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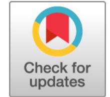

Synergistic Activation of Bovine Herpesvirus 1 Productive Infection and Viral Regulatory Promoters by the Progesterone Receptor and Krüppel-Like Transcription Factor 15

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ABSTRACT Bovine herpesvirus 1 (BoHV-1), including modified live vaccines, readily infects the fetus and ovaries, which can lead to reproductive failure. The BoHV-1 latency reactivation cycle in sensory neurons may further complicate reproductive failure in pregnant cows. The immediate early transcription unit 1 (IEtu1) promoter drives expression of important viral transcriptional regulators (bICP0 and bICP4). This promoter contains two functional glucocorticoid receptor (GR) response elements (GREs) that have the potential to stimulate productive infection following stressful stimuli. Since progesterone and the progesterone receptor (PR) can activate many GREs, we hypothesized that the PR and/or progesterone regulates productive infection and viral transcription. New studies demonstrated that progesterone stimulated productive infection. Additional studies revealed the PR and Krüppel-like transcription factor 15 (KLF15) cooperated to stimulate productive infection and IEtu1 promoter activity. IEtu1 promoter activation required both GREs, which correlated with the ability of the PR to interact with wild-type (wt) GREs but not mutant GREs. KLF15 also cooperated with the PR to transactivate the bICP0 early promoter, a promoter that maintains bICP0 protein expression during productive infection. Intergenic viral DNA fragments (less than 400 bp) containing two GREs and putative KLF binding sites present within genes encoding unique long 52 (UL-52; component of DNA primase/helicase complex), Circ, bICP4, and IEtu2 were stimulated by KLF15 and the PR more than 10-fold, suggesting that additional viral promoters are activated by these transcription factors. Collectively, these studies suggest progesterone and the PR promote BoHV-1 spread to reproductive tissues, thus increasing the incidence of reproductive failure.

IMPORTANCE Bovine herpesvirus 1 (BoHV-1) is the most frequently diagnosed cause of abortions in pregnant cows and can cause "abortion storms" in susceptible herds. Virulent field strains and even commercially available modified live vaccines can induce abortion, in part because BoHV-1 replicates efficiently in the ovary and corpus luteum. We now demonstrate that progesterone and the progesterone receptor (PR) stimulate productive infection. The BoHV-1 genome contains approximately 100 glucocorticoid receptor (GR) response elements (GREs). Interestingly, the PR can bind and activate many promoters that contain GREs. The PR and Krüppel-like transcription factor 15 (KLF15), which regulate key steps during embryo implantation, cooperate to stimulate productive infection and two viral promoters that drive expression of key viral transcriptional regulators. These studies suggest that the ability of progesterone and the PR to stimulate productive infection has the potential to promote virus spread in reproductive tissue and induce reproductive failure.

KEYWORDS BoHV-1, productive infection, progesterone receptor, regulation of gene expression

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Bovine herpesvirus 1 (BoHV-1) can cause reproductive failure in cattle following infection of the ovary and/or fetus (1), making it the most frequently diagnosed cause of viral abortion in North America. Exposure of a susceptible herd to BoHV-1 can result in "abortion storms" with between 25% and 60% of cows undergoing abortion. Ovaries and the corpus luteum are particularly susceptible to viral replication and pathogenesis (2–4). Even current modified live vaccines (5) and a virus strain lacking thymidine kinase (6) can cause abortions and infertility. Several studies also concluded that naive heifers vaccinated with an inactivated BoHV-1 vaccine are less likely to have an abnormal estrous cycle and significantly higher pregnancy rates relative to heifers vaccinated with a modified live vaccine (1, 4, 7–9).

Following acute infection, trigeminal ganglia (TG) are a primary site for lifelong latency (10, 11). It is well established that the incidence of BoHV-1 reactivation from latency is increased by stressful stimuli (11, 12). Furthermore, the synthetic corticosteroid dexamethasone (DEX) mimics the effects of stress, stimulates productive infection (13), and initiates reactivation from latency (14–21). BoHV-1 viral gene products, including the regulatory proteins bICP0 and VP16, are readily detected within hours after DEX treatment (19, 22–24). DEX-induced cellular transcription factors were identified within the first three hours in TG neurons following DEX treatment of latently infected calves, and these transcription factors stimulate certain viral promoters and productive infection (25). Corticosteroids bind and activate the glucocorticoid receptor (GR); consequently, the GR interacts with a GR response element (GRE) and stimulates transcription (26, 27). Recent studies demonstrated that DEX stimulates productive infection, in part by activating the BoHV-1 immediate early transcription unit 1 (IEtu1) promoter because there are two functional GREs in the promoter (13, 28). Corticosteroids and other nuclear hormones, including progesterone, also have potent anti-inflammatory and immunosuppressive properties (26, 29–31), which may enhance viral replication and viral spread during reactivation from latency. In summary, the ability of stress to directly stimulate viral gene expression and productive infection positively correlates with the ability of the synthetic corticosteroid to stimulate reactivation from latency.

As in other *Alphaherpesvirinae* subfamily members, BoHV-1 gene expression is operationally divided into three distinct phases during productive infection of cultured cells: immediate early (IE), early (E), and late (L) (14, 15). IE gene expression is stimulated by VP16, a tegument protein (32, 33). IEtu1 encodes two transcriptional regulatory proteins, bICP0 and bICP4, because a single IE transcript is differentially spliced and then translated into bICP0 or bICP4 (Fig. 1A) (34–36). The bICP0 protein is also translated from an E mRNA (E2.6) because a separate E promoter drives expression of the bICP0 E transcript (34–37). The bICP0 protein has properties similar to those of herpes simplex virus 1 (HSV-1)-encoded ICP0 (38), including a RING finger that is crucial for stimulating viral promoters and productive infection (39, 40) as well as interfering with activation of the beta interferon promoter (41–43). bICP4 is considered a functional orthologue of HSV-1-encoded ICP4 (35, 36).

In this study, we obtained evidence that a stress-induced transcription factor, Krüppel-like transcription factor 15 (KLF15), cooperates with the progesterone receptor (PR) to stimulate productive infection as well as IEtu1 and bICP0 early promoter activity. The GREs located in the IEtu1 promoter activity were essential for progesterone-mediated promoter activation. These studies indicate that progesterone and the PR can enhance spread of BoHV-1 to reproductive tissues and induce reproductive failure in cattle.

RESULTS

PR and KLF15 cooperate to stimulate productive infection. To test whether progesterone and the PR have a direct effect on productive infection, mouse neuroblastoma cells (Neuro-2A) were cotransfected with gC-Blue genomic DNA and the number of infected cells was determined. Neuro-2A cells were used for these studies because they can be readily transfected and BoHV-1 can replicate at low levels in these

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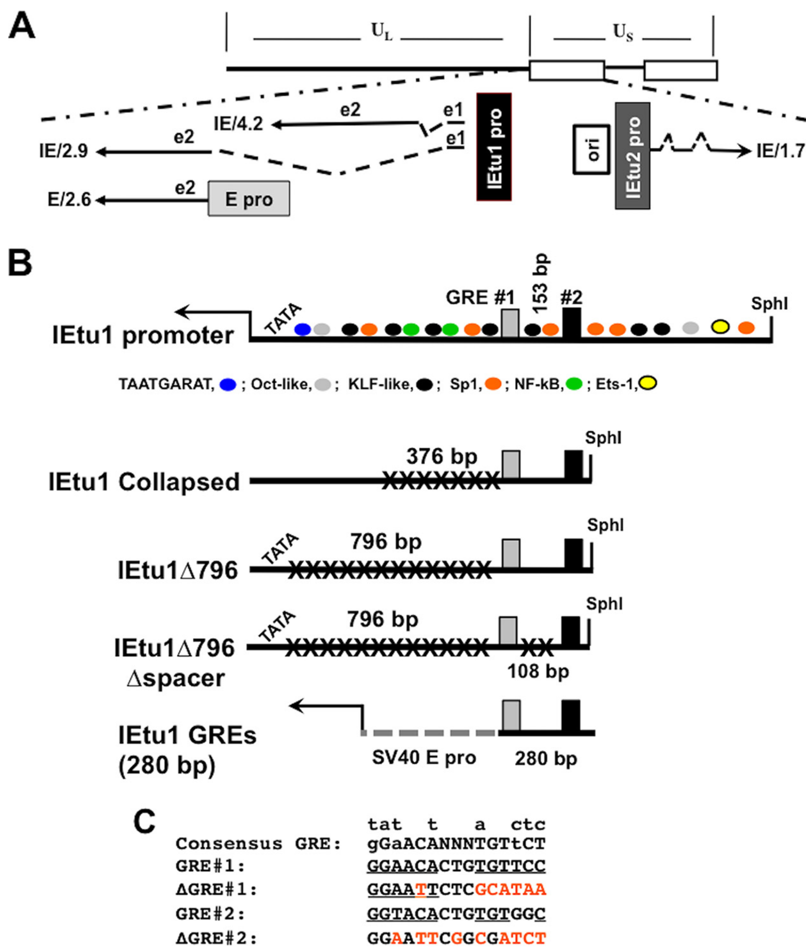

FIG 1 Schematic of BoHV-1 genome and plasmid constructs used in this study. (A) Structure of BoHV-1 genome and location of unique long (U_L) region, direct repeats (open rectangles), and unique short region (U_S). IE/4.2 mRNA encodes the bICP4 protein, and IE/2.9 mRNA encodes the bICP0 protein. A single IE promoter activates expression of IE/4.2 and IE/2.9 and is designated 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). The origin of replication (ORI) separates IEtu1 from IEtu2. IEtu2 promoter (IEtu2 pro) regulates IE1.7 mRNA expression, which is translated into 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. (B) The full-length IEtu1 promoter was cloned as an XhoI-SphI restriction site. The start site of transcription (arrow), TATA box, and binding site for VP16/Oct1 complex are indicated as TAATGARAT, and the locations of GRE1 and GRE2 are shown. Additional transcription factor-binding sites in the IEtu1 promoter are indicated. ETs-1 belongs to the ETS (erythroblast transformation-specific) transcription factor family. The IEtu1 collapsed promoter construct is inserted at the KpnI and HindIII restriction sites of pGL3-Basic Vector (Promega). The IEtu1Δ796 and IEtu1Δ796Δspacer constructs were synthesized and inserted at the KpnI and HindIII restriction sites of pGL3-Basic Vector. The X's indicate internal deleted sequences in the IEtu1 promoter constructs. A 280-bp fragment (IEtu1 GREs) was cloned into pGL3-Promoter Vector at the KpnI and XhoI restriction sites. (C) Nucleotide sequence of consensus GRE (small letters indicate the nucleotides that are the preferred base of the consensus sequence), GRE1, GRE2, and mutations that were incorporated into the IEtu1 GREs fragment and IEtu1Δ796.

cells (44). Neuro-2A cells were transfected with BoHV-1 gCblue DNA instead of infecting cells because VP16 and other regulatory proteins in the virion, including bICP4 (45), diminish the stimulatory effects of cellular genes on productive infection (data not shown). The gCblue virus contains the LacZ gene in the coding sequences of the gC locus, and using this virus allows one to accurately measure the effects of genes on productive infection. Two PR isoforms exist, PR A and PR B, which have cell-type- and promoter-specific transactivation functions (46); consequently, plasmids that express PR A and PR B were used for cotransfection with gCblue DNA. PR A and PR B increased the number of β-galactosidase-positive (β-Gal$^+$) Neuro-2A cells more than 7-fold,

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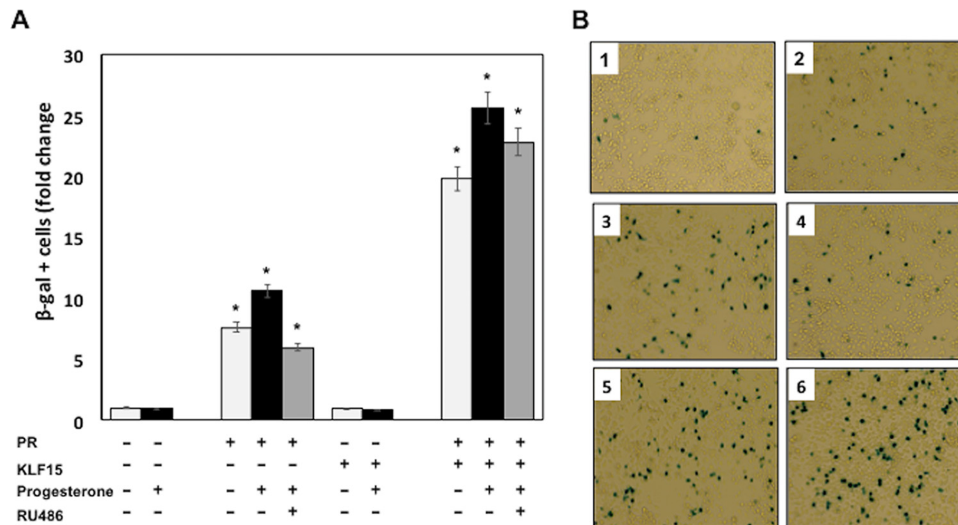

FIG 2 KLF15 and the GR cooperate to stimulate productive infection. Neuro-2A cells were transfected with 3.0 μg of BoHV-1 gCblue genomic DNA and, where indicated, plasmids that expresses the human PR protein (A and B) (1.0 μg of DNA of each) and/or KLF15 (0.5 μg of DNA). Cells were incubated with 2% stripped fetal calf serum 24 h after transfection. Stripped fetal bovine serum was used for these studies because normal serum contains steroid hormones, which activate nuclear hormone receptors (22). To maintain the same amount of DNA in each sample, empty vector was included in samples. Designated cultures were then treated with progesterone (1 μM; Tocris Bioscience; 2835), DMSO (vehicle), and, where indicated, RU486 (1 μM; Sigma). At 48 h after transfection, cells were fixed and stained for counting the β-Gal$^+$ cells. The value for the control (gCblue virus DNA treated with the DMSO vehicle after transfection) was set at 1. The results for progesterone- or RU486-treated cultures were compared to those for the control and are averages from three independent studies (A). An asterisk indicates a significant difference between the control and samples transfected with the PR and/or KLF15 and treated with progesterone or RU486 ($P < 0.05$) using the Student *t* test. Representative cultures stained for LacZ expression are shown in panel B. (1) gCblue virus DNA; (2) gCblue virus DNA plus PR; (3) gCblue virus DNA plus PR plus progesterone; (4) gCblue virus DNA plus PR plus progesterone plus RU486; (5) gCblue virus DNA plus PR plus KLF15; (6) gCblue virus DNA plus PR plus KLF15 plus progesterone.

which was significantly higher than for the gCblue virus alone (Fig. 2A and B). Addition of progesterone increased the number of β-Gal$^+$ Neuro-2A cells more than 10-fold (Fig. 2B, compare panel 1 to panel 3). RU486 antagonizes GR- and PR-mediated signaling (46–48), and RU486 reduced the ability of PR and progesterone to stimulate productive infection. However, RU486 did not reduce the number of β-Gal$^+$ Neuro-2A cells to basal levels.

A previous study demonstrated that Krüppel-like transcription factor 15 (KLF15) and the glucocorticoid receptor (GR) form a feed-forward transcriptional loop that synergistically stimulates productive infection (28), suggesting that KLF15 may cooperate with the PR to stimulate productive infection. Further credence for testing KLF15 and the PR is based on the report that KLF15 and the PR regulate embryo implantation (49). Although KLF15 alone had no effect on productive infection, cotransfection of gCblue with the PR and KLF15 stimulated productive infection approximately 20-fold (Fig. 2). When progesterone was added, productive infection was stimulated more than 25-fold. Surprisingly, RU486 only had a slight effect on KLF5- and PR-mediated activation in the presence of progesterone. In summary, these studies suggested that the PR and progesterone stimulated productive infection even when progesterone functions were antagonized by RU486, and KLF15 cooperated with the PR to stimulate productive infection.

PR stimulates IEtu1 promoter activity. Transient-transfection studies were performed with Neuro-2A cells to test whether the PR stimulates IEtu1 promoter activity because this promoter contains two functional GR binding sites (13) and a PR response element is similar to a GRE (50). Furthermore, the PR has been reported to bind and transactivate most consensus GREs (51), including GRE1 and -2. The IEtu1 promoter is critical for productive infection because it directs IE expression of bICP0 and bICP4

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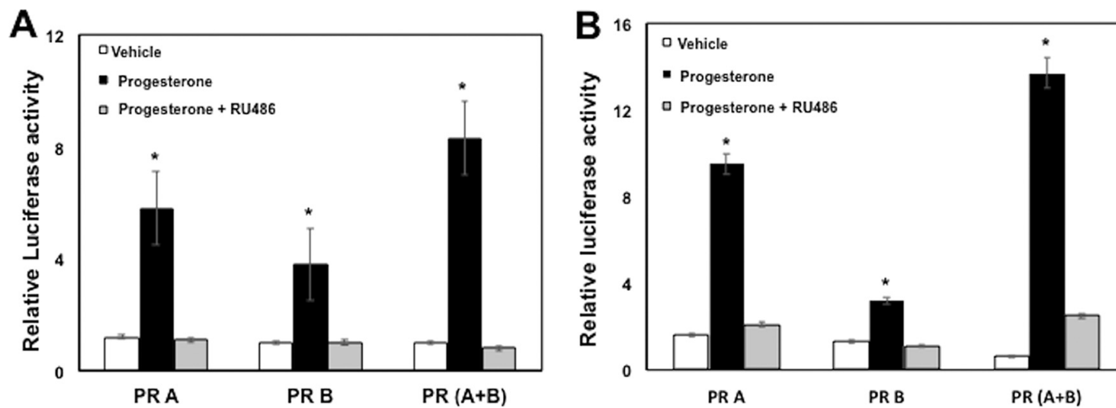

FIG 3 PR activates the IEtu1 promoter. Neuro-2A cells were transfected with the IEtu1 collapsed promoter construct containing the firefly luciferase reporter gene (0.5 μg of DNA) (A), MMTV LTR promoter construct (0.5 μg of DNA) (B), a plasmid that expresses the human PR protein (1.0 μg of DNA), and a plasmid that expresses *Renilla* luciferase (0.05 μg of DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. At 24 h after transfection, designated cultures were treated with 2% stripped fetal calf serum, and then DMSO (vehicle), progesterone (1 μM; Tocris Bioscience), or RU486 (1 μM; Sigma) was added to cultures. At 48 h after transfection, cells were harvested and protein lysate was subjected to a dual-luciferase assay as described in Materials and Methods. Levels of promoter activity in the empty luciferase vector (pGL3-Basic Vector) were normalized to a value of 1; fold activation for other samples is presented. The results are the averages from 3 independent experiments, and error bars indicate SEs. An asterisk indicates a significant difference between the control and samples transfected with the PR and treated with progesterone ($P < 0.05$) using the Student *t* test.

(34, 36). Thus, it was not surprising to find that the promoter contains many potential transcription factor-binding sites (Fig. 1A); furthermore, these transcription factor-binding sites are well conserved in field strains and modified live vaccine strains (data not shown). For example, 18 virus isolates from the respiratory tract contained the same nucleotides spanning the TATA box (TAGCTTATAAA); conversely, the two nucleotides that are underlined were C and G residues in five virus isolates obtained from the genital tract. For all 23 viral isolates that were examined (respiratory and genital isolates), GRE1 and GRE2 were identical. However, genital isolates contained 5 identical nucleotide substitutions in sequences spanning the spacer between GRE1 and GRE2, and two additional substitutions were present in 3 out of 5 genital isolates. Considering that both GREs are conserved in 23 BoHV-1 isolates, this supports our prediction that these motifs are biologically significant.

The IEtu1 collapsed promoter construct (Fig. 1B) was used to test whether sequences adjacent to the GREs were transactivated by the PR because the full-length IEtu1 promoter construct we originally examined contains extensive sequences downstream from the start site of transcription; furthermore, sequences between the TATA box and the GREs negatively regulate transcription (52). Furthermore, the goal of this study was to test whether the PR transactivated the GREs and not examine the effect of surrounding sequences. We consistently found that the PR A isoform stimulated the IEtu1 collapsed promoter more than 5-fold when progesterone was added to cultures, whereas PR B stimulated promoter activity less than 4 fold (Fig. 3A). Adding the two PR isoforms in the transfection mixture increased progesterone dependent promoter activity more than 8-fold. As a positive control, we examined the effects of PR A and PR B on the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) because this promoter is stimulated by progesterone (53). PR A and PR A plus PR B activated MMTV promoter activity more than 9-fold when progesterone was added to cultures (Fig. 3B). Conversely, PR B stimulated MMTLV promoter activity less than 4-fold. RU486 significantly reduced the ability of progesterone to transactivate the IEtu1 collapsed promoter and the MMTV LTR to basal levels of promoter activity.

Localization of IEtu1 promoter sequences important for PR-mediated activation. To localize IEtu1 promoter sequences important for PR-mediated activation of the IEtu1 promoter, two additional constructs were prepared (Fig. 1B) and compared to the IEtu1 collapsed construct. IEtu1Δ796 was stimulated more than 25-fold by progester-

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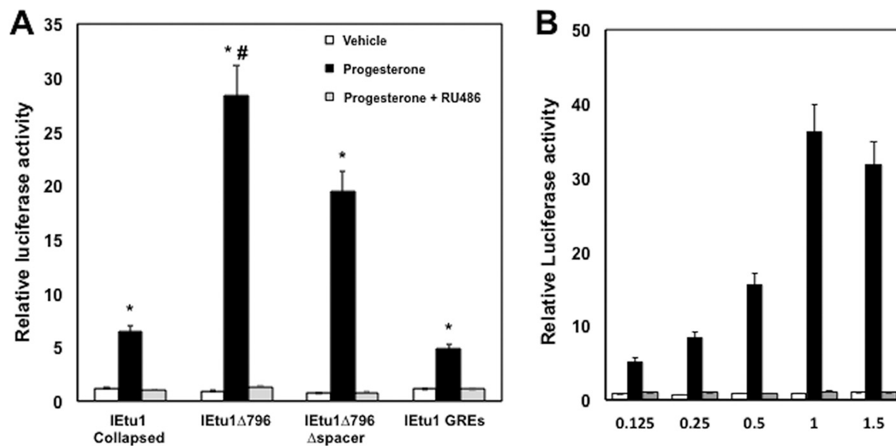

FIG 4 PR transactivates the IEtu1 promoter. Neuro-2A cells were cotransfected with the IEtu1 collapsed, IEtu1Δ796, IEtu1Δ796Δspacer, or IEtu1GREs (0.5 μg of DNA) luciferase construct, plasmids expressing PR A and B constructs (0.5 μg of DNA of each plasmid) (A), and a plasmid encoding *Renilla* luciferase (0.05 μg of DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. At 24 h after transfection, designated cultures were treated with 2% stripped fetal calf serum, and then DMSO (vehicle), progesterone (1 μM; Tocris Bioscience), and/or RU486 (1 μM; Sigma) was added to the cultures. An asterisk indicates a significant difference between the control and samples transfected with the PR and treated with progesterone ($P < 0.05$) using the Student *t* test. The pound sign indicates a significant difference between IEtu1Δ796 cotransfected with PR and treated with progesterone versus IEtu1 collapsed or IEtu1Δ796Δspacer cotransfected with PR and treated with progesterone, using the Student *t* test ($P < 0.05$). (B) Neuro-2A cells were transfected as for panel A using the IEtu1 collapsed luciferase construct and various concentrations of progesterone added. At 48 h after transfection, cells were harvested and the protein lysate was subjected to a dual-luciferase assay as described in Materials and Methods. Levels of promoter activity in the empty luciferase vector (pGL3-Basic Vector) were normalized to a value of 1, and fold activation for other samples is presented. The results are the averages from 3 independent experiments, and error bars indicate SEs.

one, and the IEtu1Δ796Δspacer construct was stimulated approximately 18-fold (Fig. 4A). Since IEtu1Δ796 lacked the TAATGARAT and Oct-like motif in the IEtu1 promoter (Fig. 1A), this result demonstrated that these motifs, which are critical for VP16-mediated activation of the IEtu1 promoter (32, 33), were not essential for PR-mediated transactivation. Spacing between the two GREs was also important because the IEtu1Δ796Δspacer construct was not as responsive to PR as was IEtu1Δ796. As another control, we examined the effect of progesterone on a 280-bp fragment of the IEtu1 promoter containing both GREs that was cloned upstream of the simian virus 40 (SV40) early promoter (IEtu1 GREs) (Fig. 1B). Progesterone stimulated the IEtu-1 GREs construct approximately 5-fold. RU486 reduced promoter activity to basal levels for all constructs, which was consistent with the other IEtu1 promoter constructs. The IEtu1Δ796 construct was further examined for PR responsiveness when increasing concentrations of progesterone were added (Fig. 4B). A 1 μM concentration of progesterone was optimal for stimulation of IEtu1Δ796 promoter activity. In summary, these studies confirmed that the IEtu1 promoter was transactivated by the PR and sequences between the TATA box and GREs influenced transactivation efficiency.

KLF15 and PR differentially regulate IEtu1 promoter activity compared to the GR. The PR and KLF15 synergistically transactivated the IEtu1 collapsed construct in a progesterone-dependent manner relative to PR alone or PR and KLF15 without progesterone treatment (Fig. 5A). Although the IEtu1Δ796 and IEtu1Δ796Δspacer constructs (see Fig. 1B for schematic of the constructs) were efficiently transactivated by the PR in a progesterone-dependent manner, KLF15 reduced promoter activity and had little effect on IEtu1Δ796 promoter activity. Relative to the IEtu1 collapsed construct, the IEtu1-GREs were not transactivated as efficiently by the PR in a progesterone-dependent manner; however, KLF15 enhanced transactivation in a progesterone-dependent fashion.

The ability of the GR and KLF15 to synergistically transactivate the respective IEtu1 collapsed promoter constructs exhibited differences from the PR and KLF15 (Fig. 5B).

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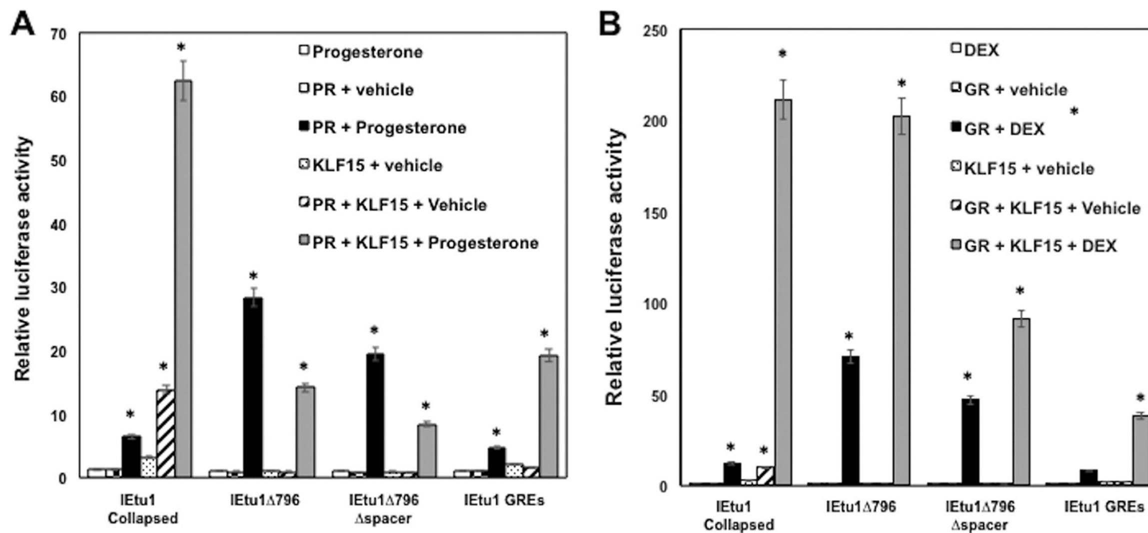

FIG 5 Localization of IEtu1 sequences necessary for activation mediated by GR or PR and KLF15. Neuro-2A cells were cotransfected with the designated IEtu1 promoter constructs containing the firefly luciferase reporter gene (0.5 μg of DNA) and, where indicated, a plasmid that expresses the human PR A and PR B (0.5 μg of DNA of each plasmid [A]), GR (1.0 μg of DNA [B]), a plasmid encoding *Renilla* luciferase (0.05 μg of DNA), and KLF15 (0.5 μg of DNA). To maintain the same amount of DNA in each sample, empty vector was included. Designated cultures were treated with 2% stripped fetal calf serum and then DMSO (vehicle), progesterone (1 μM), or DEX (10 μM; Sigma) at 24 h after transfection. At 48 h after transfection, cells were harvested and the protein lysate was subjected to dual-luciferase assays. Levels of promoter activity in the empty luciferase vector (pGL3-Basic Vector) were normalized to a value of 1, and fold activation for other samples is presented. The results are the average from 3 independent experiments, and the error bars indicate SEs. An asterisk indicates a significant difference between the control and samples transfected with the PR plus KLF15 and treated with progesterone ($P < 0.05$) using the Student *t* test.

For example, transactivation of the IEtu1 collapsed construct by the GR and KLF15 was more than 200-fold, whereas the PR and KLF15 transactivated the promoter approximately 3-fold less efficiently. Secondly, sequences missing from IEtu1Δ796 were crucial for transactivation by KLF15 and the PR regardless of progesterone addition; however, these sequences were not important for transactivation by KLF15, GR, and DEX. The IEtu1Δ796Δspacer construct removes 108 bp between GRE1 and GRE2, and these sequences were important for transactivation by the PR or GR and KLF15. In summary, these studies indicated that sequences downstream of the GREs in the IEtu1 promoter had differential effects on the ability of KLF15 to cooperate with the GR versus the PR.

GREs are essential for PR- and PR-plus-KLF15-mediated transactivation of IEtu1 promoter activity. Although a consensus PR response element is very similar to a GRE (50) and the PR can bind to and transactivate promoters containing GREs (51), it was important to directly test whether the GREs located in the IEtu1 promoter are required for PR activation. For these studies, we used the IEtu1Δ796 construct and mutated GRE1 and both GREs as previously described (13, 28) (Fig. 1C). As previously seen with the GR (13, 28), mutagenesis of both GREs significantly reduced PR-mediated transactivation in the presence of progesterone (Fig. 6A) and PR-plus-KLF15-mediated transactivation (Fig. 6B). Deletion of GRE1 has an effect, but low levels of PR- and PR-plus-KLF15-mediated transactivation were detected. Identical results were obtained with the IEtu1 GREs construct in which one or both GREs were mutated (data not shown).

To test whether the PR interacts with the GREs, chromatin immunoprecipitation (ChIP) studies were performed using the IEtu1Δ796 construct. The PR antibody (Fig. 7A and C), but not isotype control antibody, immunoprecipitated a 156-bp DNA fragment that is amplified by primers specific for the IEtu1 GREs. As expected, a detectable PCR product was not present in mock-transfected cells even in the input samples (Fig. 7A). We subsequently tested whether the PR was bound to IEtu1Δ796Δ2xGREs sequences because the two GREs in IEtu1Δ796 were required for efficient transactivation by the PR. In contrast to results obtained with the wild-type (wt) IEtu1Δ796 construct, we were unable to detect binding of the PR to sequences

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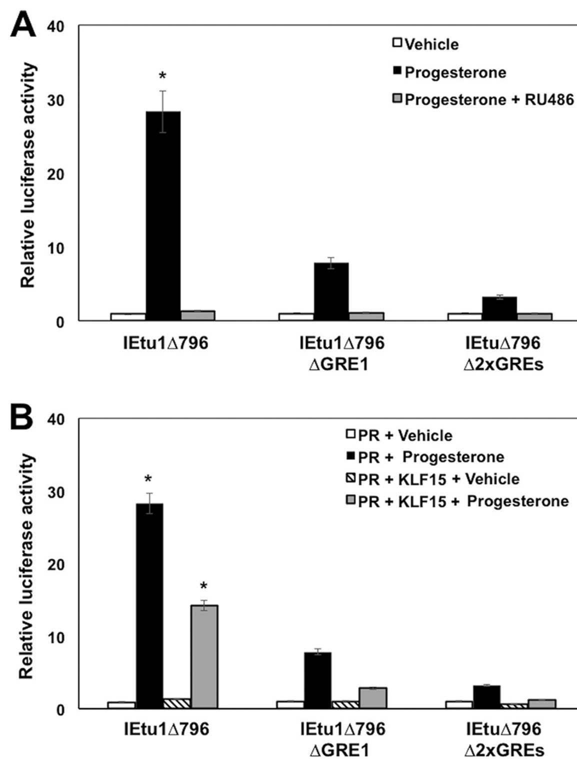

FIG 6 Intact GREs are crucial for transactivation by the PR and KLF15. (A) Neuro-2A cells were cotransfected with the designated IEtu1 promoter constructs containing the firefly luciferase reporter gene (0.5 μg of DNA) and, where indicated, a plasmid that expresses the human PR A and PR B (0.5 μg of DNA of each plasmid) and a plasmid encoding *Renilla* luciferase (0.05 μg of DNA). To maintain the same amount of DNA in each sample, empty vector was included. Certain cultures were treated with 2% stripped fetal calf serum and then DMSO (vehicle), progesterone (1 μM), and progesterone plus RU486 (1 μM) where indicated at 24 h after transfection. (B) Neuro-2A cells were cotransfected with the designated IEtu1 promoter constructs containing the firefly luciferase reporter gene (0.5 μg of DNA) and, where indicated, a plasmid that expresses human PR A and PR B (0.5 μg of DNA of each plasmid), a plasmid encoding *Renilla* luciferase (0.05 μg of DNA), and KLF15 (0.5 μg of DNA). To maintain the same amount of DNA in each sample, empty vector was included. Cultures were treated with 2% stripped fetal calf serum and, where indicated, DMSO (vehicle) and progesterone (1 μM) at 24 h after transfection. An asterisk denotes a significant difference between the control and samples transfected with the PR (A) or PR plus KLF15 (B) and treated with progesterone ($P < 0.05$) using the Student *t* test.

in the IEtu1Δ796 Δ2xGREs plasmid following transfection of Neuro-2A cells (Fig. 7B and C). As expected, these same primers, which flank the two GREs, amplified input samples transfected with the IEtu1Δ796Δ2xGREs construct (Fig. 7B).

To test whether the PR bound the BoHV-1 IE GRE region during productive infection, bovine kidney cells (MDBK) were mock infected or infected with BoHV-1 and treated with dimethyl sulfoxide (DMSO) vehicle or progesterone for 4 and 8 h postinfection, and ChIP assays were performed using an isotype control antibody or PR-specific antibody. PCRs of ChIP DNAs were done using a primer set that amplifies a 107-bp fragment containing the two IE GREs. As shown in Fig. 7D (PR) and Fig. 7E, the PR occupied the IE gene region containing the GREs in BoHV-1-infected MDBK cells at 8 h after infection. No product was seen in immunoprecipitates from mock-infected cells (Fig. 7D, lanes m) or in the isotype control immunoprecipitates. Similar to the results obtained using transfected promoter constructs, progesterone treatment had no impact on the PR occupancy of the viral IE GRE region (Fig. 7D and E). In summary, these studies demonstrated that the PR interacted with the GREs located within the IEtu1 promoter, and sequences encompassing the GREs in the IEtu1 promoter were bound by the PR in productively infected bovine cells.

KLF15 and the PR transactivated the bICP0 E promoter in a cooperative fashion. Relative to HSV-1 and HSV-2, the organization of the BoHV-1 bICP0- and bICP4-coding

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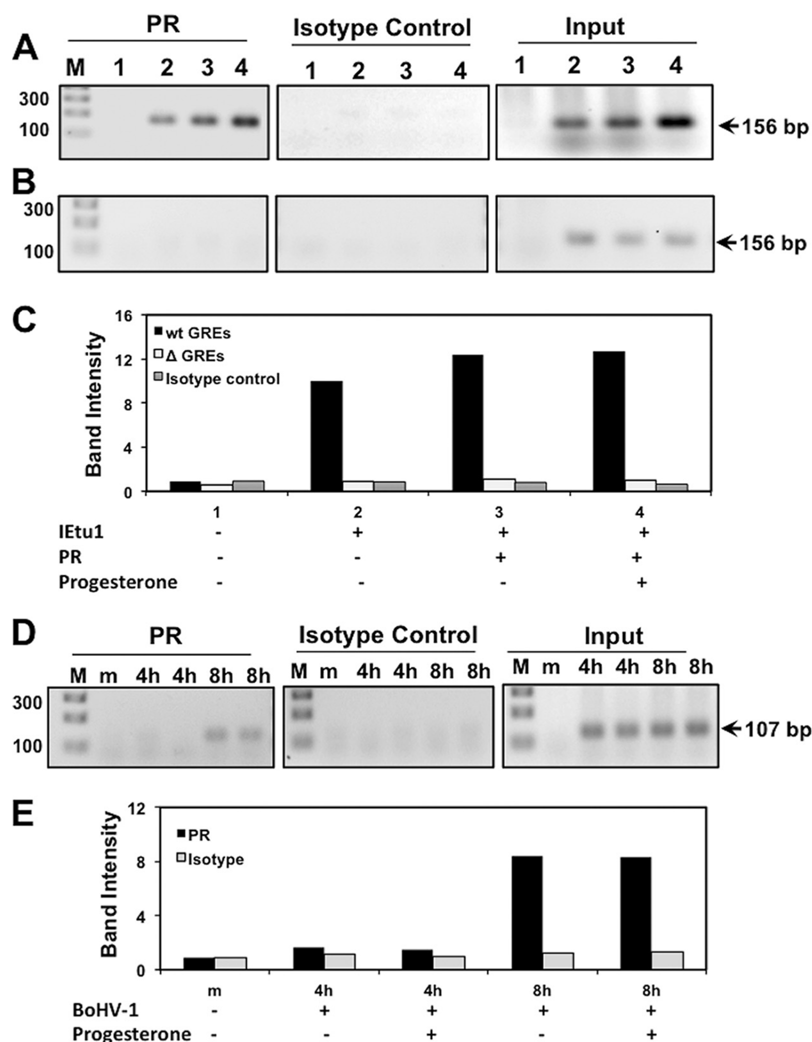

FIG 7 The PR interacts with GREs located in the IEtu1 promoter. (A and B) Neuro-2A cells were cotransfected with the IEtu1Δ796 construct (A) or IEtu1Δ796Δ2xGREs (B) (3.0 μg of DNA) and PR A and PR B plasmid (1.5 μg of DNA of each PR construct). Empty vector was added to maintain the same concentration of DNA in each transfection assay. Lane 1, Neuro-2A cells transfected with no plasmid; lane 2, Neuro-2A cells transfected with IEtu1Δ796 construct; lane 3, Neuro-2A cells transfected with IEtu1Δ796 construct and the PR (no progesterone treatment); lane 4, Neuro-2A cells transfected with IEtu1Δ796 construct and PR. Where indicated, cultures were treated with progesterone for 1 h before harvesting of cells. Transfected cells were processed for ChIP as described in Materials and Methods, and immunoprecipitation (IP) was conducted using the PR antibody or isotype control antibody. Input was 10% of the total DNA-protein complexes that were used for IP and PCR performed as described in Materials and Methods. (C) Comparison of DNA for the wt and mutated IEtu1Δ796 construct amplified by the IEtu1GREs specific primers that was immunoprecipitated with the PR antibody relative to the isotype control antibody shown in panels A and B. The intensity of bands in panel E was quantified using the Bio-Rad ChemiDoc system. These results are representative of those from three independent experiments. (D) MDBK cells were mock infected (m) or infected with BoHV-1 (MOI = 1) and treated with vehicle or progesterone for 4 h and 8 h postinfection. The PR occupancy was assessed by ChIP assay using the IEtu1 GRE primer set. Input consisted of 10% of total DNA. M, DNA size markers. (E) The intensity of bands was quantified as for panel C. These results are representative of those from three independent experiments.

regions has two dramatic differences. As mentioned above, there is a single promoter (IEtu1) that drives IE expression of two mRNAs, IE/2.9 and IE/4.2, from an alternatively spliced transcript. IE/2.9 encodes bICP0, and IE/4.2 encodes bICP4 (34–36) (Fig. 1A). Second, an E promoter drives expression of an early transcript (E/2.6) that is translated into bICP0. Since PR-mediated activation of productive infection was partially resistant to the effects of RU486 but transactivation of the IEtu1 promoter by PR was inhibited by RU486, the PR and KLF15 may regulate other promoters in a progesterone-

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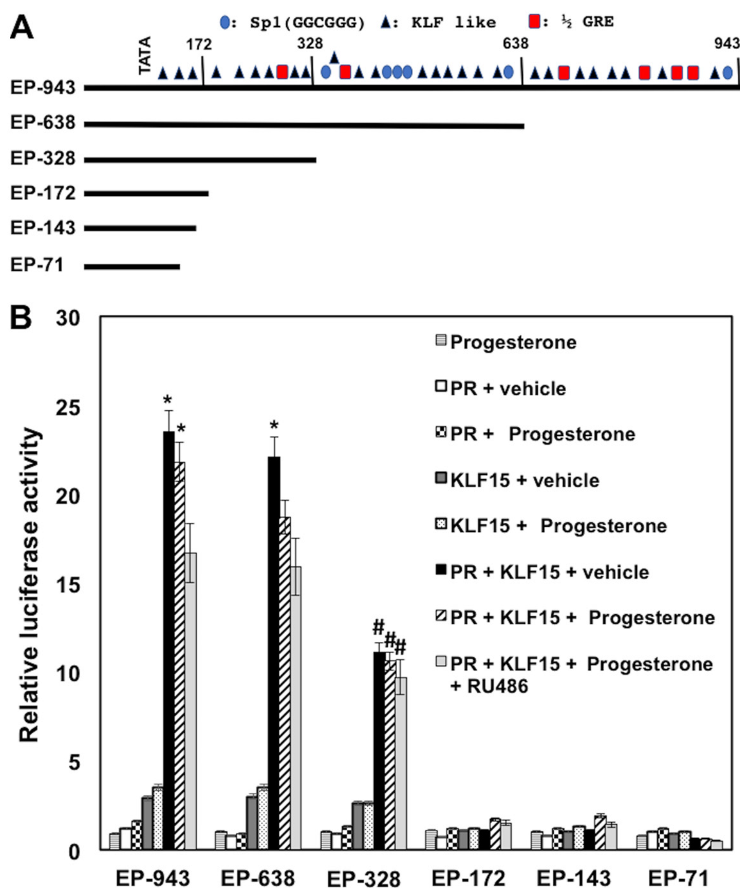

FIG 8 The bICP0 early promoter is transactivated by the PR and KLF15. (A) Schematic diagram of bICP0 early promoter and promoter deletion constructs used in this study. Positions of Sp1 binding sites, half-GREs, putative KLF binding sites, TATA boxes, and start site of transcription (arrow) are shown. (B) Neuro2-A cells were cotransfected with 0.5 μg of the designated bICP0 E promoter constructs containing the firefly luciferase gene downstream, 0.05 μg of a plasmid encoding *Renilla* luciferase, human PR A and B expression plasmid (0.5 μg of DNA of each plasmid), and 0.5 μg of KLF15 expression plasmid. DNA concentrations were equalized for all transfections by using an empty expression vector. Neuro-2A cells were treated with 2% stripped fetal calf serum 24 h after transfection, and then cultures were treated with DMSO (vehicle), progesterone (1 μM; Tocris Bioscience), RU486 (1 μM; Sigma), or RU486 plus progesterone. At 48 h posttransfection, cells were collected and processed for the dual-luciferase assay. Levels of promoter activity in the empty luciferase vector (pGL3-Basic Vector) were normalized to a value of 1, and fold activation for other samples is presented. The results are the averages from 3 independent experiments, and error bars indicate SEs. An asterisk indicates significant differences ($P < 0.05$) in cells transfected with the EP-943 cotransfected with PR plus KLF15 relative to all other EP-943 samples except when EP-943 was cotransfected with PR plus KLF15 and treated with progesterone. The EP-638 sample cotransfected with KLF15 plus PR was significantly different relative to all other samples transfected with EP-638; the results of EP-638 or EP-943 cotransfected with KLF15 plus PR were not significantly different. With respect to EP-328, the pound sign indicates a significant difference from the other EP-328 samples. The results for EP-943 or EP-638 cotransfected with KLF15 and GR were significantly different than EP-328 cotransfected with the same constructs. The Student *t* test was used for analyzing the results.

independent manner. Consequently, we tested whether the PR and KLF15 regulated bICP0 E promoter activity using a series of promoter deletion constructs previously described (54), as shown in Fig. 8A. These studies revealed KLF15 and PR A plus B synergistically stimulated EP-943 and EP-638 promoter activity, but progesterone treatment did not dramatically increase promoter activity (Fig. 8B). Furthermore, PR plus KLF15 plus progesterone did not further stimulate EP-943 or EP-638 promoter activity, and addition of RU486 reduced promoter activity less than 2-fold. Although EP-328 was stimulated more than 10-fold by KLF15 and the PR, the efficiency of transactivation was significantly different from those of EP-943 and EP-638. EP-172, EP-143, and EP-71 were not transactivated by the PR and KLF15. Relative to the IEtu1 promoter, two striking

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differences were observed. First, PR- and KLF15-mediated transactivation of IEtu1 promoter activity was significantly reduced by RU486; conversely, RU486 had little effect on transactivation of the bICP0 early promoter. Second, the bICP0 early promoter does not contain consensus "whole" GREs with dyad symmetry; however, six putative half-GREs were identified (Fig. 8A). Four of the half-GREs were located between 638 and 943, and deletion of these sequences had little effect on PR-mediated transactivation. However, there are half-GREs located between 328 and 638 and between 172 and 328, implying these motifs may play a role in PR-mediated transactivation of the bICP0 promoter. Each potential half-GRE was flanked by consensus Sp1 and/or potential KLF binding sites, which makes it difficult to predict which site is important. In summary, bICP0 early promoter sequences from 172 to 638 were crucial for transactivation by the PR and KLF15.

The PR and KLF15 can transactivate specific intergenic regions containing GREs. The BoHV-1 genome contains approximately 100 putative GREs (13) (Fig. 9A), suggesting that some are activated by the PR. Thirteen regions in the viral genome contain at least 2 putative GREs and a potential KLF binding site within 400 bp (Fig. 9A and B). No preference was given to whether these sequences were contained in a known viral promoter because of the close proximity of viral genes to each other. Sequences containing these regions were synthesized and cloned upstream of a minimal promoter (pGL3-Promoter Vector) to test whether these sequences were transactivated by the PR and KLF15. As with the GR (28), the PR only had a modest effect transactivating the respective intergenic fragment when progesterone was added (Fig. 9C). Four fragments, CIRC, UL52, bICP4, and IEtu2, were transactivated more than 10-fold by KLF15 plus PR (A and B) plus progesterone (Fig. 9C). Although RU486 reduced transactivation of the CIRC, UL52, IEtu2, and US intergenic fragments, RU486 had virtually no effect on the bICP4 intergenic fragment. Addition of progesterone to the bICP4 construct also had little effect on KLF15- and PR-mediated transactivation. Strikingly, the UL52 fragment was the only intergenic fragment transactivated 10-fold by the GR plus KLF15 and DEX (28).

DISCUSSION

Based on studies presented in this report and previously published studies (1, 6, 55), we hypothesize that the ability of progesterone and the PR to stimulate productive infection and activate crucial viral promoters influences reproductive complications, including abortion (Fig. 10). During acute infection of pregnant cows, the PR and progesterone have the potential to stimulate viral gene expression and replication. Consequently, viral spread to reproductive tissues—the ovaries, corpus luteum, and fetus—would occur with an increased frequency (1). Strikingly, susceptibility to genital herpes infection is increased by progesterone, in part due to decreased immune responses (56), which is relevant to this model. During latency, stress as mimicked by the synthetic corticosteroid DEX is a molecular switch that activates the GR and induces reactivation from latency (17–20, 25, 57–61) (Fig. 10). We predict that stress and progesterone, by virtue of interfering with immune responses or stimulating productive infection, may enhance the frequency of reactivation in pregnant cows, culminating in virus spread to ovaries or a developing fetus. Progesterone is considered a neurosteroid because it is synthesized in the nervous system (62, 63) and the PR has been reported to be expressed in certain central nervous system (CNS) and sensory neurons (64–66), which suggests that progesterone may influence viral transcription in latently infected TG neurons. Unlike most transcription factors, the PR is a "pioneer" transcription factor that remodels silent chromatin and activates transcription (67). The BoHV-1 genome contains more than 100 putative GREs (13), suggesting that cells expressing activated PR can transform a quiescent viral genome into an actively transcribing genome. Additional studies are necessary to test whether progesterone has the potential to directly stimulate reactivation from latency or enhance the ability of stress to increase the incidence of reactivation from latency.

In response to stress, the GR and KLF15 regulate gene expression dynamics and

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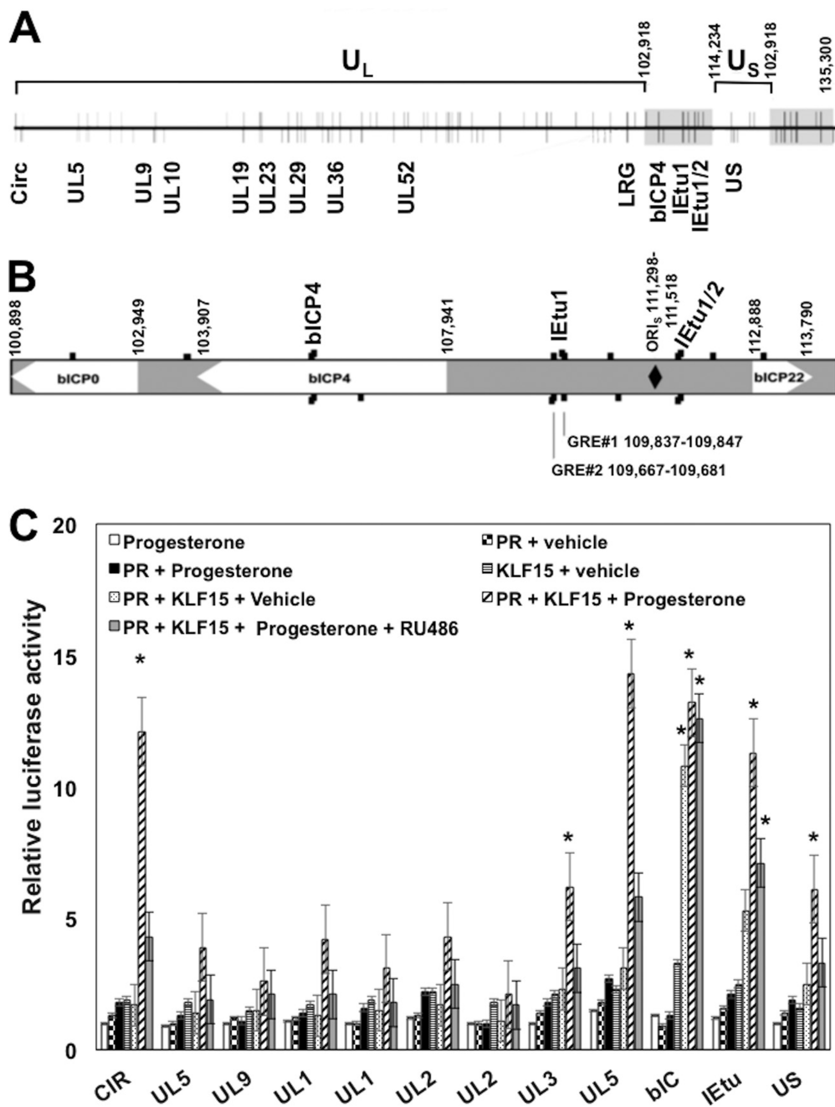

FIG 9 Schematic of GREs in BoHV-1 IE genome and activation of intergenic regions by the PR and KLF15. (A) Diagram of linear BoHV-1 genome (horizontal line) with predicted GREs denoted by vertical lines. Lines above the genome represent GREs on the positive/forward DNA strand, while lines below indicate GREs on the negative strand. The terminal repeats are indicated by gray rectangles. The locations of the unique long region and unique short region are indicated. Genomic regions that contain at least two putative GREs and potential KLF binding sites and are 400 bp or less are indicated by the gene in which they are located. These sequences were synthesized (GeneScript), cloned into pGL3-Promoter Vector as previously described (28) and then used for studies described below. (B) Expanded genomic region that includes bICP0, bICP4, and bICP22. Genomic sequences located between bICP4 and bICP22 contain the origins of replication (ORIs [black diamond]) and IEtu1 and IEtu2 to the left and right of the ORIs, respectively (not highlighted). Black boxes indicate enlarged GREs on positive (above) or negative strands (below). GRE1 and GRE2 within the IEtu1 promoter and the genomic region are indicated for the beginning and end of the sequences. (C) Neuro-2A cells were cotransfected with the designated plasmid constructs (0.5 μg of DNA) containing the firefly luciferase gene downstream and indicated BoHV-1 genomic sequences, a plasmid encoding *Renilla* luciferase (0.05 μg of DNA), KLF15 expression plasmid (0.5 μg of DNA), and human PR A and B expression plasmids (0.5 μg of each plasmid). To maintain equal plasmid amounts in the transfection mixtures, the empty expression vector was added as needed. For these studies, Neuro-2A cells were treated with 2% stripped fetal calf serum 24 h after transfection and then cultures were treated with DMSO (vehicle), progesterone (1 μM; Tocris Bioscience), and/or RU486 (1 μM; Sigma). At 48 h after transfection, cells were harvested and protein extracts subjected to dual-luciferase assay. Promoter activity in the empty luciferase vector (pGL3-Promoter Vector) was normalized to a value of 1, and fold activation for other samples was calculated. The results are the averages from 3 independent experiments, and error bars indicate SEs. An asterisk indicates a significant difference between the control ($P < 0.05$) using the Student *t* test; only the constructs stimulated more than 10-fold were analyzed.

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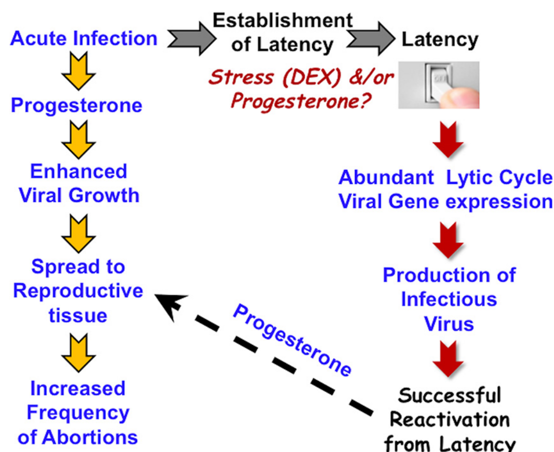

FIG 10 Hypothetical model summarizing how BoHV-1 replication and spread is enhanced by progesterone. For details, see text.

integrate signals by a feed-forward transcription loop (68–70) because the GR stimulates KLF15 expression (25, 68–73), KLF15 and the GR stably interact, and these transcription factors synergistically transactivate specific promoters (69, 70, 74). The PR and KLF15 also appear to fit the criteria for a feed-forward loop because the PR and progesterone stimulate KLF15 expression, which is required for maximal activation of E2F1 expression (75), and regulation of embryo implantation (49). Preliminary studies suggest that there is a weak or indirect interaction between KLF15 and the PR (data not shown).

The IEtu1 promoter contains several potential binding sites for cellular transcription factors (Fig. 1B), including negative regulatory elements (52). Interestingly, the TATA box and single TAATGARAT motif are more than 700 bases from the first GRE (GRE2 [Fig. 1B]) and the GREs are not required for VP16-mediated activation of the IEtu1 promoter (32). The ability of the PR plus progesterone or the GR plus DEX to transactivate the IEtu1 collapsed promoter is affected by surrounding sequences because the IEtu1Δ796 construct was transactivated more efficiently. Conversely, IEtu1Δ796 was not transactivated by the PR plus KLF15 plus progesterone but was synergistically activated by the GR plus KLF15 plus DEX, indicating that cooperation between KLF15 and GR versus PR requires different sequences and/or transcriptional cofactors. The 420 bp deleted from the IEtu1 collapsed construct to generate IEtu1Δ796 contain several cellular transcription factor-binding sites (Fig. 1B). Considering that each cell type possesses qualitative and quantitative differences in cellular transcription factors, the presence of many cellular transcription factor-binding sites in the IEtu1 promoter may facilitate transcriptional activation in the absence of viral regulatory proteins.

In the absence of progesterone, the ability of KLF15 to cooperate with the PR to stimulate productive infection was similar to the effect observed with the PR and progesterone treatment. Although one could argue that there are low levels of progesterone in stripped serum, addition of RU486 did not reduce productive infection to basal levels. These observations suggest that the PR and KLF15 stimulated productive infection, in part, by an "unliganded" mechanism, which has been reported to occur and is dependent on PR phosphorylation (76). The finding that KLF15 and the PR stimulated the bICP0 E promoter and bICP4 intergenic region in an unliganded fashion (in the presence of RU486) was different relative to the effects of RU486 on IEtu1 promoter activity. The bICP0 E promoter, unlike the IEtu1 promoter, has no "whole" GREs: however, six putative half-GREs and GC-rich elements exist. KLF family members interact with GC, CA-rich sequences, and certain Sp1 binding sites (77–84); thus, it is difficult to predict where KLF15 may interact with BoHV-1 sequences. Furthermore, KLF family members, including KLF15, are effectors of nuclear receptor signaling (85), suggesting that interactions between GR or PR and KLF15 influence KLF binding to

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DNA. In summary, the ability of KLF family members to cooperate with the GR and PR to regulate BoHV-1 gene expression is complex but may have important effects on productive infection and key viral promoters in certain circumstances.

MATERIALS AND METHODS

Cells and virus. Mouse neuroblastoma cells (Neuro-2A) and bovine kidney cells (MDBK) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum (FCS), penicillin (10 U/ml), and streptomycin (100 μg/ml).

A BoHV-1 mutant containing the β-Gal gene in place of the viral gC gene was obtained from S. Chowdury (LSU School of Veterinary Medicine) (gCblue virus), and stocks of this virus grown in MDBK cells. The gCblue virus grows to titers similar to those of the wt parental virus and expresses the LacZ gene. Procedures for preparing genomic DNA were described previously (87).

Quantification of β-Gal-positive cells. Neuro-2A cells grown in 60-mm plates were cotransfected with 3 μg of the gCblue viral genome and the desired amounts of plasmid expressing PRA and PRB and KLF15 using Lipofectamine 3000 (L3000075; Invitrogen). At 48 h after transfection, cells were fixed with a solution containing 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) and then stained with a solution containing 1% Bluo-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.5 M $MgCl_2$ in PBS. The number of β-galactosidase (β-Gal)-positive cells was determined as described previously (17, 39, 86, 87). Progesterone (1 μM; Tocris Bioscience; 2835) or RU486 (1 μM; Sigma) was added to certain cultures to examine the effects KLF15 and PR A and B on productive infection. The results are expressed as fold induction relative to the control because this minimized the differences in cell density, Lipofectamine 3000 lot variation, and transfection efficiency.

Plasmids. The human progesterone receptor A and B isoforms in the pSG5 expression vector were obtained from Pierre Chambon (University of Strasbourg, Strasbourg, France). A mouse GR expression vector was obtained from Joseph Cidlowski, NIH. The KLF15 expression vector was obtained from Deborah Otteson (University of Houston).

A 280-bp fragment contains the two GREs located in the IEtu1 promoter and was previously described (28). These sequences were synthesized by GeneScript and cloned into pGL3-Promoter Vector at the unique KpnI and XhoI restriction (IEtu1 GREs).

The IEtu1 collapsed promoter construct was inserted at the KpnI and HindIII restriction sites of pGL3-Basic Vector. The IEtu1Δ796, IEtu1Δ796Δspacer, IEtu1Δ796ΔGRE1 and IEtu1Δ796Δ2×GREs constructs were synthesized by GeneScript and inserted at the KpnI and HindIII restriction sites of pGL3-Basic Vector (see Fig. 1B for a schematic of these constructs). The construction and characteristics of the BoHV-1 bICP0 E promoter and deletion constructs (EP-943, EP-638, EP-328, EP-172, EP-143, and EP-71) used in the present study were described previously (61). Numbers in the plasmid names refer to the length of the bICP0 E promoter fragment cloned into pGL3-Basic Vector (Promega). Truncations to the promoter were made from the 5′ terminus. All plasmids were prepared from bacterial cultures by alkaline lysis and 2 rounds of cesium chloride centrifugation.

Transfection and dual-luciferase reporter assay. Neuro-2A cells (8×10^5) were seeded into 60-mm dishes containing EMEM with 10% FCS 24 h prior to transfection. Two hours before transfection, medium was replaced with fresh growth medium lacking antibiotics. Cells were cotransfected with a plasmid containing the firefly luciferase gene downstream of the SV40 early promoter (0.5 μg of plasmid DNA) and a plasmid encoding *Renilla* luciferase under the control of a minimal herpesvirus thymidine kinase (TK) promoter (0.05 μg of DNA). To maintain equal plasmid amounts in the transfection mixtures, the empty expression vector was added as needed. Neuro-2A cells were incubated in 2% charcoal stripped fetal calf serum after transfection. At 24 h after transfection, Neuro-2A cultures were treated with progesterone (1 μM; Tocris Bioscience; 2835) and/or RU486 (1 μM; Sigma). In certain studies, water-soluble DEX (Sigma; P7556) was added to cultures. Forty hours after transfection, cells were harvested and protein extracts were subjected to a dual-luciferase assay using a commercially available kit (E1910; Promega). Luminescence was measured by using a GloMax 20/20 luminometer (E5331; Promega).

ChIP assay. Neuro-2A cells were grown on 100-mm dishes and cotransfected with the desired promoter constructs. Cultures were treated with progesterone (1 μM; Tocris Bioscience; 2835) in 2% stripped serum medium for 1 h before harvesting cells. MDBK cells were mock infected or infected with BoHV-1 (multiplicity of infection [MOI] = 1). As indicated, cultures were treated with DMSO vehicle or progesterone for 4 and 8 h after infection. All steps involved with chromatin immunoprecipitation (ChIP) studies were performed as previously described (28) using 5 μl of PR antibody (alpha PR-22; Thermo Fisher Scientific; MA1-412) or isotype-specific antibody. PCR was performed using primers that amplify the IEtu1Δ796: forward primer 5′-CAAATAGGCAAAAGCAACG-3′ and reverse primer 5′-ATAATAGGAGC GGCTGTCGG-3′. These primers are adjacent to the two GREs and thus yield a 156-bp product even when the GREs are mutated. For productively infected cells, different primers were used: forward primer 5′-CCCACTTTTGCCTGTGTG-3′ and reverse primer 5′-TTTTCCTCCTCCTTCCCC-3′. These primers yield a product of 107 bp and were previously described (28).

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