Chapter 3

The Glucocorticoid Receptor and Certain KRÜPPEL-Like Transcription Factors have the Potential to Synergistically Stimulate Bovine Herpesvirus 1 Transcription and Reactivation from Latency

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Abstract

Bovine herpesvirus 1 (BoHV-1), an important bovine pathogen, establishes life-long latency in sensory neurons within trigeminal ganglia (TG). Stress, as mimicked by the synthetic corticosteroid dexamethasone, consistently induces reactivation in calves latently infected with BoHV-1. Dexamethasone induces expression of several transcription factors in TG neurons during early stages of reactivation, including Krüppel-like transcription factors (KLF): KLF4, KLF6, KLF15, and promyelocytic leukemia zinc finger. Furthermore, the glucocorticoid receptor (GR) is consistently detected in TG neurons expressing viral regulatory proteins during reactivation from latency. The viral immediate early transcription unit 1 (IEtu1) promoter that drives expression of two viral transcription factors (bICP0 and bICP4) contains two GR response elements (GREs) and is stimulated by DEX. KLF15 and the GR form a feed forward transcription loop that synergistically stimulates productive infection and IEtu1 promoter activity. New studies demonstrate the GR and KLF6 synergistically stimulate productive infection and IEtu1 promoter activity if the GREs are intact. Furthermore, the GR and KLF6 interact with wild-type GREs within the IEtu1 promoter, but not GRE mutants. These studies suggest that certain KLF family members and the GR can convert a silent viral genome in latently infected neurons into an actively transcribing genome during reactivation from latency.

Keywords: bovine herpesvirus 1, immediate early transcription, reactivation from latency, sensory neurons, glucocorticoid receptors, Krüppel-like transcription factors

1. Introduction

Bovine herpesvirus 1 (BoHV-1), an alpha-herpesvirinae subfamily member, is an important bovine pathogen that causes conjunctivitis and/or upper respiratory tract disease [1–3]. BoHV-1 is a significant cofactor in the polymicrobial disease referred to as bovine respiratory disease complex (BRDC), which is the most important disease of cattle. BoHV-1 infection impairs cell-mediated immunity, CD8+ T cell recognition of infected cells, and induces apoptosis in CD4+ T cells [4]. Viral proteins, VP8, bICP0 and bICP27, inhibit interferon dependent transcription [4–8]. Infection also erodes mucosal surfaces of the upper respiratory tract, which promotes establishment of the bacterial pathogen, *Mannheimia haemolytica (MH)* in the lower respiratory tract [9]. BoHV-1 productive infection increases neutrophil adhesion and activation [10], thus amplifying the pathogenic potential of *MH. MH,* a gram negative bacterium, exists as normal flora within the upper respiratory tract of healthy ruminants [11]. Stress and/or co-infections disrupt this commensal relationship; consequently *MH* becomes the predominant organism that causes life-threatening bronchopneumonia in BRDC cases [9]. BRDC is the most important disease in cattle because it costs the US cattle industry more than one billion dollars in losses each year [9, 12, 13]. A BoHV-1 entry protein is a BRDC susceptibility gene for Holstein calves [14] confirming BoHV-1 is a significant BRDC cofactor.

Like most alpha-herpesvirinae subfamily members, including human herpes simplex virus 1 (HSV-1) and HSV2, BoHV-1 initiates acute infection on mucosal surfaces [1–3]. High levels of infectious virus are produced; consequently BoHV-1, HSV-1, or HSV-2, spread to the peripheral nervous system via cell-to-cell spread. Latency is subsequently established in sensory neurons, but periodically reactivates from latency, and thus is widespread in cattle throughout the world. Reactivation of the virus from the latent state is initiated by external stimuli (e.g. stress and immunosuppression). During reactivation, viral gene expression is stimulated and infectious virus is produced and transported back to mucosal surfaces. The ability of alpha-herpesvirinae subfamily members to reactivate from the latent state is critical for virus transmission. Regulation of the complex virus host interactions controlling the latency-reactivation cycle is not well understood, which hinders developing therapeutic strategies that prevent reactivation from latency.

BoHV-1 is an excellent model to study these events because the natural host can be used and the synthetic corticosteroid dexamethasone (DEX) consistently initiates reactivation from latency in infected calves [2]. We have used experimentally infected calves treated with DEX to initiate reactivation from latency in order to identify virus-host interactions important for the latency-reactivation cycle. These studies identified host cellular factors and pathways that may be crucial for latency maintenance [15] and reactivation [16]. The following discussion focuses on the mechanisms by which BoHV-1 "escapes" a latent infection following a stressful stimulus and subsequently successfully reactivates from latency. Certain steps during BoHV-1 reactivation from latency are likely to be similar during reactivation of latency of other alpha-herpesvirinae subfamily members.

2. Acute infection leads to a life-long latent infection in sensory neurons

Acute infection of calves induces programmed cell death, inflammation and high levels of virus production [1–3]. BoHV-1 genes are expressed in three distinct phases during acute infection or productive infection of cultured cells: immediate early (IE), early (E), or late (L) [1–3]. IE gene expression is specifically stimulated by viral protein 16 (VP16), a tegument protein. IE transcription unit 1 (IEtu1) encodes two transcriptional regulatory proteins, BoHV-1 infected cell protein 0 (bICP0) and bICP4, because a single IE transcript is differentially spliced and subsequently translated into bICP0 or bICP4 (**Figure 1**). The bICP0 protein is also translated from an E mRNA (E2.6) because a separate E promoter drives expression of the bICP0 E transcript.

During acute infection of calves, infectious virus particles enter the peripheral nervous system via cell–cell spread. If infection is initiated within the oral, nasal, or ocular cavity, the primary site for latency is sensory neurons located in trigeminal ganglia (TG) [1–3]. Viral gene expression and infectious virus are detected in TG from 2 to 6 days after infection. Lytic cycle viral gene expression is then extinguished, a significant number of infected neurons survive, and these neurons harbor viral genomes, which is operationally defined as the establishment of latency. Abundant expression of the BoHV-1 encoded latency related (LR) gene occurs in latently infected neurons, but infectious virus is not detected (maintenance of latency) [1–3]. LR-RNA is anti-sense to and overlaps the BoHV-1 infected cell protein 0 (bICP0) gene. The LR gene has two open reading frames (ORF1 and ORF2), and two reading frames lacking an initiating methionine (RF-B and RF-C). In addition, the LR gene encodes two micro-RNAs that interfere with bICP0 expression in transfected cells [17]. A LR mutant virus strain with three stop codons at the N-terminus of ORF2 exhibits diminished clinical symptoms, and reduced virus shedding from

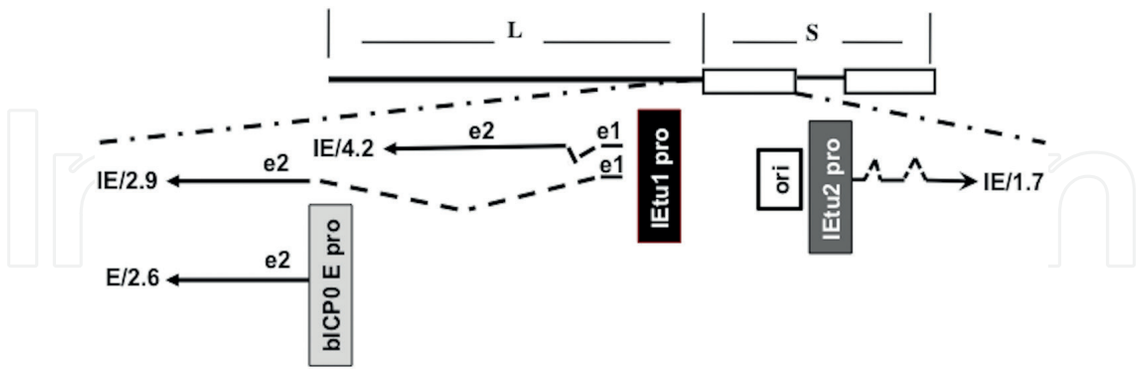

Figure 1. Location of IE transcripts and promoters actively expressed during productive infection. The mRNA IE/4.2 encodes the bICP4 protein and IE/2.9 encodes the bICP0 protein [58, 59, 72]. A single IE promoter activates expression of IE/4.2 and IE/2.9 and is designated IEtu1 (black rectangle). E/2.6 is the early transcript that encodes bICP0 and an early promoter activates expression of this transcript (bICP0 E pro; gray rectangle). All bICP0 protein-coding sequences are contained in Exon 2 (e2). The origin of replication (ORI) separates IEtu1 from IEtu2. The IEtu2 promoter (IEtu2 pro) regulates expression of the IE1.7 mRNA that is translated into the bICP22 protein. Solid lines in the transcript position map represent exons (e1, e2, or e3) and dashed lines denote introns.

the eye, TG, or tonsils of infected calves [1–3]. ORF1, ORF2, and RF-C are expressed when bovine cells are infected with wild-type or the LR-rescued virus, but these proteins have reduced or no expression following infection with the LR mutant virus [1–3]. Wild-type (wt) BoHV-1, but not the LR mutant virus, reactivates from latency after treatment with the synthetic corticosteroid DEX. The anti-apoptosis activity of ORF2 is predicted to increase the survival of infected neurons and thus would be important for the latency-reactivation cycle [1–3].

Recent studies demonstrated that during latency, the canonical Wnt/β-catenin signaling is active and ORF2 appears to be important for maintaining this important signaling pathway [15, 18]. Although dysregulation of the Wnt/β-catenin signaling is frequently associated with many types of cancer [19, 20], this signaling pathway has the potential to promote the establishment and maintenance of latency in sensory neurons because it enhances cell survival, axonal growth, and directs axons to their proper synaptic targets [21–25].

3. Stress-induced reactivation from latency

Increased corticosteroid levels, due to increased stress, correlates with increasing the incidence of BoHV-1 reactivation from latency [1–3]. DEX can also stimulate productive infection [26], and initiate reactivation from latency in calves or rabbits latently infected with BoHV-1 [1–3]. Six hours after DEX treatment lytic cycle viral RNA expression is detected in neurons of latently infected calves [27, 28]. Certain lytic cycle viral proteins, bICP0 and VP16 for example, are readily detected in TG neurons within hours after DEX treatment [29, 30]. The glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), which are present in subpopulations of sensory neurons [31, 32], are activated by interacting with corticosteroids. The GR is frequently detected in TG neurons that express bICP0 or VP16 [31, 32]. IEtu1 promoter activity is stimulated by the GR and the synthetic corticosteroid DEX because there are two consensus GREs in the promoter [26] suggesting this promoter is activated by the GR and/or MR following stressful stimuli. Since the IEtu1 promoter drives expression of two viral transcriptional regulatory proteins (bICP0 and bICP4; **Figure 1**), activation of this promoter may stimulate productive infection in certain latently infected neurons.

DEX treatment of latently infected calves induces apoptosis of T cells that persist in TG after infection [27]. T cells also persist in TG of humans or mice latently infected with HSV-1 and may promote maintenance of latency [33–37]. Within 3 h after DEX treatment, 11 cellular genes are induced more than ten fold in TG [16]. Pentraxin 3, a regulator of innate immunity and neurodegeneration, is stimulated 35–63 fold at 3 or 6 h after DEX treatment. Furthermore, expression of a soluble Wnt antagonist, Dickkopf-1 is induced more than 10 fold [15, 16]. Dickkopf-1 is responsible for stress-induced neuronal death [38, 39] indicating there is a correlation between disrupting the Wnt signaling pathway and activation of lytic cycle viral gene expression during reactivation. Two transcription factors, promyelocytic leukemia zinc finger (PLZF) and Slug are induced more than 15-fold 3 h after DEX treatment. PLZF or Slug stimulates BoHV-1 productive infection 20-fold or 5-fold respectively, and Slug stimulates the late glycoprotein C promoter more than 10-fold. Additional DEX induced transcription factors, SPDEF (Sam-pointed domain containing Ets transcription factor), Kruppel-like transcription factor 15 (KLF15), KLF4, KLF6, and GATA6, stimulate productive infection and certain key viral promoters.

The finding that four KLF family members (KLF4, KLF6, KLF15, and PLZF) are stimulated during DEX induced reactivation from latency is intriguing because KLF family members resemble the SP1 transcription factor family and both family of transcription factors interact with guanine-cytosine (GC) rich motifs, reviewed in [40, 41]. Genomes of alpha-herpesvirinae subfamily members, including BoHV-1, are GC rich and many viral promoters contain Sp1 consensus binding sites as well as other GC rich motifs [40]. In fact, HSV-1 gene expression is activated by Sp1 [42]. HSV-1 and probably BoHV-1 genomes exist as silent chromatin during latency, [43]: however, HSV-1 DNA is associated with unstable chromatin during productive infection [44–46]. Regardless of the stimulus that initiates reactivation from latency, silent viral heterochromatin must be converted into an actively transcribing template for reactivation from latency to be successful suggesting cellular transcription factors initially stimulate lytic cycle viral gene expression.

To test whether the GR and certain stress-induced transcription factors can cooperate to stimulate viral transcription, the IEtu1 promoter and BoHV-1 DNA fragments (less than 400 bp) containing potential GR and KLF binding sites were identified and examined for transcriptional activation by stress-induced transcription factors. The rational for testing intergenic regions of the BoHV-1 genome is the viral genome contains more than 100 putative GRE binding sites [26] and a subset of GREs in cellular chromatin can activate transcription from greater than 5 kb to the nearest promoter [47]. KLF15 cooperated with the GR to stimulate the IEtu1 promoter activity and productive infection [48]. Furthermore, intergenic regions within the unique long 52 gene (UL-52; component of DNA primase/helicase complex), bICP4, IEtu2 that expresses the regulatory protein (bICP22), and unique short region were stimulated by KLF15 and the GR. In contrast to KLF15, the other stress-induced transcription factors only have a modest effect on IEtu1 promoter activity. The GR and KLF15 interact with sequences within wild-type IEtu1 GREs and UL-52 fragment, but not GRE mutants. Co-immunoprecipitation studies indicated that KLF15 and the GR are stably associated with each other. Interestingly, the GR and KLF15 can synergistically regulate gene expression by a feed-forward transcription loop [49–51]. Hallmarks of a feed-forward loop are a primary factor (GR in this example) induces expression of a second factor, KLF15 [16, 49–54], and the two factors synergistically activate expression of genes in a specific pathway. Adipogenesis [55] and amino acid metabolizing enzymes are also synergistically regulated by the GR and KLF15 [50, 51]. In summary, these studies suggest that activation of BoHV-1 gene expression during DEX induced reactivation from latency is, in part, regulated by a feed-forward transcription loop containing the GR and KLF15.

4. The GR and KLF6 cooperate to stimulate productive infection

To test whether KLF6 and the activated GR have a cooperative effect on productive infection, a mouse neuroblastoma cell line (Neuro-2A) was cotransfected with gCblue genomic DNA and KLF6 and/or the GR. The gCblue virus grows to similar titers as the wt parental virus and expresses the Lac Z gene from the gC locus during productive infection (**Figure 2A**). Neuro-2A cells were used for these studies because they have neuronal like properties [56], can be readily transfected, and are semi-permissive for BoHV-1 [57]. Neuro-2A cells were transfected with gCblue DNA instead of infecting cells because VP16 and other viral regulatory proteins in the virion particle can diminish the stimulatory effects of DEX on productive infection (data not shown). KLF6 and the GR plus DEX treatment increased the number of β-Gal+ Neuro-2A cells

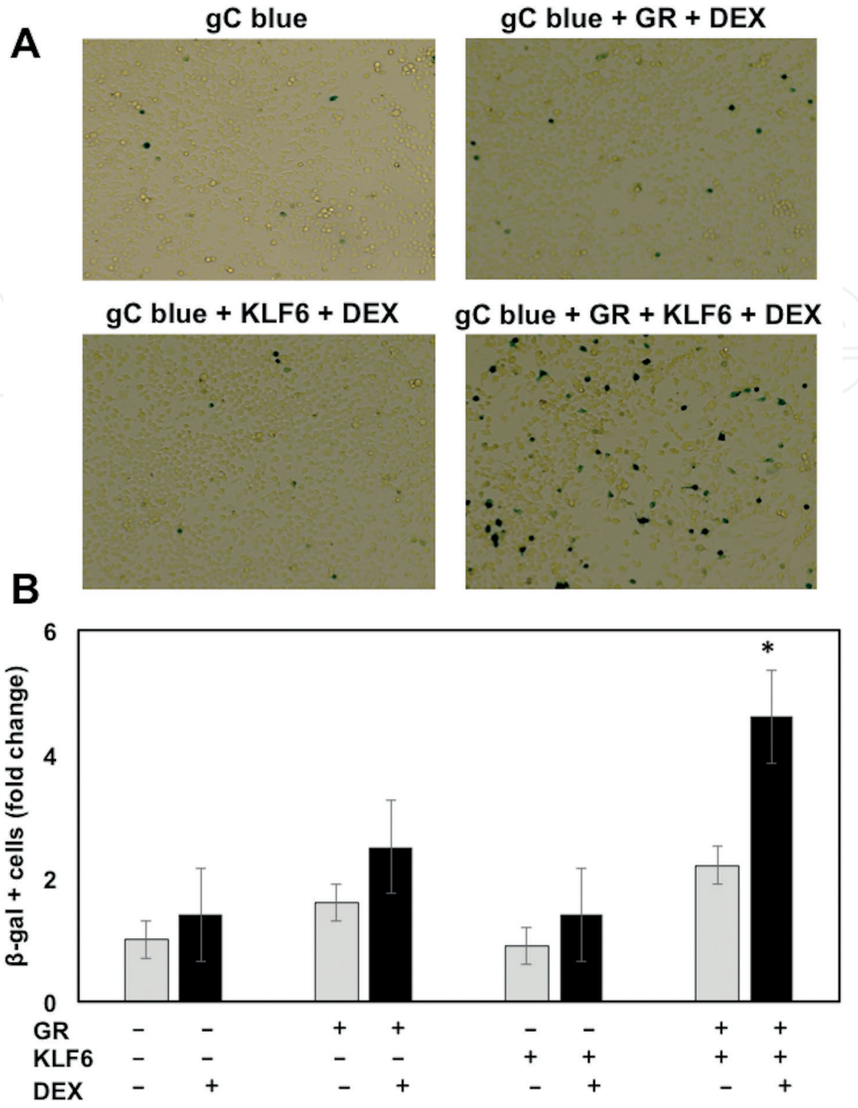

Figure 2. KLF6 and the GR cooperate to stimulate productive infection. Neuro-2A cells were transfected with 2 ug BoHV-1 gCblue genomic DNA and where indicated a plasmid that expresses the mouse GR protein (1.0 ug DNA) and/or KLF6 (0.5 ug DNA) using Lipofectamine 3000 (catalog no. L3000075; Invitrogen). A mouse GR expression vector was obtained from Dr. Joseph Cidlowski, NIH and the KLF6 expression vector was obtained from Bin Guo (North Dakota State University). Neuro-2A cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% FCS, penicillin (10 U/ml), and streptomycin (100 μg/ml). The 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 bovine kidney cells (CRIB). 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 [73]. To maintain the same amount of DNA in each sample, empty vector was included in samples. Cells were incubated with stripped fetal calf serum 24 h after transfection and then treated with water soluble DEX (10 μM; Sigma, D2915). At 40 h after transfection, cells were fixed and stained for counting the number of β-Gal+ cells as previously described [48]. Representative cultures stained for Lac Z expression are shown in (**Panel A**). The value for the control (gCblue virus DNA treated with PBS after transfection) was set at 1. The results from DEX treated cultures were compared to the control and are an average of three independent studies (**Panel B**). The asterisk denotes a significant difference between the control and samples transfected with the GR or KLF6 and treated with DEX ($P < 0.05$) using the student's T test.

more than 4-fold, which was significantly higher than GR + DEX and the GR or KLF6 alone (**Figure 2A** and **B**). Cotransfection of gCblue and the GR + KLF6 stimulated productive infection 2-fold even when cultures were not treated with DEX, which was similar to the effects observed when gCblue genomic DNA was cotransfected with the GR and DEX treatment.

5. KLF6 and GR synergistically trans-activates the IEtu1 promoter

Transient transfection studies were performed in Neuro-2A cells to test whether KLF6 and the GR synergistically trans-activate the IEtu1 promoter because this promoter contains two consensus GR binding sites (**Figure 3A**) required for DEX mediated transactivation [26]. The

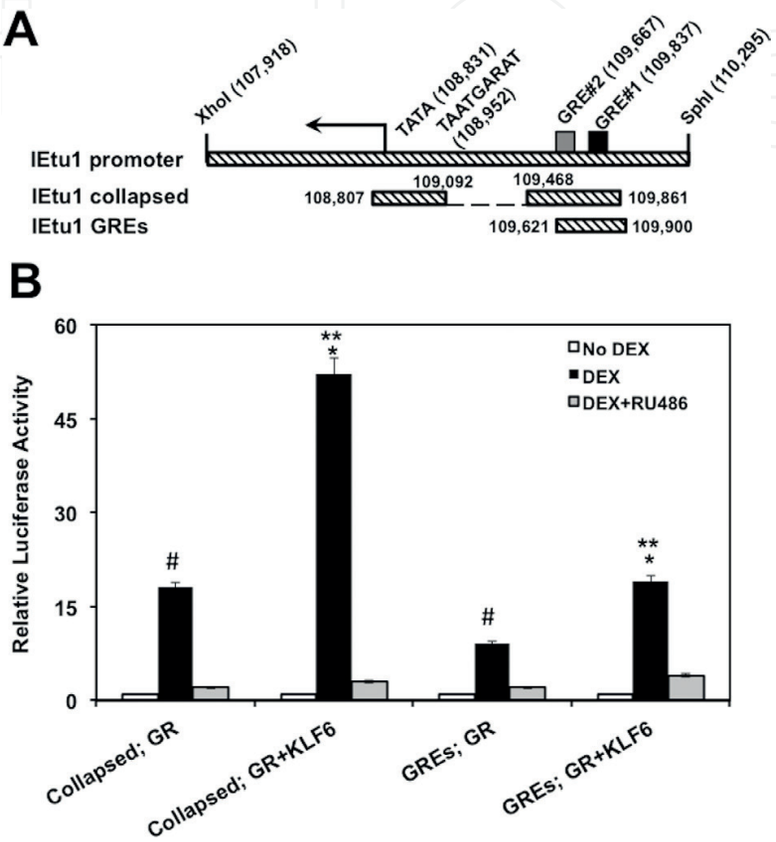

Figure 3. KLF6 and the GR cooperatively transactivate the IEtu1 promoter. **Panel A:** The full length IEtu1 promoter was cloned as an XhoI-SphI restriction site. Start site of transcription (arrow), TATA box, binding site for VP16/Oct1 complex is denoted as TAATGARAT [74], and location of GRE#1 and GRE#2 (black and grey rectangles) are shown. Numbers are genomic coordinates of the first nucleotide of each respective motif or restriction enzyme site. GenScript synthesized the IEtu1 collapsed promoter construct and genomic coordinates are included: this fragment is inserted at KpnI and HindIII restriction sites of pGL3-Basic Vector. A 280 bp fragment (IEtu1 GREs) was cloned into the pGL3-Promoter Vector at unique KpnI and XhoI restriction sites [48]. **Panel B:** Neuro-2A cells were transfected with 0.5 ug DNA of the IEtu1 collapsed promoter (Collapsed) or IEtu1 GREs plasmid (GREs) and where indicated a plasmid that expresses the mouse GR protein (1.0 ug DNA) and/or KLF6 (0.5 ug DNA). To maintain equal plasmid amounts in the transfection mixtures, the empty expression vector was added as needed. Designated cultures were treated with water-soluble DEX (10 uM; Sigma) or DEX + RU486 (10 uM; Sigma) at 24 h after transfection. At 48 h after transfection, cells were harvested, and protein lysate subjected to dual-luciferase assay using a commercially available kit (E1910; Promega). Luminescence was measured by using a GloMax 20/20 luminometer (E5331; Promega). All transfections contained a plasmid encoding *Renilla* luciferase under the control of a minimal herpesvirus thymidine kinase (TK) promoter (0.050 ug DNA) as an internal control. Promoter activity in the empty luciferase vector (pGL3-Promoter Vector) was normalized to a value of 1 and fold activation for other samples presented. The results are the average of three independent experiments and error bars denote the standard error. A single asterisk denotes a significant difference ($P < 0.05$) between the IEtu1 collapsed or IEtu1 GREs when cotransfected with GR and KLF6 plus DEX treatment when compared to promoter activity of the respective promoter construct cotransfected with GR plus DEX treatment. Two asterisks denote a significant difference ($P < 0.05$) between the IEtu1 collapsed or IEtu1 GREs when cotransfected with GR and KLF6 and DEX treatment versus the same study conducted but treated with DEX+ RU486 or no DEX. A (#) denotes a significant difference between IEtu1 collapsed or IEtu1 GREs cotransfected with the GR and treated with DEX when compared to the same luciferase reporter cotransfected with GR and treated with DEX+ RU486 or no DEX. Statistical analysis was performed using the Student *t* test.

IEtu1 promoter drives IE expression of bICP0 and bICP4, the most important viral transcriptional regulatory proteins encoded by BoHV-1 [58–60] (**Figure 1**). The IEtu1 collapsed promoter construct (**Figure 3A**) was initially used to test whether sequences adjacent to the GREs were trans-activated by KLF6 and the GR. The full-length IEtu1 promoter construct contains extensive sequences downstream from the start site of transcription and has sequences between the TATA box and the GREs that are important for KLF trans-activation [16]: consequently the collapsed IEtu1 collapsed promoter construct was used for these studies. We have consistently found that the GR+ DEX stimulated promoter activity more than 15 fold and GR + KLF6 + DEX stimulated promoter activity more than 50 fold (**Figure 3B**). RU486 antagonizes corticosteroid/GR signaling [61, 62] and as expected RU486 significantly reduced the ability of KLF6 and GR to transactivate the IEtu1 collapsed construct.

A 280 bp fragment containing both GREs within the IEtu1 promoter and flanking sequences was cloned upstream of the minimal SV40 early promoter and designated IEtu1 GREs [48] (**Figure 3A**). This construct was examined for its ability to be activated by KLF6 and the GRE as a comparison to the IEtu1 collapsed promoter construct. KLF6 and the GR consistently stimulated the IEtu1 GREs construct approximately 16-fold whereas the GR + DEX stimulated this construct only 6-fold (**Figure 3B**). RU486 also significantly reduced the ability of KLF6 and GR to transactivate the IEtu1 GREs construct. Although the IEtu1 collapsed construct was trans-activated more by the GR + KLF6+ DEX relative to the IEtu1 GREs construct, the overall trends were similar.

6. The GREs are necessary for transactivation by the GR and KLF6

To identify sequences in the IEtu1 GREs that mediate transactivation by KLF6 and the GR, constructs containing site-specific mutations in GRE#1, GRE#2, and KLF like binding sites were compared to the wt IEtu1 GREs (**Figure 4A–C**) [48]. Mutagenesis of GRE1 (ΔGRE1) or both GREs and the KLF binding sites (Δ2xGREΔKLF) significantly reduced cooperative activation by KLF6 and the GR when DEX was added to the cultures (**Figure 4D**). Mutagenesis of the 2 putative KLF sites (ΔKLF) had no effect on trans-activation by KLF6 and the GR when cultures were treated with DEX. As previously reported [48] and shown in **Figure 4D**, the effect of DEX and the GR was significantly reduced when GRE#1 (ΔGRE1) was mutated and abolished when both GREs and putative KLF sites (Δ2xGREΔKLF) were mutated. In summary, mutagenesis of the GRE#1 significantly reduced synergistic transactivation by KLF6 and the GR when cultures were treated with DEX.

7. KLF6 and the GR interact with sequences located in the IEtu1 GREs

To test whether KLF6 and the GR interact with sequences located in the IEtu1 GREs, chromatin immuno-precipitation (ChIP) studies were performed in Neuro-2A cells. Cells were transfected with the promoter construct containing the IEtu1 GREs followed by treatment with Vehicle

or DEX. As shown in **Figure 5A**, ChIP studies demonstrated that the GR and KLF6 occupied the GRE region of the IEtu1 GREs (lanes 2–4). No specific PCR product was amplified from ChIPs of cells transfected with the IEtu1 GREs from IPs using the control IgG (IgG C Panel) or cells transfected with the Δ2XGREΔKLF construct (**Figure 5C** and **D**). Treatment with DEX had little effect on the levels of GR bound to IEtu1 GRE sequences (**Figure 5A** and **B**); however, we detected an increase in KLF6 bound to the IEtu1 GREs when cotransfected with KLF6 and GR in the absence of DEX when compared to DEX treatment. At least three reasons may have led to this unexpected result. First, we suggest that low levels of corticosteroids in media containing

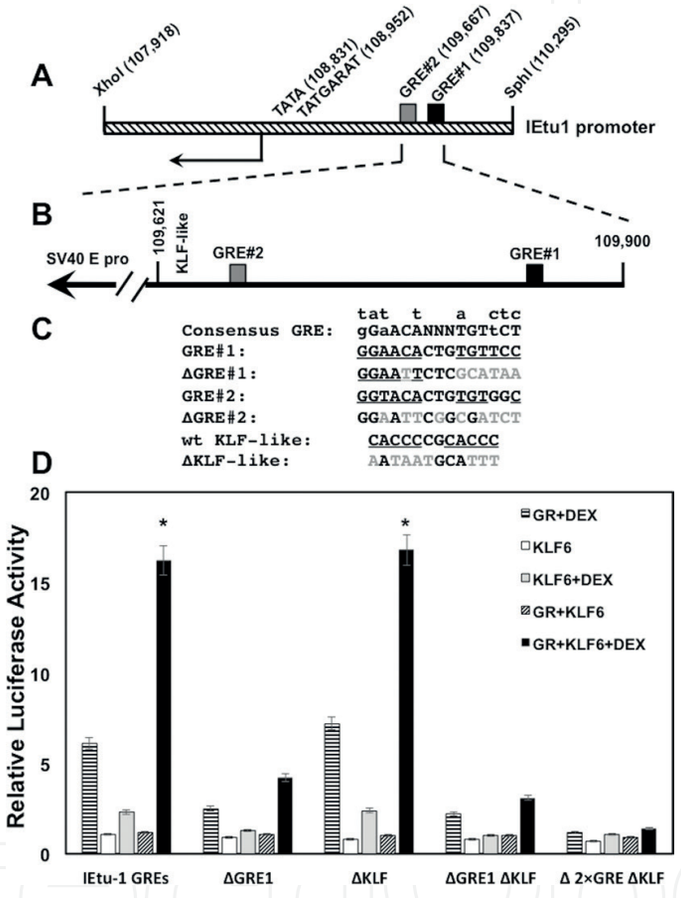

Figure 4. Identification of sequences in the IEtu1 GREs that are responsive to KLF6 and the GR. **Panel A:** Schematic of IEtu1 promoter and location of TATA box, TAATGARAT motif, and the two GREs. Numbers denote the genomic location of the first nucleotide of each motif. **Panel B:** Schematic of 280 bp fragment that contains the IEtu1 GREs and putative KLF-binding sites. **Panel C:** Nucleotide sequence of motifs in the IEtu1 GREs and mutations that were prepared. Mutations in GRE#1 and GRE#2 were previously described and were shown to disrupt trans-activation by the GR in transient transfection studies [26, 48]. **Panel D:** Neuro-2A cells were transfected with the designated luciferase plasmid (0.5 ug DNA) and where indicated a plasmid that expresses the mouse GR protein (1.0 ug DNA) and/or KLF6 (0.5 ug DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. Cultures were then treated with 2% "stripped" fetal calf serum and then water soluble DEX (10 uM; Sigma) at 24 h after transfection. At 48 h after transfection, cells were harvested, and protein lysate was subjected to dual-luciferase assay as described in **Figure 3B**. The level of promoter activity in the empty luciferase vector (pGL3-Promoter Vector) was normalized to a value of 1 and the fold activation values for other samples are presented. The results are the average of three independent experiments and error bars denote the standard error. The asterisks denote a significant difference ($P < 0.05$) between IEtu1 GREs (wt) and the ΔKLF mutant when compared to the other mutants (ΔGRE1, ΔGRE1ΔKLF and Δ2XGREΔKLF) after cotransfection with GR + KLF6 and treated with DEX, as determined by the Student *t* test.

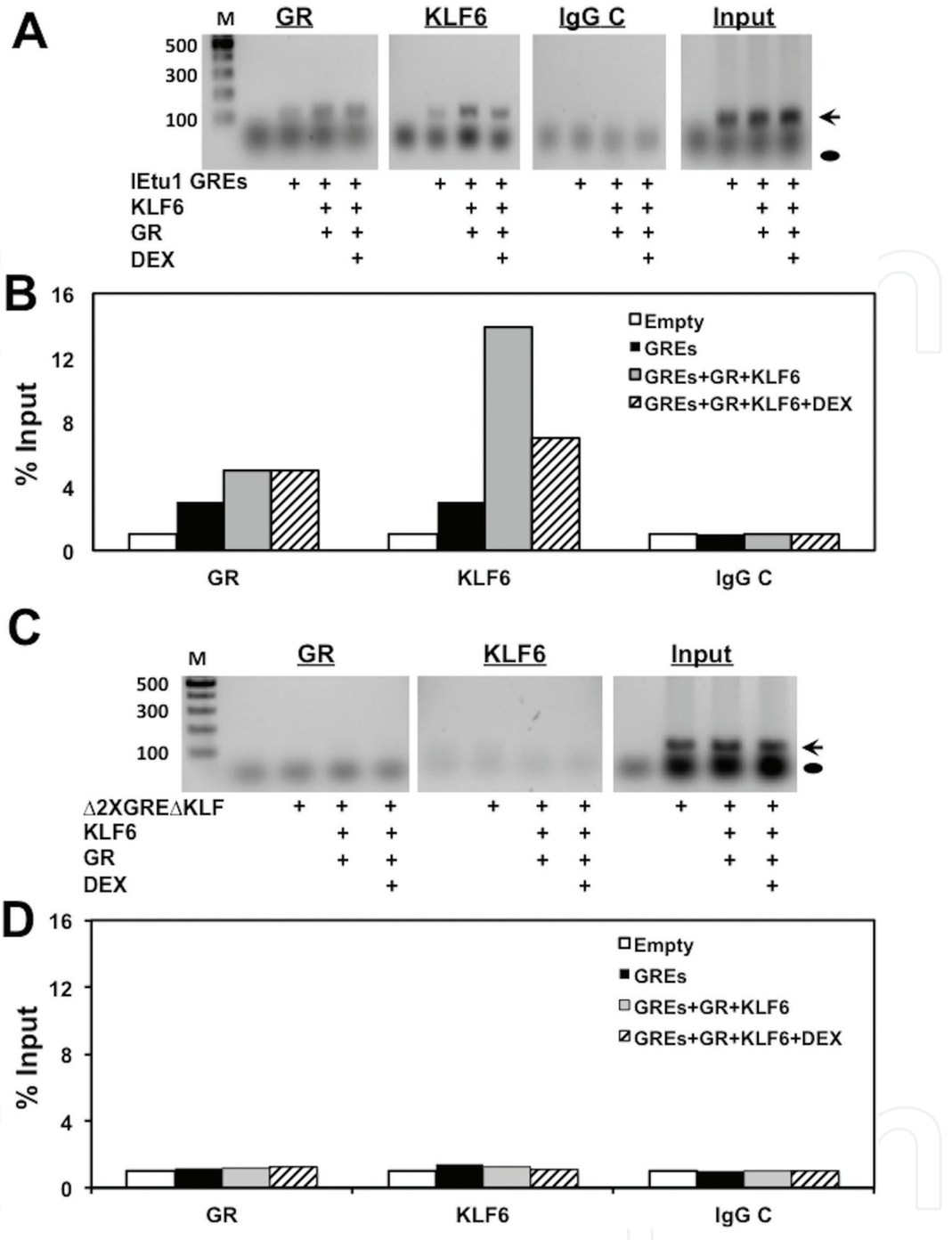

Figure 5. Interaction between GR and KLF6 with IEtu1 GREs. Neuro-2A cells were cotransfected with the IEtu1 GREs construct (**Panel A**; 4 ug DNA) or Δ2XGREΔKLF fragment (**Panel C**; 4 ug DNA), KLF6 expression plasmid (1.5 ug DNA) and/or the GR plasmid (2 ug DNA). Empty vector was added to maintain the same concentration of DNA in each transfection assay. Designated cultures were treated with DEX (10 uM; Sigma) 4 h before cells were harvested. ChIP studies were performed as previously described in Neuro-2A cells [48]. Polymerase chain reaction (PCR) was performed using primers that amplify the IEtu1 GREs and Δ2XGREΔKLF: forward primer is 5′- CCCACTTTTGCCTGTGTG-3′ and reverse primer is 5′-TTTTCCTCCTCCTTCCCC-3′. These primers yield a product of 107 base pairs. Input was 10% of the total DNA: protein complexes that used for IP and then PCR performed using PCR primers described in the materials and methods. Arrows denote the specific PCR product, 107 bp for IEtu1 GREs or for Δ2XGREΔKLF, and the circle denotes the position of primer dimers. Estimation of the level of binding to wild-type IEtu1 GREs sequences (**Panel B**) or Δ2XGREΔKLF (**Panel D**) is shown. The results are representative of three independent studies.

2% stripped fetal bovine serum may be a reason why the GR was associated with the IEtu1 GREs in the absence of DEX. Secondly, independent studies concluded that the GR can be associated with GREs in the absence of corticosteroids [61, 63]. Thirdly, treatment of cells with DEX reduces GR levels and the availability of GR to bind to DNA [26, 64]. All input samples (whole lysate prior to IP) yielded the specific 107 bp PCR product except Neuro-2A cells not transfected with the IEtu1 GREs construct (**Figure 5A**, Input panel, lane 1). In summary, the GR and KLF6 were specifically recruited to IEtu1 GRE sequences when the GREs were intact.

8. The GR does not stably interact with KLF6

Co-immunoprecipitation (co-IP) studies were used to test whether GR and KLF6 physically interact. Neuro-2A cells were cotransfected with plasmids that express KLF6 and the GR. Following IP with the GR antibody, we were unable to detect KLF6 in the immunoprecipitate regardless of DEX treatment (**Figure 6**). As expected, both proteins were detected in whole cell lysate (input). Furthermore, the GR was detected in the immunoprecipitate after IP was performed with the GR antibody. When KLF6 was used to IP whole cell lysate, the GR was not detected in the immunoprecipitate (data not shown). The same experimental conditions revealed that KLF15 and the GR were stably associated in transfected Neuro-2A cells [48]. In summary, co-IP studies suggested KLF6 and the GR were not stably associated with each other.

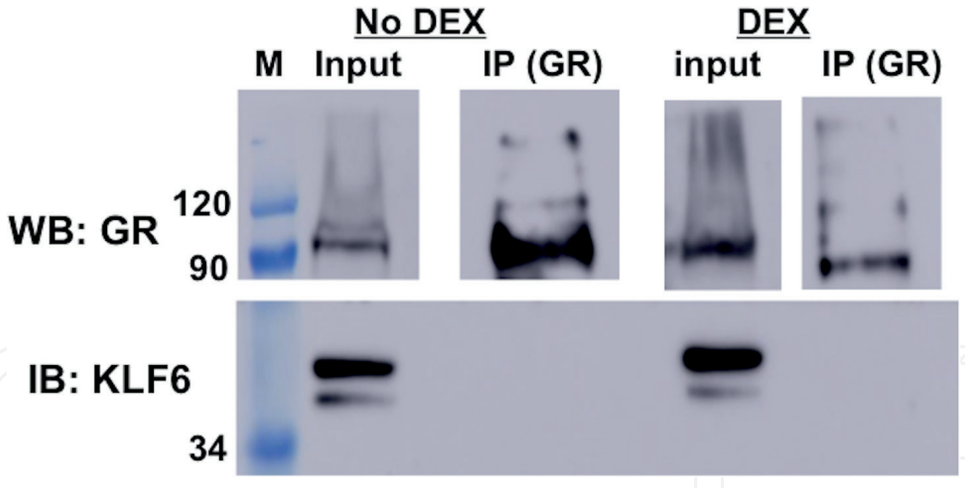

Figure 6. The GR does not physically interact with KLF6. Neuro-2A cells were grown to confluence on 100 mm dishes. Cells were cotransfected with plasmids that express KLF6 (1.5 μg) and the GR (2 μg). Cultures were treated with DEX (10 μM) in 2% stripped serum medium for 4 h before harvesting of transfected cells and other cultures were not treated DEX. Whole cell lysate was prepared with RIPA lysis buffer with 1× Protease Inhibitor cocktail (Thermo-scientific, cat. No: 78430) and protein concentration quantified. Protein extracts (500 μg) were combined with anti-GR (Cell Signaling; 3660) and /or anti-KLF6 (5 μg) antibodies (Thermo Fisher Scientific, 39–6900) and reactions were incubated for overnight at 4°C on rotator. Co-IP and Western blot studies were performed as described previously [48]. The secondary donkey anti-rabbit antibody (NA9340V) was purchased from GE Healthcare and secondary sheep anti-mouse antibody was purchased from GE Healthcare. Following immunoprecipitation with the GR antibody, KLF6 was not detected in the immune-precipitate by western blotting in samples treated with or without DEX. Input lanes are (whole cell lysate) used as positive controls for expression of the both proteins. Molecular weight markers (lane M) are shown to the left of the panels.

9. Discussion and summary

In this study, we provided evidence that KLF6 and the GR synergistically stimulate productive infection and IEtu1 promoter activity. The IEtu1 promoter must be activated for productive infection because it encodes two viral transcriptional regulators, bICP0 and bICP4 (**Figure 7A**)

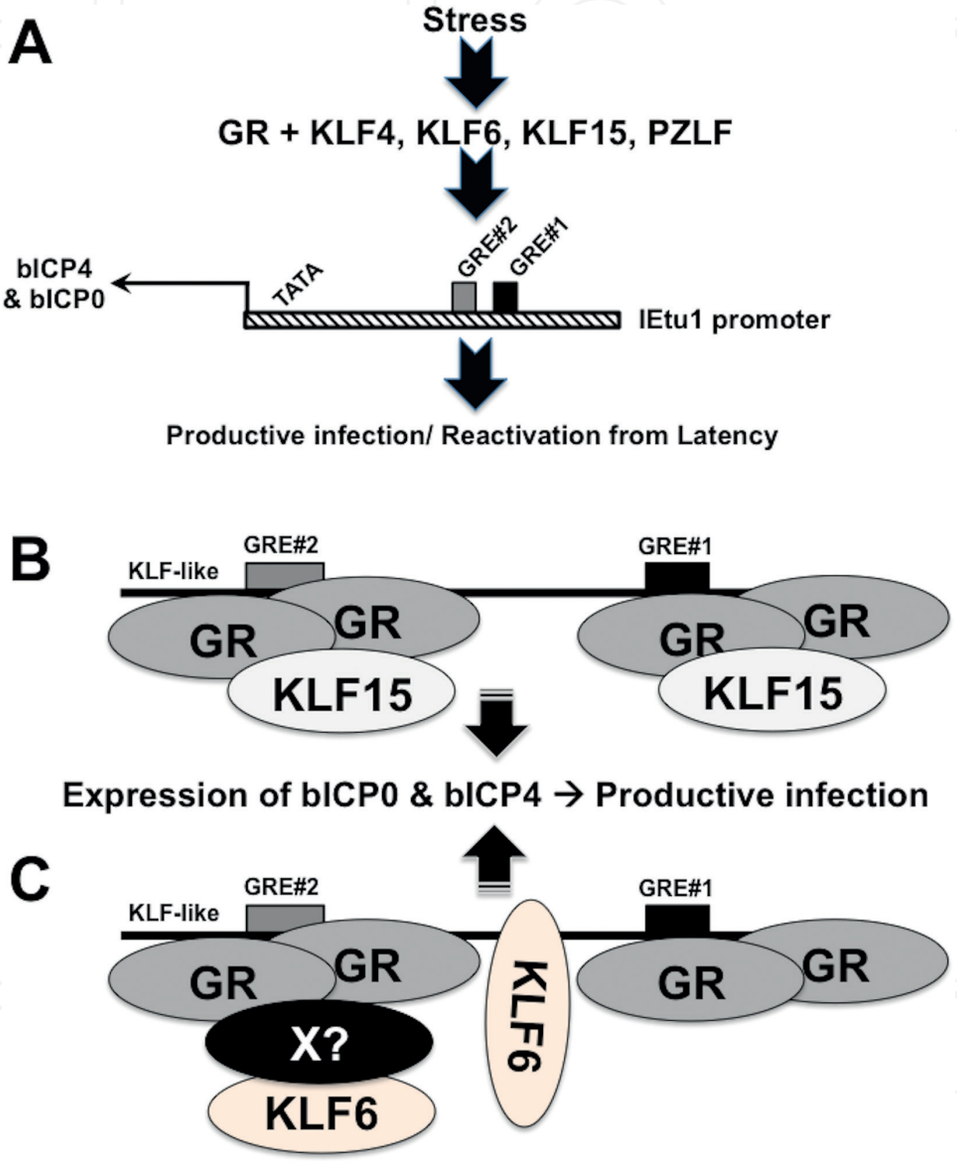

Figure 7. The GR and certain KLF family members stimulate BoHV-1 replication and IEtu1 promoter activity. **Panel A:** Stress activates the GR, which in turn stimulates expression of four stress-induced KLF family members in TG neurons [16]. Recent studies demonstrated that stress, as mimicked by DEX plus the GR, activates IEtu1 promoter activity because two GREs are located in the promoter [26]. KLF6 and KLF15 cooperate with the GR to activate IEtu1 promoter activity. Stress mediated activation of the IEtu1 promoter is crucial for productive infection because this promoter drives expression of two viral regulatory proteins (bIC0 and bICP4). **Panel B:** KLF15 stably interacts with the GR: consequently, this complex synergistically stimulates IEtu1 promoter activity by binding to the GREs [48]. **Panel C:** KLF6 and the GR cooperate to stimulate expression of IEtu1 promoter activity and productive infection. In contrast, to KLF15, KLF6 did not stably interact with the GR. Consequently, we propose that a KLF6 indirectly interacts with the GR via an unknown GR coactivator (X) or binding of the GR to a GRE promotes KLF6 interactions with sequences between GRE#1 and GRE#2. This schematic does not suggest that the interactions occur at independent GREs within the IEtu1 promoter; it merely suggests that these are the two likely mechanisms by which KLF6 cooperates with the GR to stimulate IEtu1 promoter activity.

[2]. During reactivation from latency, stress, as mimicked by the synthetic corticosteroid DEX, activates the GR and induces expression of several KLF family members (KLF4, KLF6, KLF15, and PLZF) [16]. A previous study demonstrated that KLF15, but not KLF4, and the GR synergistically stimulate IEtu1 promoter activity [48]. In contrast to KLF6, KLF15 stably interacts with the GR to establish a feed-forward transcriptional loop [48, 51, 53, 65, 66] (**Figure 7B**). Although KLF6 and KLF15 can both positively regulate promoter activity, they also can repress transcription in a promoter-specific manner [67, 68]. One study concluded there is a synergistic effect between the GR and transcriptional factors that recognize CACCC motifs [69], a known KLF6 binding site [70, 71]. There are no CACCC motifs on the positive strand of the IEtu1 GREs fragment; however, there are 2 CACCC motifs on the negative strand (KLF-1 like; **Figure 7B**). When these motifs were mutated (ΔKLF mutant), there was no difference in KLF6 and GR mediated trans-activation suggesting there may be KLF binding sites located between GRE#2 and GRE#1.

Relative to GRE#2, mutating GRE#1 was more important for GR mediated trans-activation [26, 48]. To ablate DEX induction of the IEtu1 promoter or the IEtu1 GREs, both GREs must be mutated [26, 48]. This is consistent with the results demonstrating there are cooperative effects between KLF15 [48] or KLF6 and the GR. ChIP results demonstrated that mutagenesis of both GREs interfered with KLF6 binding to sequences spanning the IEtu1 GREs, suggesting: 1) an unknown GR or KLF6 coactivator functions as a bridge between the GR and KLF6, which allows interactions between these two transcription factors (**Figure 7C**; left scenario at GRE#2), or 2) GR interactions with GRE#1 and/or GRE#2 influence adjacent sequences that are necessary for KLF6 to bind DNA **Figure 7B**; right scenario at GRE#1). Since KLF family members can bind to several GC or CA rich motifs, it is difficult to predict which sequences adjacent to GRE#1 or GRE#2 are important for interacting with KLF6 and/or KLF15.

The BoHV-1 genome contains approximately 100 putative GREs [26]. We identified 13 intergenic regions in the viral genome that contain at least 2 putative GREs and potential KLF binding sites within 400 base pairs. KLF15 and the GR significantly transactivate fragments present in unique long (UL)-52, bICP4, IEtu2, and Us fragments when DEX was added to cultures [48]. In contrast, KLF6 and the GR were unable to transactivate these intergenic fragments in the presence or absence of DEX (data not shown) confirming KLF15 has novel properties relative to KLF6.

KLF4, KLF6, and KLF15 expression are induced in TG neurons of calves that are latently infected with BoHV-1 during early stages of DEX induced reactivation from latency [16]. Cellular, not viral encoded, transcription factors are predicted to be crucial for initiating viral transcription during initial stages of reactivation from latency because lytic cycle viral gene expression is not readily detected in TG of latently infected calves [29, 30]. Thus, activation of the IEtu1 promoter by the GR and DEX-induced transcription factors, KLF6 and KLF15 for example, may be sufficient to trigger lytic cycle viral gene expression in a subset of latently infected neurons following a stressful stimulus, as shown in **Figure 7B** and **C**.

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