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The bovine herpesvirus 1 regulatory proteins, bICP4 and bICP22, are expressed during the escape from latency

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Abstract

Following acute infection of mucosal surfaces by bovine herpesvirus 1 (BoHV-1), sensory neurons are a primary site for life-long latency. Stress, as mimicked by the synthetic corticosteroid dexamethasone, consistently induces reactivation from latency. Two viral regulatory proteins (VP16 and bICP0) are expressed within 1 hour after calves latently infected with BHV-1 are treated with dexamethasone. Since the immediate early transcription unit 1 (IEtu1) promoter regulates both bICP0 and bICP4 expression, we hypothesized that the bICP4 protein is also expressed during early stages of reactivation from latency. In this study, we tested whether infected cell protein 4 (bICP4) and bICP22, the only other BoHV-1 protein known to be encoded by an immediate early gene, were expressed during reactivation from latency by generating peptide specific antiserum to each protein. bICP4 and bICP22 protein expression were detected in trigeminal ganglionic (TG) neurons during early phases of dexamethasone induced reactivation from latency, operationally defined as the escape from latency. Conversely, bICP4 and bICP22 were not readily detected in TG neurons of latently infected calves. In summary, it seems clear that all proteins encoded by known BoHV-1 IE genes (bICP4, bICP22, and bICP0) were expressed during early stages of dexamethasone-induced reactivation from latency.

Keywords

bovine herpevirus 1; stress-induced reactivation from latency; ICP4; ICP22

INTRODUCTION

Acute infection of cattle by bovine herpesvirus 1 (BoHV-1) can result in clinical disease in the upper respiratory tract, nasal cavity, and ocular cavity (Jones and Chowdhury, 2007). Furthermore, BoHV-1 can cause reproductive failure in cattle following infection of the ovary and/or fetus (Chase et al., 2017), making it the most frequently diagnosed cause of viral abortion in North America. Following acute infection, a primary site of BoHV-1

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latency is sensory neurons in trigeminal ganglia (TG). Periodically, reactivation from latency occurs: consequently, the virus is widespread in cattle (Jones, 1998, 2003, Jones et al., 2006, Jones, 2009). The incidence of BoHV-1 reactivation from latency is increased following stressful stimuli that increase corticosteroid levels, reviewed in (Jones et al., 2011; Jones and Chowdhury, 2007; Perng and Jones, 2010). Administration of the synthetic corticosteroid dexamethasone (DEX) to latently infected calves or rabbits consistently induces BoHV-1 reactivation from latency (Inman et al., 2002; Jones, 1998, 2003, Jones et al. 2006; Jones et al., 2000; Rock et al., 1992). Six hours after DEX treatment lytic cycle viral RNA expression is readily detected in a subset of trigeminal ganglionic neurons of latently infected calves (Winkler et al., 2002; Winkler et al., 2000). Within 1–3 hours after dexamethasone treatment of latently infected calves, which is operationally defined as the escape from latency, two viral regulatory proteins are detected (VP16 and bICP0) (Frizzo da Silva et al., 2013; Kook et al., 2015a). Interestingly, bICP0 and VP16+ neurons detected during reactivation from latency, also frequently express the glucocorticoid receptor (GR). In contrast to VP16, other late viral proteins (gC and gD) were not detected until later after dexamethasone-induced reactivation from latency.

In contrast to herpes simplex virus 1 (HSV-1), BoHV-1 expresses only 3 proteins encoded by IE genes: bICP0, bICP4, and bICP22 (Jones, 2003). Furthermore, expression of bICP0 is complex because two distinct promoters regulate its expression. For example, the IE transcription unit 1 (IEtu1) promoter drives expression of bICP0 and bICP4 because a single IE transcript is differentially spliced and then translated into bICP0 or bICP4 (Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991) and (Figure 1A and B). The bICP0 protein is also translated from an E mRNA (E2.6) because a separate E promoter drives expression of the bICP0 E transcript (Fraefel et al., 1994; Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991), Figure 1A and B. IEtu1 promoter activity is stimulated by the GR and the synthetic corticosteroid dexamethasone because two consensus GR response elements (GREs) are located in the promoter (El-Mayet et al., 2017; Kook et al., 2015b) suggesting this promoter is activated by stress induced transcription factors during reactivation from latency. If the IEtu1 promoter is activated by cellular factors during reactivation from latency, bICP4 should also be expressed during the escape from latency.

Although we previously developed antiserum that recognizes bICP4 during productive infection by Western blot studies, this serum yielded high levels of background when formalin fixed and paraffin embedded TG sections prepared from latently infected calves or during reactivation from latency were incubated with the same antiserum (data not shown). Consequently, we developed new antiserum directed against two bICP4 peptides to test whether its expression was induced during reactivation from latency. We also developed antiserum directed against the other IE protein (bICP22) to compare its expression during the latency-reactivation cycle. bICP04+ and bICP22+ TG neurons were detected at 3 hours after dexamethasone treatment, but were not readily detected in TG prepared from latently infected calves prior to DEX treatment or uninfected TG. Consecutive sections suggested that a subset of bICP4+ or bICP22+ TG neurons were also GR+. In summary, similar to bICP0, these studies suggested bICP4 and bICP22 were expressed during the escape from latency.

MATERIALS AND METHODS

Cells, virus, and antibodies:

Bovine kidney (CRIB) cells, which are unable to be infected by bovine viral diarrhea virus (BVDV), (Flores et al., 1996) were obtained from ATCC (Manassas, VA). CRIB cells were maintained in Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum, penicillin (10 U/ml), and streptomycin (100 μg/ml). The Cooper strain of BoHV-1 (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services (Ames, IA) and was grown in CRIB cells. Pierce Custom Services, Thermo Scientific (Rockford, IL) identified potential antigenic peptides and then generated peptide-specific rabbit polyclonal antibodies directed against bICP4 and bICP22 (Program No. TA2422X and TA2422X). For details of the peptides used and the location of these peptides, see Figure 1B and legend of Figure 1.

Bovine TG samples:

Unvaccinated male calves (primarily Holsteins) from dairy farms in Nebraska were bled and the presence of BoHV-1 antibodies examined by an enzyme-linked immunosorbent assay (ELISA) performed by the University of Nebraska Diagnostic Center. Calves (~200 kg) that lacked BoHV-1 specific antibodies were inoculated with 10^7 PFU of BoHV-1 into ocular and nasal cavities as described previously (Inman et al., 2001, 2002; Lovato et al., 2003; Perez et al., 2005; Perez et al., 2006; Perez et al., 2008). Calves were housed under strict isolation and given antibiotics to prevent bacterial pneumonia. At 60 days post-inoculation (dpi), calves were considered to be latently infected, and were injected intravenously (jugular vein) with 100 mg of water-soluble DEX (Sigma, D2915, St Louis, MO) to initiate reactivation from latency. The latently infected and DEX treated calves were then anesthetized with XylaMed (Bimeda, Inc, LE Sueur, MN) followed by electrocution. After decapitation, TG were collected, and samples from each TG were formalin fixed and paraffin embedded. The remainder of both TG were minced into small pieces were then stored at −80 °C. TG used in this study were derived from earlier studies (Kook et al., 2015; Workman et al., 2012). Experiments were performed in accordance with the American Association of Laboratory Animal Care guidelines and the University of Nebraska IACUC committee.

Immunohistochemistry:

Immunohistochemistry was performed using the Ultra-Sensitive ABC Rabbit IgG Staining Kit (32054, Pierce; Thermo Scientific; Rockford, IL) essentially as previously described (Frizzo da Silva et al., 2013; Kook et al., 2015; Liu et al., 2016; Workman et al., 2018; Zhu et al., 2017). Briefly, thin sections (4 to 5 μm) were cut from each TG and mounted onto slides and processed as described previously (Liu et al., 2016; Sinani et al., 2013). After blocked with the animal-free blocking solution (15019L, Cell Signaling; Danvers, MA) for 1 h at room temperature, tissue sections were subsequently incubated with the bICP4 (1:250 dilution; TA2422X; Pierce Custom Services, Thermo Scientific, Rockford, IL) or bICP22 peptide-specific rabbit polyclonal antibody (1:100 dilution; TA2424X; Pierce Custom Services, Thermo Scientific, Rockford, IL), or GR-specific rabbit monoclonal antibody (1:250 dilution; 12041; Cell Signaling; Danvers, MA) overnight in a humidified chamber at 4 °C. Three washes in 1×TBS (pH 7.6) containing 0.025% Triton X 100 (TBST) were

performed between each step. The next day, slides were incubated with biotinylated goat anti-rabbit IgG (32054, Pierce Custom Services, Thermo Scientific, Rockford, IL) for 30 min at room temperature in a humidified chamber followed by Avidin-biotinylated enzyme complex (32054, Pierce Custom Services, Thermo Scientific, Rockford, IL) incubation for 30 min at room temperature. After washing in 1×TBS, slides were incubated with freshly prepared substrate (SK-4800; Vector Laboratories; Cole-Parmer; Vernon Hills, IL), rinsed with distilled water, and lightly counterstained with hematoxylin (51275; Sigma-Aldrich, St Louis, MO). Thin sections from latently infected calves were used as a negative control.

Western blot analysis:

Confluent CRIB cells grown in 60-mm dishes were infected with a multiplicity of infection (MOI) of 2 PFU/cell of BoHV-1. At the designated times after infection, cells were washed with PBS and lysed with 0.2 ml of radioimmunoprecipitation assay (RIPA) buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor (Roche Molecular Biochemicals; Indianapolis, IN) in 10 ml buffer). The respective samples were boiled in Laemmli sample buffer for 5 min, and all samples were separated on a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore-Sigma; St Louis, MO) and blocked for 2 h in 5% nonfat dry milk with Tris-buffered saline-0.1% Tween 20 (TBS-T). Membranes were then incubated with bICP4 or bICP22 peptide-specific rabbit polyclonal antibody (1:1000 dilution) overnight at 4°C. An antibody directed against Glyceraldehyde 3-phosphate dehydrogenase (GAPHD) (1:20,000 dilution; sc-365062; Santa Cruz Biotechnology; Santa Cruz, CA) was used as a loading control. After washing with TBS-T (5 times for 5 minutes each), blots were incubated with anti-rabbit IgG, HRP-linked antibody (7074S; Cell Signaling; Danvers, MA) or ECL™ anti-mouse IgG horseradish peroxidase linked whole antibody (from sheep) GPR (NXA931V; GE Healthcare UK Limited), which was diluted 1:2,000 in 5% nonfat milk in TBS-T. Blots were then washed with TBS-T as above, exposed to Clarity™ Western ECL Substrate (1705061; Bio-Rad; Hercules, CA), and autoradiography performed.

RESULTS

Expression of bICP4 and bICP22 in productively infected bovine cells

Peptide specific antibodies directed against bICP4 and bICP22 were generated in rabbits using two peptides for each ORF (see Figure 1B for location of the individual peptides and legend of Figure 1 for amino acids used for each peptide). To test the specificity of the bICP4 and bICP22 antibodies, CRIB cells were infected with BoHV-1 (MOI of 2) for 12 hours and then viral protein expression examined by Western blot analysis (Figure 1C). bICP4 protein expression (approximately 180 kD protein) was detected in infected cells, but not in mock-infected cells. The bICP22 anti-serum in infected, but not uninfected cell lysate specifically recognized a 53 kD protein. Based on the size of the bICP4 and bICP22 open reading frames, the proteins migrated at the expected size. Additional studies compared bICP4 and bICP22 expression at 1.5, 3, 6, 12, and 24 hours post-infection (hpi) by Western blots analysis. As shown in Fig. 1D, bICP4 was readily detected in infected cells as early as 3 hpi and at 6, 12, and 24 hpi. In contrast to bICP4, bICP22 was not readily detected until 6

hpi and also at 12 and 24 hpi. As expected, bICP4 and bICP22 were not detected in uninfected cells: conversely, similar levels of GAPDH were detected in uninfected and infected cells. In summary, these results indicated that both bICP4-and bICP22-peptide specific antibodies recognized the expected protein during productive infection.

Detection of bICP4 and bICP22 during reactivation from latency

To test whether bICP4 and bICP22 protein expression was detected during reactivation from latency, immunohistochemistry (IHC) studies were performed using TG sections from latently infected calves, and calves treated with DEX to induce reactivation from latency. As expected, bICP4-positive (bICP4+) TG neurons were not readily detected in calves latently infected with wt BoHV-1 (Figure 2) or uninfected calves (data not shown). In contrast, bICP4+ TG neurons were detected in the latently infected TG neurons that were treated with DEX for 3 and 6 h (arrows denote TG neurons that were specifically stained): however, bICP4+ TG neurons were not detected at 1.5 hours after DEX treatment.

As with the bICP4 anti-serum, the bICP22 specific anti-serum did not readily detect TG neurons from latently infected (Figure 3) or uninfected calves (data not shown). Conversely, bICP22+ TG neurons were readily detected in latently infected TG that had been treated with DEX for 3 and 6 h. In some TG neurons, the nucleus was heavily stained with the bICP22 antibody. bICP22+ TG neurons were also not readily detected at 1.5 hours after DEX treatment.

The percentage of bICP4+ and bICP22+ TG neurons was estimated by counting neurons in many sections (Figure 4). At 3 and 6 h after DEX treatment, approximately 19 and 25% TG neurons were bICP4. For bICP22, approximately 11% of TG neurons were bICP22+ and 26% at 6 h after DEX treatment. Since it is impossible to section the entire bovine TG and perform IHC on all sections, these numbers are only an estimation of TG neurons expressing bICP4 or bICP22. Furthermore, bICP4+ or bICP22+ TG neurons were not uniformly expressed in each section. With that said, it was clear there were significantly more bICP4+ or bICP22+ TG neurons at 3 and 6 h after DEX treatment relative to latently infected TG or at 1.5 h after DEX treatment.

Certain TG neurons that express bICP4 or bICP22 also express the GR.—Since glucocorticoid receptor positive (GR^+) TG neurons increase during reactivation from latency, and TG neurons that express bICP0 during early stages of reactivation frequently express the GR (Frizzo da Silva et al., 2013; Kook et al., 2015a), we further tested whether GR and bICP4 or bICP22 were expressed in the same neuron following DEX treatment. Consecutive sections were prepared from TG samples at 6 h after DEX treatment, and each section was stained with the GR, bICP4 or bICP22 specific antibody. GR+ and bICP4+ (Figure 5A) or bICP22+ (Figure 5B) TG neurons were detected in a subset of TG neurons at 6 h after DEX treatment. We found that 14 out of 15 bICP4+ TG neurons were also GR+. Seven out of 10 bICP22+ TG neurons were also GR+. These studies suggested that most neurons expressing bICP4 or bICP22 also expressed the GR.

DISCUSSION

In this study, we provided evidence that bIC4 and bICP22 were expressed during the escape from latency. We have now provided evidence that all three BoHV-1 proteins encoded by IE genes are expressed during the escape from latency. Since bICP0 and bICP4 expression are regulated by the same IE promoter (Figure 1A), we further suggest that the IEtu1 promoter is active during the escape from latency. Interestingly, the IEtu1 promoter contains two functional GREs that can be activated by the synthetic corticosteroid DEX (El-Mayet et al., 2017; Kook et al., 2015b; Sawant et al., 2018) suggesting activation of this promoter is an early response to a stressful stimulus. bICP0 and bICP4 are crucial for activating early and late viral gene expression and productive infection (Boutell and Everett, 2013; Parkinson and Everett, 2000, 2001; Saira et al., 2008), functions crucial for reactivation from latency. Although the exact function of bICP22 is unknown, ICP22 encoded by HSV-1 is a general transcriptional regulator of cellular and viral mRNAs, in part by mediating changes on the host RNA polymerase II (Frase and Rice, 2007; Orlando et al., 2006). Thus, bICP22 expression during reactivation may also stimulate lytic cycle viral gene expression.

A previous study demonstrated that bICP0 and VP16 was detected in TG neurons of latently infected calves at 30 and 90 minutes after DEX treatment (Kook et al., 2015a). Conversely, we were unable to detect bICP4 and bICP22 at 90 minutes after DEX-induced reactivation from latency. Although this could be because the bICP0 and VP16 antiserum we used had greater avidity for their targets relative to the antiserum developed against the bICP4 or the bICP22 protein, we cannot exclude the possibility that the bICP0 E promoter initially stimulates bICP0 protein expression during the escape from latency. Thus, it is possible that bICP0 and/or VP16 are expressed earlier than bICP4 during reactivation from latency. Studies designed to test whether the bICP0 E promoter is stimulated by stress-induced transcription factors are currently in progress.

In contrast to two late viral proteins (gC and gD) that are not readily detected during BoHV-1 reactivation from latency (Frizzo da Silva et al., 2013), VP16 was readily detected in TG neurons during the escape from latency, as early as 30 or 90 minutes after DEX treatment of latently infected calves (Frizzo da Silva et al., 2013; Kook et al., 2015a). With respect to HSV-1, VP16 was reported to play a crucial role during reactivation from latency (Camarena et al., 2010; Kim et al., 2012; Sawtell and Thompson, 2016; Thompson et al., 2009). Considering VP16 is a tegument protein that stimulates IE gene expression (Misra et al., 1994; Misra et al., 1995), VP16 may initiate IE gene expression in a subset of TG neurons during reactivation in latency. Conversely, in a distinct subset of TG neurons, the IEtu1 or bICP0 E promoter may be initially activated by the GR and stress-induced transcription factors. Regardless of which scenario is correct, production of infectious virus particles during reactivation from latency will likely require all of these cellular and viral transcription factors. In addition to stimulating viral gene expression, the ability of corticosteroids to suppress inflammation and immune responses (Rhen and Cidlowski, 2005; Smoak and Cidlowski, 2004) would be expected to enhance the efficiency of virus shedding and spread to peripheral sites.

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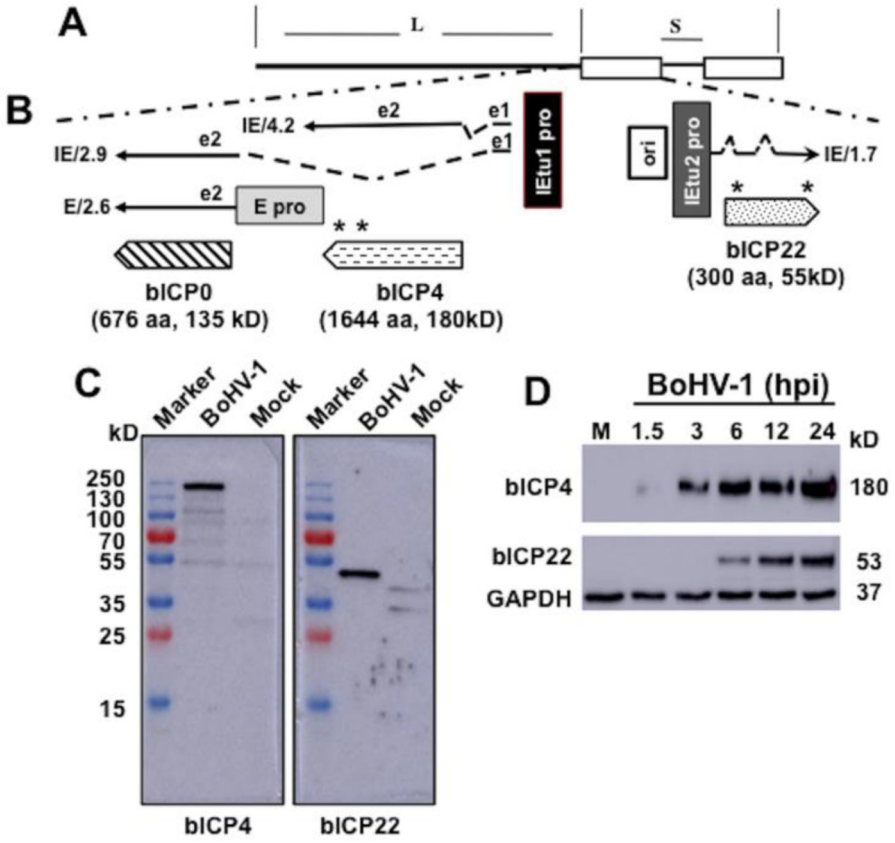

Figure 1: Schematic of IE region and location of the bICP0, bICP4, and bICP22 genes.

Panel A: Structure of BoHV-1 genome and location of unique long (L) region, direct repeats (open rectangles), and unique long region (S).

Panel B: The IE/4.2 mRNA encodes the bICP4 protein and IE/2.9 mRNA encodes the bICP0 protein. A single IE promoter activates expression of IE/4.2 and IE/2.9 (IEtu1; black rectangle). E/2.6 is the early bICP0 mRNA and is regulated by the bICP0 early promoter (E pro; gray rectangle). bICP0 protein coding sequences are in Exon 2 (e2). Origin of replication (ORI) separates IEtu1 from IEtu2. IEtu2 promoter (IEtu2 pro) regulates IE1.7 mRNA expression, which is translated into the bICP22 protein. Solid lines in IE/2.9, IE/4.2, and IE/1.7 are exons (e1, e2, or e3) and dashed lines introns. Location and size of the three proteins encoded by IEtu1 and IEtu2 are shown: arrows denote directionality of the proteins. The bICP4 peptides synthesized for producing the peptide specific antibody are: amino acids 1461–1478 (RRAGQAPGREAREGRGRG) and amino acids 1604–1621 (GVSPWGSRGVRAFRRPPG). The peptides synthesized for producing the bICP22 antibody are: amino acids 26–40 (GPAPADEHARRGPGA) and amino acids 281–296 (GSPSGRARARPAPAKR). An asterisk denotes the location of the peptides within the bICP4 and bICP22 ORFs.

Panel C: Monolayers of CRIB cells were mock infected or infected with BoHV-1 (MOI=2) and whole-cell lysate prepared at 12 hours after infection. Proteins (50 ug protein in each lane) were separated in a 10% SDS-PAGE and detected by Western blotting using the rabbit anti-bICP4 or bICP22 peptide antibody as described in Materials and Methods. The marker

lane is a Thermo Scientific Page Ruler pre-stained protein marker and the size of these proteins is denoted on the left.

Panel D: As described above, CRIB cells were mock infected (lane M) or infected with BoHV-1 (MOI=2) for the denoted time (hours after infection). Whole-cell lysate (50 ug protein in each lane) was separated in a 10% SDS-PAGE and detected by Western blotting using the rabbit anti-bICP4 or bICP22 peptide antibody. GAPDH protein levels were analyzed in the respective samples as a loading control. Approximate size of the respective proteins is denoted on the right of the Western Blots.

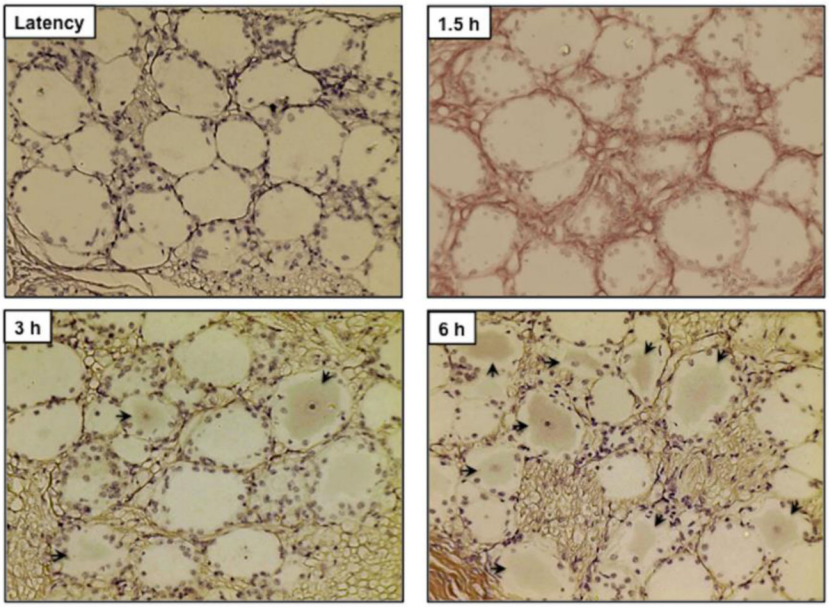

Figure 2. bICP4 is expressed in TG neurons following DEX treatment to induce reactivation from latency.

Thin sections were cut from formalin-fixed, paraffin-embedded TG sections of the latently infected claves and those treated with DEX for the designated times (1.5 h, 3 h and 6 h) after infection. IHC was performed using rabbit anti-bICP4 peptide antibody (1:250) and Biotinylated goat anti-rabbit IgG (Vector Laboratories) were used as the primary and secondary antibodies as described in Materials and Methods. Arrows denote neurons that were recognized by the bICP4 antibody. Magnification is approximately 400X.

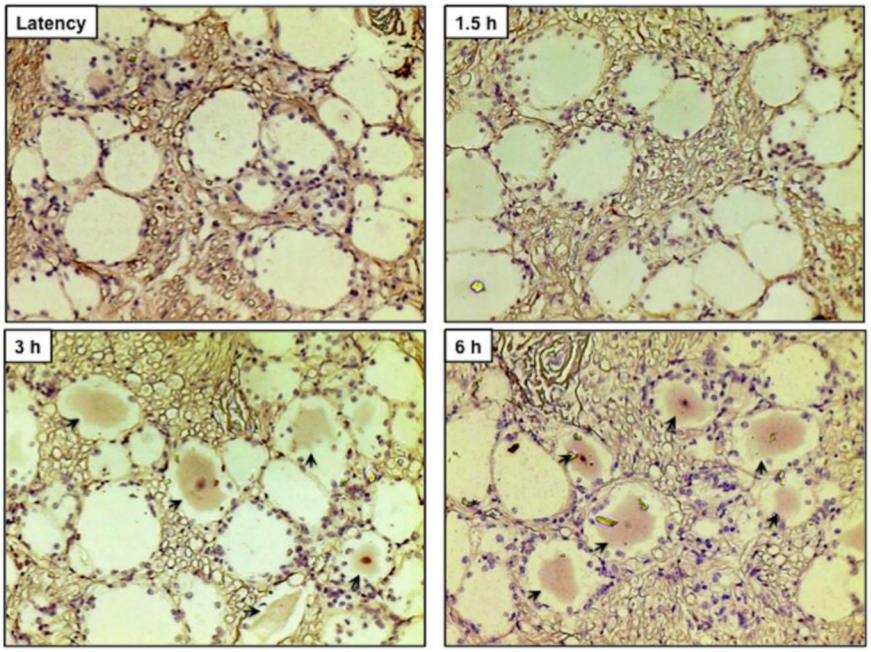

Figure 3. bICP22 is expressed in TG neurons following DEX treatment to induce reactivation from latency.

Thin sections were cut from formalin-fixed, paraffin-embedded TG sections of the latently infected claves and these treated with DEX for the designated times (1.5 h, 3 h and 6 h) after infection. IHC was performed using rabbit anti-bICP22 peptide antibody (1:100) and Biotinylated goat anti-rabbit IgG (Vector Laboratories) were used as the primary and secondary antibodies as described in Materials and Methods. Arrows denote neurons recognized by the bICP22 antibody. Magnification is approximately 400X.

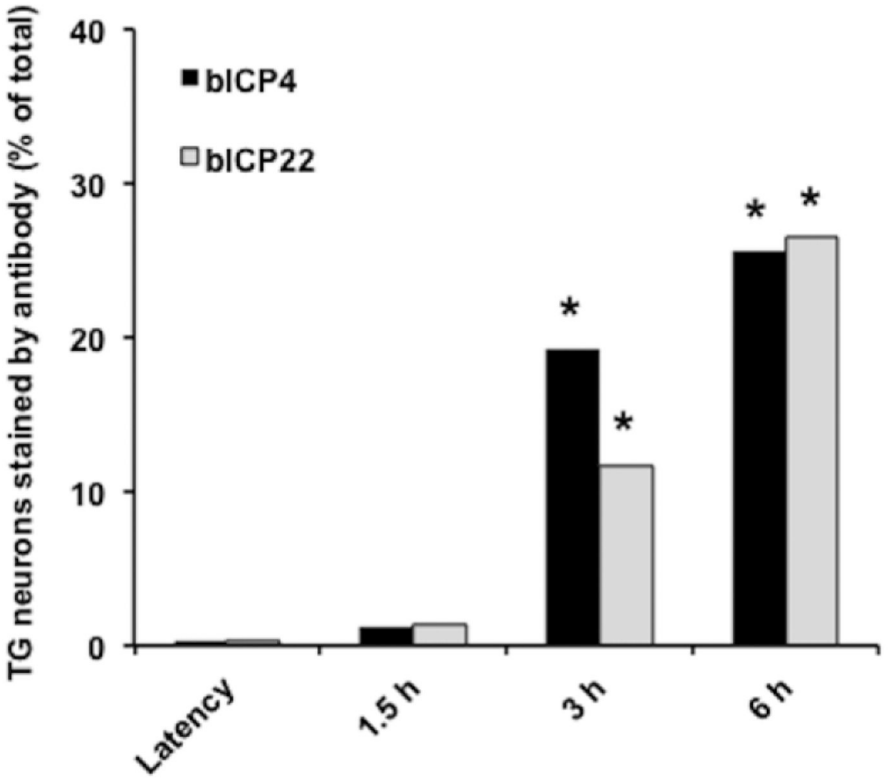

Figure 4. Estimating the number of neurons that express bICP4 and bICP22 during reactivation from latency.

The % of bICP4+ TG neurons was estimated by counting the following number of TG neurons: latency (538), 1.5 h after DEX treatment (515), 3 h after DEX treatment (668), and 6 hours after DEX treatment (546). The % of bICP22^{+} TG neurons was estimated by counting the following numbers of TG neurons: latency (582), 1.5 h after DEX (497), 3 h after DEX treatment (734), and 6 h after DEX treatment (597). An asterisk denotes a significant increase in bICP4+ or bICP22+ TG neurons relative to latency. The asterisks denote significant differences ($P<0.001$) in the numbers of bICP4+ neurons as determined by a Student t test.

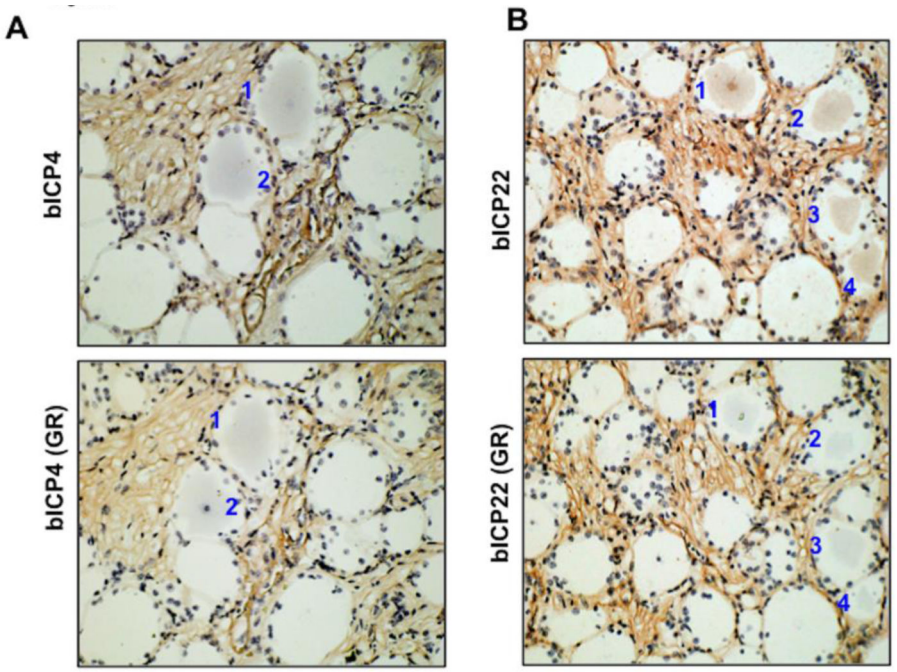

Figure 5. Identification of GR+ TG neurons that express bICP4 or bICP2 in the same TG neuron.

IHC was performed using the bICP4 or bICP22 antibodies as described in Figures 2 and 3 using sections cut from TG of latently infected calves at 6 h after DEX treatment to initiate reactivation from latency. IHC was also performed using a GR-specific antibody on consecutive sections. Numbers denote TG neurons that were bICP4+ or bICP22+ and GR+.