).

(i) SF9 cells (infected or uninfected) were suspended in native binding buffer (20 mM NaPO\textsubscript{4}, 500 mM NaCl, 10 mM imidazole [pH 8.0]), followed by three freeze-thaw cycles and a final 10-min spin at 3,000 \texttimes g. Eleven milligrams of protein was loaded onto the Ni\textsuperscript{7} column equilibrated with native binding buffer and allowed to bind for 1 h at 4°C. Three washes of 10 ml each were performed using native wash buffer (50 mM NaPO\textsubscript{4}, 500 mM NaCl, 20 mM imidazole [pH 8.0]).

(ii) Lysis of SF9 cells (infected or uninfected) was conducted under denaturing conditions by suspending cells in a solution of 6 M guanidine-HCl, 20 mM NaPO\textsubscript{4}, 7.8, and 500 mM NaCl. The cell lysate was passed through an 18-gauge needle to ensure total lysis. Eleven milligrams of total protein was loaded onto the Ni\textsuperscript{7} column equilibrated with denaturing binding buffer (8 M urea, 20 mM NaPO\textsubscript{4}, 500 mM NaCl [pH 7.8]) and allowed to bind for 1 h at 4°C. The column was washed with denaturing binding buffer (pH 7.8 or 6 or 5.6) (two washes for each pH 4 ml in each wash).

After SF9 proteins bound under denaturing conditions, the Ni\textsuperscript{7} column was equilibrated in native binding buffer (see above) and blocked for 30 min with 0.01% bovine serum albumin (BSA) in native binding buffer at 4°C. SK-N-SH cells were lysed in NP-40 lysis buffer, and 3 mg of protein was applied to the column, which was incubated for 1 h at 4°C. Finally, native wash buffer was applied to the column (three 10-ml washes).

In all cases, the contents of the column were analyzed by boiling the beads in sodium dodecyl sulfate (SDS) reducing sample buffer, and the released proteins were loaded on a 12% SDS-polyacrylamide gel electrophoresis (PAGE) gel. After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore), and Western blotting was performed using an anti-C/EBP-\(\alpha\) specific antibody (1:4AA; Santa Cruz Biotechnology).

**SDS-PAGE and Western blotting.** Standard 12% SDS-PAGE gels were prepared using the Laemmli system. Proteins were transferred to PVDF membrane (Millipore), blocked for 1 h by using 5% nonfat dry milk with phosphate-buffered saline–0.05% Tween 20 (PBS-T), and incubated with primary antibody overnight at 4°C. The C/EBP-\(\alpha\) antibody was diluted 1:500 in the blocking solution. After 30 min of washing with PBS-T, the blots were incubated with donkey anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (Amersham Biosciences), which was diluted 1:1,000 in nonfat dry milk with PBS-T. Blots were washed for 30 min with PBS-T and exposed to Amersham ECL reagents, and autoradiography was performed.

**Immunohistochemistry.** wt-infected calves were euthanized, and their TG were fixed in neutral buffered formalin and then embedded in paraffin. Thin sections (4 to 5 \textmu m) were cut and mounted onto slides. Tissue sections were incubated for 20 min at 65°C, followed by two incubations of 10 min in xylene and rehydration in graded alcohols. Tissue sections were then incubated with 3% hydrogen peroxide in PBS (pH 7.4) for 20 min at room temperature to block endogenous peroxidase. After three washes in Tris-buffered saline (TBS; 5 min each) at room temperature, tissue sections were digested with 0.05% Protease XIV (P-5147; Sigma) in Tris buffer (0.05 M Tris, pH 7.6) for 20 min at 37°C. Tissue sections were then blocked with 5% normal goat serum diluted in TBS containing 0.25% BSA for 45 min at room temperature in a humidified chamber. Primary anti-C/EBP-\(\alpha\) antibody (sc-14AA; Santa Cruz Biotechnology) was used at a 1:250 dilution in 1% blocking solution in PBS-T. The antibody was incubated for 1 h at room temperature in a humidified chamber. After three washes in TBS, slides were incubated with freshly prepared substrate (Vector NovaRed substrate kit for peroxidase; Vector Labs), rinsed with distilled water, and counterstained with hematoxylin. Thin sections from mock-infected calves were used as a negative control.

**RNA extraction.** Extraction of RNA was performed as described by Chomczynski and Sacchi (9). Briefly, TG were minced into small pieces, placed into 10 ml of solution D (4 M guanidine thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 14 mM \(\beta\)-mercaptoethanol), and homogenized. After two phenol extractions were performed, RNA concentrations were determined spectrophotometrically (260 nm) and RNA was reprecipitated in 3 volumes of ethanol.

RNA was prepared from MDBK cells using TRizol reagent as described previously (49, 50).

**DNase I treatment and reverse transcription.** Three micrograms of total RNA was treated with 1 U of DNase I (Invitrogen) for 15 min at room temperature in the presence of a RNase inhibitor (RNasin; Promega, Madison, WI). After DNase I treatment, samples were incubated at 65°C for 7.5 min in the presence of 2 mM EDTA to eliminate DNase I activity. One microgram of total RNA (DNase I treated) plus 1 \mu g of random hexamers were incubated at 65°C for 7.5 min and then chilled on ice. Sixteen microliters of ice-cold reverse transcription mix (20 mM Tris-HCl [pH 8.3], 50 mM KCI, 2.5 mM MgCl\textsubscript{2}, 100 \mu g BSA/ml, 1 mM dithiothreitol, 0.5 mM each deoxynucleoside triphosphates, 10 U of RNasin, and 100 U of reverse transcriptase (Invitrogen) was added. The reaction mixture was incubated for 10 min at 25°C and then for 50 min at 42°C. As a control for DNA contamination in RNA samples, 2 \mu g of RNA (DNase I treated) was mixed with ice-cold reverse transcription mix lacking RT.

**PCR:** An aliquot (2 \mu l) of the reverse transcription reaction mixture was used for a PCR using primers specific for bovine C/EBP-\(\alpha\) (5′-AACCTCCATAACAAAGACAGA and -GTGCTATTGCACTGCTGGC), Bovine growth hormone (GH) primers (5′-GCTTTCTCGGTCTCTGCGC and -TCTTGCCTCCTCCACCCCTA) were used as an internal control. PCRs were carried out in 50-\mu l reactions containing 5 mM Mg\textsubscript{2+}, 200 \mu M concentrations of each deoxynucleoside triphosphate, 1 \mu M concentrations of each primer, and 1 U Taq polymerase. Five microliters of GC melt (Advantage GC-2; BD Biosciences) was then added to the reaction mixture. Amplification was carried out with 37 cycles of denaturing at 95°C for 45 s, annealing at 53°C for 45 s, and extending at 72°C for 1 min. Upon completion of the last cycle, reactions were further incubated at 72°C for 10 min to ensure complete extension of the amplified product. PCR products were electrophoresed by using a 2% agarose gel.

**Cell lysis and protein quantitation.** MDBK or SK-N-SH whole-cell lysate was prepared using NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl [pH 8], 150 mM NaCl). The NP-40 lysis buffer also contained 1 tablet of protease inhibitor cocktail (11 8361 53001; Roche) for each 10 ml of buffer. Protein concentrations were measured using a Bradford reagent kit (500-0006; Bio-Rad).

**Analysis of C/EBP-\(\alpha\) expression on plaque formation.** The gC\textsubscript{blue} virus grows to titers similar to those for wt BHV-1 and was grown in MDBK cells. Procedures for preparing BHV-1 genomic DNA have been described previously (18, 30–32). 9.1.3 cells were cotransfected with the gC\textsubscript{blue} viral DNA and one of plasmids encoding bICP0, C/EBP-\(\alpha\), or C/EBP-\(\beta\) (pcDNA3.1−) using Lipofectamine 2000 (Invitrogen). β-Galactosidase-negative (β-Gal+) cells were counted at 24 h after transfection as described previously (18, 30–32). The number of β-Gal+ cells in cultures transfected with pcDNA3.1− and gC\textsubscript{blue} genomic DNA was set at a value of 1 for each experiment. All other transfections were examined for β-Gal+ cells and are expressed as induction (\(\text{r}=\text{fold}\)) relative to the control. This representation of the data minimizes differences in cell density, variation in Lipofectamine 2000 lots, and transfection efficiency.

**RESULTS**

**Identification of cellular proteins that interact with the LR fusion protein.** The bacterial two-hybrid system was used to identify cellular proteins that bind to the LR fusion protein. A fragment containing the 7d cDNA (11) (Fig. 1) was cloned into the pBT vector such that the major ORF was in frame with Acl coding sequences (pBT7day). Since BHV-1 can replicate in cottontail rats (48), a commercially available rat brain cDNA library was used as target sequences. Cotransformation of E. coli with the pBT7day and pTRG plasmids resulted in several positive colonies. Rat cDNA inserts were sequenced, and BLAST analysis was performed to identify cellular proteins...
TABLE 1. Rat proteins that interact with the LR fusion protein encoded by 7d cDNA

<table>
<thead>
<tr>
<th>Description of protein</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptosis regulator</td>
<td></td>
</tr>
<tr>
<td>BH3 domain from Bid</td>
<td>BC023963</td>
</tr>
<tr>
<td>Cdc42 (GTPase)</td>
<td>NM009861</td>
</tr>
<tr>
<td>BCR-related gene</td>
<td>BC043673</td>
</tr>
<tr>
<td>NADH dehydrogenase 4, mitochondrial</td>
<td>BC020382</td>
</tr>
<tr>
<td>Transcription factor</td>
<td></td>
</tr>
<tr>
<td>CCAAT enhancer binding protein</td>
<td>BC011319</td>
</tr>
<tr>
<td>High mobility group nuclear protein 2 (Hmgn2)</td>
<td>NM016937</td>
</tr>
<tr>
<td>Topoisomerase 1 binding protein (Btid2)</td>
<td>XM354550</td>
</tr>
<tr>
<td>Other</td>
<td></td>
</tr>
<tr>
<td>Elongation factor 1-α1 homologue</td>
<td>AK088041</td>
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<tr>
<td>Ribosomal phosphoprotein P0</td>
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</tr>
<tr>
<td>Ubiquitin C</td>
<td>NM019639</td>
</tr>
<tr>
<td>B-cell and monocyte-activating chemokine precursor</td>
<td>AF144754</td>
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<tr>
<td>RIKEN cDNA 2610510H1q gene</td>
<td>BC005803</td>
</tr>
<tr>
<td>RIKEN cDNA D130012J15 gene</td>
<td>NM172271</td>
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</tbody>
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* Procedures for performing the BacterioMatch II two-hybrid screening are described in Materials and Methods.

that interacted with the LR fusion protein (Table 1). As expected, we did not obtain positive colonies when bacteria were transformed with pTRG or pBT7day alone.

Two cellular genes that encode proapoptotic proteins (the BH3 domain of Bid and Cdc42) and two other genes that encode proteins with potential roles in apoptosis (NADH dehydrogenase 4 and a BCR-related gene) were identified in the two-hybrid screening (Table 1). The Bcr protein is a GTPase-activating protein (14), suggesting that it activates the GTPase activity of Cdc42. Since the LR gene can inhibit apoptosis in cultured cells (10, 25) and TG of infected calves (43), we expected that LR proteins would interact with proapoptotic proteins. Determining the significance of the other binding proteins will require additional studies. We began by examining the significance of the LR fusion protein interacting with C/EBP-α.

The LR fusion protein interacts with human and insect C/EBP-α. The interaction between C/EBP-α and the BHV-1 LR fusion protein (Table 1) was of particular interest because Epstein-Barr virus (EBV), human herpesvirus 8 (HHV-8), and herpesvirus saimiri (HVS) productive infections are stimulated by C/EBP-α (28, 60, 62, 67). To confirm the interaction between the LR fusion protein and C/EBP-α, a recombinant baculovirus that expresses the LR fusion protein as a histidine-tagged protein (recombinant baculovirus His66) (33) was used. Because mammalian C/EBP-α shares significant homology with the Drosophila melanogaster homolog (64), we tested whether the insect C/EBP-α homolog was bound to the Histagged LR fusion protein. SF9 insect cells were infected with the recombinant baculovirus His66, and infected cells were lysed and then loaded onto an Ni²⁺ affinity column using native conditions to preserve protein-protein interactions. A Western blot analysis of proteins bound to the column demonstrated that insect C/EBP-α was retained in the Ni²⁺ column only when SF9 cells were infected with the recombinant baculovirus His66 (Fig. 2A, lane 4). We also noted that the insect C/EBP-α (approximately 27 kDa) (Fig. 2A) was smaller than the human homolog (approximately 33 kDa) (Fig. 2B).

To test whether the LR fusion protein interacted with mammalian C/EBP-α, the recombinant LR fusion protein was bound to the Ni²⁺ column under denaturing conditions. Whole-cell extract prepared from SK-N-SH cells was subsequently incubated with the LR fusion protein that was immobilized on the Ni²⁺ column. Under denaturing conditions, the insect C/EBP-α protein was not retained in the Ni²⁺ column (Fig. 2B, lane 2). When SK-N-SH cell extract was incubated with the column containing the LR fusion protein, a band of the expected size of human C/EBP-α (approximately 33 kDa) was...
was not bound to the column (Fig. 2B, lane 6). As expected, cell extract was incubated with the column, human C/EBP- 
protein interacted with human or insect C/EBP-
summary, these results demonstrated that the LR fusion pro-
retained in the Ni²⁺ column and was recognized by the 
C/EBP-α antibody (Fig. 2B, lane 5). In contrast, when only SF9 
cell extract was incubated with the column, human C/EBP-α was 
not bound to the column (Fig. 2B, lane 6). As expected, the 
human C/EBP-α protein was not retained on the Ni²⁺ 
column lacking the LR fusion protein (Fig. 2B, lane 4). In 
summary, these results demonstrated that the LR fusion protein 
interacted with human or insect C/EBP-α protein.

**Analysis of C/EBP-α protein expression following infection of bovine cells.** Since the LR fusion protein interacted with the 
C/EBP-α protein, we tested whether C/EBP-α protein levels were altered during viral infection. During the course of produc-
tive infection, a prominent 41-kDa protein in infected cells was 
induced; it was recognized by the C/EBP-α antibody (Fig. 3, lanes V). This 41-kDa protein is the approximate size of the 
long translational form of C/EBP-α (41, 47). The levels of a 
33-kDa band recognized by the C/EBP-α antibody increased in 
mock-infected MDBK cells as a function of time after plating 
(Fig. 3, lanes M). Induction of the 41-kDa protein recognized 
by the C/EBP-α antibody was detected at 24 h after infection 
when cells were infected at a high (5) or low (0.01) multiplicity 
of infection (Fig. 3 and data not shown). In summary, these 
studies suggested that BHV-1 infection of cultured cells led to 
induction of the long form of C/EBP-α.

**Analysis of C/EBP-α expression in TG of infected calves.** To examine C/EBP-α protein expression in TG of infected calves, 
immunohistochemistry was performed using the C/EBP-α spe-
cific antibody. C/EBP-α protein expression was readily de-
tected in TG neurons of calves that were infected for 4 or 7 
days (Fig. 4). At 4 days after infection, C/EBP-α protein expression was primarily localized to the perinuclear region, 
whereas at 7 days after infection, C/EBP-α was detected in the 
nucleus of certain neurons. In contrast, C/EBP-α protein expression was not detected in TG neurons of an uninfected calf 
(Fig. 4). However, a few nonneuronal cells in all calves, including 
mock-infected calves, expressed C/EBP-α. At 14 days after infection, there appeared to be slight neuronal staining (Fig. 4), 
which was not observed in mock-infected calves or at 60 
days after infection (latency) (data not shown). Calves latently 
infected with BHV-1 undergo reactivation from latency following 
administration of DEX, a synthetic corticosteroid (54). At 
24 (Fig. 4) or 48 (data not shown) h after a single dose of DEX, 
C/EBP-α protein expression was readily detected in a subset of 
TG neurons. In contrast, when a mock-infected calf was ad-
ministered the same dose of DEX for 24 h, C/EBP-α protein expression was not detected in TG neurons (Fig. 4).

RT-PCR was performed on TG samples of calves infected with 
wt BHV-1 or left uninfected. C/EBP-α, but not GH, RNA levels were higher at 6 days after infection than at 10 or 14 days 
after infection (Fig. 5). Although C/EBP-α RNA was detected 
in mock-infected calves (Fig. 5), we assume expression oc-
curred in nonneuronal cells or C/EBP-α was unstable in neu-
rons, because it was not detected in TG neurons prepared from 
mock-infected calves (Fig. 4). In summary, these studies indi-
cated that C/EBP-α protein expression was detected in TG 
neurons of acutely infected calves or following DEX-induced 
reactivation from latency.

**wt C/EBP-α, but not a DNA binding mutant, increases plaque formation.** Since C/EBP-α stimulates HHV-8, HVS, or 
EBV gene expression and productive infection (28, 60, 62, 67), 
we hypothesized that C/EBP-α may enhance BHV-1 produc-
tive infection. To test whether the overexpression of C/EBP-α-
enhanced plaque formation, bovine cells were cotransfected 
with gCblue genomic DNA and increasing concentrations of wt 
pC/EBP-α or a DNA binding mutant (pC/EBP-αADB) (Fig. 6). Transfection of 9.1.3 cells with BHV-1 genomic DNA yields 
low levels of infectious virus. Cotransfection of BHV-1 DNA 
with a plasmid expressing bICP0 enhances productive infection 
and virus yield (Fig. 6) (32). At 24 h after transfection, cells 
were fixed and β-Gal + cells identified. This time point was 
used as the time to count β-Gal + cells to minimize the number 
of virus-positive cells that result from virus spread. Further-
more, at later times, many of the β-Gal + cells lift off the dish, 
making it difficult to count virus-positive cells (18, 32). The 
number of β-Gal + cells directly correlates with the number of 
plaques produced following transfection with the BHV-1 blue 
virus (17, 18, 32). When a 16:1 molar ratio of pC/EBP-α to 
gCblue viral DNA was cotransfected into 9.1.3 cells, a 
threefold increase in plaques was observed relative to pc/EBP-
αDB or the blank expression vector (Fig. 6). At high concen-
trations of pC/EBP-α, the number of plaques was slightly lower 
than the numbers obtained with the empty expression vector or 
with equivalent amounts of pC/EBP-αDB, pC/EBP-α was less efficient than bICP0 at activating productive infection, 
even when transfected at four times the level of bICP0.

**DISCUSSION**

In this study, we identified cellular proteins that interact with the 
major ORF encoded by an alternatively spliced polyade-
ylated LR transcript that is abundantly expressed in TG of 
calves at 7 days after infection (11, 12). Seven days after infec-
tion is important with respect to establishing latency in calves, 
because after this time most viral gene expression is extin-
guished in TG (57). Only the LR gene (40) and ORF-E (29) 
are abundantly expressed after 7 days postinfection. Therefore, 
we predicted that LR proteins, in part, regulate the transition 
between productive infection and establishment of latency.
The ability of the LR gene (10, 25) and products encoded by the 7d cDNA (data not shown) to inhibit apoptosis correlates with the LR gene being necessary for reactivation from latency after DEX treatment (31). During the transition from acute infection to establishment of latency, an LR mutant virus induces higher levels of apoptosis in TG of infected calves, which reduces establishment from latency (43). Furthermore, insertion of the wt LR gene (52), but not the LR gene containing stop codons at the 5’ terminus of ORF-2 (45), into a herpes simplex virus type 1 latency-associated transcript minus mutant virus restores high levels of spontaneous reactivation in the rabbit eye model and explant-induced reactivation. Finally, the ability of herpes simplex virus type 1 latency-associated transcript to inhibit apoptosis is crucial for wt levels of reactivation from latency (34). Taken together, these observations suggest that the ability of the LR gene to inhibit apoptosis is one function that promotes the establishment of latency in a pool of neurons that can subsequently reactivate from latency.

The results from this study suggested that the antiapoptotic functions of the LR gene rely on the ability of LR proteins to interact with Bid or Cdc42. Bid links the extrinsic pathway of apoptosis to the intrinsic pathway; following cleavage by caspase 8, Bid interacts with mitochondria (42), inducing cytochrome C and Smac/Diablo release (63). Bid is also specifically cleaved by granzyme B (3, 4, 53), suggesting that the ability of LR proteins to interact with Bid impairs cytotoxic T-lymphocyte-induced death of infected neurons. Neuronal stress, including removal of nerve growth factor from neuronal...
cultures (39, 68), induces activity of Cdc42, a Rho GTPase family member (24). When PC12 cells (rat neuron-like cell line) are treated with nerve growth factor, they differentiate and behave like mature sympathetic neurons (19, 20). If PC12 cells or primary sympathetic neurons are deprived of nerve growth factor, Cdc42 is activated, resulting in apoptosis (39, 68). Since DEX reproducibly induces BHV-1 reactivation from latency (1, 2, 7, 26, 37, 54, 58, 66) and DEX is a potent stressor, Cdc42 may be activated in TG.

C/EBPs belong to the bZIP (basic and leucine zipper domain) family of transcription factors (64). The D. melanogaster genome contains 27 bZIP genes, of which 21 have a mammalian counterpart (15). Two of these bZIP proteins have homology with human C/EBP-/H9251 outside the bZIP region, showing 76% and 36% identity in the basic region and 23% and 28% identity in the leucine zipper (15). The C/EBP-/H9251 mRNA is translated into a 30- or 42-kDa protein, because there is an in-frame ATG codon downstream of the first ATG and both ATGs can be used as start codons. The shorter form of C/EBP-/H9251 retains dimerization and DNA binding domains but possesses altered trans-activation potential (41), because the N-terminal domain contains trans-activating domains (41, 47). C/EBP-/H9251 regulates cell cycle regulation and differentiation (41), suggesting that interactions with the LR fusion protein helps neurons recover from infection.

The interaction between the LR fusion protein and C/EBP-/H9251 may also influence viral gene expression, because C/EBP-/H9251 stimulates lytic gene transcription of EBV, HHV-8, and HVS (28, 60, 62, 67). C/EBP-/H9251 binds to the major lytic viral transactivators of EBV and HHV-8, Zta and Rta proteins, respectively. Binding of viral activators stabilizes C/EBP-/H9251, and the protein complex directly binds to viral IE promoters to activate viral transcription (28, 62, 67). Several lines of evidence suggested that C/EBP-/H9251 enhanced BHV-1 productive infection. First, a BHV-1-encoded or -induced product led to the induction of the full-length C/EBP-/H9251 isoform, or C/EBP-/H9251 was posttranslationally modified (Fig. 3). Secondly, there was a positive correlation between viral gene expression in TG and

![FIG. 5. Detection of C/EBP-/H9251 RNA in TG of BHV-1-infected cattle. RT-PCR for C/EBP-/H9251 was performed using RNA prepared from TG on the indicated days after infection or from a mock-infected calf (Mock). RNA extraction, RT-PCR, and PCR were performed as described in Materials and Methods. The cDNA for the respective samples was generated with random primers. Bovine GH was used as an internal control for cDNA amplification and DNA contamination. Positive (GH+) and negative (GH−) RT-PCRs (without reverse transcriptase) are shown. The first lane contains a 100-bp DNA ladder (New England BioLabs).]

![FIG. 6. The DNA binding domain of C/EBP-/H9251 is necessary to enhance productive infection. Bovine cells (9.1.3) were cotransfected with increasing amounts of pC/EBP-/H9251 or pC/EBP-/H9251/ΔDB (13 ng, 52 ng, 0.21 μg, 0.83 μg, or 3.32 μg) or bICP0 (52 ng), and the gCblue virus genome (0.83 μg DNA). Ratios of pC/EBP-/H9251 or pC/EBP-/H9251/ΔDB DNA molecules to viral genomic DNA molecules are 1:1, 4:1, 16:1, 64:1, or 256:1, respectively. The ratio between bICP0 molecules and viral DNA molecules is 4:1. An empty expression vector (pcDNA3.1) was used to maintain equivalent amounts of DNA. At 24 h after transfection, cells were fixed and stained, and the number of β-Gal− cells was counted. The number of β-Gal+ cells in the vector control (pcDNA3.1−) was set at onefold. The number of β-Gal+ cells in each well was calculated as the change (n-fold) compared to the vector control. The results are the averages from three independent experiments.]

C/EBP-α protein expression (Fig. 4) because C/EBP-α was detected in TG neurons at times (4 and 7 days after infection) when viral gene expression (57) and infectious virus (31) are detected. Finally, wt C/EBP-α, but not a DNA binding mutant, enhanced BHV-1 plaque formation (Fig. 6). These studies suggested that C/EBP-α activates certain key BHV-1 promoters, directly or indirectly, which is consistent with the role that C/EBP-α plays in EBV, HHV8, or HVS productive infection. We hypothesize that, in addition to inhibiting apoptosis, interactions between the LR fusion protein and C/EBP-α repress productive infection, thus promoting the establishment of latency in neurons that can support reactivation from latency. Studies designed to test these predictions are in progress.

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