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Pioneer transcription factors, progesterone receptor and Krüppel like transcription factor 4, cooperatively stimulate the bovine herpesvirus 1 ICP0 early promoter and productive late protein expression.

Laximan Sawant,

Nishani Wijesekera,

Clinton Jones*

Oklahoma State University, College of Veterinary Medicine, Department of Veterinary Pathobiology, Stillwater, OK 74078

Abstract

Bovine herpesvirus 1 (BoHV-1), including commercially available modified live vaccines, readily infect the fetus and ovaries, which can cause reproductive failure. The BoHV-1 latency-reactivation cycle in sensory neurons further complicates reproductive failure because progesterone sporadically induces reactivation from latency. The progesterone receptor (PR) and Krüppel-like transcription factor 15 (KLF15) cooperatively stimulate productive infection and the immediate early transcription unit 1 (IEtu1) promoter. In addition to the IEtu1 promoter, the bICP0 gene also contains a separate early (E) promoter. In this study, we tested the hypothesis that PR and KLF family members transactivate the bICP0 E promoter. PR and KLF4 stimulated bICP0 E promoter activity and expression of late productive viral protein expression in a cooperative manner. Additional studies revealed three enhancer domains within the bICP0 E promoter were responsive to PR and KLF4. Chromatin immunoprecipitation studies demonstrated PR and KLF4 occupy bICP0 E promoter sequences in transfected Neuro-2A cells and at late times following infection of bovine kidney cells. Co-immunoprecipitation studies indicated PR and KLF4 stably

*Corresponding author.

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- Laximan Sawant, Nishani Wijesekera, and Clinton Jones: Data curation
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- Laximan Sawant and Nishani Wijesekera: Visualization and Investigation
- *Clinton Jones:* Supervision
- Clinton Jones: Writing- Reviewing and Editing

interact with each other. These studies suggest cooperative activation of the bICP0 E promoter by PR and KLF4 correlate with interactions between these pioneer transcription factors.

Keywords

abortions; progesterone; bovine herpesvirus 1 (BoHV-1); Krüppel-like transcription factor 4; viral replication and gene expression

INTRODUCTION

Following infection of the ovary and/or fetus, bovine herpesvirus 1 (BoHV-1) can cause reproductive failure in cattle (Chase et al., 2017). Detection of viral replication in ovaries and corpus luteum correlates with reproductive complications (Miller and van der Maaten, 1984; Miller et al., 1989). BoHV-1 can also cause “abortion storms” with significant numbers of cows undergoing abortion: hence, BoHV-1 is the most frequently diagnosed cause of viral abortion in the United States. Commercially available modified live vaccines (Miller et al., 1995), including a virus strain lacking thymidine kinase (Chase et al., 2017) can also cause reproductive failure. Strikingly, naive heifers vaccinated with an inactivated BoHV-1 vaccine have significantly reduced abortion rates relative to heifers vaccinated with a modified live vaccine (Chase et al., 2017; Miller et al., 1995).

Following acute infection, sensory neurons in trigeminal ganglia (TG) are a primary site for lifelong latency after acute infection (Jones, 2013; Jones et al., 2011). BoHV-1 reactivation from latency is increased by stressful stimuli and the synthetic corticosteroid dexamethasone (DEX) consistently triggers reactivation from latency (Jones, 1998), in part by stimulating viral replication (Kook et al., 2015). Interestingly, rabbits latently infected with BoHV-1 also reactivate from latency following treatment with progesterone (P4) (El-mayet et al., 2020), a hormone crucial for maintaining pregnancy (Boomsma and Paoletti, 2002). DEX (El-mayet et al., 2020; Rock et al., 1982), but not P4 (El-mayet et al., 2020), induces reactivation in all rabbits latently infected with BoHV-1. Viral transcriptional regulatory proteins (bICP0, bICP4, and VP16) are detected in TG neurons within 3 hours after DEX treatment (Frizzo da Silva et al., 2013; Rock et al., 1992b; Winkler et al., 2000). Cellular transcription factors are also induced in TG of calves latently infected with BoHV-1 within hours after DEX treatment (Workman et al., 2012). Promyelocytic leukemia zinc finger (PLZF) and Slug are induced more than 10-fold within three hours after DEX treatment. Four Krüppel-like transcription factor 4, (KLF4), KLF6, KLF15, PLZF, SPDEF (Sam-pointed domain containing Ets transcription factor), and GATA6 are also induced during early stages of DEX-induced reactivation. These stress-induced transcription factors stimulate productive infection and certain key viral promoters. While it is currently unclear whether P4 induces expression of the same cellular transcription factors during reactivation from latency, we suggest DEX and P4 promote reactivation from latency by stimulating expression of cellular transcription factors that transactivate key viral promoters: consequently, productive infection ensues. The ability of PR and GR to suppress immune responses (Rhen and Cidlowski, 2005; Szekeres-Bartho and Poberts, 2010) is also predicted to enhance virus replication and spread during later stages of reactivation from latency.

The glucocorticoid receptor (GR) and PR belong to the Type I family of nuclear hormone receptors; thus, PR can bind most GR response elements (GREs) and stimulate promoters containing GREs (Claessens and Helsen, 2017; Szekeres-Bartho and Polgar, 2017; Oakley and Cidlowski, 2013). GR-corticosteroid complexes or PR-P4 complexes translocate the nucleus, interact with a GRE, and then stimulates transcription. The BoHV-1 genome contain more than 100 GREs (Kook et al., 2015) suggesting DEX and P4 regulate expression of certain viral promoters. Two functional GREs are present in the BoHV-1 immediate early transcription unit 1 (IEtu1) promoter (El-Mayet et al., 2017; Kook et al., 2015). The IEtu1 promoter drives immediate early expression of two key viral transcription regulatory proteins, infected cell protein 0 (bICP0) and ICP4 (Wirth *et al.*, 1989, 1991, 1992). GR or PR cooperates with KLF15 to stimulate the IEtu1 promoter, and the two GREs are crucial for cooperative transactivation of IEtu1 promoter activity (El-mayet et al., 2020; El-mayet et al., 2019; El-Mayet et al., 2017). Furthermore, GR or PR and KLF15 cooperatively transactivate the bICP0 early (E) promoter (El-mayet et al., 2019; El-Mayet et al., 2017).

For these studies, we tested whether PR cooperates with KLF4 to cooperatively activate the bICP0 E promoter. The rational for this study comes from previous studies that demonstrate GR and KLF4 cooperatively transactivate the bICP0 E promoter (El-mayet et al., 2020) and PR and GR bind and transactivate many of the same GREs. For these studies, we define "cooperative transactivation" as the ability of GR and KLF4 to work or act together. KLF4 and PR are pioneer transcription factors that can specifically bind silent chromatin, and in conjunction with other transcriptional coactivators remodel silent chromatin and activate transcription (Iwafuchi-Doi and Zaret, 2014; Mayran and Doulin, 2018; Soufi et al., 2015; Zaret and Carrol, 2011). In contrast, most transcription factors bind DNA in an open or relaxed chromatin confirmation. Since HSV-1, and presumably BoHV-1, exists as silent chromatin during latency (Knipe and Cliffe, 2008), cellular pioneer transcription factors may stimulate viral gene expression during early stages of reactivation from latency.

MATERIALS AND METHODS

Cells and Virus

Mouse neuroblastoma cells (Neuro-2A) and bovine kidney cells (CRIB) were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 ug/ml).

The wild-type Cooper strain of BoHV-1 was used for certain studies. 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 CRIB cells. The gCblue virus grows to similar titers as the wt parental virus and expresses the Lac Z gene. Procedures for preparing genomic DNA were described previously (Inman et al., 2001).

Quantification of β-Gal positive cells

Neuro-2A cells grown in 60 mm plates were cotransfected with 1.5 μg of the gCblue viral genome and the designated amounts of plasmid expressing PR or KLF4 using Lipofectamine 3000 (catalog no. L3000075; Invitrogen). At 48 h after transfection, cells were fixed with a solution containing 2% formaldehyde and 0.2% glutaraldehyde in phosphatebuffered 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 βgalactosidase (β-Gal)-positive cells was determined as described previously (El-mayet et al., 2020; El-Mayet et al., 2017). The number of β-Gal positive cells in cultures expressing the blank vector was set at 1 for each experiment. To calculate fold change of β-Gal positive cells, the number of blue cells in cultures transfected with the plasmids of interest were divided by the number of blue cells in cultures transfected with the blank vector. The effect KLF4, P4 and PR had on the number of β-Gal+ cells is expressed as fold induction relative to the control. This approach minimized the differences in cell density, Lipofectamine 3000 lot variation, and transfection efficiency.

Plasmids

The human PR-A and PR-B expression plasmids are in the pSG5 expression vector and were obtained from Pierre Chambon (University of Strasbourg, Strasbourg, France). The KLF4 expression vector was obtained from Dr. Jonathan Katz (University of Pennsylvania). The KLF9 expression vector was purchased from Neogen Bio-system. The KLF6 expression vector was obtained from Dr. Bin Guo (North Dakota State University). The PLZF expression vector was obtained from Dr. Derek Sant'Angelo (Sloan-Kettering Cancer Center). The Slug expression vector was obtained from Paul Wade (NIEHS, Research Triangle Park, NC).

The construction and characteristics of the BoHV-1 bICP0 E promoter and deletion constructs (EP-943, EP-638, EP-328, and EP-172) were described previously (Workman et al, 2009). Numbers in the plasmid name refer to the length of the bICP0 E promoter fragment cloned into pGL3-Basic Vector (Promega) (See Figure 3A and 4A for schematics of these constructs). The ½ GRE mutants depicted in Figure 5A were previously described (El-mayet et al., 2020). All plasmids were prepared from bacterial cultures by alkaline lysis and 2 rounds of cesium chloride ultracentrifugation.

Transfection and dual-luciferase reporter assay

Neuro-2A cells (8×10^5) were seeded into 60-mm dishes containing MEM with 10% FBS at 24 h prior to transfection. Two hours before transfection, MEM was replaced with fresh growth MEM lacking antibiotics. Cells were cotransfected with the designated plasmid containing the firefly luciferase gene (0. 5 ug plasmid DNA) and a plasmid that encodes *Renilla* luciferase under the control of a minimal herpesvirus thymidine kinase (TK) promoter (50 ng DNA). To maintain equal plasmid amounts in the transfection mixtures, the empty expression vector was added as needed. Neuro-2A cells were incubated in MEM containing 2% charcoal stripped FBS after transfection. At 24 h after transfection, Neuro-2A cultures were treated with P4 (100 nM; Tocris Bioscience; 2835) and/or RU486 (1 μM; Sigma). 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).

Chromatin immunoprecipitation (ChIP) assay

ChIP studies were performed using standard procedures as previously described (El-mayet et al., 2020; El-mayet et al., 2019; El-Mayet et al., 2017; Kook et al., 2015; Sawant et al., 2018;). In brief, Neuro-2A cells were grown in 100-mm dishes and cotransfected with the designated bICP0 E promoter constructs (3 μg DNA) and a plasmid that expresses PR-A and PR-B (1.5 μg of DNA of each PR construct) and/or KLF4 (1.5 μg DNA). Cells were transfected with the indicated plasmids using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, Neuro-2A cells were cultured in MEM containing 2% stripped FBS. Cultures were treated with vehicle (DMSO) or P4 (100 nM; Tocris) as denoted. Cells were harvested for ChIP analysis at 40 hours after transfection. ChIP analysis was also performed using CRIB cells that were mock infected or infected with BoHV-1 (multiplicity of infection [MOI] of 1), followed by P4 treatment for 4 h, 8 h, 16 h and 24 h after infection prior to harvesting cells. Formaldehyde cross-linked cells (0.72 % final concentration formaldehyde) were lysed in buffer A (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Cross-linking was stopped by addition of 2.5 ml of 2.5 M glycine and then incubating at 48° C for 5 min. Following sonication to generate DNA fragments of approximately 500 bp, cell lysate containing sheared DNA was precleared using salmon sperm DNA-agarose (Millipore) to remove DNA that non-specifically sticks to agarose. Input samples were collected (10 ul) from the pre-cleared sonicated DNA protein complexes (500 ul sample).

Cleared lysate (1/3 of the total lysate) was incubated in 5 ug of PR antibody (alpha PR-22; Thermo Fisher Scientific; MA1–412), 5 ug anti-KLF4 (ab72543; Abcam), or 5 ug control rabbit IgG (18140; Sigma) in buffer B (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA [pH 8.0], 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for 12 h at 4C. Immunoprecipitates containing sheared DNA fixed to the designated transcription factor were collected using protein A Dynabead beads (Life Technologies) and washed extensively with buffer C (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS). Samples were extracted twice with phenol: chloroform: Isoamyl alcohol to remove proteins associated with sheared DNA bound to a specific transcription factor. This DNA was then subjected to PCR using previously described primers (El-Mayet, 2017) that amplify sequences encompassing the GREs within the IEtu1 promoter (5'-TAGCCGCTCCATTCTCTC-3'and 5'-AAAAGTGGGGAAGCAGGG-3') that yields a 218-bp fragment. The bICP0 E promoter specific primers (5'-TCCGCCCCCCCCCAAAAAC-3' and 5'-GAAACCCCCCACGCAAGG-3') yield a 127 bp bICP0 E promoter fragment (El-mayet et al., 2020): for location of bICP0 E primers, see Figure 6A. DNA bands were quantified using Image Lab software and presented as percent input. Input samples represented 2% of total cell lysate.

Co-immunoprecipitation studies and Western blot analysis

Neuro-2A cells grown on 100 mm dishes were cotransfected with plasmids that express KLF4 (1.5 μg DNA) and 1ug of PR-A and PR-B expression plasmid. Designated cultures were treated with P4 (100 nM; Tocris Bioscience; 2835) in MEM containing 2% stripped FBS and P4 for 4 hours before harvesting of transfected cultures. Whole cell extracts were prepared with RIPA lysis buffer with 1× Protease Inhibitor cocktail (Thermo-scientific) and protein concentration quantified. Protein lysate (500 μg) was combined with anti-PR and /or anti-KLF4 (5 μg) antibodies and the reactions were incubated for overnight at 4°C on a rotator. Protein A Dynabeads® (cat. No 10001D, Life Technologies) were added and incubated for 2 h at 4° C with rotation. Immunoprecipitates were collected using a magnet (DynaMag™) (cat. No.12321D, Life Technologies), supernatants removed and the Dynabeads® -Ag-Ab complexes washed 3 times with 1ml of washing buffer (20 mM Tris HCL pH (8.0), 500 mM NaCl, 2 mM EDTA, 1% Triton X100, 0.1% SDS). Proteins were eluted from Dynabeads® by incubating with 30 μl of elution buffer (1%SDS, 100 mM $NaHCO_3$), the samples were vortexed, and then incubated in a water bath at 42° C for 30 minutes.

For SDS-PAGE, proteins were mixed with an equal amount of 2X sample loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 50 mM dithiothreitol, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. Proteins were separated in a SDS–10% PAGE gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) and blocked for 1 h in 5% w/v nonfat dry milk with 1× Tris-buffered saline–0.1% Tween 20 (TBS-T). Membranes were then incubated with the designated primary antibody at 4°C with gentle shaking overnight. The primary antibody was diluted 1:1,000 in the blocking solution. After 45 min of washing with TBS-T, the blots were incubated with secondary antibodies (peroxidase-conjugated immunoglobulin G (Amersham Biosciences), which was diluted 1: 2,000 in 5% nonfat milk in TBS-T for 1h. Blots were washed 45 min with TBS-T and exposed to Amersham ECL reagents, and then autoradiography performed. Primary antibodies were purchased from Thermo Fisher Scientific: anti PR (MA1–412) and Proteintech: anti- KLF4 antibody (11880–1-AP). The secondary anti-rabbit IgG antibody (7074S) was purchased from Cell Signaling.

Statistical analysis

Analyses of all data sets was performed, and graphs constructed using GraphPad Prism (version 7.0d). Ordinary one-way analysis t test with no correction for multiple comparisons was performed.

RESULTS

KLF4 and PR cooperate to stimulate productive infection

To examine the possibility that PR and KLF4 influence late viral protein expression, mouse neuroblastoma cells (Neuro-2A) were cotransfected with gC-Blue genomic DNA. We used Neuro-2A cells for these studies because they have neuronal like properties (Tremblay et al., 2010), approximately 50% of the cells are transfected, and BoHV-1 replicates at low levels in these cells (Thunuguntla et al., 2017). Neuro-2A cells were transfected with BoHV-1

gCblue DNA versus infecting these cells because VP16 and other regulatory proteins are present in the virion, bICP4 for example (Barber et al., 2017). Consequently, the stimulatory effects of cellular or viral proteins on productive infection are reduced or eliminated. β-Gal expression directly correlates with virus replication in highly permissive bovine or rabbit cells because the gC promoter is a late promoter and its expression is low prior to viral DNA replication. Time points between 24–48 h after transfection were used to count cells expressing β-Gal+ cells to minimize the number of virus-infected cells that resulted from virus spread, in essence secondary infection of surrounding cells (Geiser et al. 2012). However, it is possible that the number of β-Gal+ cells in Neuro-2A cells may not directly reflect productive infection because of the inefficient replication of BoHV-1 in these cells (Thunuguntla et al., 2017). Hence, for these studies we refer to an increase in the number of β-Gal+ Neuro-2A cells as increasing late viral protein expression. After transfection, cultures were incubated in MEM + 2% stripped FBS in the presence or absence of P4. FBS passed through a column containing “activated” charcoal removes hormones, lipid-based molecules, certain growth factors, and cytokines yielding stripped FBS: however, this process does not remove salts, glucose, and most amino acids. Cotransfection of gCBlue and PR+KLF4 stimulated the number of β-Gal+ Neuro-2A cells approximately 29-fold even when P4 was not added to cultures, which was significantly different compared to the effects seen by PR+ P4 treatment or when transfected with KLF4 alone (Figure 1). P4 treatment did not significantly increase the effects of PR+KLF4. Cultures were treated with RU486 because it is a PR specific antagonist and is commonly used to address whether transcriptional activation by PR is ligand dependent: in essence P4 must bind PR to activate transcription (Pandit et al., 2002; Schulz et al., 2002). RU486 decreased the number of β-Gal+ Neuro-2A cells suggesting productive late viral protein expression was stimulated by KLF4+PR in a ligand dependent manner. While we believe over-expressing KLF4 and PR directly transactivates the bICP0 E promoter, it is also possible that KLF4 and PR activate cellular genes, which then stimulate productive late viral gene expression.

PR cooperates with KLF4 to transactivate the bICP0 E promoter

The organization of BoHV-1 bICP0 and bICP4-coding regions is unique relative to the HSV-1 genome (Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991). For example, the IEtu1 promoter drives IE expression of IE/2.9 and IE/4.2 mRNAs, which are derived from a single alternatively spliced transcript (Figure 2A). IE/2.9 mRNA is translated into the bICP0 protein and IE/4.2 mRNA is translated into the bICP4 protein. Secondly, an E promoter drives expression of E/2.6, an early transcript translated into the bICP0 protein (Fraefel et al., 1994; Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991). bICP0 RNA is detected more frequently than bICP4 RNA in TG of calves during DEX induced reactivation from latency (Workman et al., 2009) suggesting the bICP0 E promoter is active during early stages of reactivation. Of note, previous studies also demonstrated GR and KLF4 cooperatively transactivated the full-length bICP0 E promoter (EP-943) (El-mayet et al., 2019). Based on these observations, we tested whether PR cooperates with stress-induced KLF family members to transactivate EP-943 promoter activity.

PR+KLF4 transactivated EP-943 activity approximately 16-fold in transfected Neuro-2A cells whereas PR alone stimulated promoter activity less than 2-fold (Figure 2B). P4

significantly reduced PR+KLF4 mediated transactivation of EP-943 to approximately 7 fold suggesting PR+KLF4 mediated transactivation occurred via a ligand independent manner (Ritter et al., 2012). Relative to KLF4 and PR, EP-943 was not cooperatively transactivated by PR and KLF6, KLF9, PLZF or SLUG regardless of P4 treatment. These studies demonstrated PR+KLF4 transactivated EP-943 significantly more than other stress-induced KLF family members.

Localization of bICP0 E promoter sequences important for PR+KLF4 mediated transactivation

bICP0 E promoter deletion mutants (Figure 3A) were used to localize sequences important for PR+KLF4 mediated promoter activation. PR+KLF4 mediated transactivation of EP-638 and EP-398 was significantly reduced relative to EP-943 (Figure 3B). P4 treatment reduced PR+KLF4 mediated transactivation of EP-638 and EP-328: however, RU486 treatment did not significantly influence promoter activity. The EP-172 construct was not significantly transactivated by PR and/or KLF4 regardless of P4 treatment. The studies in Figure 3 suggested separate enhancer domains upstream of the TATA box and promoter proximal sequences were transactivated by PR+KLF4 in a cooperative fashion.

Additional bICP0 E promoter mutants were prepared to further test the premise that cooperation occurred between these putative enhancer domains (Figure 4A). Deleting sequences between 172–328 (EP-943Δ172–328) significantly reduced PR+KLF4 mediated transactivation (Figure 4B). However, the Δ172–328 promoter construct was still transactivated by PR+KLF4 more than 10-fold and P4 treatment reduced promoter activity. Although PR+KLF4 mediated transactivation of EP-638 was not significantly different than EP-328, deleting sequences between 638–328 (EP943Δ328–638, Figure 4A) significantly reduced the effects of PR+KLF mediated transactivation regardless of P4 or RU486 treatment (Figure 4B). Collectively, these studies suggested PR+KLF4 mediated transactivation of the bICP0 E promoter required cooperation between different enhancer domains located between nucleotides 172–328, 328–368, and 638–943.

½ GREs in the bICP0 E promoter are not required for PR+KLF4 mediated transactivation

The bICP0 E promoter contains two ½ GRE-like motifs between nucleotides 638 and 943 (Figure 4A: yellow circles). These motifs have one nucleotide difference from a consensus ½ GRE that is transactivated by a GR monomer (Schiller et al., 2014). We tested whether mutating these ½ GREs (Figure 5A) influenced PR+KLF4 mediated transactivation when compared to EP-943 and EP-638. A construct containing one or both ½ GREs was transactivated by PR+KLF4 with similar efficiency as EP-943 (Figure 5B). There was also no significant difference between PR+KLF4 mediated transactivation of EP-943 and the ½ GRE mutants when P4 was added to cultures. Finally, the ½ GRE mutants were transactivated by PR+KLF4 more efficiently relative to EP-638: however, transactivation was similar to wt EP-943. These studies indicated the ½ GREs were not required for transactivation of the EP-943 promoter by PR+KLF4.

PR and KLF4 occupy bICP0 E promoter sequences in transfected or productively infected cells

To test whether PR and KLF4 directly interact with bICP0 E promoter sequences, chromatin immunoprecipitation (ChIP) studies were performed. Location of primers used for these studies and the size of the PCR product (127 bp) are shown in Figure 6A. Neuro-2A cells were cotransfected with EP-943, the PR expression plasmid, and/or KLF4 followed by treatment with P4. Relative to the isotype control antibody and mock-transfected cells, occupancy of bICP0 E promoter sequences was significantly higher when ChIP studies was performed with the PR antibody (Figure 6B). When Neuro-2A cells were transfected with EP-943 and PR was not over-expressed, PR occupied the promoter significantly more than the isotype control. However, PR and KLF4 over-expression did not significantly increase levels of binding to promoter sequences.

Occupancy of the bICP0 E promoter with KLF4 was significantly higher relative to the isotype control antibody and mock transfected cells even when KLF4 was included the transfection. KLF4 binding was slightly increased when KLF4 or PR+KLF4 was over-expressed: however, binding was not increased significantly. In general, KLF4 binding was not significantly influenced by P4 treatment. While these studies clearly demonstrated direct interactions occur between the bICP0 E promoter, PR, and KLF4 in the absence of viral proteins, they did not reveal cooperative binding between PR and KLF4 and over-expression of PR or KLF4 did not significantly increase promoter occupancy.

During productive infection of bovine kidney cells (CRIB), PR and KLF4 occupancy of the bICP0 E promoter was significantly higher at 16 and 24 hours after infection relative to the isotype control antibody (Figure 6C). Occupancy of the bICP0 E promoter by KLF4 was also significantly higher at 16 and 24 hours when cultures were not treated with P4 compared to the isotype control antibody. As a comparison to the bICP0 E promoter, we used the same DNA from infected CRIB cells to examine PR and KLF4 occupancy of the IEtu1 promoter. These studies revealed that significantly more PR occupied IEtu1 promoter sequences at 8, 16 and 24 hours after infection using primers that amplified a fragment encompassing the two GREs in this promoter (Figure 6D). KLF4 also occupied the IEtu1 promoter at 8, 16, and 24 hours after infection regardless of P4 treatment, which was earlier than observed for the bICP0 E promoter.

The inability to detect cooperative binding of KLF4 and PR to the bICP0 promoter in transfected or infected cells may have been influenced by the location of primers used in this study. For example, most of the putative KLF4 binding sites are upstream of the PCR product (Figure 6A). Considering the average size of sonicated DNA for these studies is 500 bp, the effect of these upstream binding sites may have been under-represented when ChIP was performed with the primers we used. We have tested other primer pairs between nucleotides 328 to 943. However, these primers yielded smeary bands above the product band and unexpected bands near the product making them unsuitable for ChIP studies. The GC content between nucleotide 328 to 943 of EP-943 is 87.5% GC, which has been problematic for developing suitable primers. Consequently, we quantified the PCR product rather than perform qPCR. In summary, PR and KLF4 occupied bICP0 E promoter sequences in transfected and productively infected cells. KLF4 also occupied the IEtu1

promoter prior to the bICP0 E promoter during productive infection of CRIB cells, which was similar to a study demonstrating GR and KLF15 occupied the IEtu1 promoter prior to the bICP0 E promoter (El-mayet et al., 2020). These studies also indicated the IEtu1 promoter is organized to express viral mRNAs prior to the bICP0 E promoter.

PR is associated with KLF4 in transfected Neuro-2A cells

To test whether PR is stably associated with KLF4, Neuro-2A cells were cotransfected with plasmids that express KLF4 and PR, and co-immunoprecipitation (co-IP) studies performed. Following IP with the PR antibody, we consistently detected KLF4 in the immunoprecipitate when cultures were not treated with P4 (Figure 7A) and when cultures were treated with P4 (Figure 7B). When IP was performed with KLF4 and the Western Blot probed with the PR specific antibody, the immunoprecipitate contained PR in the absence of P4 (Figure 7C) or presence of P4 (Figure 7D). In summary, these studies revealed KLF4 and PR were associated with each other in Neuro-2A cells when these proteins were over-expressed. Furthermore, the association between KLF4 and PR was independent of P4 treatment.

DISCUSSION

In this study, we examined the effects PR and stress-induced transcription factors have one bICP0 E promoter activity. These studies revealed PR and KLF4 cooperatively transactivated the bICP0 E promoter in transient transfection studies. Strikingly, KLF4 cooperated with PR to stimulate bICP0 E promoter activity significantly higher than PR, KLF4 and P4 treatment (Figures 3 and 4). Furthermore, P4 did not dramatically increase late viral gene expression when PR and KLF4 were over-expressed (Figure 1). These observations suggest PR and KLF4 stimulated bICP0 E promoter activity and productive late viral protein infection without P4 activating the PR, which is commonly referred to as an “unliganded” mechanism. Interestingly, unliganded PR activation requires phosphorylation (Li and O’Malley, 2003). P4 stimulates KLF4 expression (Shimizu *et al.*, 2010), in part by repressing expression of a miRNA that specifically binds KLF4 mRNA and reduces KLF4 protein expression (Cittelly *et al.*, 2013). Based on these published observations, we suggest that interactions between PR and KLF4 (Figure 7) in cells where there is limiting P4 levels or in the absence of P4 triggers bICP0 E promoter activity. The IEtu1 promoter is activated by PR via a P4 dependent mechanism. In summary, P4-dependent and independent mechanisms are predicted to promote virus replication and spread in the context of reproductive tissues of cattle. It should also be pointed out that PR and KLF4 may activate expression of cellular genes that activate bICP0 E promoter activity.

Three enhancer-like domains located between nucleotides 638–943, 328–638, and 172–328 were important for PR+KLF4 mediated transactivation (Figure 4A). These distinct enhancer domains contain numerous Sp1 binding sites, three nucleosome enriched KLF4 binding sites (Soufi et al., 2015), four KLF-like binding sites, and two potential CACC rich KLF binding sites. Additional GC and CA-rich motifs in the bICP0 E promoter may also be crucial because KLF family members were reported to bind GC and/or CA rich sequences (Black et al., 2001; Kaczynski et al., 2003; McConnell and Yang, 2010; Uchida et al., 2000). Since the bICP0 E promoter lacks “whole” GREs and the ½ GREs were not important for

PR+KLF4 mediated transactivation (Figure 5B). Consequently, we predict KLF4 guides PR to KLF, Sp1, or other CA/GC rich motifs in the bICP0 E promoter, which do not resemble consensus PR binding sites. Future studies will focus on identifying bICP0 E promoter sequences important for PR+KLF4 cooperative transactivation and testing whether mutating these motifs impair KLF4 and PR binding.

DEX stimulates reactivation from latency in all rabbits latently infected with BoHV-1 (El-mayet et al., 2020; Rock et al., 1992a): conversely, P4 sporadically induces reactivation (El-mayet et al., 2020). Since PR and GR cooperate with KLF15 to activate the IEtu1 promoter (El-mayet et al., 2019; El-Mayet et al., 2017) and PR and GR cooperate with KLF4 to transactivate the bICP0 E promoter (El-mayet et al., 2020), merely activating these two key viral regulatory promoters are not the only events necessary for triggering reactivation from latency. The BoHV-1 genome contains more than 100 putative GREs (Kook et al., 2015) suggesting DEX and PR differentially induce expression of certain viral and/or cellular genes. While the mechanisms by which nuclear hormones regulate BoHV-1 pathogenesis and reactivation from latency are unclear, fluctuations in DEX and P4 in cattle have the potential to facilitate virus transmission and survival in nature. Understanding how PR and GR mediate viral replication and reactivation from latency can lead to development of new modified live vaccines that do not reactivate from latency or replicate in certain tissues as well as wild-type BoHV-1. These vaccines would likely reduce economic losses caused by BoHV-1 acute infections and reactivation from latency.

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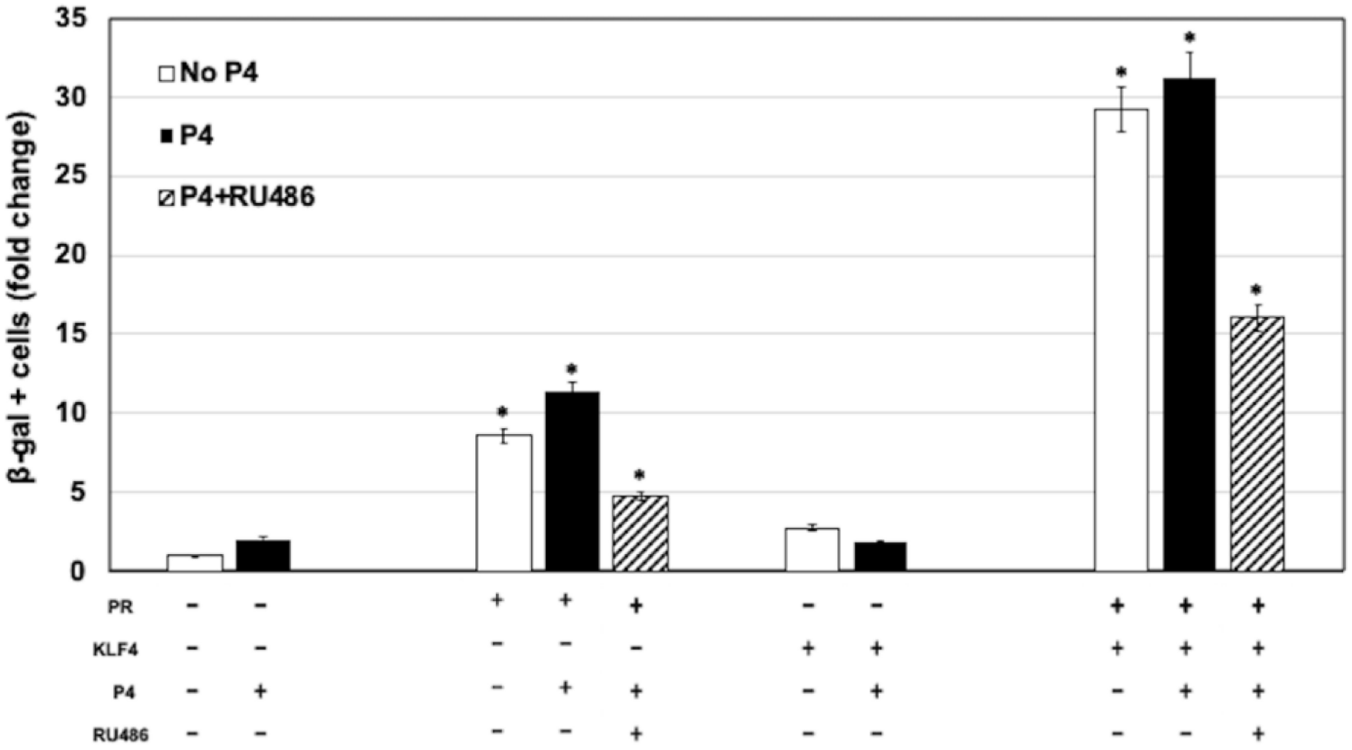

Figure 1. PR+KLF4 cooperate to stimulate late productive protein infection.

Neuro-2A cells were transfected with 1.5 ug BHV-1 gC-Blue and where indicated a plasmid that expresses the human PR protein (PR-A and PR-B) (1.0 ug DNA of each plasmid) and KLF4 (0.5 ug DNA). To maintain the same amount of DNA in each sample, empty vector was included in samples. Cells were incubated with MEM containing 2% stripped FBS 24 h after transfection. Designated cultures were treated with vehicle (DMSO), P4 (100 nM), DMSO (vehicle), and, where indicated, RU486 (1 uM). At 48 h after transfection, cells were fixed and stained for counting β-Gal+ cells. The value for the control (gC-Blue virus cotransfected with empty vector, treated with the DMSO vehicle after transfection) was set at 1. The results for P4 or RU486-treated cultures were compared to those for the control. The number of β-Gal+ Neuro-2A cells was counted from 4 independent quadrants/plate. The results are the average of 3 independent experiments. An asterisk indicates a significant difference between the control and samples transfected with the PR and/or KLF4 and treated with or without P4 or RU486 ($P < 0.05$) using the Student *t* test.

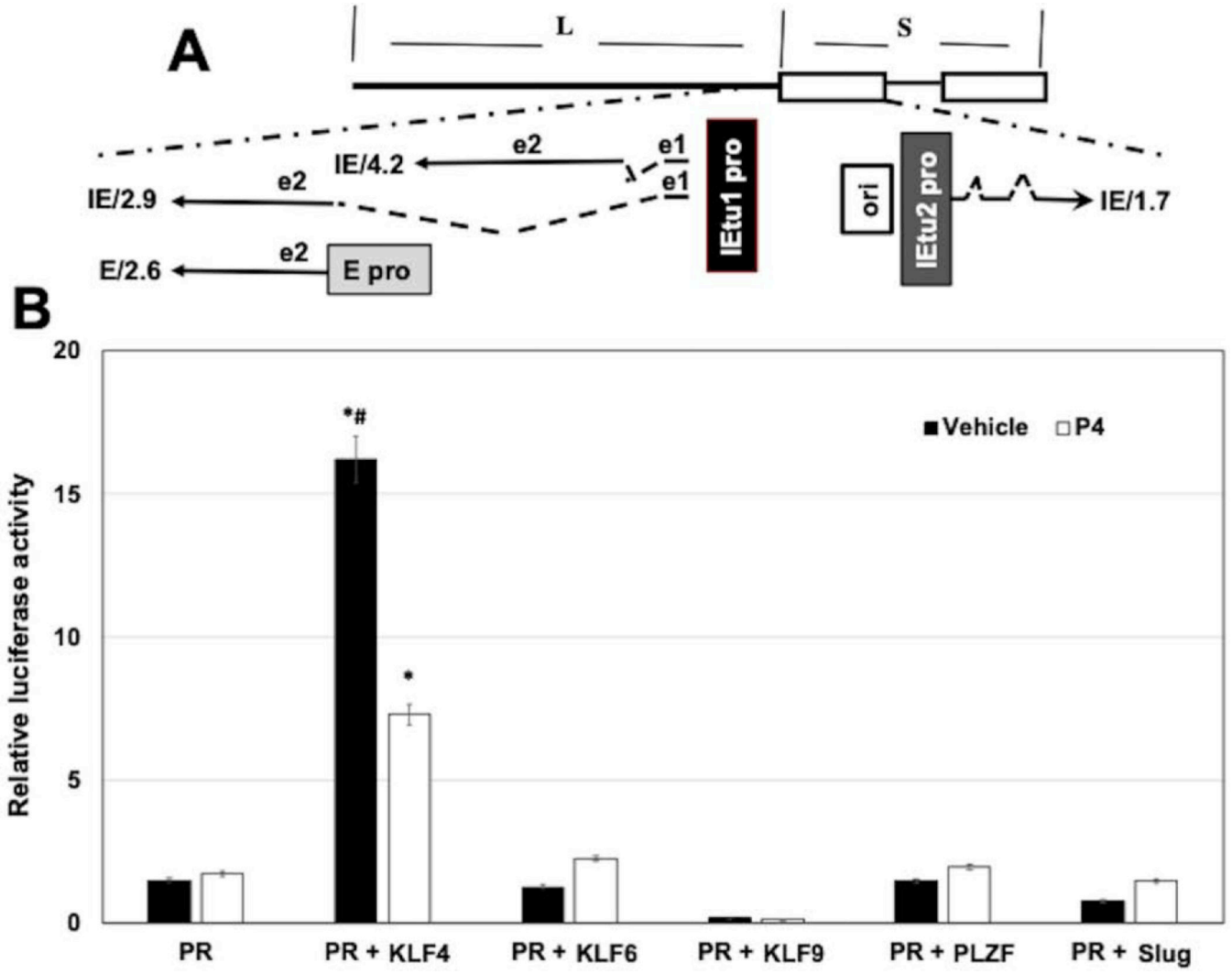

Figure 2. PR+KLF4 cooperate to transactivate the bICP0 E promoter

Panel A: Schematic of BoHV-1 genome and location of unique long (L) region, direct repeats (open rectangles), and unique short region (S). 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 and is designated IEtu1 (black rectangle). E/2.6 is the early bICP0 mRNA and is regulated by the bICP0 E promoter (E pro; gray rectangle). bICP0 protein coding sequences are in Exon 2 (e2). Origin of replication (ORI) separates IEtu1 from IEtu2. The 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.

Panel B: Neuro-2A cells were transfected with the designated bICP0 E promoter construct containing the firefly luciferase reporter gene (0.5 ug DNA) and where indicated a plasmid that expresses the human PR protein (A and B) (1.0 ug of DNA of each) and/or KLF-4, KLF-6, KLF-9, KLF-15, PLZF or SLUG (0.5 ug 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 FBS, and then vehicle

(DMSO) or P4 (100 nM) added to cultures. At 48 hours after transfection, cells were harvested and protein lysate subjected to dual-luciferase assay. Levels of promoter activity in the empty luciferase vector EP-943 were normalized to a value of 1 and fold activation for other samples presented. The results are the average of 3 independent experiments and error bars denote the standard error. A pound sign indicates significant differences ($P < 0.05$) in cells transfected with the EP-943 cotransfected with PR plus KLF4 relative to EP-943 sample cotransfected with PR and treated with P4. An asterisk sign indicates a significant difference between EP-943 cotransfected with KLF4 and PR and EP-943 cotransfected with the other stress-induced transcription factors (KLF6, KLF9, PLZF, Slug) with or without P4 treatment. The Student *t* test was used for analyzing the results.

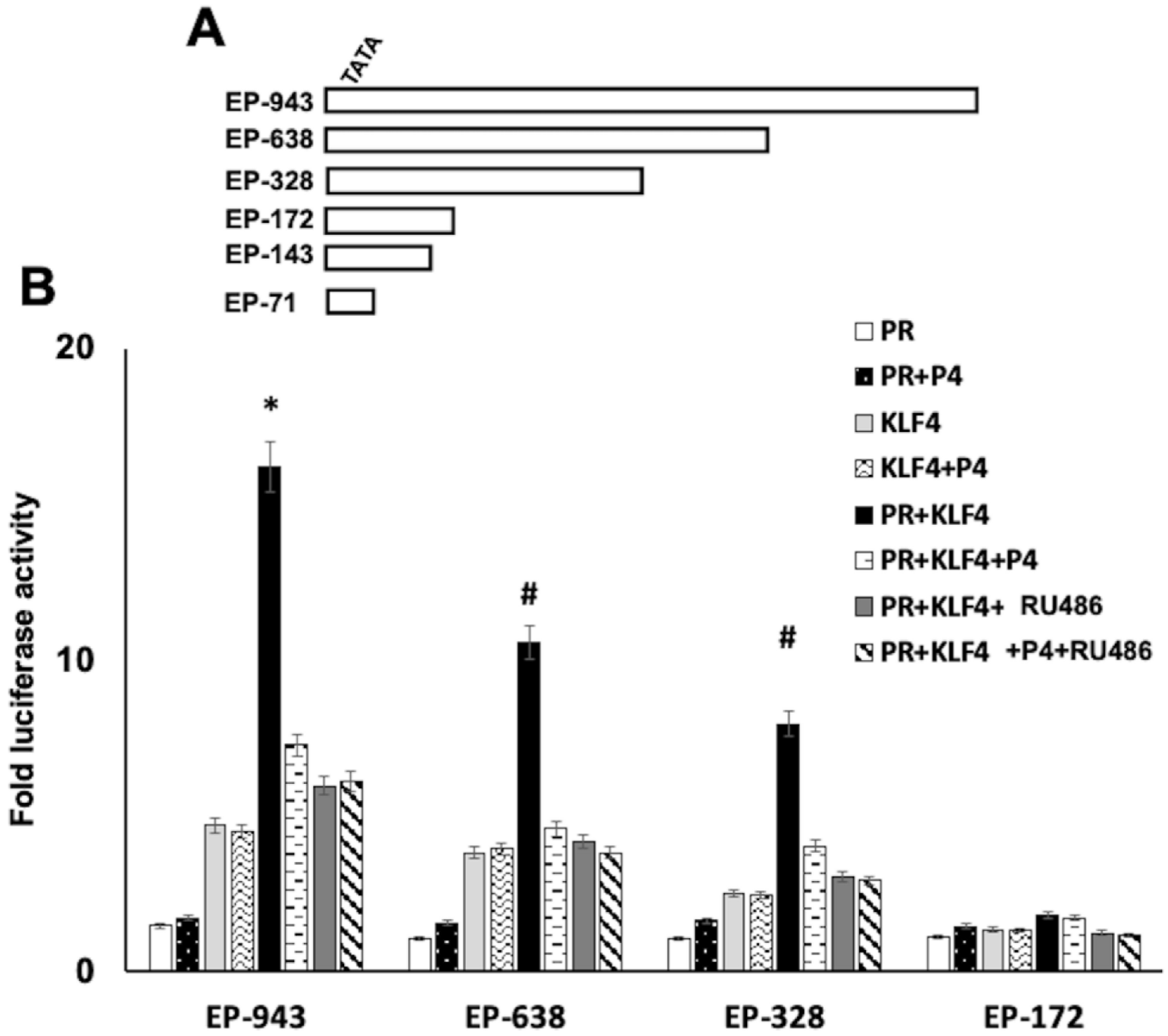

Figure 3. Localization of bICP0 E promoter sequences important for PR+KLF4 mediated transactivation

Panel A: The full-length bICP0 E promoter (EP-943) and deletion constructs were prepared as previously described (Workman et al., 2011). These fragments were cloned upstream of the luciferase vector (pGL3-Basic Vector, Promega) as SacI-HindIII fragments. The position of the TATA box is shown, and fragments extend to the nucleotide prior to the ATG.

Panel B: Neuro-2A cells were transfected with the designated bICP0 E promoter constructs containing the firefly luciferase reporter gene (0.5 ug DNA; see Figure 3A for schematic of constructs) and where indicated a plasmid that expresses human PR protein (A and B) (1.0 ug of DNA of each) and/or KLF4 (0.5 ug DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. At 24 h after transfection, cultures were incubated with MEM containing 2% stripped FBS, and then DMSO (vehicle) or P4 (100 nM; Tocris Bioscience; 2835) was added to cultures. At 48

hours after transfection, cells were harvested and protein lysate subjected to a dual-luciferase assay. Levels of promoter activity in the empty luciferase vector (pGL3-Promoter Vector) were normalized to a value of 1 and fold activation for other samples are presented. The results are the average of 3 independent experiments and error bars denote the standard error. An asterisk indicates significant differences ($P < 0.05$) in cells transfected with the EP-943 cotransfected with PR plus KLF4 relative to other EP-943 samples. The EP-638 sample cotransfected with KLF4 plus PR was significantly different relative to all other samples transfected with EP-638; the results of EP-638 or EP-943 cotransfected with KLF4 plus PR were significantly different (denoted by pound sign). With respect to EP-328, the pound sign indicates a significant difference from the other EP-328 samples. The results of EP-638 or EP-328 cotransfected with KLF4 plus PR were not significantly different. The results for EP-943 cotransfected with KLF4 and PR were significantly different than EP-328 cotransfected with the same constructs. The Student *t* test was used for analyzing the results.

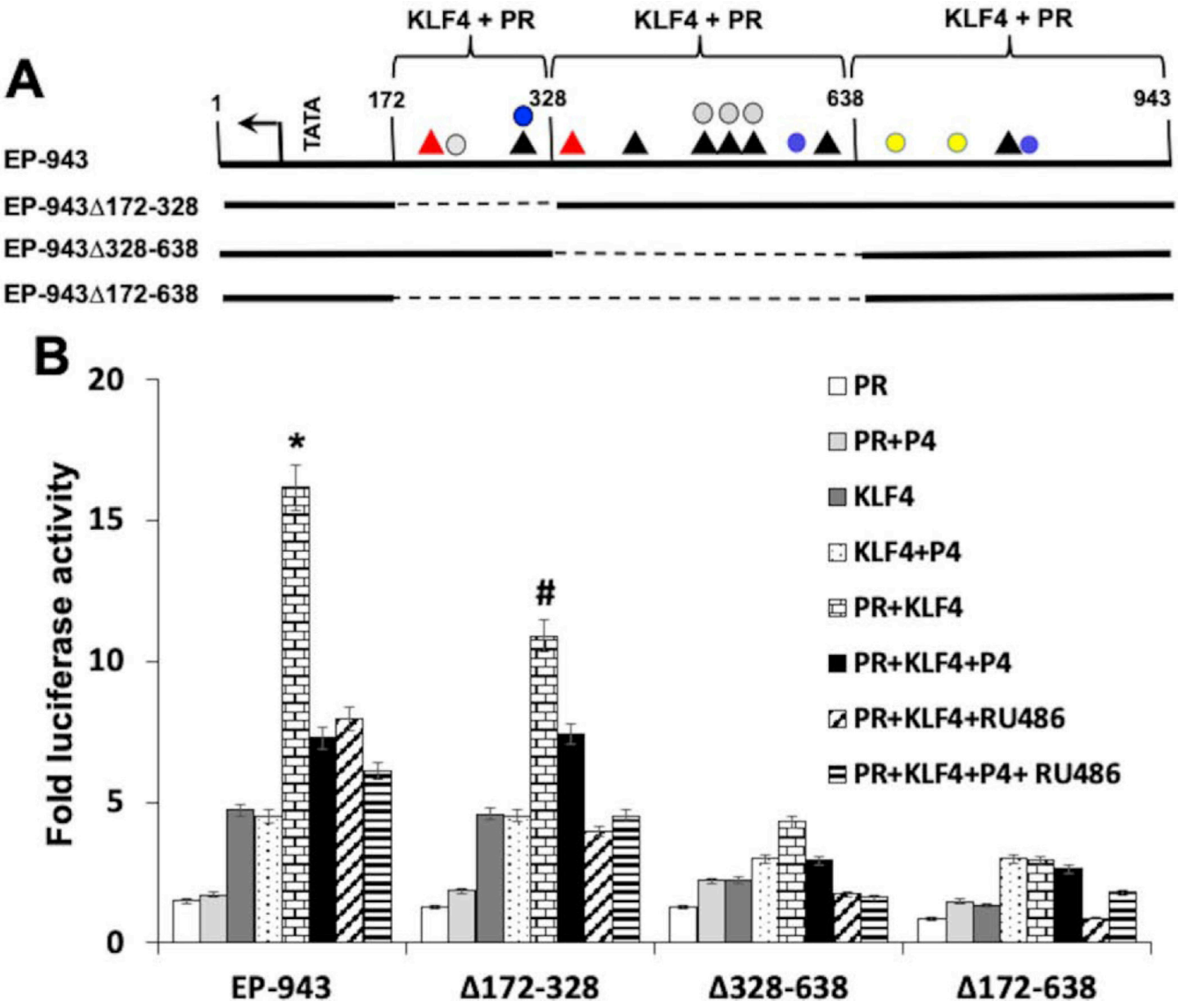

Figure 4. Internal bICP0 E promoter deletions identify unique enhancer domains.

Panel A: Schematic of internal bICP0 E promoter deletion constructs. Black triangles denote consensus Sp1 binding sites, red triangles are consensus KLF CACCC-rich motifs, blue circles are consensus KLF4 binding sites, gray circles are KLF4-like binding sites, and yellow circles are ½ GRE-like binding sites. The location of the TATA box and the three major start sites of transcription (denoted by arrow) are 22–24 nucleotides downstream of the TATA box (Wirth et al., 1992). The number 1 denotes the last nucleotide prior to the initiating methionine of the bICP0 protein.

Panel B: Neuro-2A cells were transfected with the designated bICP0 E promoter constructs (0.5 ug DNA) and where indicated a plasmid that expresses the human PR protein (A and B) (1.0 ug of DNA of each) and where indicated KLF4 (0.5 ug 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 FBS, and then P4 (100 nM), DMSO (vehicle) and/or RU486 where indicated (1 uM) was added to designated

cultures. At 48 hours after transfection, cells were harvested and protein lysate subjected to dual-luciferase assay. The results are the average of 3 independent experiments and error bars denote the standard error. An asterisk denotes a significant difference ($P<0.05$) between the results obtained using the EP-943 construct cotransfected with PR+KLF4 using the Student *t* test ($P<0.05$). PR+KLF4 mediated transactivation of Δ172–328 construct was significantly different than the Δ328–638 and Δ172–638 deletion constructs, denoted by the #.

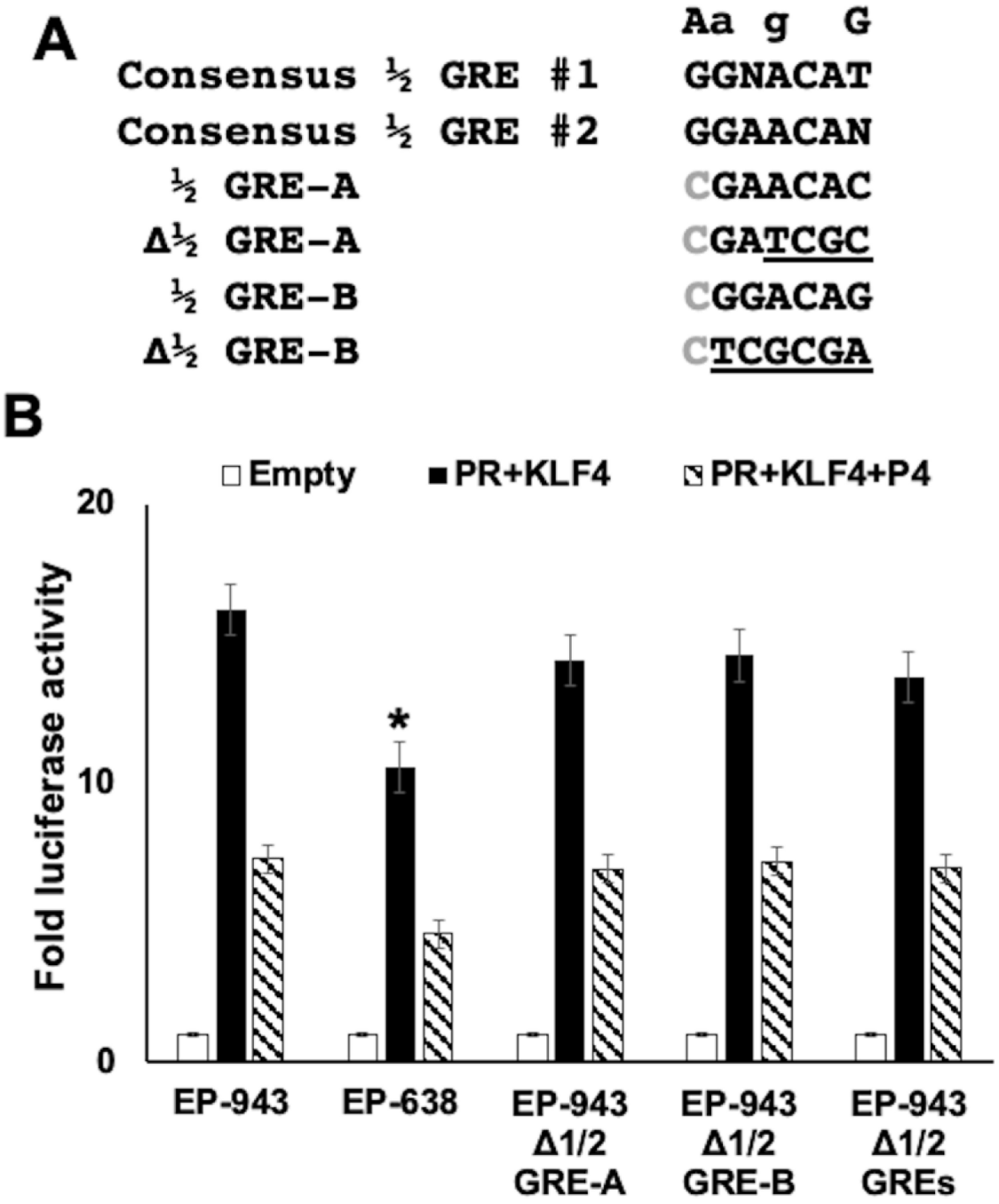

Figure 5. PR+KLF4 mediated transactivation of bICP0 E promoter does not require ½ GREs.

Panel A: Consensus ½ GRES previously shown to be transactivated by GR in a DEX dependent manner: nucleotides above the consensus sequences reflect variants in the ½ GREs (N denotes any nucleotide can be at that particular position) (Schiller et al., 2014). Sequence of putative ½ GREs in bICP0 promoter (wt and mutant) located between 638–943. Grey nucleotides denote differences in the consensus ½ GRE sequences. A NruI restriction enzyme site (TCGCGA) was used to introduce mutations into each of the ½ GRE sites (underlined nucleotides).

Panel B: Neuro-2A cells were transfected with the designated bICP0 E promoter constructs (0.5 ug DNA) and where indicated a plasmid that expresses the human PR protein (A and B) (1.0 ug of DNA of each) and KLF4 (0.5 ug 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 incubated with MEM containing 2% stripped FBS, and then vehicle (DMSO) or P4 (100 nM) added to designated cultures. At 48 hours after transfection, cells were harvested and protein lysate subjected to dual-luciferase assay. The results are the average of 3 independent experiments and error bars denote the standard error. An asterisk denotes a significant difference ($P<0.05$) between the results obtained using the EP-943 constructs cotransfected with PR+KLF4 versus EP-638 using the Student *t* test (P <0.05).

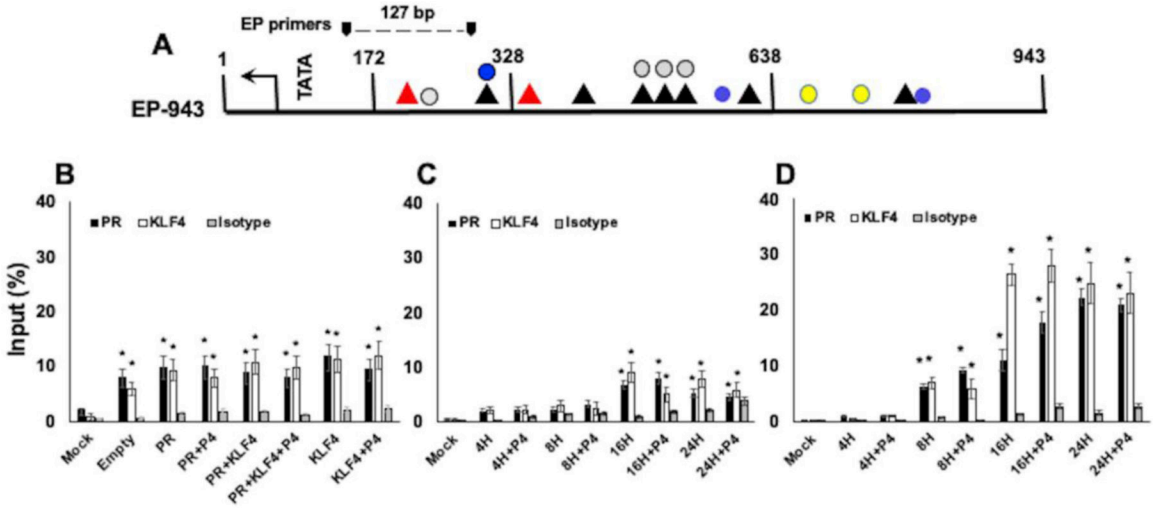

Figure 6. KLF4 and the PR interact with sequences located in the bICP0 early promoter.

Panel A: Schematic of bICP0 EP-943 promoter region. Black triangles denote consensus Sp1 binding sites, red triangles are consensus KLF CACCC-rich motifs, blue circles are consensus KLF4 binding sites, gray circles are KLF4-like binding sites, and yellow circles are ½ GRE-like binding sites. The location of the TATA box and the three major start sites of transcription (denoted by arrow) are 22–24 nucleotides downstream of the TATA box (Wirth et al., 1992). The number 1 is the last nucleotide prior to the bICP0 E initiating methionine codon. The location of the early promoter (EP) primers are denoted by black pentagons. The size of the PCR product is 127 bp and the hatched line denotes the region of EP-943 that is amplified.

Panel B: Neuro-2A cells were cotransfected with the bICP0 E promoter (EP-943), plasmids that expresses the human PR protein (PR-A and PR-B: 1.5 ug of DNA of each PR construct) and/or KLF4 (1.5 ug DNA). Empty vector was added to maintain the same concentration of DNA in each transfection assay. Mock samples were Neuro-2A cells not transfected with DNA. After transfection, MEM containing 2% stripped FBS was added to cultures. Transfected cells were processed for ChIP at 40 hours after transfection as described in Materials and Methods, and immunoprecipitation (IP) performed using the PR, KLF 4, or isotype control antibody. Detection of immunoprecipitated DNA was performed using the EP primer set that specifically amplifies the bICP0 E promoter.

Panel C: CRIB cells were mock infected or infected with BoHV-1 (MOI of 1) and treated with vehicle or P4 for 4 h, 8h, 16h and 24h. Infected cells were processed for ChIP as described in materials and methods, and immunoprecipitation (IP) conducted using PR, KLF4, or isotype control antibody. Detection of immunoprecipitated DNA was performed using the EP primer set that specifically amplifies the bICP0 E promoter.

Panel D: The same DNA from infected CRIB cells used for the bICP0 E promoter ChIP studies were used to compare occupancy of the IEtu1 promoter. ChIP was performed using the 3'-DRR primer set that amplifies a fragment containing the two GREs within the IEtu1 promoter. For all studies in this figure, the results are the average of 3 independent infections and ChIP studies. An asterisk denotes statistically significant differences ($P < 0.05$) from ChIP samples immunoprecipitated with isotype control IgG using the Student *t* test.

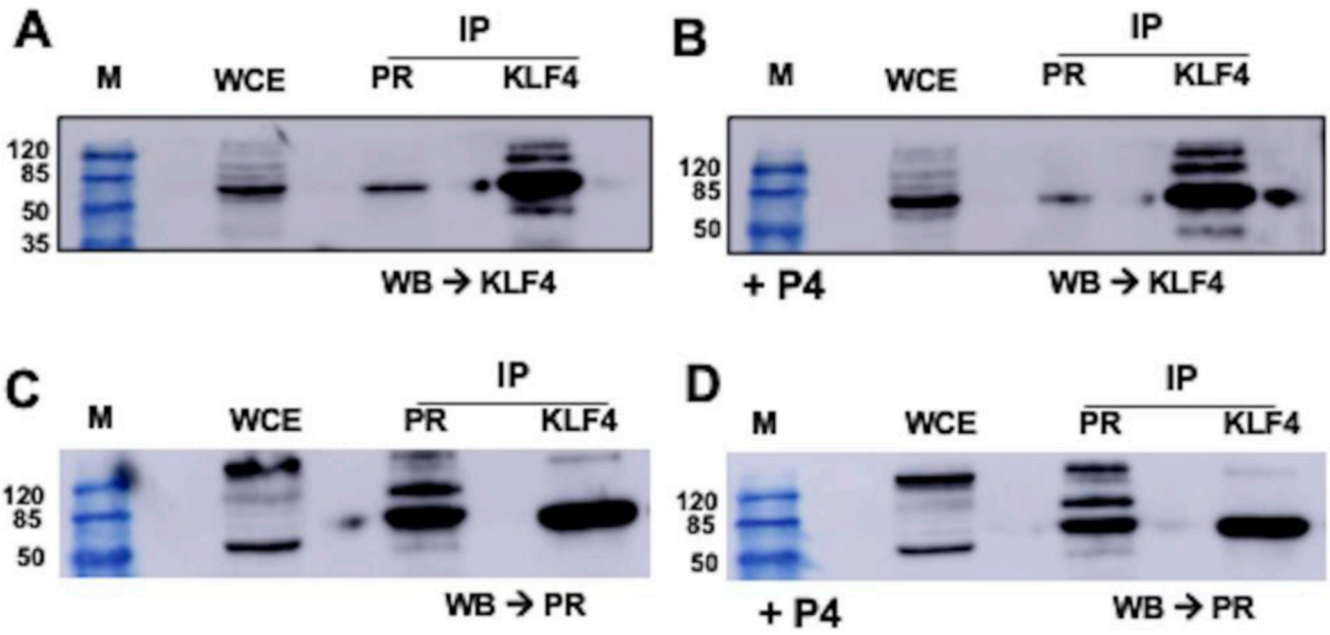

Figure 7. PR and KLF4 are associated with each other in transfected Neuro-2A cells.

Neuro-2A cells were grown to 80% confluence on 100 mm dishes in stripped FBS. Cells were cotransfected with plasmids that express the human PR protein (PR-A and PR-B) (1μg of DNA of each PR construct) and/or KLF4 (1.5 ug DNA). After transfection cultures were incubated with MEM containing 2% stripped FBS **(panel A and C)**. Cultures were treated with P4 at 24 hours after transfection (100 nM; Tocris Bioscience; 2835) in MEM containing 2% stripped FBS for 4 hours **(panel B and D)**. Whole cell extract (WCE) was prepared and co-IP studies performed using the PR or KLF4 antibody as described in material and methods. Following IP with the designated antibody, KLF4 or PR was detected in immunoprecipitates by Western blotting. WCE (50 ug protein) was used as a positive control.