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GENOME REPLICATION AND REGULATION OF VIRAL GENE EXPRESSION

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Two Pioneer Transcription Factors, Krüppel-Like Transcription Factor 4 and Glucocorticoid Receptor, Cooperatively Transactivate the Bovine Herpesvirus 1 ICP0 Early Promoter and Stimulate Productive Infection

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ABSTRACT An important site for bovine herpesvirus 1 (BoHV-1) latency is sensory neurons within trigeminal ganglia (TG). The synthetic corticosteroid dexamethasone consistently induces BoHV-1 reactivation from latency. Expression of four Krüppel-like transcription factors (KLF), i.e., KLF4, KLF6, PLZF (promyelocytic leukemia zinc finger), and KLF15, are induced in TG neurons early during dexamethasone-induced reactivation. The glucocorticoid receptor (GR) and KLF15 form a feed-forward transcription loop that cooperatively transactivates the BoHV-1 immediate early transcription unit 1 (IEtu1) promoter that drives bovine infected cell protein 0 (bICP0) and bICP4 expression. Since the bICP0 gene also contains a separate early (E) promoter, we tested the hypothesis that GR and KLF family members transactivate the bICP0 E promoter. GR and KLF4, both pioneer transcription factors, cooperated to stimulate bICP0 E promoter activity in a ligand-independent manner in mouse neuroblastoma cells (Neuro-2A). Furthermore, GR and KLF4 stimulated productive infection. Mutating both half GR response elements did not significantly reduce GR- and KLF4-mediated transactivation of the bICP0 E promoter, suggesting that a novel mechanism exists for transactivation. GR and KLF15 cooperatively stimulated bICP0 activity less efficiently than GR and KL4: however, KLF6, PLZF, and GR had little effect on the bICP0 E promoter. GR, KLF4, and KLF15 occupied bICP0 E promoter sequences in transfected Neuro-2A cells. GR and KLF15, but not KLF4, occupied the bICP0 E promoter at late times during productive infection of bovine cells. Collectively, these studies suggest that cooperative transactivation of the bICP0 E promoter by two pioneer transcription factors (GR and KLF4) correlates with stimulating lytic cycle viral gene expression following stressful stimuli.

IMPORTANCE Bovine herpesvirus 1 (BoHV-1), an important bovine pathogen, establishes lifelong latency in sensory neurons. Reactivation from latency is consistently induced by the synthetic corticosteroid dexamethasone. We predict that increased corticosteroid levels activate the glucocorticoid receptor (GR). Consequently, viral gene expression is stimulated by the activated GR. The immediate early transcription unit 1 promoter (IEtu1) drives expression of two viral transcriptional regulatory proteins, bovine infected cell protein 0 (bICP0) and bICP4. Interestingly, a separate early promoter also drives bICP0 expression. Two pioneer transcription factors, GR and Krüppel-like transcription factor 4 (KLF4), cooperatively transactivate the bICP0 early (E) promoter. GR and KLF15 cooperate to stimulate bICP0 E promoter activity but significantly less than GR and KLF4. The bICP0 E promoter contains enhancer-like domains necessary for GR- and KLF4-mediated transactivation that are distinct from those for GR and KLF15. Stress-induced pioneer transcription factors are proposed to

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activate key viral promoters, including the bICP0 E promoter, during early stages of reactivation from latency.

KEYWORDS bovine herpesvirus 1, gene expression, pioneer transcription factor, stress response

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Bovine herpesvirus 1 (BoHV-1) can induce abortions in pregnant cows following infection of ovaries and/or the fetus (1). Infection of the upper respiratory tract erodes mucosal surfaces and causes conjunctivitis (2, 3). Infection also suppresses host immune responses and increases the incidence of life-threatening bacterial pneumonia (4, 5). A BoHV-1 entry protein encoded by the poliovirus receptor related 1 gene is an important bovine respiratory disease complex susceptibility gene for Holstein calves (6). In summary, BoHV-1 can induce abortions and is a cofactor during the development of bovine respiratory disease complex, an important polymicrobial disease of cattle (3).

Trigeminal ganglia (TG) are a primary site for lifelong latency following acute infection of the oral, nasal, or ocular cavity (7, 8). Increased stress, food and water deprivation during shipping of cattle, weaning, and/or dramatic weather changes increase corticosteroid levels and reactivation from latency (8, 9). Corticosteroids, including dexamethasone (DEX), bind and activate the glucocorticoid receptor (GR) (10), suggesting that GR regulates certain aspects of reactivation from latency. The synthetic corticosteroid DEX, which mimics the effects of stress, stimulates productive infection (11) and initiates reactivation from latency (9). Interestingly, the immediate early transcription unit 1 (IEtu1) promoter, which drives expression of two crucial viral regulatory proteins (bovine infected cell protein 0 [bICP0] and bICP4), contains consensus GR response elements (GREs) in the promoter, and mutating these GREs interferes with DEX-induced promoter activity (11). In addition to GR directly stimulating BoHV-1 gene expression, the immunosuppressive properties of increased corticosteroid levels also promote virus spread during later stages of reactivation from latency (12).

For successful reactivation from latency to occur, the quiescent viral genome in latently infected neurons must be remodeled into an actively transcribing genome to produce infectious virus. BoHV-1 proteins encoded by all three immediate early (IE) genes (bICP0, bICP4, and bICP22) and the viral tegument protein that specifically activates IE promoters (VP16) are detected in TG neurons within hours after DEX treatment (13–15). Conversely, other late viral proteins (gC and gE, for example) are difficult to detect during reactivation. DEX-induced cellular transcription factors have been identified within a few hours in TG neurons following DEX treatment of latently infected calves: a subset of these transcription factors stimulate certain viral promoters and productive infection (16). Strikingly, four members of the Krüppel-like transcription factor (KLF) family, i.e., KLF4, KLF6, KLF15, and promyelocytic leukemia zinc finger (PLZF), were identified in this study. KLF family members belong to a large family of transcription factors that include the Sp1 transcription factor family; both transcription factor families interact with GC- or CA-rich motifs (17, 18). Many herpesvirus promoters contain an Sp1 binding site, and Sp1 activates herpes simplex virus (HSV) IE promoters (19). Recent studies have demonstrated that GR and KLF15 form a feed-forward transcription loop to transactivate the IEtu1 (20) and HSV-1 ICP0 (21) promoters, which supports our prediction that GR and stress-induced transcription factors stimulate key promoters and productive infection following stress.

In this study, we provide evidence that GR and KLF4 cooperatively transactivate the bICP0 E promoter and stimulate productive infection. GR and KLF15 also transactivated the bICP0 E promoter but not as efficiently as GR and KLF4. Surprisingly, DEX was not required for transactivation by GR and KLF4 or GR and KLF15, indicating that these factors stimulated promoter activity via an unliganded mechanism. Chromatin immunoprecipitation (ChIP) studies revealed that KLF4, KLF15, and GR occupied bICP0 E promoter sequences: however, only GR and KLF15 occupied ICP0 E promoter sequences late during productive infection. KLF4 and GR are pioneer transcription factors

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(22), suggesting that they can activate a chromatinized bICP0 E promoter following a stressful stimulus.

RESULTS

GR cooperates with KLF4 and KLF15 to transactivate the bICP0 E promoter. The organization of BoHV-1 bICP0 and bICP4 coding regions is unique relative to HSV-1. For example, a single promoter (IEtu1) drives IE expression of IE/2.9 and IE/4.2 mRNAs, which are derived from an alternatively spliced transcript. IE/2.9 mRNA is translated into the bICP0 protein, and IE/4.2 mRNA is translated into the bICP4 protein (23–25). Second, an E promoter drives expression of E/2.6, an early transcript that is translated into the bICP0 protein because exon 1 located in IE/2.9 is noncoding (23–26). Recombinant BoHV-1 viruses that contain point mutations in the RING finger of bICP0 or a deletion of the amino terminus of bICP0 grow less efficiently than the rescued mutant or wild-type (wt) BoHV-1 (27, 28). Furthermore, the deletion mutant essentially established a quiescent infection in a small subset of bovine kidney cells (27), and when these mutants were used to infect calves, virus shedding and antibody responses were not readily detected (C. Jones, unpublished results). Finally, reverse transcription-PCR (RT-PCR) studies revealed that bICP0 RNA was detected more frequently than bICP4 RNA in TG of calves during DEX-induced reactivation from latency (29). Collectively, these observations indicated that bICP0 is essential for productive infection and plays an important role during reactivation from latency, and the bICP0 E promoter is predicted to maintain high levels of bICP0 expression.

Initial studies tested whether GR can cooperate with stress-induced KLF family members to regulate full-length bICP0 early promoter activity (see description of promoter construct EP-943 below). For these studies, mouse neuroblastoma (Neuro-2A) cells were incubated with medium containing stripped fetal bovine serum (FBS). 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. FBS contains bioactive corticosteroids, because nearly all Neuro-2A cells incubated with minimal essential medium (MEM) plus 10% FBS contained nuclear-localized GR: conversely, GR is present in the cytoplasm of Neuro-2A cells incubated with 2% stripped FBS (11). Neuro-2A cells were used for these studies because they have neuron-like properties, can be differentiated into dopamine like neurons (30), are readily transfected, and yield low levels of infectious BoHV-1 virus after infection (31). GR and KLF4 transactivated bICP0 E promoter activity more than 30-fold in transfected Neuro-2A cells, whereas KLF4 alone stimulated promoter activity approximately 6-fold (Fig. 1A). GR alone stimulated EP-943 promoter activity with or without DEX treatment less efficiently than KLF4 (Fig. 1A, 2, and 3). Surprisingly, DEX significantly reduced GR- and KLF4-mediated transactivation to levels similar to those of KLF4 alone. Cultures were also treated with RU486 because it is a GR and progesterone receptor-specific antagonist commonly used to assess whether transcriptional activation by GR is ligand dependent (32, 33). Addition of RU486 to GR- and KLF4-transfected cells (with DEX or with no DEX) slightly increased promoter activity. In general, nuclear hormone receptors, including GR, are activated by a ligand, in this case DEX (34). However, under certain circumstances, GR can be translocated from the cytoplasm to the nucleus and activate gene expression by ligand-independent mechanisms (35, 36). In fact, the promoter that drives BRCA1 (breast cancer-associated gene 1) expression is positively regulated by GR via an unliganded mechanism (37). Similar to the bICP0 E promoter, BRCA1 promoter activity is reduced when cultures are treated with corticosteroids. While we do not understand the mechanism by which GR and KLF4 transactivated the bICP0 E promoter by a ligand-independent manner, there is precedence for GR-mediated activation of promoters by an unliganded mechanism.

GR and KLF15 also cooperatively transactivated the bICP0 E promoter, but promoter activation was significantly less than that of GR plus KLF4. In contrast to the effects on GR- plus KLF4-mediated transactivation, the addition of DEX and/or RU486 had only

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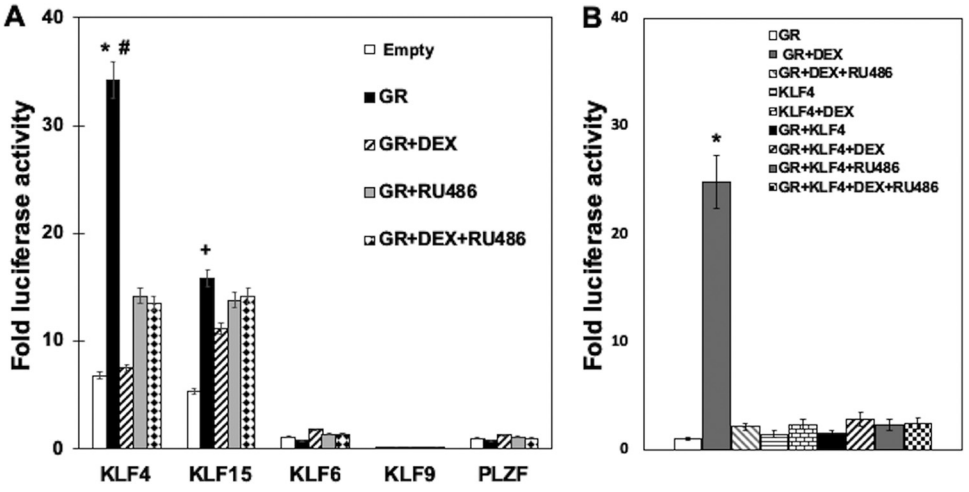

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FIG 1 GR and KLF4 cooperate to efficiently transactivate the bICP0 E promoter. (A) Neuro-2A cells were transfected with the designated bICP0 E promoter (0.5 μg DNA) and, where indicated, a plasmid expressing mouse GR protein (1.0 μg DNA) and/or KLF-4, KLF-15, KLF-6, KLF-9, or PLZF (0.5 μg DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. After transfection, 2% stripped FBS was added to the medium. Designated cultures were treated with water-soluble DEX (10 μM; Sigma) or 1 μM RU486 at 24 h after transfection. At 48 h after transfection, cells were harvested and protein lysate was subjected to the dual-luciferase assay. The results are the average of three independent experiments, and error bars denote the standard error. An asterisk indicates significant differences ($P < 0.05$) in results for cells transfected with EP-943 cotransfected with GR plus KLF4 relative to results for all other EP-943 samples cotransfected with KLF4. A pound sign indicates a significant difference in results for cells when EP-943 was cotransfected with KLF4 and GR relative to EP-943 cotransfected with GR and KLF15. A plus symbol indicates that GR and KLF15 significantly increased EP-943 promoter activity relative to the effects of GR and KLF6, KLF9, or PLZF ($P < 0.05$). Student's *t* test was used for analyzing the results. (B) Neuro-2A cells were transfected with a MMTV LTR construct (0.5 μg DNA) and, where indicated, a plasmid expressing mouse GR protein (1.0 μg DNA) and/or KLF-4. An asterisk indicates significant differences ($P < 0.05$) in results for cells transfected with the MMTV LTR cotransfected with GR relative to all other samples.

slight effects on GR- and KLF15-mediated transactivation. EP-943 promoter activity was not significantly stimulated by GR plus KLF6, KLF9, or PLZF. In fact, these KLF family members did not readily transactivate the bICP0 E promoter nor was there a cooperative effect with GR.

Additional studies tested whether the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) was cooperatively transactivated by GR and KLF4. The stimulatory effects of GR on the MMTV LTR are well documented, because this promoter/enhancer contains multiple well-defined GREs (38). As expected, GR and DEX significantly increased MMTV LTR promoter activity in Neuro-2A cells (Fig. 1B). Strikingly, KLF4 reduced promoter activity to basal levels regardless of the addition of DEX or RU486, indicating that unique regulatory sequences in EP-943 are crucial for GR- and KLF4-mediated transactivation.

Localization of bICP0 E promoter sequences important for transactivation mediated by GR and KLF4 or by GR and KLF15. bICP0 E promoter deletion mutants were used to localize sequences important for GR- and KLF4-mediated promoter activation. GR- and KLF4-mediated transactivation of EP-638 and EP-328 was significantly reduced relative to that of EP-943 (Fig. 2). DEX treatment reduced GR- and KLF4-mediated transactivation of EP-638 and EP-328. Furthermore, RU486 slightly increased promoter activity, consistent with the effects on EP-943. In comparison to EP-638 and EP-328, EP-172, EP-143, and EP-71 were not significantly transactivated by GR and KLF4.

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GR- and KLF15-mediated transactivation of bICP0 E promoter deletion constructs showed striking differences compared to that of GR and KLF4. For example, GR and KLF15 transactivated EP-943, EP-638, and EP-328 at similar levels regardless of the addition of DEX and/or RU486 (Fig. 3). However, GR and KLF15 did not significantly transactivate EP-172, EP-143, and EP-71. In comparison to GR and KLF4, GR plus KLF4

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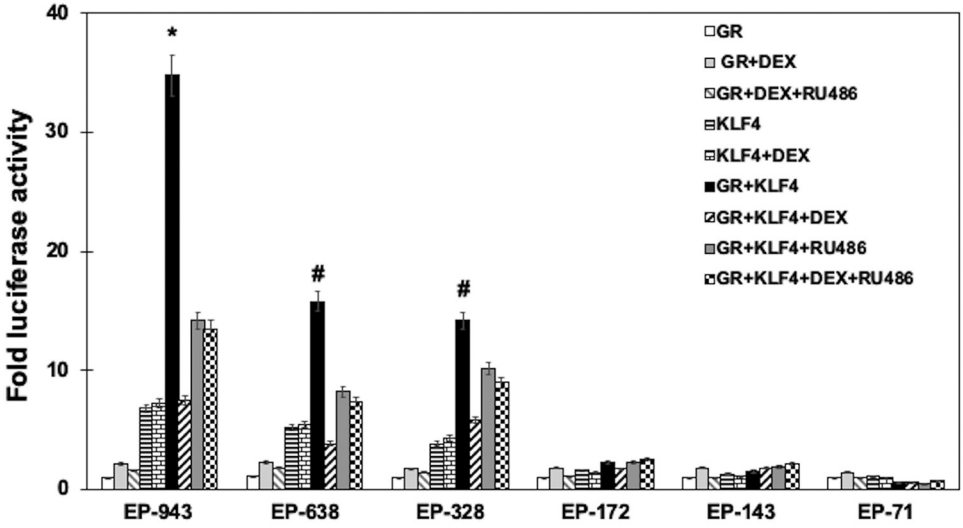

FIG 2 Localization of bICP0 E promoter sequences important for GR- and KLF4-mediated transactivation. Cells were transfected with the designated bICP0 E promoter constructs (0.5 μg DNA) that were previously described (29) and, where indicated, a plasmid that expresses mouse GR protein (1.0 μg DNA) and/or KLF4 (0.5 μg DNA). After transfection, 2% stripped FBS was added to the medium. Designated cultures were treated with water-soluble DEX (10 μM; Sigma) and/or 1 μM RU486 at 24 h after transfection. At 48 h after transfection, the cells were harvested and the dual-luciferase assay performed. The results are the average of three independent experiments, and error bars denote the standard error. An asterisk indicates significant differences ($P < 0.05$) in results for cells transfected with EP-943 cotransfected with GR and KLF4 relative to all other EP-943 samples. Results for EP-638 and EP-328 cotransfected with GR and KLF4 were not significantly different. Promoter activity of EP-638 and EP-328 cotransfected with GR and KLF4 was significantly different ($P < 0.05$) from that for EP-172, EP-143, and EP-71 (denoted by a pound sign). Student's *t* test was used for analyzing the results.

plus KLF15 did not synergistically or additively transactivate any of the bICP0 E promoter constructs regardless of DEX or RU486 treatment (data not shown). In summary, these studies confirmed that the bICP0 E promoter was cooperatively transactivated by GR and KLF4 more efficiently than by GR and KLF15.

Cooperative transactivation of the bICP0 E promoter by GR and KLF4 versus GR and KLF15 requires different enhancer-like elements. The simplest explanation for the bICP0 E promoter deletion studies described in Fig. 2 and 3 was that separate enhancer domains upstream of promoter-proximal sequences were transactivated by GR and KLF4 in a cooperative fashion and that sequence requirements for GR- and KLF15-mediated transactivation are different (Fig. 4A). For example, sequences necessary for GR- and KLF4-mediated transactivation appear to contain two separate enhancer-like domains, nucleotides 638 to 943 and nucleotides 172 to 328. GR- and KLF4-mediated transactivation of EP-638 and that of EP-328 were not significantly different, suggesting that a deletion of sequences between nucleotides 638 and 328 did not significantly reduce promoter activity: however, in the context of EP-943, deletion of sequences between nucleotides 638 and 328 may be important. GR- and KLF15-mediated transactivation was not affected by deleting nucleotides 638 to 943, whereas the cumulative deletion of nucleotides 172 to 943 was important. Sequence analysis of these regions revealed numerous Sp1 binding sites, two putative nucleosome-enriched binding sites for KLF4 (39), and two potential half GREs that may be transactivated by a GRE monomer (40). Furthermore, there are many GC- and CA-rich motifs in the bICP0 E promoter that may be important for KLF4- and/or KLF15-mediated transactivation (18, 41). These individual fragments were cloned upstream of a simple promoter luciferase construct (pGL3 promoter vector or pGL4.24[luc2P/minP] vector; Promega), and transactivation by GR and KLF4 was examined in Neuro-2A cells. This approach did not recapitulate the results obtained with EP promoter deletion constructs (data not shown), adding support to the premise that cooperation occurred between different enhancer domains located within EP-943.

Additional bICP0 E promoter mutants were prepared to further test the premise that cooperation occurred between the putative enhancer domains. Deleting sequences

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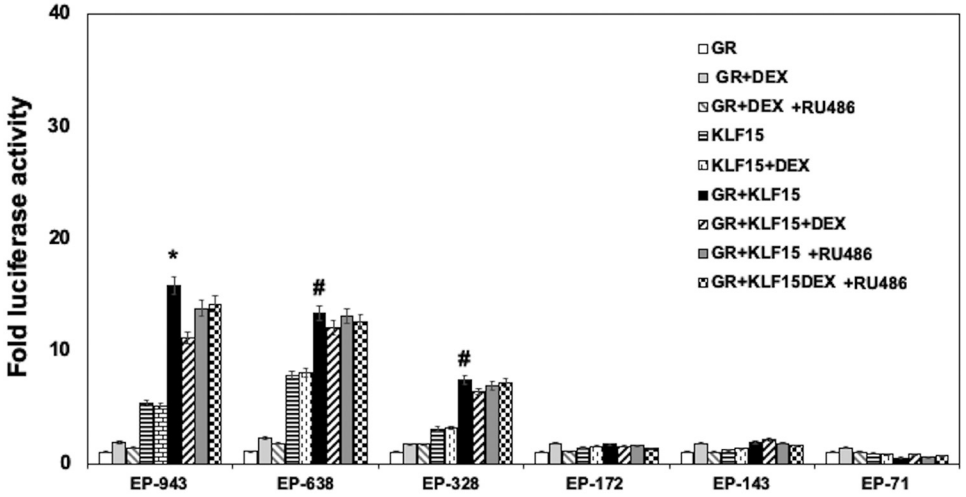

FIG 3 Localization of bICP0 E promoter sequences important for GR- and KLF15-mediated transactivation. Neuro-2A cells were transfected with the designated bICP0 E promoter constructs (0.5 μg DNA) that were previously described (29) and, where indicated, a plasmid that expresses mouse GR protein (1.0 μg DNA) and/or KLF15 (0.5 μg DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. After transfection, 2% stripped FBS was added to the medium. Designated cultures were then treated with water-soluble DEX (10 μM; Sigma) and/or 1 μM RU486 at 24 h after transfection. At 48 h after transfection, cells were harvested and protein lysate was subjected to the dual-luciferase assay. The results are the average of three independent experiments, and error bars denote the standard error. An asterisk indicates significant differences ($P < 0.05$) between results for cells transfected with EP-943 cotransfected with GR and KLF15 and the EP-943 sample transfected with GR or KLF15 treated with or without DEX and RU486. Results for EP-638 cotransfected with GR and KLF15 were significantly different from those for the EP-638 sample transfected with GR or KLF15 treated with or without DEX and RU486 (denoted by a pound sign). The results for EP-328 cotransfected with GR and KLF15 were significantly different from those for EP-328 transfected with GR or KLF15 with or without DEX and RU486 (denoted by a pound sign). Results for EP-943, EP-638, and EP-328 cotransfected with GR and KLF15 were not significantly different from each other. Results for EP-943 cotransfected with GR and KLF15 were significantly different from those for EP-172, EP-143, or EP-71 cotransfected with the same constructs. Student's *t* test was used for analyzing the results.

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between nucleotides 172 and 328 (EP-943Δ172-328) significantly reduced GR- and KLF4-mediated transactivation (Fig. 4B) but not GR- and KLF15-mediated transactivation (Fig. 4C). As expected, EP-943Δ328-638 and EP-943Δ172-638 were not efficiently transactivated by GR and KLF4 or by GR and KLF15. Collectively, these studies suggested that GR- and KLF4-mediated transactivation required cooperation between different enhancer domains in the bIPC0 E promoter (nucleotides 172 to 328 plus nucleotides 638 to 943), which was different from GR- and KLF15-mediated transactivation.

Half GREs in the bICP0 E promoter are not required for GR- and KLF4-mediated transactivation. The bICP0 E promoter contains two half GREs between nucleotides 638 and 943 (Fig. 4A) that have one nucleotide difference from consensus half GREs that can be transactivated by a GR monomer (40) (Fig. 5A). To investigate if these half GREs are important for GR-mediated transactivation, mutant promoter constructs lacking one or both half GREs were prepared. These promoter constructs were compared to EP-943 and EP-638 for their ability to be transactivated by GR and KLF4. A construct containing one or both half GREs was transactivated by GR and KLF4 with similar efficiency as EP-943 (Fig. 5B). There was also no significant difference between GR- and KLF4-mediated transactivation of EP-943 and the half GRE mutants when DEX was added to cultures. Finally, the half GRE mutants, like EP-943, were transactivated by GR and KLF4 more efficiently than EP-638. Collectively, these studies revealed that efficient transactivation of the EP-943 promoter by GR and KLF4 does not require the half GREs.

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KLF4 cooperates with GR to stimulate productive infection. A recent study demonstrated that KLF15 cooperates with GR and DEX to stimulate productive infection (20). To examine the effect GR and KLF4 have on productive infection, Neuro-2A

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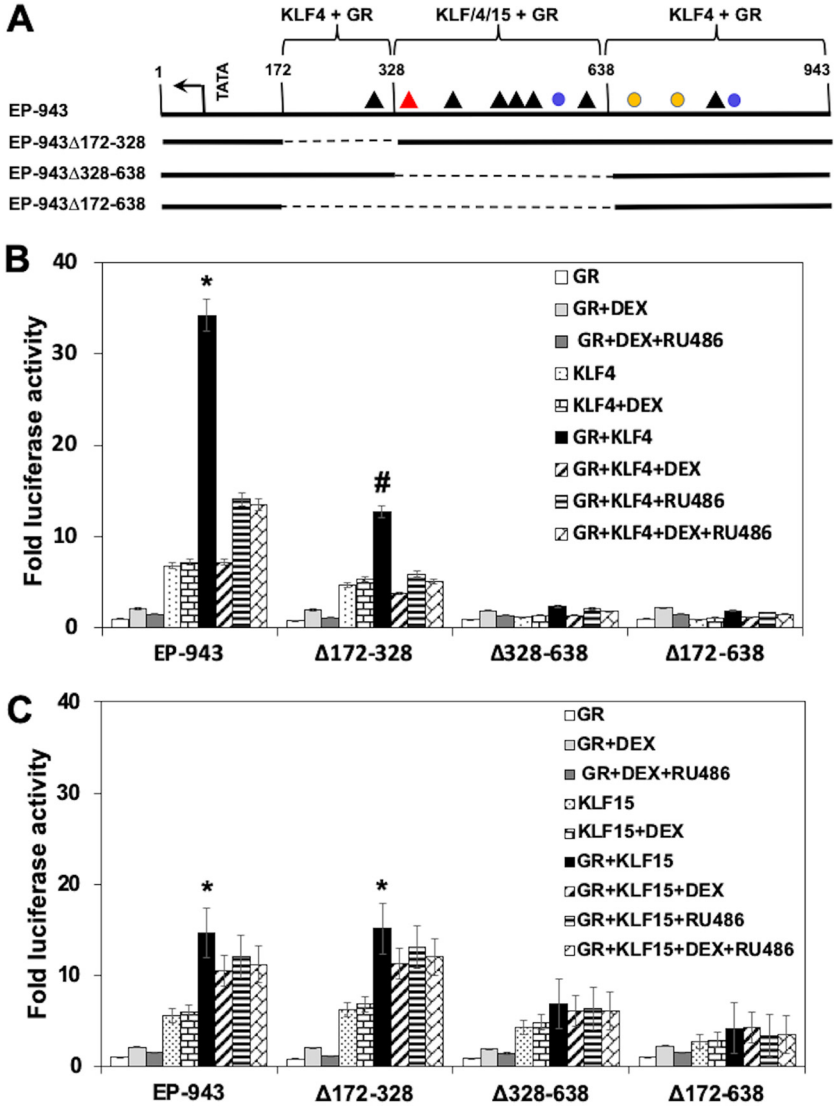

FIG 4 Internal bICP0 E promoter deletions identify unique enhancer domains. (A) Schematic of additional bICP0 E promoter deletion constructs. Black triangles denote consensus Sp1 binding sites, a red triangle denotes a consensus KLF CACCC-rich motif, blue circles denote KLF4-like binding sites, and orange circles denote half GRE-like binding sites. (B and C) Neuro-2A cells were transfected with the designated bICP0 E promoter constructs (0.5 μg DNA) and, where indicated, a plasmid that expresses mouse GR protein (1.0 μg DNA), KLF4 (B), or KLF15 (C) (0.5 μg DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. After transfection, 2% stripped FBS was added to the medium. Designated cultures were then treated with water-soluble DEX (10 μM; Sigma) and/or 1 μM RU486 at 24 h after transfection. At 48 h after transfection, the dual-luciferase assay was performed. The results are the average of three 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 GR and KLF4 and that cotransfected with GR and KLF15 (Student's *t* test; $P < 0.05$). GR- and KLF4-mediated transactivation of the Δ172-328 construct was significantly different from that of the Δ328-638 and Δ172-638 deletion constructs (denoted by a pound sign).

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cells were cotransfected with gCblue genomic DNA. Neuro-2A cells were transfected with BoHV-1 gCblue DNA instead of being infected because VP16 and other regulatory proteins in the virion, bICP4 for example (34), diminish the stimulatory effects of DEX on productive infection. GR plus KLF4 plus DEX stimulated the number of β-galactosidase (B-Gal)-positive Neuro-2A cells more than 6-fold, which was significantly higher than that stimulated by GR plus DEX or by GR or KLF4 alone (Fig. 6A and B). Cotransfection of gCBlue and GR and KLF4 stimulated productive infection more than 5-fold even when DEX was not added to cultures, which was significantly

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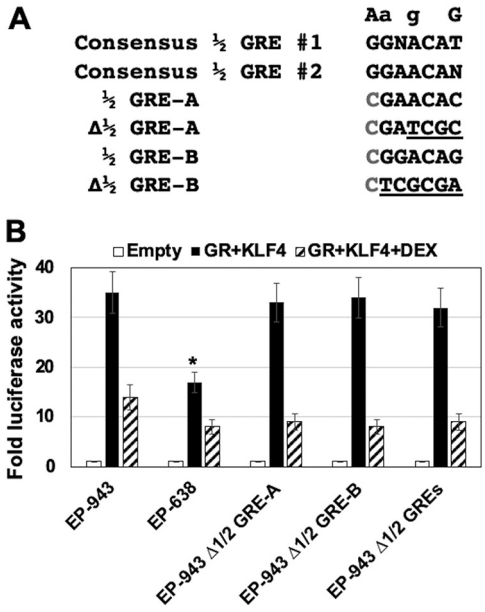

FIG 5 GR- and KLF4-mediated transactivation of bICP0 E promoters was not affected by mutating the half GREs. (A) Consensus half GREs previously shown to be transactivated by GR in a DEX-dependent manner: nucleotides above the consensus sequences reflect variants in the half GREs (N denotes any nucleotide at that particular position) (40). The sequences of putative half GREs in the bICP0 promoter (wt and mutant) are located between nucleotides 638 and 943. Gray nucleotides denote differences in the consensus half GRE sequences. An NruI restriction enzyme site (TCGCGA) was used to introduce mutations into each of the half GRE sites (underlined nucleotides). (B) Neuro-2A cells were transfected with the designated bICP0 E promoter constructs (0.5 μg DNA) and, where indicated, a plasmid that expresses mouse GR protein (1.0 μg DNA) and KLF4 (0.5 μg DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. After transfection, 2% stripped FBS was added to the medium. Designated cultures were then treated with water-soluble DEX. At 48 h after transfection, the dual-luciferase assay was performed. The results are the average of three 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 GR and KLF4 and those using EP-638 (Student's t test; $P < 0.05$).

different than the effects seen by GR plus DEX treatment or with transfection with KLF4 alone. DEX treatment slightly increased the effects of GR and KLF4, whereas RU486 reduced this effect, suggesting that other viral promoters were stimulated by KLF4 and GR via a ligand-dependent manner.

Occupancy of GR, KLF4, and KLF15 with bICP0 E promoter sequences in transfected or productively infected cells. To test whether GR, KLF4, and KLF15 were recruited to the bICP0 E promoter, chromatin immunoprecipitation (ChIP) studies were performed. Neuro-2A cells were cotransfected with EP-943, GR expression plasmid, KLF4, or KLF15, followed by treatment with DEX or a vehicle. Following ChIP with the designated antibodies, bICP0 E promoter sequences were amplified with a specific primer set described in Materials and Methods. Relative to the isotype control antibody and mock-infected cells, the occupancy of bICP0 E promoter sequences was significantly higher when IP was performed with the GR antibody (Fig. 7): however, neither DEX treatment nor transfection with KLF4 or KLF15 (Fig. 7) dramatically increased GR binding. Occupancy of the bICP0 E promoter with KLF4 and KLF15 was also significantly higher than that of the isotype control antibody or mock-infected cells. However, binding of KLF4 or KLF15 was not dramatically affected by DEX or overexpression of GR, indicating that ChIP studies did not reveal cooperative binding between GR and KLF4 or KLF15. In summary, these studies demonstrated that direct interactions occur between the bICP0 E promoter, GR, and KLF4 in the absence of viral protein expression.

F7

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Following productive infection of bovine kidney cells (CRIB), GR and KLF15 occupancy of the bICP0 E promoter was significantly higher at 8 and 16 h after infection than that of the isotype control antibody (Fig. 8A). Occupancy by KLF15, but not KLF4, of the bICP0 E

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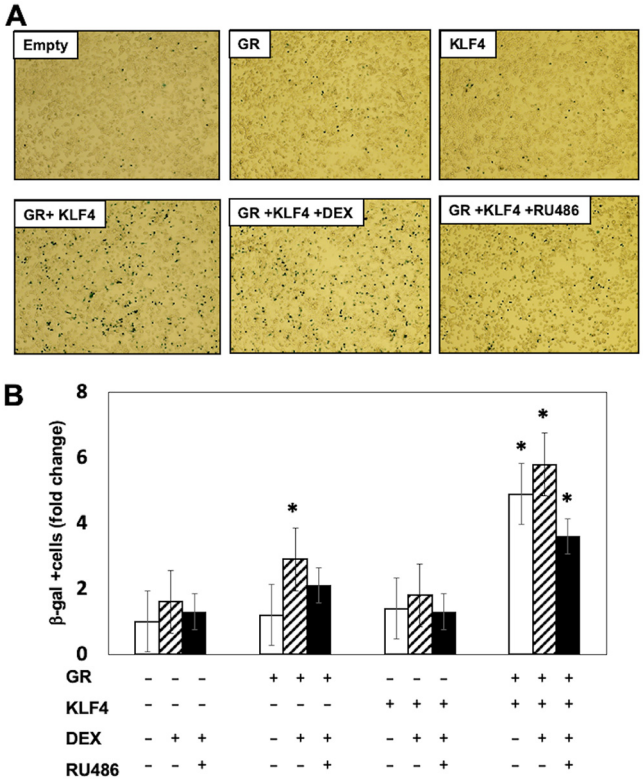

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FIG 6 GR and KLF4 cooperate to stimulate productive infection. Neuro-2A cells were transfected with 1.5 μg BoHV-1 gCblue and, where indicated, a plasmid expressing mouse GR protein (1.0 μg DNA) and KLF4 (0.5 μg DNA). To maintain the same amount of DNA in each sample, empty vector was included in the samples. After transfection, 2% stripped FBS was added to the medium. Designated cultures were then treated with water-soluble DEX (10 μM; Sigma) and/or 1 μM RU486. At 48 h after transfection, the number of β-Gal$^+$ cells were counted. The value for the control (gCblue virus cotransfected with empty vector and then treated with PBS after transfection) was set at 1. The results from DEX-treated cultures were compared to those from the control and are an average of three independent studies. (A) Representative photographs of cultures following staining for β-Gal expression are shown. (B) The number of β-Gal$^+$ Neuro-2A cells from four independent quadrants/plate was counted. The results are the average of three independent experiments. An asterisk indicates a significant difference between control and samples transfected with GR and/or KLF4 and treated with DEX or RU486 (Student's *t* test; $P < 0.05$).

promoter was significantly higher at 16 h after infection when DEX was added to cultures than occupancy by the isotype control antibody. In contrast to the bICP0 E promoter, significantly more GR occupied IEtu1 promoter sequences at 4, 8, and 16 h after infection when primers that amplified a fragment encompassing the two GREs in this promoter were used (Fig. 8B). KLF15 was detected at 4 and 8 after infection when cultures were not treated with DEX. By 16 h after infection, KLF15 occupied the IEtu1 promoter regardless of DEX treatment. KLF4 occupied the IEtu1 promoter at 16 h after infection when cultures were treated with DEX, consistent with the finding that GR and KLF4 transactivated the IEtu1 promoter (20). These studies revealed promoter-specific occupancy of GR and KLF15 with the IEtu1 promoter throughout productive infection; however, KLF15 occupancy of the bICP0 E promoter did not increase until 16 h after infection. Furthermore, KLF4 occupied the IEtu1 promoter at 16 h after infection in the presence of DEX but did not occupy bICP0 E promoter sequences during productive infection.

DISCUSSION

The KLF4 protein is present in more TG neurons during DEX-induced reactivation from latency than during latency or in uninfected calves (16), implying that it is part of the normal stress response in TG. Additional stimuli relevant to reactivation, heat stress, p53 induction, DNA repair, and the Wnt signaling pathway also stimulate KLF4 expression (41). Previous studies concluded that GR and sKLF4 coregulate anti-inflammatory

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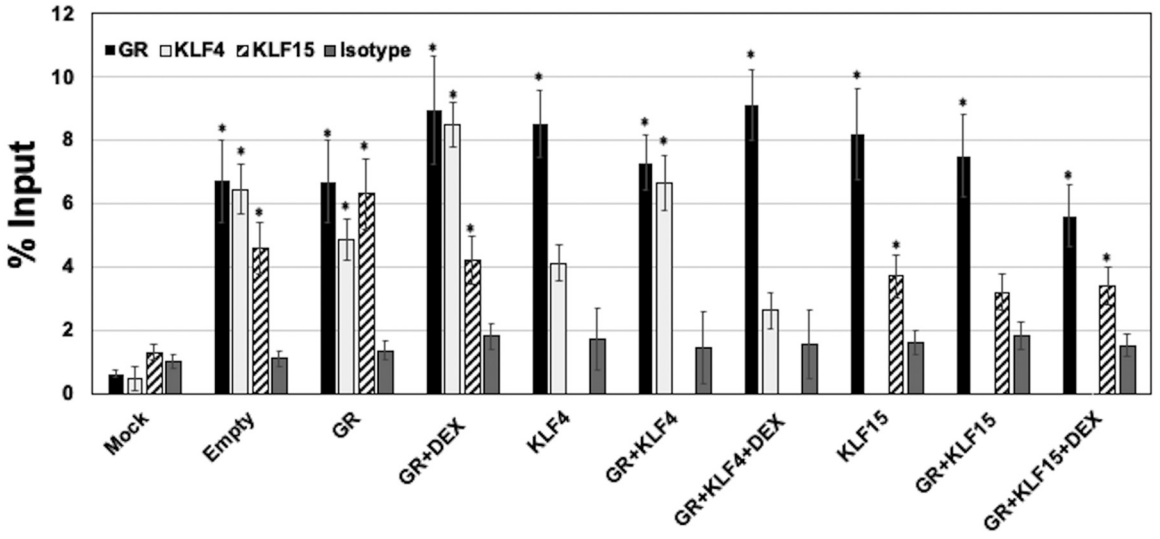

FIG 7 KLF4, KLF15, and the GR interact with sequences located in the bICP0 early promoter. Neuro-2A cells were cotransfected with the bICP0 E promoter EP-943, GR expression plasmid (3.0 μg DNA), KLF4 (1.5 μg DNA), and/or KLF15 (1.5 μg DNA). Empty vector was added to maintain the same concentration of DNA in each transfection assay. Mock samples were Neuro-2A cells not transfected with DNA. After transfection, 2% stripped FBS was added to the medium. Transfected cells were processed for ChIP as described in Materials and Methods, and immunoprecipitation (IP) was conducted using GR, KLF15, KLF4, or isotype control antibody. Detection of immunoprecipitated DNA was performed using the EP-943 primer set that specifically amplifies the bICP0 E promoter. The results are the average of three independent experiments. An asterisk denotes statistically significant differences ($P < 0.05$) from ChIP samples immunoprecipitated with isotype control IgG (Student's *t* test).

gene expression in keratinocytes (42) and common genes that accelerate epidermal barrier acquisition *in utero* (43). KLF4 is a pioneer transcription factor because it can access target sites in silent chromatin and interacts with GR or other transcriptional coactivators to remodel silent chromatin and stimulate transcription (22, 39, 44, 45). Of note, KLF4 and three other transcription factors, Oct4, c-Myc, and Sox2, reprogram fibroblasts into induced pluripotent stem cells (46). GR is also a pioneer transcription factor because it can bind a subset of GREs in silent chromatin (47, 48) and induces a nuclease-hypersensitive site culminating in transcriptional activation (10, 45). We suggest that KLF4 does not readily occupy bICP0 E promoter sequences during productive infection because the BoHV-1 viral genome is likely organized as unstable chromatin during productive infection, as is the case for HSV-1 (49), and KLF4 preferentially interacts with silent chromatin (39). The inability to readily detect KLF4 occupying bICP0 E promoter sequences was not related to a host shutoff of KLF4 protein expression, because similar levels of KLF4 were detected throughout productive infection (data not shown). Since KLF4 occupied bICP0 E promoter sequences in transfected Neuro-2A cells, we suggest that virus-encoded or induced factors restrict KLF4 interactions during productive infection or that novel factors in Neuro-2A cells facilitate KLF4 binding.

GR is a modular transcription factor that contains a well-defined DNA binding domain (DBD), steroid binding domain (SBD), and at least two transactivation domains (TADs) (10, 50). The MMTV LTR is strongly stimulated by activated GR (38), the GR SBD must interact with corticosteroids (in this case DEX), and this hormone-GR homodimer enters the nucleus. GR, via a DBD-dependent mechanism, specifically binds GREs in the MMTV LTR. GR, mediated by both TADs, interacts with transcriptional coactivators and specific transcription factors (Fig. 9A, denoted by X, Y, and Z), including certain KLF family members (51): these interactions culminate in transcriptional activation. In contrast to the MMTV LTR, the bICP0 E promoter has no consensus GREs and mutating the half GREs did not significantly reduce GR- and KLF4-mediated transactivation, suggesting that GR does not directly bind to viral promoter sequences. Thus, we predict that KLF4 directly binds one of the seven consensus Sp1 binding sites, GC-rich motifs,

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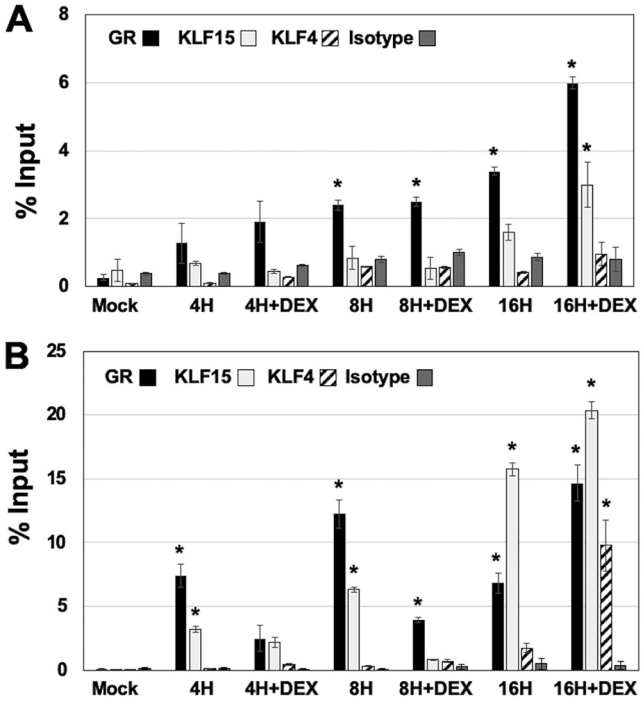

FIG 8 Interactions between GR, KLF4, and KLF15 with bICP0 E and IEtu1 promoters during productive infection. Bovine kidney (CRIB) cells were mock infected or infected with BoHV-1 (MOI of 1) and treated with vehicle or DEX for 4 h, 8 h, and 16 h. Infected cells were processed for ChIP as described in Materials and Methods, and immunoprecipitation (IP) was conducted using GR, KLF15, KLF4, or isotype control antibody as described in Materials and Methods. (A) Detection of immunoprecipitated DNA was performed using the EP-943 primer set that specifically amplifies the bICP0 E promoter. (B) Detection of immunoprecipitated DNA was performed using the 3′DRR primer set that amplifies the GREs within the IEtu1 promoter. The results are the average of three independent experiments. An asterisk denotes statistically significant differences ($P < 0.05$) from ChIP samples that were immunoprecipitated with isotype control IgG (Student's *t* test).

CA-rich motifs, or putative KLF4 binding sites in the bICP0 E promoter and recruits GR to the promoter via one of two distinct mechanisms (Fig. 9B and C). The model in Fig. 9A predicts that KLF4 directly interacts with GR, and therefore GR can subsequently associate with viral DNA (Fig. 9B). The model in Fig. 9C predicts that KLF4 is stably associated with a transcriptional coactivator (W, for example) that also interacts with

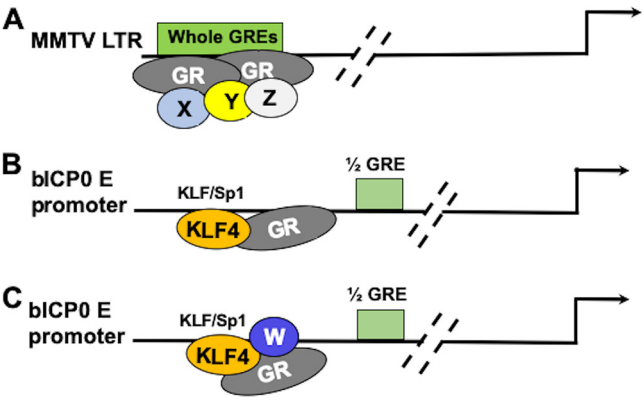

FIG 9 Hypothetical model summarizing differences between GR-mediated transactivation of MMTV LTR versus GR- and KLF4-mediated transactivation of the bICP0 E promoter. Arrows denote start sites of transcription. Dashed lines denote sequences not shown from the GREs (A) or half GRES (B and C) to the start site of transcription. In panel A, X, Y, and Z denote putative transcriptional cofactors associated with the GR transcription complex that transactivates the MMTV LTR. In panel C, W denotes a putative transcriptional coactivator that associates with the GR and KLF4 transcription complex. For additional details, see the text.

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GR; hence, GR is not directly associated with viral DNA. While we have readily detected stable interactions between GR and KLF15 (20), GR does not appear to stably interact with KLF4 (data not shown), suggesting that the model in Fig. 9C is correct. If the scenario in Fig. 9C is correct, the GR DBD may not be essential, because direct contact with DNA by GR is not required, but GR TADs would be essential. Furthermore, the GR SBD may also not be essential because GR- and KLF4-mediated transactivation occurred by a ligand-independent manner (35, 36). We further suggest that binding of DEX to GR may obstruct interactions between crucial transcriptional coactivators, perhaps KLF4 or W (Fig. 9C), which are required for GR- and KLF4-mediated transactivation.

In the context of a latent infection, BoHV-1 lytic cycle regulatory genes are not abundantly expressed during latency in calves, as judged by *in situ* hybridization studies (52) and immunohistochemistry studies (13–15). Thus, cellular transcription factors are predicted to trigger viral gene expression during the transition from latency to reactivation from latency. We predict that transactivation of the IEtu1 promoter by the GR and KLF15 feed-forward loop (20) is a critical step that must occur during very early stages of reactivation. The half-life of cortisol in plasma is only 66 min at normal hormone levels: with high steroid levels, the half-life can increase to 120 min (53), suggesting that corticosteroid levels in TG neurons are limiting following a reactivation stimulus. Thus, the ability of GR and KLF4 to stimulate the bICP0 promoter in a ligand-independent manner may maintain viral gene expression long enough for the complete complement of lytic cycle genes to be expressed and to produce infectious virus in a small subset of latently infected neurons. Enhancer elements within the bICP0 E promoter that are important for GR- and KLF4-mediated transactivation are downstream of the ICP4 IE4.2 mRNA (24, 25), suggesting that they may also sustain IEtu1 promoter activity. Understanding the effects that GR and KLF4 have on stimulation of viral gene expression and where these pioneer transcription factors bind in the context of the entire genome may provide insight into how GR and KLF4 sustain viral gene expression following a stressful stimulus.

AQ: Q

MATERIALS AND METHODS

Cells and virus. Mouse neuroblastoma (Neuro-2A) cells, rabbit skin (RS) cells, and bovine kidney (CRIB) cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% FBS, penicillin (10 U/ml), and streptomycin (100 μg/ml).

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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 were grown in CRIB 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 (54).

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 GR or KLF14 using Lipofectamine 3000 (catalog no. L3000075; Invitrogen). After transfection, 2% stripped FBS was added to the medium. 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-Gal-positive cells was determined as described previously (20). In brief, the number of β-Gal-positive cells in cultures expressing the blank vector was set at 1 for each experiment. To calculate the 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 that KLF14, DEX, and overexpression of GR had on productive infection is expressed as fold induction relative to that of the control. This representation of the data minimizes differences in cell density, Lipofectamine 3000 lot variation, and transfection efficiency.

Plasmids. A mouse GR expression vector was obtained from Joseph Cidlowski (NIH). The KLF4 expression vector was obtained from Jonathan Katz (University of Pennsylvania). The KLF15 expression vector was obtained from Deborah Otteson (University of Houston). The KLF9 expression vector was purchased from Neogen Bio-system. The KLF6 expression vector was obtained from Bin Guo (North Dakota State University). The PLZF expression vector was obtained from Derek Sant'Angelo (Sloan-Kettering Cancer Center).

AQ: S

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 (36). The 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. Constructs with internal mutations (EP-943Δ172-328, EP-943Δ328-638, and EP-943Δ172-638) were synthesized by GenScript and inserted into pGL3 basic vector (see Fig. 4A for a schematic of these constructs).

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The half GRE mutants depicted in Fig. 5A were synthesized from nucleotides 639 to 943 so that the respective fragments contained 5′-end KpnI and 3′-end SacI restriction sites (GenScript). The plasmid EP-638 was constructed such that it was cloned into the unique SacI-HindIII sites of the pGL3 basic vector (Promega). EP-638 was then cleaved with KpnI and SacI, and each half GRE 639–943 fragment (KpnI-SacI fragment) was cloned into the double-digested EP-638 vector, which reconstituted the EP-943 that contains the respective half GRE mutants.

The MMTV LTR luciferase reporter construct (pGL3-MMLV-LTR-Luc) was obtained from Stephen Goff (Addgene catalog no. 67831). All plasmids were prepared from bacterial cultures by alkaline lysis and two 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, the medium was replaced with fresh growth medium lacking antibiotics. Cells were cotransfected with the designated plasmid containing the designated promoter firefly luciferase gene reporter constructs (0.5 μg plasmid DNA), a plasmid encoding *Renilla* luciferase under the control of a minimal herpesvirus thymidine kinase (TK) promoter (50 ng DNA). Where indicated, expression plasmids expressing GR, KLF4, and/or KLF15 were included in the plasmid mixture. To maintain equal plasmid amounts in transfection mixtures, empty expression vector was added as needed. Neuro-2A cells were incubated in 2% stripped FBS after transfection. At 24 h after transfection, Neuro-2A cultures were treated with water-soluble DEX (10 μM; Sigma, D2915). Forty hours after transfection, cells were harvested and protein extracts were subjected to the dual-luciferase assay using a commercially available kit (E1910; Promega). Luminescence was measured using a GloMax 20/20 luminometer (E5331; Promega).

ChIP assay. In brief, chromatin immunoprecipitation (ChIP) studies were performed as previously described (20, 55, 56). Neuro-2A cells were grown in 100-mm dishes and cotransfected with the designated bICP0 E promoter construct (4 μg DNA) and plasmids that express GR, KLF4, and/or KLF15 (3 μg DNA of each). For these studies, cells were transfected with the indicated plasmids using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. After transfection, Neuro-2A cells were cultured in MEM containing 2% charcoal-stripped FBS. Designated cultures were treated with vehicle (PBS) or DEX (10 μM; Sigma) for 4 h. For ChIP assays of BoHV-1-infected cells, CRIB cells were mock infected or infected with BoHV-1 (multiplicity of infection [MOI] of 1), followed by incubation with medium containing 2% stripped FBS, and then cultures were treated with DEX for 4 h prior to harvesting cells. Formaldehyde cross-linked cells 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. 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 nonspecifically sticks to agarose. Input samples were collected (10 μl) from the precleared sonicated DNA-protein complexes (500 μl sample). Cleared lysate (one-third of the total lysate) was incubated with 2 μg of anti-GR antibody (3660S; Cell Signaling), anti-KLF4 antibody (ab72543; Abcam), anti-KLF15 antibody (ab2647; Abcam), or 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 4 C. This process specifically immunoprecipitated the designated transcription factor bound to sheared DNA. Immunoprecipitates containing sheared DNA fixed to the designated transcription factor were collected using Dynabeads protein A 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 (56) that amplify the 3′-DRR region within the lEtu1 promoter (5′-TAGCCGCTCCATTCTCTC-3′ and 5′-AAAAGTGGGGAAGCAGGG-3′), which yields a 218-bp fragment. The bICP0 E promoter-specific primers (5′-TCCGCCCCCCCCCAAAAAC-3′ and 5′-GAAACCCCC CACGCAAGGC-3′) were also used and yield a 127-bp bICP0 E promoter fragment. DNA bands were quantified using Image Lab software and are presented as percent input. The input samples represented 2% of the total cell lysate.

ACKNOWLEDGMENTS

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AUTHOR QUERIES

Below are queries from the copy editor indicating specific areas of concern. Please respond in-line in the main text above, either by marking a change or indicating "ok."

1

AQau—Please make certain that all authors' names are spelled correctly, and confirm the given-names and surnames are identified properly by the colors (this is important for how the names are indexed).
■= Given-Name, ■= Surname

AQfund—The table below includes funding information that you provided on the submission form when you submitted the manuscript. This funding information will not appear in the article, but it will be provided to CrossRef and made publicly available. Please check it carefully for accuracy and mark any necessary corrections. If you would like statements acknowledging financial support to be published in the article itself, please make sure that they appear in the Acknowledgments section. Statements in Acknowledgments will have no bearing on funding data deposited with CrossRef and vice versa.

Funder	Grant(s)	Author(s)	Funder ID
HHS | National Institutes of Health (NIH)	R21NS102290	Clinton J. Jones	https://doi.org/10.13039/100000002
HHS | National Institutes of Health (NIH)	P20GM103648	Clinton J. Jones	https://doi.org/10.13039/100000002
USDA | National Institute of Food and Agriculture (NIFA)	2016-09370	Clinton J. Jones	https://doi.org/10.13039/100005825
USDA | National Institute of Food and Agriculture (NIFA)	2018-06668	Clinton J. Jones	https://doi.org/10.13039/100005825

AQA—Note that ASM style does not allow the use of a plus symbol in lieu of "and" or the word "plus" in combinations such as "GR+KLF15".

AQB—"1/2 GR" change to "half GR" throughout correct?

AQC—Sentence beginning "The bICP0 promoter ..." correct as edited?

AUTHOR QUERIES

Below are queries from the copy editor indicating specific areas of concern. Please respond in-line in the main text above, either by marking a change or indicating "ok."

AQD—Please proofread the entire article carefully, as not all minor edits have been queried. To ensure sequential order, references have been renumbered in the text and References (in the original manuscript, reference 51 was a duplicate of reference 38). Please check and correct the renumbering if necessary. If any reference should be deleted from the References list, please mark "Reference deleted" in the margin next to that entry; do not renumber subsequent references.

AQE—"also transactivated the bICP0 E promoter" correct as edited? "promoter" was added.

AQF—If RT-PCR is not defined correctly, please provide.

AQG—"(see description of promoter construct EP-943 below)" addition here correct? Since this is the first mention of one of the constructs in the paper, some explanation is needed here. Reword if necessary.

AQH—Please proofread all figure legends carefully, as many minor edits were made to clarify the sense.

AQI—"Results for EP-943, EP-638, and EP-328 cotransfected with GR and KLF15 were not significantly different from each other." correct as edited? "from each other" was added. If not correct, please explain what is being compared.

AQJ—Sentence beginning "In contrast to the effects on ..." correct as edited?

AQK—Sentence beginning "GR- and KLF15-mediated transactivation" correct as edited?

AQL—"that can be transactivated by a GR monomer" correct as edited? "that" was added/

AQM—"instead of infecting cells" change to "instead of being infected" correct? If not, please reword for clarity.

AQN—In the paragraph beginning "Following productive infection of ...," please confirm that the text and parenthetical figure citations match the results shown in Fig. 8A and B.

AQO—Sentence beginning "Occupancy by KLF15 ..." correct as edited?

AQP—"than in latency or in uninfected calves" correct as edited? Original was "when compared to latency and uninfected calves".

AQQ—"and to produce infectious virus in a small subset of latently infected neurons" correct as edited?

AUTHOR QUERIES

Below are queries from the copy editor indicating specific areas of concern. Please respond in-line in the main text above, either by marking a change or indicating "ok."

3

AQR—"BHV" change to "BoHV" here correct (done for consistency).

AQS—"The KLF9 expression vector was purchased from Neogen Bio-system" correct as edited? Please confirm company name.

AQT—Original citation of reference 63 at the end of the sentence beginning "The construction and characteristics .." has been changed to 36. Correct?

AQU—Please clarify what is meant by "each half GRE 639–943 fragment". Do you mean "each half GRE fragment from nucleotides 639 to 943"? Reword for clarity if necessary.

AQV—Is reference 28 a Ph.D. dissertation?