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A cell cycle regulator, E2F2, and glucocorticoid receptor cooperatively transactivate the bovine alphaherpesvirus 1 immediate early transcription unit 1 promoter

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ABSTRACT Bovine alphaherpesvirus 1 (BoHV-1) infection causes respiratory tract disorders and immune suppression and may induce bacterial pneumonia. BoHV-1 establishes lifelong latency in sensory neurons after acute infection. Reactivation from latency consistently occurs following stress or intravenous injection of the synthetic corticosteroid dexamethasone (DEX), which mimics stress. The immediate early transcription unit 1 (IEtu1) promoter drives expression of infected cell protein 0 (bICP0) and bICP4, two viral transcriptional regulators necessary for productive infection and reactivation from latency. The IEtu1 promoter contains two glucocorticoid receptor (GR) responsive elements (GREs) that are transactivated by activated GR. GC-rich motifs, including consensus binding sites for specificity protein 1 (Sp1), are in the IEtu1 promoter sequences. E2F family members bind a consensus sequence (TTTCCCGC) and certain specificity protein 1 (Sp1) sites. Consequently, we hypothesized that certain E2F family members activate IEtu1 promoter activity. DEX treatment of latently infected calves increased the number of E2F2^{+} TG neurons. GR and E2F2, but not E2F1, E2F3a, or E2F3b, cooperatively transactivate a 436-bp *cis*-regulatory module in the IEtu1 promoter that contains both GREs. A luciferase reporter construct containing a 222-bp fragment downstream of the GREs was transactivated by E2F2 unless two adjacent Sp1 binding sites were mutated. Chromatin immunoprecipitation studies revealed that E2F2 occupied IEtu1 promoter sequences when the BoHV-1 genome was transfected into mouse neuroblastoma (Neuro-2A) or monkey kidney (CV-1) cells. In summary, these findings revealed that GR and E2F2 cooperatively transactivate IEtu1 promoter activity, which is predicted to influence the early stages of BoHV-1 reactivation from latency.

IMPORTANCE Bovine alpha-herpesvirus 1 (BoHV-1) acute infection in cattle leads to establishment of latency in sensory neurons in the trigeminal ganglia (TG). A synthetic corticosteroid dexamethasone consistently initiates BoHV-1 reactivation in latently infected calves. The BoHV-1 immediate early transcription unit 1 (IEtu1) promoter regulates expression of infected cell protein 0 (bICP0) and bICP4, two viral transcriptional regulators. Hence, the IEtu1 promoter must be activated for the reactivation to occur. The number of TG neurons expressing E2F2, a transcription factor and cell cycle regulator, increased during early stages of reactivation from latency. The glucocorticoid receptor (GR) and E2F2, but not E2F1, E2F3a, or E2F3b, cooperatively transactivated a 436-bp *cis*-regulatory module (CRM) in the IEtu1 promoter that contains two GR responsive elements. Chromatin immunoprecipitation studies revealed that E2F2 occupies IEtu1 promoter sequences in cultured cells. GR and E2F2 mediate cooperative transactivation of IEtu1 promoter activity, which is predicted to stimulate viral replication following stressful stimuli.

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Bovine alphaherpesvirus 1 (BoHV-1) is a significant pathogen of cattle and cofactor for bovine respiratory disease, a poly-microbial disease in beef and dairy cattle that can lead to life-threatening pneumonia, reviewed in references (1, 2). BoHV-1 is also associated with reproductive failure and is the most frequently diagnosed cause of viral abortion in North American cattle (3). BoHV-1 belongs to the family *Herpesviridae* and the subfamily *Alphaherpesvirinae*. Members of this subfamily produce high levels of virus production during acute infection, which leads to apoptosis and inflammation, reviewed in references (1, 2). Viral gene expression occurs in three well-defined phases: immediate early (IE), early (E), and then late (L). The IE transcription unit 1 (IEtu1) encodes two key transcriptional regulatory proteins (bICP0 and bICP4), which stimulate productive infection (4, 5). The IEtu1 promoter contains two consensus glucocorticoid receptor (GR) response elements (GREs) and is stimulated by the synthetic corticosteroid dexamethasone (DEX) (6, 7). Stress-mediated activation of IEtu1 promoter activity is predicted to mediate early steps during reactivation from latency (8, 9). Consequently, identification of cellular factors that cooperate with GR to stimulate IEtu1 promoter activity will provide insight into the mechanism that mediates early stages of reactivation from latency.

After BoHV-1 acute infection in mucosal membranes of the oral, nasal, or ocular regions, latency is established in sensory neurons located in the trigeminal ganglia (TG) (1, 2), central nervous system (10), and unidentified cells within the pharyngeal tonsil (8, 9). The only viral transcript abundantly expressed in latently infected TG neurons is the latency-related (LR) transcript, which encodes at least two proteins, two micro-RNAs, and other non-coding RNAs (11). LR gene products support the survival of infected neurons by impairing apoptosis (12), repressing productive infection (13), activating the canonical Wnt/β-catenin signaling pathways (14, 15), and impairing stress-induced transcription (16, 17). DEX mimics stress and consistently induces BoHV-1 reactivation from latency in TG (1, 2) and pharyngeal tonsil (8, 9).

The E2F family of transcription factors are cell cycle regulatory proteins that contain a conserved DNA-binding domain, an acidic transcriptional activation domain, and a retinoblastoma (Rb) protein binding site (18). Phosphorylation of Rb family members by cyclin-dependent kinase-cyclin complexes releases E2F family members. A subset of E2F family members (E2F1, E2F2, and E2F3) activate transcription, whereas other family members repress or have little effect on stimulating transcription (19–21). Notably, consensus E2F binding sites are present in promoters of many genes that regulate cell cycle progression (19, 20). Previous studies revealed that E2F family members mediate replication and gene expression of α-herpesvirinae subfamily members. For example, silencing E2F1 impairs BoHV-1 and HSV-1 replication in cultured cells, and E2F1 or E2F2 transactivates IEtu1 and early bICP0 promoter activity (22, 23). Interestingly, E2F1 also activates the HSV-1 thymidine kinase (TK) promoter via a GC-rich motif, not a consensus E2F binding site (24).

The focus of this study was to test whether (i) E2F2 is expressed during the early stages of reactivation from latency; and (ii) E2F2 cooperates with GR to transactivate IEtu1 promoter activity. Interestingly, E2F2 protein expression was detected in more TG neurons during the early stages of DEX-induced reactivation. Furthermore, GR and E2F2 cooperatively transactivate a *cis*-regulatory module (CRM) containing a 436-bp fragment from the IEtu1 promoter that contains both GREs. These studies suggest that GR and E2F2 can potentially enhance BoHV-1 viral gene expression and accelerate reactivation from latency.

RESULTS

E2F2 expression is induced in TG neurons during the early stages of reactivation from latency

Initial studies compared E2F2 expression in TG neurons during DEX-induced reactivation from latency (Fig. 1). E2F transcription factors are essential regulators of genes that drive cell cycle entry and DNA synthesis (25). The positive transcriptional activators of the E2F family include E2F1, E2F2, and E2F3, whereas E2F4, E2F5, and E2F6 impair transcription (20). All IE proteins (bICP0, bICP4, and bICP22) and VP16 are readily detected in TG neurons of latently infected calves treated with DEX for 6 h, but not in latently infected calves (26–28). Trigeminal ganglia neurons derived from latently infected calves expressed E2F2 6 h after DEX treatment (Fig. 1A). E2F2 over-expression can induce S phase entry in cultured cells; hence, E2F2 staining was not expected to be at high levels in TG neurons 6 h after DEX treatment. Notably, E2F2 was not detected in TG obtained from latently infected (Fig. 1A). Approximately threefold more TG neurons expressed E2F2 6 h after DEX treatment of latently infected calves (Fig. 1B). Conversely, E2F1 was not readily detected in TG neurons of calves latently infected with BoHV-1 regardless of DEX treatment (F. S. El-mayet, unpublished data). This was surprising because E2F1 protein expression is induced in established bovine kidney (CRIB) (22) or rabbit skin

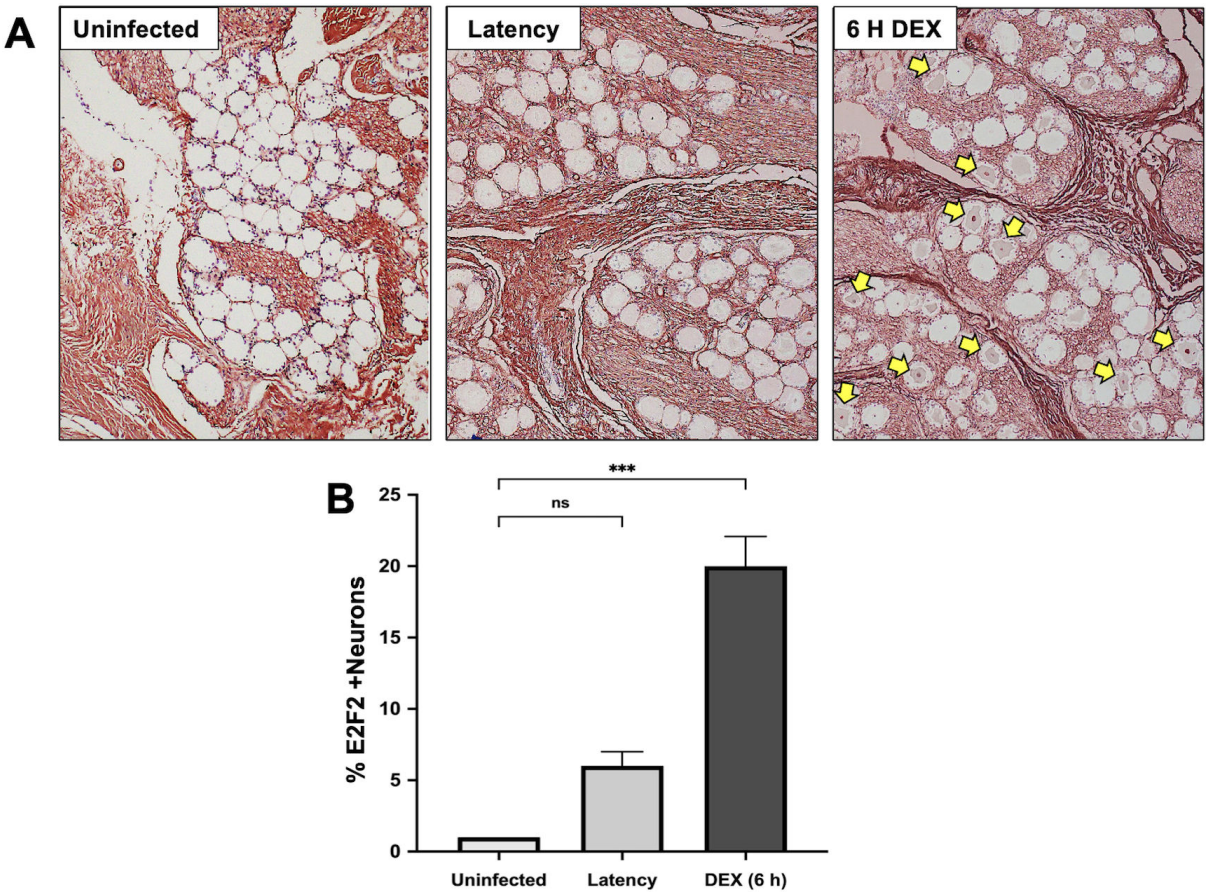

FIG 1 E2F2 protein levels increase in TG neurons during the early stages of BoHV-1 reactivation from latency. (A) Immunohistochemistry was performed to detect E2F2 expression in bovine TG neurons from uninfected, latently infected calves (Latency), or latently infected calves treated with DEX for 6 h (6H DEX) as described in Materials and Methods. Arrowheads denote TG neurons stained by the E2F2 antibody: magnification of sections is 400×. (B) Quantification of TG neuron expressing E2F2 in uninfected, latently infected, or latently infected calves treated with DEX for 6 h. The number of $E2F2^{+}$ neurons was estimated from approximately 115 total neurons. The data are shown as mean ± SEM of percent positive out of the total neurons counted. Asterisks indicate significant differences compared to latency or uninfected as determined by Student's *t* test (***$P < 0.001$; ns, non-significant). These results are representative of TG sections from three different calves.

cells (23) infected with BoHV-1. In contrast, E2F2 protein expression is not increased in CRIB cells during BoHV-1 productive infection (22). E2F2 is a DNA binding protein that is primarily located in the nucleus (29, 30). Consequently, we suggest that DEX treatment increased E2F2 expression not re-localization of E2F2 to nuclei of TG neurons.

E2F2 and GR cooperatively transactivate BoHV-1 IEtu1 promoter activity

The IEtu1 promoter drives the expression of IE mRNAs that are translated into two transcriptional regulatory proteins, bICP0 and bICP4, that are important for productive infection (see Fig. 2A for the schematic of IEtu1, IEtu1 promoter, and mRNAs in the BoHV-1 genome). The transcription factor binding sites in the IEtu1 promoter, including GRE#1 and GRE#2, are well conserved in 23 respiratory and genital isolates (31). Although the TATA box in 18 viruses obtained from the respiratory tract (TAGCTTATA AA) is identical, the two underlined nucleotides are C and G residues in five isolates obtained from the genital tract. Furthermore, three out of five genital isolates contained two substitutions in sequences between the two GREs.

To test whether E2F2 regulates IEtu1 promoter activity, dual-luciferase assays were performed using different CRMs that span the IEtu1 promoter. It should be noted that the 3′-CRM does not contain the IEtu1 promoter TATA box (Fig. 2B). The four CRM fragments (Fig. 2B) were cloned upstream of a firefly luciferase reporter plasmid that contains a minimal promoter that has a TATA box (pGL4.24[*luc*2P/minP]). Previous studies demonstrated that the IEtu1 promoter construct is regulated by the GR and certain stress-induced factors (7, 32). The denoted IEtu1 CRM constructs were cotransfected into mouse neuroblastoma cells (Neuro-2A) or monkey kidney (CV-1) cells with plasmids that express GR-α, E2F2, and a *Renilla* luciferase gene driven by a minimal TK promoter. These studies were performed in minimal essential medium (MEM) plus 2% stripped fetal bovine serum (FBS) in the presence or absence of DEX. FBS passed through a column containing "activated" charcoal removes hormones, lipid-based molecules, certain growth factors, and cytokines yielding stripped FBS. This process does not remove salts, glucose, and most amino acids. Neuro-2A cells were used for this study because they have neuronal-like properties and can be differentiated into dopamine-like neurons, and approximately 50% of Neuro-2A cells are transfected (7, 33). However, Neuro-2A cells do not support high levels of BoHV-1 replication (34). These characteristics of Neuro-2A cells make them a useful model for investigating the impact of stress-induced transcription factors on the activation of BoHV-1 regulatory promoters. CV-1 cells were used because they do not express GR (35) (data not shown), which allows one to test whether E2F2 transactivates the respective IEtu1 CRM constructs in the absence of GR. Eight possible GR isoforms can be expressed because of alternative splicing of the primary transcript, and GR-α has the highest transactivation ability, reviewed in reference (36). For all studies, a mouse GR-α expression plasmid was used, and hereafter GR-α is referred to as GR. Endogenous GR in Neuro-2A cells does not efficiently transactivate GR-responsive promoters, and this protein is smaller than GR-α (7, 37). It is not known if the endogenous GR protein is a GR isoform with reduced transcriptional activation potential or if the GR gene contains deletions of protein-coding sequences important for activating GRE-containing promoters.

Strikingly, GR, DEX, and E2F2 increased IEtu1GREs construct activity 29-fold in Neuro-2A cells (Fig. 2C) and ~780-fold in CV-1 cells (Fig. 2D) relative to the basal activity of the denoted CRM luciferase construct. When E2F2 and GR were cotransfected with the IEtu1GREs construct (no DEX treatment), promoter activity was significantly less compared to when DEX was added in Neuro-2A and CV-1 cells. E2F2 alone increased IEtu1GREs construct activity 12- and 160-fold in Neuro-2A and CV-1 cells, respectively. E2F2 and GR exhibited little to no transactivation of the other three CRM constructs (5′-CRM, 3′-CRM, or 87-bp repeats) in both cell types. In summary, only the IEtu1GREs CRM construct was cooperatively transactivated by E2F2, GR, and DEX in Neuro-2A and CV-1 cells.

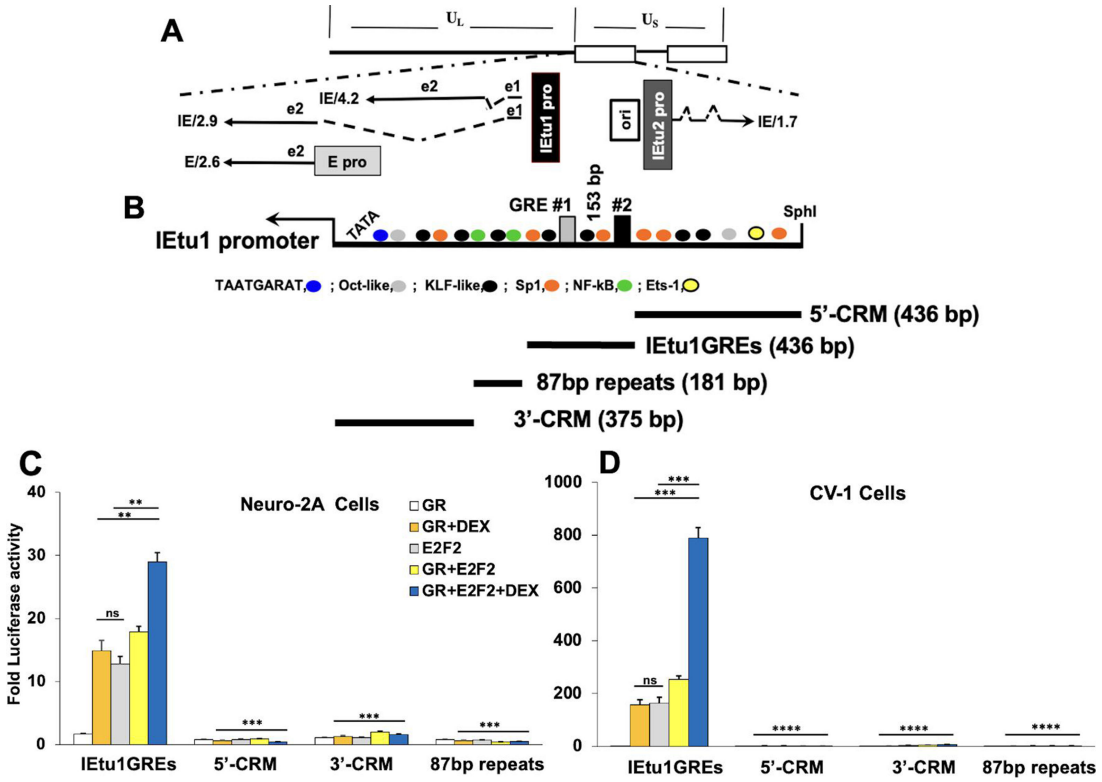

FIG 2 IEtu1GREs CRM is cooperatively transactivated by GR and E2F2. (A) Schematic of BoHV-1 genome. U_L refers to unique long and U_S unique short. The open rectangles are the repeats. Location of IEtu1 promoter and bICP0 early (E) promoter. IE/4.2 denotes the IE transcript that encodes bICP4, IE/2.9 the IE transcript that encodes bICP0, and E/2.6 that encodes bICP0 produced by the bICP0 E promoter. Location of the origin of replication (ORI), IEtu2 promoter, and IE/1.7 mRNA that encodes bICP22. Solid lines in the transcripts denote exons and dashed lines denote introns. (B) Schematic of full-length IEtu1 promoter and IEtu1 CRM constructs used in this study. Location of GREs, Sp1 binding sites, Oct-like motif, KLF-like binding sites, NF-κB binding site, TAATGARAT box, TATA box, and start site for transcription is denoted by arrow. Neuro-2A cells (C) or CV-1 cells (D) were transfected with the denoted IEtu1 CRM constructs containing the firefly luciferase reporter gene (0.5 µg DNA), a plasmid that expresses the mouse GR protein (1.0 µg DNA), and/or E2F2 (0.5 µg DNA). All samples contained a plasmid that expresses *Renilla* luciferase (0.05 µg DNA). To maintain the same amount of DNA in each sample, empty vectors were included in certain samples. At 24 h after transfection, cultures were treated with 2% "stripped" fetal bovine serum, solvent (No DEX), or water-soluble DEX (10 µM) as denoted. At 48 h after transfection, cells were harvested, and protein lysate was subjected to a dual-luciferase assay as described in Materials and Methods. Promoter activity in the denoted IEtu1 CRM constructs inserted into the pGL4.23[luc2/minP] vector and cotransfected with just the GR construct was normalized to a value of 1, and fold activation for other samples is presented. The results are the average of three independent experiments, and error bars denote the standard error. Asterisks denote a significant difference between the control and samples transfected with the GR or GR + E2F2 and treated with DEX using the Student's *t* test (ns, non-significant; **P* < 0.05; ***P* < 0.01; and ****P* < 0.001).

Based on the studies in Fig. 2, we predicted GREs in the IEtu1 promoter play a significant role in the ability of GR and E2F2 to cooperatively transactivate the IEtu1GREs CRM construct. To test this prediction, both GREs in the IEtu1GREs CRM construct were mutated (IEtu1ΔGREs; Fig. 3A). These studies revealed that E2F1, GR, and DEX did not cooperatively transactivate the IEtu1GREs CRM construct. Notably, cooperative transactivation of E2F2, GR, and DEX of the IEtu1ΔGREs construct was significantly reduced in Neuro-2A (Fig. 3B) or CV-1 (Fig. 3C) cells. In summary, cooperative transactivation of the IEtu1GREs construct by E2F2, GR, and DEX required the two consensus GREs.

IEtu1GREs promoter activation by GR and E2F family members

Since E2F2 transactivated the IEtu1GREs construct more efficiently than E2F1 (Fig. 3), we tested whether other E2F family members that are transcriptional activators (29) cooperate with GR to stimulate the IEtu1GREs construct. The IEtu1GREs construct was cotransfected into Neuro-2A or CV-1 cells with a plasmid that expresses GR, an E2F

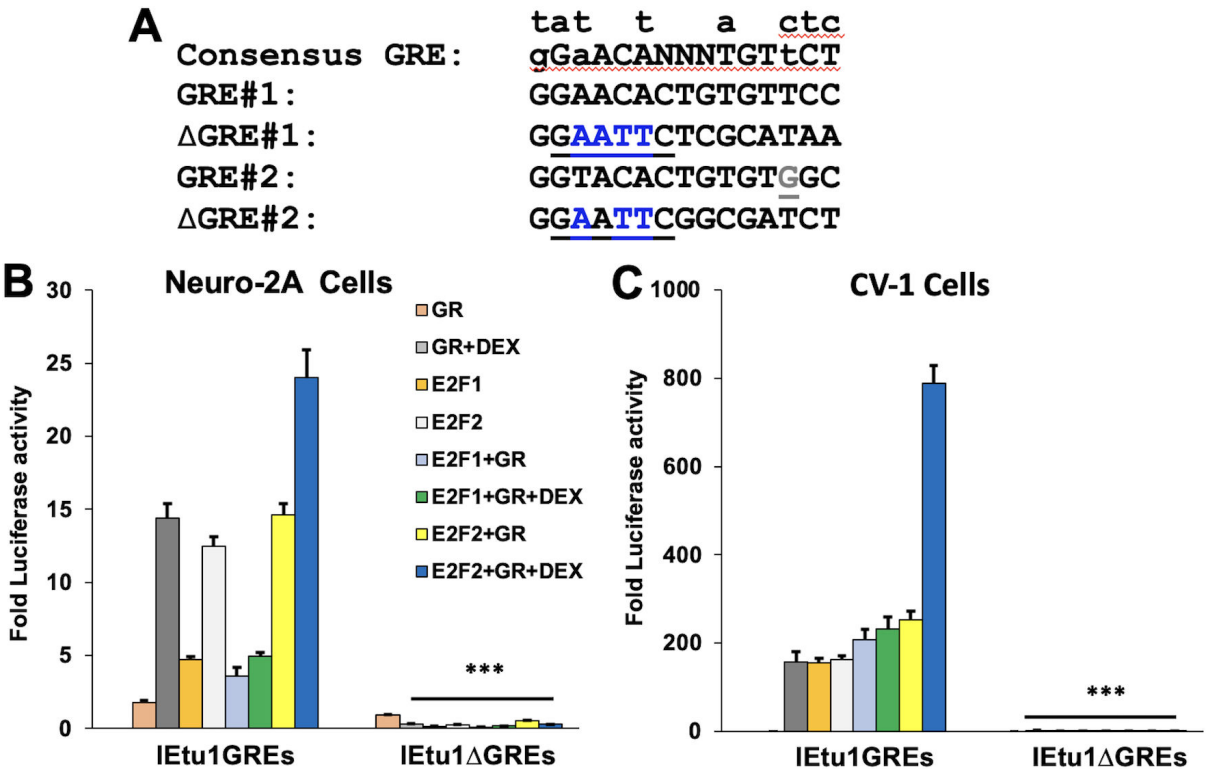

FIG 3 Mutagenesis of GREs in IEtu1GREs CRM construct abolished GR- and E2F2-mediated transactivation. (A) Sequence of consensus GRE. Small letters above a capital letter denote this nucleotide is less preferred than nucleotides in capitals. Two small letters denote that both nucleotides are present in certain consensus sequences but are less preferred relative to capital letters. N is anything. This consensus was previously described (38). Nucleotides used to mutate the GREs are an EcoRI site (GAATTCC): blue nucleotides denote changes in GRE#1 and GRE#2. The underlined gray nucleotide in GRE#2 is a mismatch of the consensus GRE. Neuro-2A cells (B) or CV-1 cells (C) were transfected with IEtu1GREs or IEtu1ΔGREs constructs containing the firefly luciferase reporter gene (0.5 µg DNA), a plasmid that expresses the mouse GR-α protein (1.0 µg DNA), E2F1, or E2F2 (0.5 µg DNA). All samples contained a plasmid that expresses *Renilla* luciferase (0.05 µg DNA). At 24 h after transfection, cultures were treated with 2% "stripped" FBS, solvent (No DEX), or water-soluble DEX (10 µM) as denoted. At 48 h after transfection, cells were harvested, and protein lysate was subjected to a dual-luciferase assay as described in Materials and Methods. Promoter activity in the denoted IEtu1 CRM constructs inserted into the pGL4.23[luc2/minP] vector and cotransfected with the GR construct was normalized to a value of 1, and fold activation for other samples is presented. The results are the average of three independent experiments, and error bars denote the standard error. Asterisks denote a significant difference between the control and samples transfected with the GR, GR + E2F1, or GR + E2F2 and treated with DEX using the Student's *t* test (***$P < 0.001$).

family member, and a plasmid containing a *Renilla* luciferase gene driven by a minimal thymidine kinase promoter. In Neuro-2A cells, GR, E2F2, and DEX transactivated the IEtu1GREs construct significantly higher relative to the effect of cotransfecting the same construct with GR, E2F1, E2F3a, or E2F3b and DEX treatment (Fig. 4A). Transactivation of IEtu1GREs by GR, E2F2, and DEX was also significantly higher in CV-1 cells treated with DEX when compared to E2F1, E2F3a, or E2F3b and DEX (Fig. 4B). In summary, these studies revealed that E2F2, but not the other E2F transcriptional activators, cooperatively transactivated the IEtu1GREs construct at significantly higher levels when DEX was added to Neuro-2A or CV-1 cultures.

Sp1 binding sites are important for E2F2-mediated transactivation

Additional studies tested whether sequences adjacent to the GREs were important for E2F2- and GR-mediated transactivation of the IEtu1GREs. The rationale for this study came from the finding that atypical E2F2 binding sites comprising two tandem consensus Sp1 binding sites (GGCGGG) (30) are present in Wt-Sp1 CRM sequences (Fig. 5A). Mutants of the Wt-Sp1 CRM sequences were generated in one of the tandem Sp1 motifs or both. Basal transcriptional activity of the respective constructs was initially

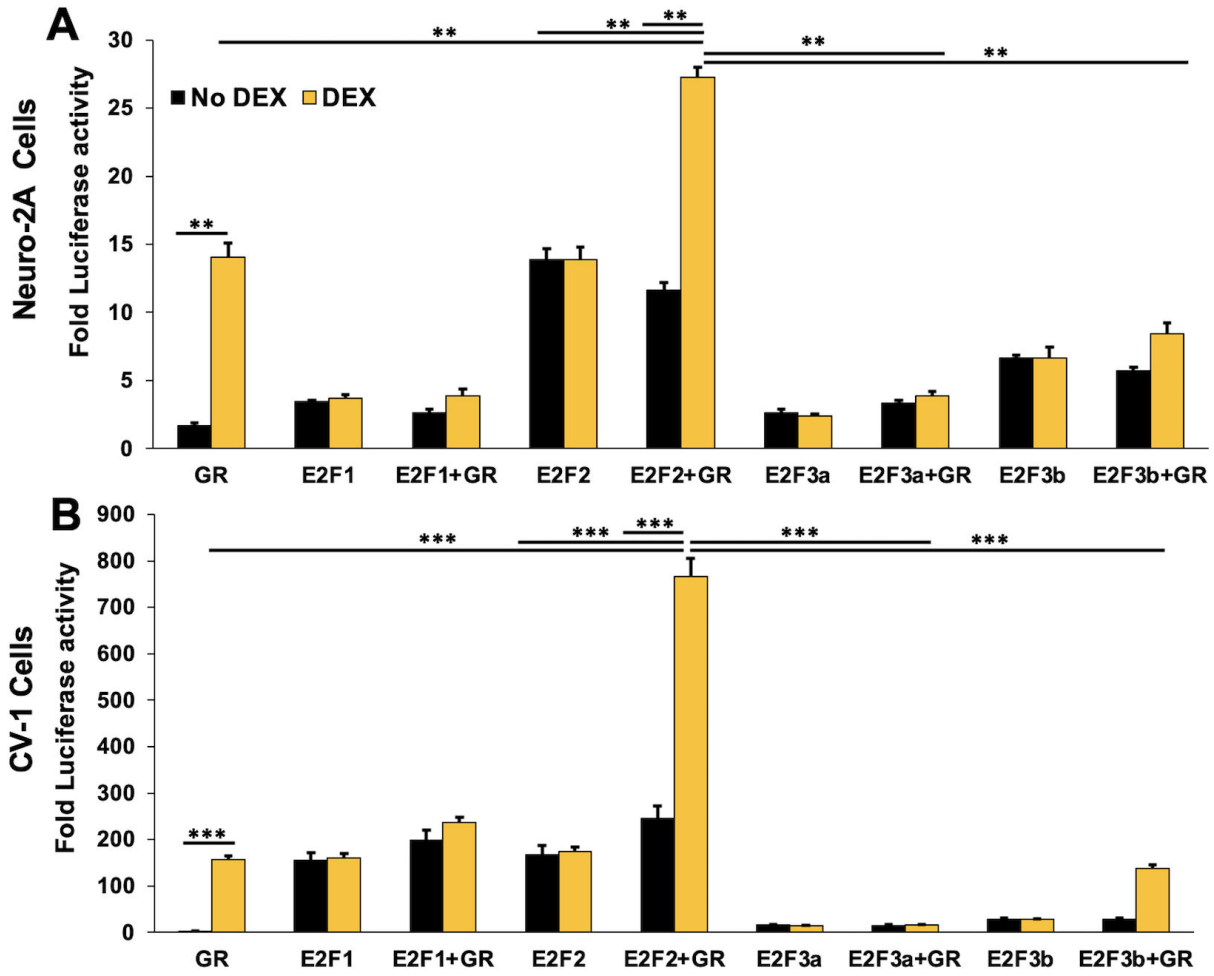

FIG 4 Comparison of IEtu1GREs transactivation by GR and E2F family members. Neuro-2A cells (A) or CV-1 cells (B) were cotransfected with the IEtu1GREs construct (0.5 µg DNA), a plasmid that expresses the mouse GR-α protein (1.0 µg DNA), E2F1, E2F2, E2F3a, or E2F3b (0.5 µg DNA). All samples contained a plasmid that expresses *Renilla* luciferase (0.05 µg DNA). At 24 h after transfection, cultures were treated with 2% "stripped" FBS, solvent (No DEX), or water-soluble DEX (10 µM) as denoted. At 48 h after transfection, cells were harvested, and protein lysate was subjected to dual-luciferase assay as described in Materials and Methods. Promoter activity of the IEtu1GREs fragment inserted into the pGL4.23[luc2/minP] vector was normalized to a value of 1, and fold activation for other samples is presented. The results are the average of three independent experiments, and error bars denote the standard error. Asterisks denote a significant difference between the control and samples transfected with just GR or GR + E2F family member and treated with DEX using the Student's *t* test (**$P < 0.01$ and ***$P < 0.001$).

examined in Neuro-2A and CV-1 cells. Relative to the Wt-Sp1 construct, transcriptional activity of the respective Sp1 mutants was not significantly different in CV-1 cells (Fig. 5B). Surprisingly, the Δ3′-Sp1 construct, but not the other two mutants, consistently had approximately twofold higher transcriptional activity than the Wt-Sp1 construct in Neuro-2A cells.

In Neuro-2A cells (Fig. 5C), the Wt-Sp1 construct was transactivated eightfold by E2F2, but not E2F1, regardless of co-transfection with GR and treatment with DEX in Neuro-2A cells . Interestingly, E2F1 and E2F2 transactivated the Wt-Sp1 CRM construct approximately 40-fold regardless of GR or DEX treatment in CV-1 cells (Fig 5D). Treating cultures with DEX did not increase E2F2-mediated transactivation of the Wt-Sp1 CRM construct in CV-1 and Neuro-2A cells. Mutating both Sp1 binding sites (ΔAll-Sp1) significantly reduced E2F2-mediated transactivation in Neuro-2A and CV-1 cells. The Δ5′-Sp1 or Δ3′-Sp1 construct exhibited reduced transactivation by E2F1 or E2F2 in Neuro-2A and CV-1 cells regardless of cotransfection with GR or DEX addition (Fig. 5C and D). Since the GREs are absent in the Wt-Sp1 CRM, we expected GR and DEX would not significantly

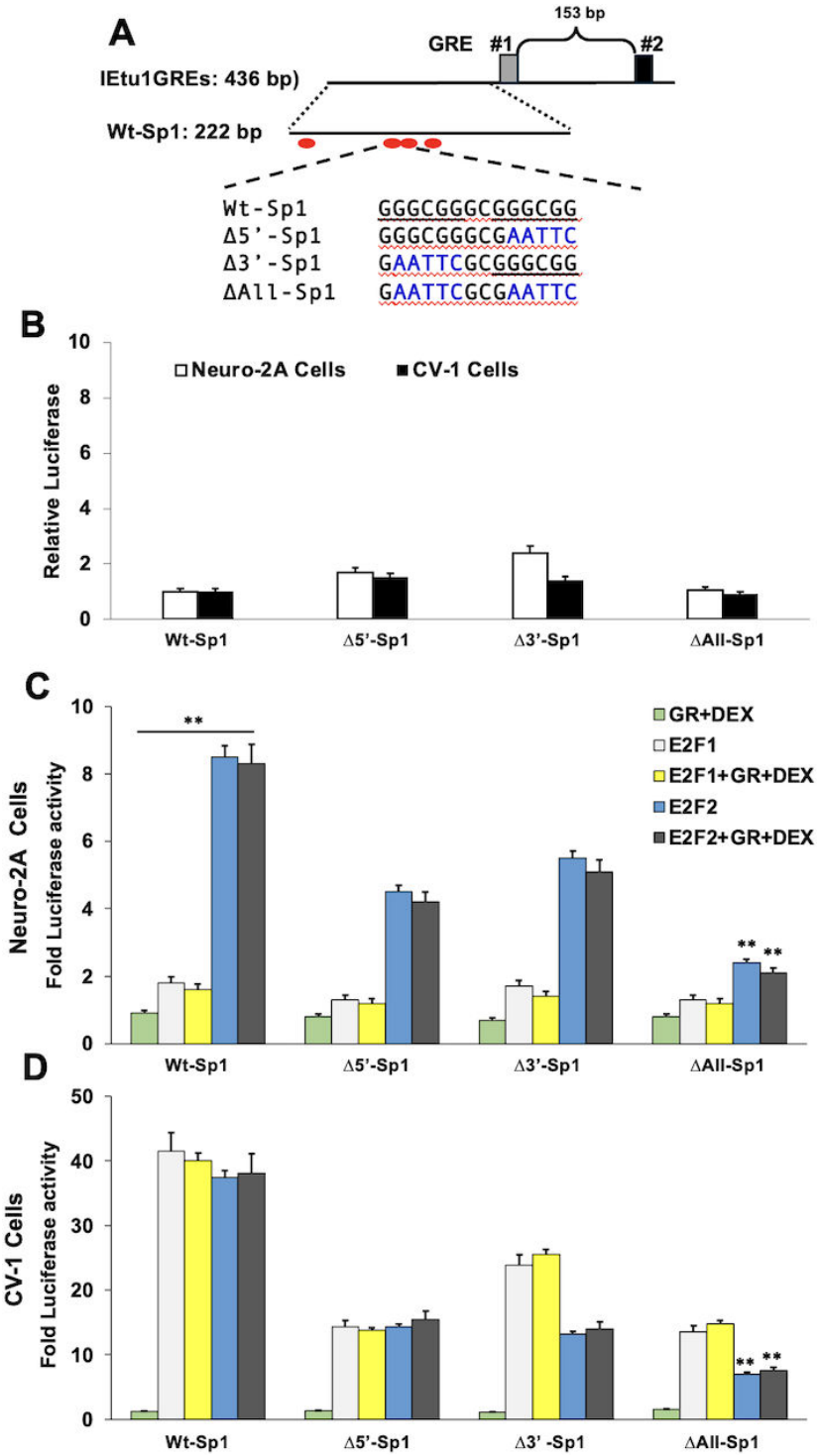

FIG 5 Sp1 binding sites within the IEtu1GREs CRM are important for E2F2-mediated transactivation. (A) Schematic of IEtu1GREs and a 222-bp fragment within the 436-bp fragment. DNA sequence of the tandem Sp1 sites in the Wt-Sp1 CRM construct and the respective mutants prepared for this study. An EcoRI site (GAATTC) was used to mutate the denoted Sp1 binding sites. Blue nucleotides denote changes in the Wt-Sp1 fragment. As with the Wt-Sp1 fragment, the mutants were inserted into the pGL4.23[luc2/minP] vector (Promega, Madison, WI, USA) between the unique SacI and HindIII restriction enzyme sites. (B) Basal transcriptional activity of Wt-Sp1 was compared to the respective Sp1 mutant constructs. Neuro-2A or CV-1 cells were transfected with 0.5 µg DNA of the denoted CRM constructs

(Continued on next page)

FIG 5 (Continued)

and 0.05 µg of the *Renilla* luciferase construct. Luciferase activity of Wt-Sp1 was set at 1 for panel B, and the other constructs were compared to these values. Neuro-2A cells (C) or CV-1 cells (D) were used to examine the effect E2F2 and GR had on the Sp1 mutants. Wt-Sp1, Δ5′-Sp1, Δ3′-Sp1, or ΔAll-Sp1 CRM constructs containing the firefly luciferase reporter gene (0.5 µg DNA) were cotransfected with a plasmid that expresses the mouse GR-α protein (1.0 µg DNA), E2F1, or E2F2 (0.5 µg DNA). All samples contained a plasmid that expresses *Renilla* luciferase (0.05 µg DNA). At 24 h after transfection, cultures in panels C and D were treated with 2% "stripped" FBS, solvent (No DEX), or water-soluble DEX (10 µM) as denoted. At 48 h after transfection, cells were harvested, and protein lysate was subjected to a dual-luciferase assay as described in Materials and Methods. Since the lEtu1 Sp1 constructs (wt or mutants) were not increased by the GR construct when cultures were treated with DEX, this was normalized to a value of 1 and then compared to the other samples in panels C and D. The results are the average of three independent experiments, and error bars denote the standard error. Asterisks denote a significant difference between the control and samples transfected with GR, GR + E2F member and treated with DEX using the Student's *t* test (**$P < 0.01$).

influence the transactivation of the Wt-Sp1 CRM construct. In summary, mutating the tandem Sp1 binding sites (ΔAll-Sp1) significantly reduced E2F2-mediated transactivation in Neuro-2A and CV-1 cells.

E2F2 and GR are localized to the nucleus when cells are treated with DEX

To compare the subcellular localization of GR and E2F2, CV-1 cells were cotransfected with plasmids that express GR and E2F2. In CV-1 cells treated with solvent, GR was localized in the cytoplasm of most cells (Fig. 6A; data not shown). A small subset of cells (CV-1 and Neuro-2A) contained GR in the nucleus prior to DEX treatment, suggesting phosphorylation of GR by certain protein kinases led to nuclear localization; this process is referred to as ligand-independent GR activation (39). Although E2F2 was detected in the nucleus of most cells, a few E2F2+ cells contained cytoplasmic localization. Similar results were obtained in Neuro-2A cells (El-mayet, unpublished). Following DEX treatment of CV-1 cultures, both GR and E2F2 were localized in the nucleus (Fig. 6B). As expected, the results in Fig. 6 demonstrated that GR and E2F2 were primarily localized to the nucleus after DEX treatment.

Additional studies tested whether over-expression of E2F2 influenced GR steady-state protein levels and vice versa in CV-1 cells. In mock-transfected CV-1 cells, GR was not readily detected (35), which was expected (Fig. 7). In cells transfected with the mouse GR-α expression construct, the prominent band migrated at approximately 100 kDa, and the predicted size is 97 kDa (36). A GR-specific band migrating with a molecular weight of approximately 130 kDa is presumably a phosphorylated isoform. Steady-state GR protein levels were reduced when cells were cotransfected with E2F2 and DEX added to cultures. Furthermore, E2F2 steady-state protein levels were increased slightly when cultures were cotransfected with GR and E2F2 plus DEX treatment. Similar results were obtained in Neuro-2A cells (El-mayet, unpublished), confirming GR expression did not dramatically increase steady-state E2F2 protein levels in CV-1 or Neuro-2A cells. The finding that GR, E2F2, and DEX treatment reduced GR-α steady-state protein levels underscored the conclusion that cooperative effects on lEtu1CRM transactivation were not due to increased GR levels when E2F2 was over-expressed.

GR and E2F2 interact with lEtu1 promoter sequences in transfected cells

To test whether E2F2 occupies lEtu1 promoter sequences, chromatin immunoprecipitation (ChIP) studies were performed in Neuro-2A or CV-1 cells transfected with the lEtu1GREs construct or BoHV-1 genomic DNA. It is well established that GR occupies lEtu1GREs during productive infection and following transfection of cells with plasmids containing the lEtu1 promoter (7, 31, 32, 40). Consequently, BoHV-1 DNA was transfected rather than infecting cells because this dramatically reduces any effects the

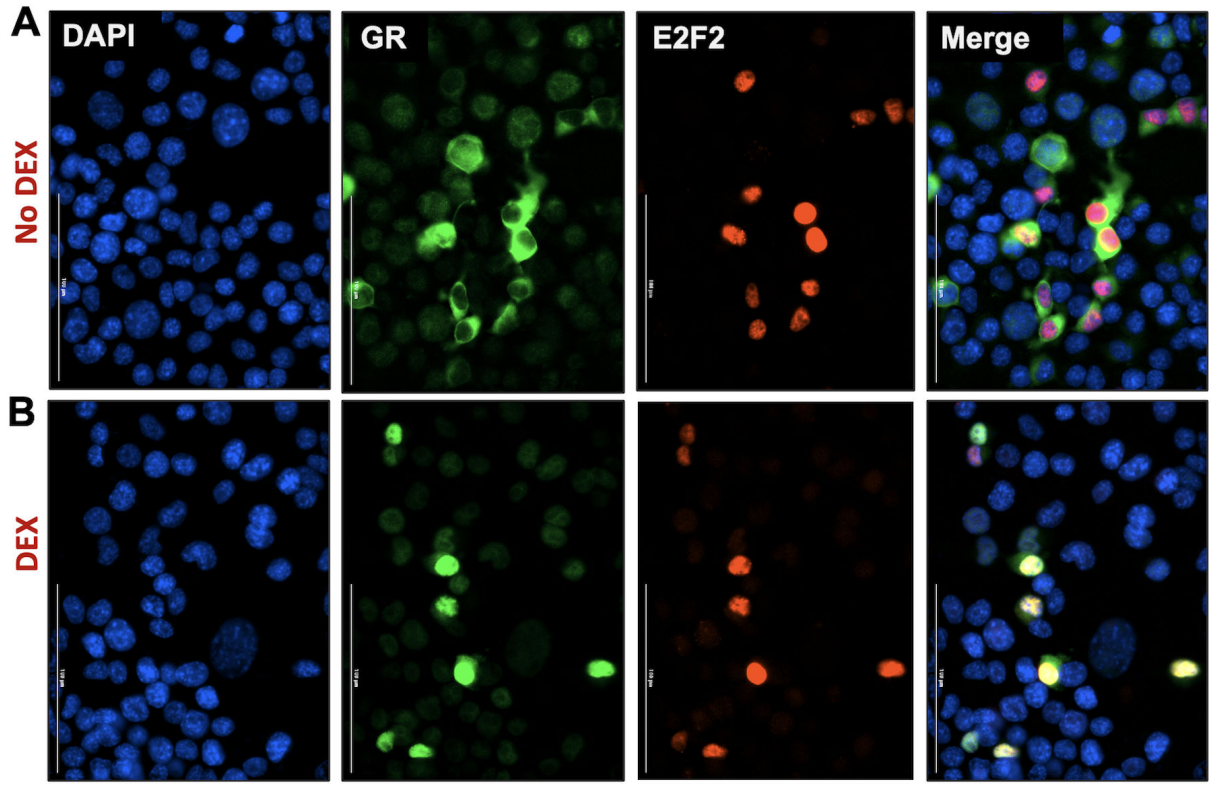

FIG 6 GR and E2F2 are present in the nucleus after DEX treatment. CV-1 cells were seeded on a multi-well cell culture slide and cotransfected with plasmids expressing GR and HA-tagged E2F2 in MEM supplemented with 2% stripped serum for 36 h. Cells were treated with solvent (No DEX) (A) or 10 µM of DEX for 1 h (B) followed by indirect immunofluorescence analysis to detect GR (green signal) and/or E2F2 (red signal). Cell nuclei were visualized using 4′,6-diamidino-2-phenylindole (DAPI) (blue signal). The image is a representative result of three experiments. Images were examined using the BioTek Cytation 5 Cell Imaging Reader, and each panel is 40× magnification. The white lines denote 100 µm.

viral transcriptional regulatory proteins have on interactions between E2F2 or GR and IEtu1GREs. In Neuro-2A cells transfected with the IEtu1GREs CRM construct, significantly more GR and E2F2 occupied IEtu1GRE sequences following IP with a GR- or E2F2-specific antibody when compared to the isotype antibody (Fig. 8A). It was also clear that E2F2 occupied IEtu1GRE sequences following transfection of Neuro-2A cells with BoHV-1 DNA (Fig. 8B).

Significantly more GR and E2F2 were also bound to the IEtu1GREs CRM construct in transfected CV-1 cells following IP with the GR- or E2F2-specific antibody when compared to the isotype control antibody (Fig. 8C). Furthermore, E2F2 occupied IEtu1GRE sequences following transfection of CV-1 cells with BoHV-1 DNA (Fig. 8D). DEX treatment did not significantly increase E2F2 occupancy of IEtu1GRE sequences when GR-α and E2F2 expression plasmids were cotransfected with the IEtu1GREs CRM construct or BoHV-1 DNA in Neuro-2A or CV-1 cells. In summary, this study demonstrated that E2F2 occupies IEtu1 promoter sequences in CV-1 and Neuro-2A cells.

DISCUSSION

Unlike IE promoters of other neurotropic alpha-herpesvirinae subfamily members, the BoHV-1 IEtu1 promoter regulates the immediate early expression of two alternatively spliced mRNAs that are translated into bICP0 and bICP4 (5, 41, 42). bICP0 expression is also regulated by a separate early promoter that sustains expression throughout productive infection (4). The BoHV-1 genome likely exists as silent chromatin during latency because lytic cycle viral proteins are not readily detected (26–28). The presence of two functional GREs in the IEtu1 promoter is one reason why stress-mediated GR

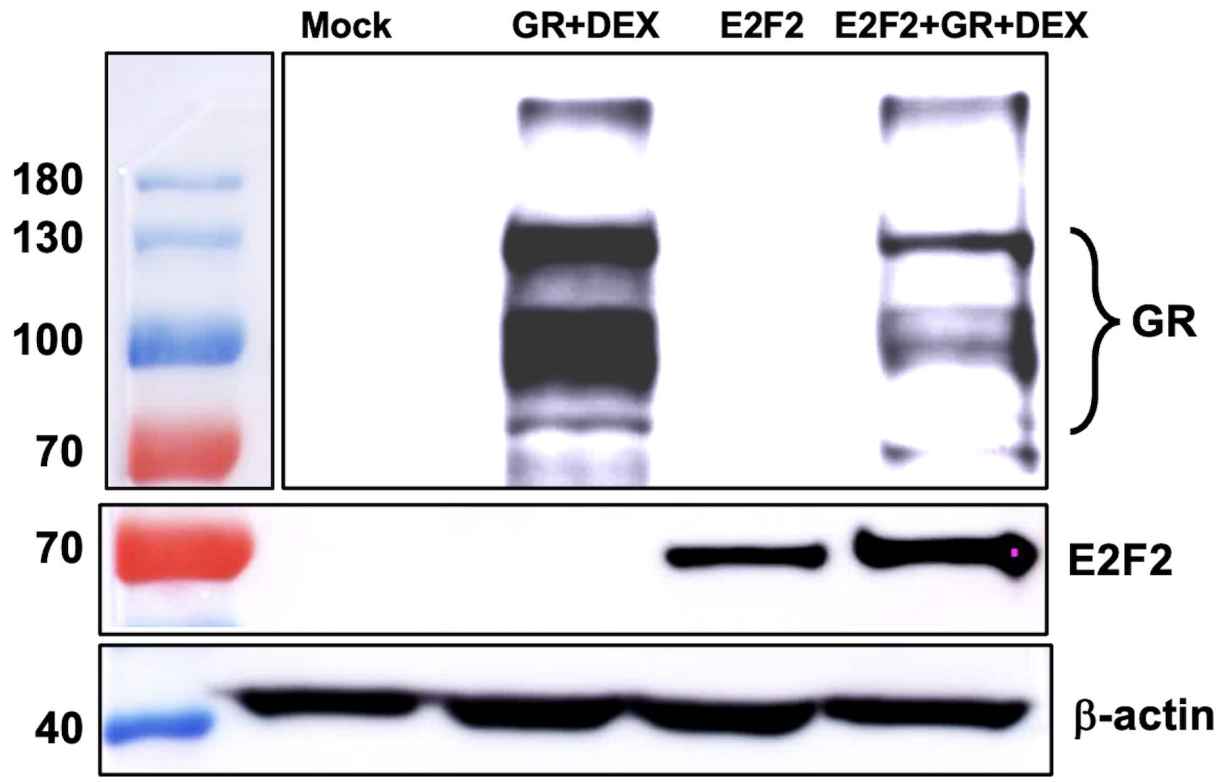

FIG 7 Examination of GR-α and E2F2 steady-state protein levels in CV-1 cells. CV-1 cells were cotransfected with plasmids that express GR-α (1.0 µg) and/or E2F2 (0.5 µg). At 24 h after transfection, cultures were treated with 2% "stripped" FBS, solvent (No DEX), or water-soluble DEX (10 µM) as denoted. At 48 h after transfection, cells were harvested, protein lysate was collected, and Western blot studies were performed using the GR and E2F2 antibodies as described in Materials and Methods. Molecular weight of the pre-stained markers is shown on the left of the Western blot. As a loading control, β-actin steady-state protein levels were examined. For each lane, 30 µg of proteins was loaded.

activation consistently initiates reactivation from latency. The finding that GR and E2F2, but not E2F1, cooperatively transactivated the IEtu1CRM construct is intriguing because more E2F2 + TG neurons were detected in latently infected calves treated with DEX for 6 h. Interestingly, there was no differential transactivation of the bICP0 E promoter by E2F1 or E2F2 (22, 23). By 6 h after latently infected calves are treated with DEX, bICP0, bICP4, and VP16 are readily detected, whereas glycoprotein E (gE), a late protein, is not (26–28). These studies imply that the IEtu1 promoter is activated during the early stages of reactivation from latency because bICP0 and bICP4 proteins are readily detected in TG neurons.

Following stressful stimuli, cortisol levels are increased and cortisol diffuses into cells. Cortisol interacts with GR bound to the heat shock 90 (Hsp90)/Hsp70 complex associated with the plasma membrane, reviewed in reference (43) (Fig. 9A). The GR-hormone complex subsequently enters the nucleus, and a GR-hormone homodimer interacts with GREs, including both IEtu1GREs (Fig. 9B). Hence, we predict that GR binding to GREs in the IEtu1 promoter is an early step during reactivation from latency because *de novo* protein synthesis of GR is not required. Furthermore, GR can function as a "pioneer" transcription factor that binds and remodels heterochromatin; consequently, transcription occurs (44). Previous studies identified certain stress-induced cellular transcription factors that we believe cooperate with GR to stimulate IEtu1 promoter activity during the early stages of reactivation from latency. For example, GR interacts with Krüppel-like factor 15 (KLF15) to cooperatively stimulate IEtu1 promoter activity (7) via a feed-forward transcription loop (45, 46) (Fig. 9C). E2F2 via interactions with the tandem Sp1 sites in the IEtu1 promoter may also enhance cooperative transactivation of the IEtu1 promoter. Recently, we reported that a complex comprising GR and Sp1

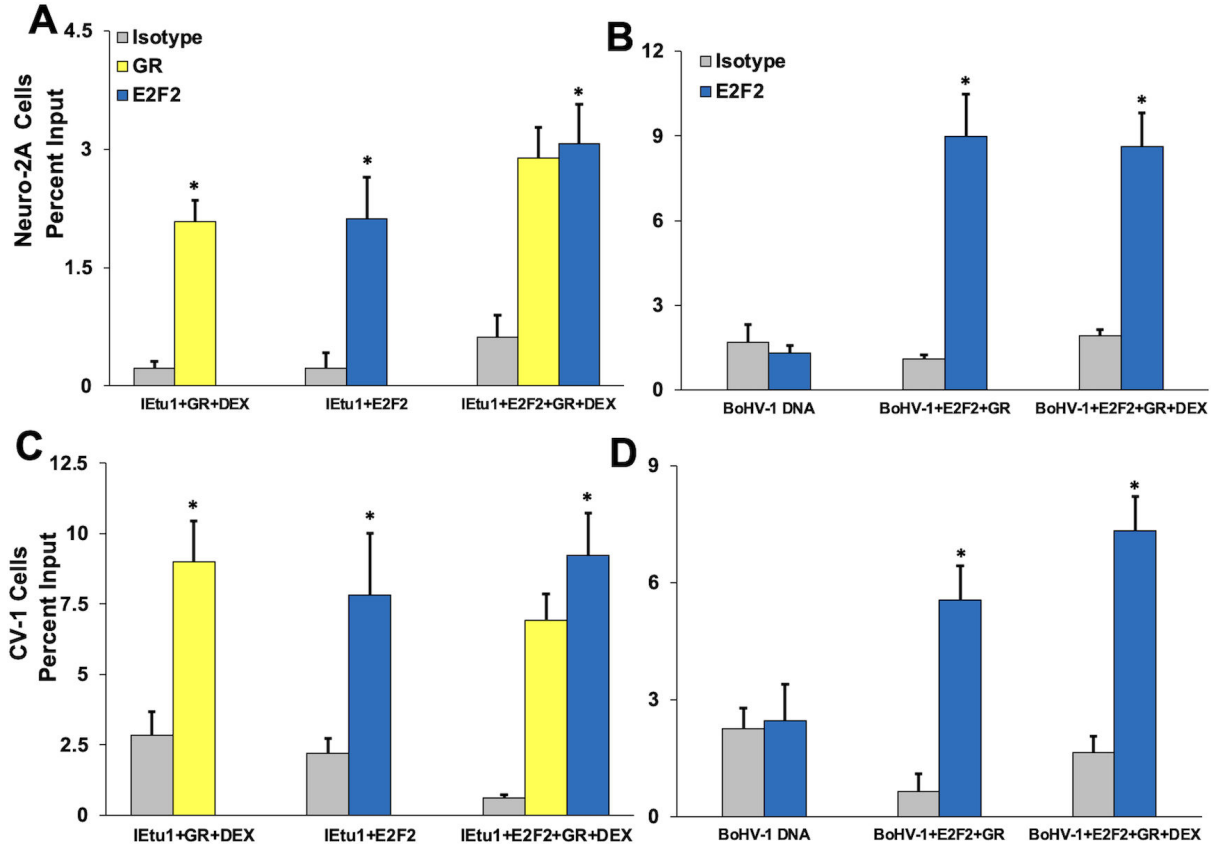

FIG 8 GR and E2F2 occupy IEtu1 promoter sequences in transfected cells. Neuro-2A cells (A and B) or CV-1 cells (C and D) were cultured in 2% stripped FBS following transfection with the IEtu1GREs construct (A and C) or BoHV-1 DNA (B and D) and either mouse GR, E2F2, or empty vector. At 48 h, the cultures were treated with DEX for 2 h. Cells were cross-linked with 16% paraformaldehyde and harvested for ChIP studies. ChIP was performed, as described in Materials and Methods. PCR was performed using the IEtu1 primers described in Materials and Methods. DNA was electrophoresed on a 1% agarose gel to separate DNA fragments and stained with ethidium bromide. Bands were quantified using Image Lab software and graphed as percent input. These results are the means from three independent experiments. Samples designated by an asterisk denote statistically significant differences from isotype control IgG by the Student's *t* test (*$P < 0.05$).

cooperatively transactivates the IEtu1 promoter (37), suggesting Sp1 also promotes IEtu1 promoter activation during reactivation from latency. Notably, host cell factor 1 (HCF-1), a cellular transcriptional coactivator, enhances GR-mediated transactivation of the IEtu1 promoter (32). The viral protein VP16 is also detected during the early stages of BoHV-1 reactivation from latency (26, 27) and specifically transactivates IE promoters, in part because it interacts with HCF-1 and Oct-1 (32). Thus, if VP16 is present, it would likely stimulate IEtu1 promoter activity. In summary, GR activation by stress is predicted to initially activate the IEtu1 promoter during the early stages of reactivation from latency. Additional stress-induced cellular transcription factors and/or VP16 are predicted to augment IEtu1 promoter activity, which enhances the incidence of successful reactivation from latency. Finally, we suggest neuronal subsets may not contain all stress-induced cellular transcription factors or VP16 expression does not occur early in all latently infected neurons. Hence, we predict several distinct mechansims exist, which triggers IEtu1 promoter activity following stressful stimuli and induces reactivation from latency.

The most frequent E2F2 binding motif identified in genomic sequences (30,47) contains a single ncleotide mismatch with the tandem Sp1 binding sites important for E2F2-mediated transactivation. This mismatch is the second C between the two Sp1 binding sites (Fig. 5A), which implies this mismatch is not essential for E2F2-mediated transactivation. The finding that GR and E2F2, but not E2F1, E2F3a, or

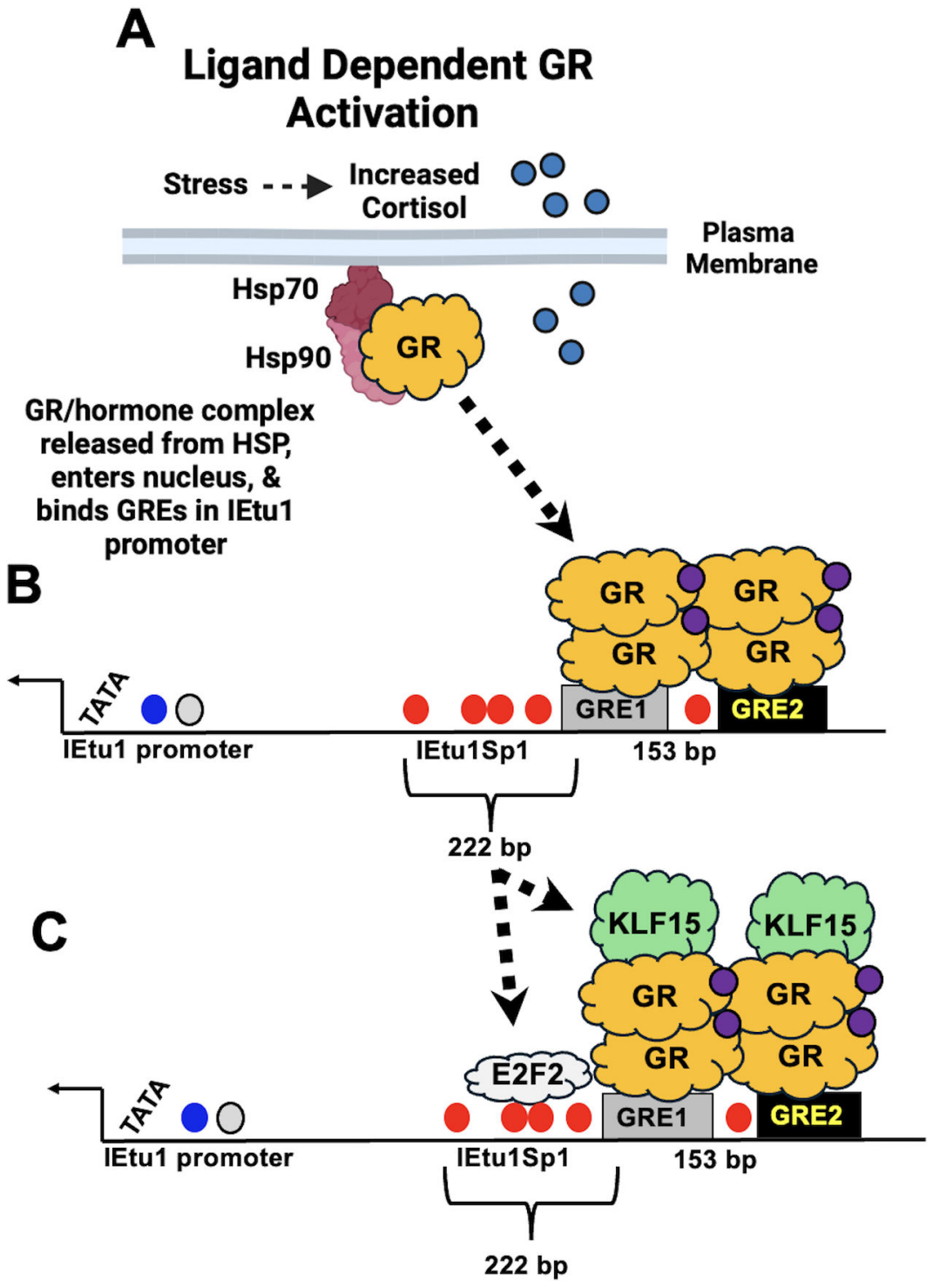

FIG 9 Schematic of how GR and certain stress-induced cellular transcription factors are predicted to regulate the early stages of BoHV-1 latency. (A) Schematic of ligand-induced GR activation in mammalian cells, reviewed in reference (43). GR is sequestered in the cytoplasm by heat shock protein 70 (Hsp70) and Hsp90. Increased stress leads to increased levels of cortisol (denoted by blue circles), which diffuses across the plasma membrane and binds GR. The GR-hormone complex is consequently released from the Hsp70/Hsp90 complex and enters the nucleus. (B) A GR-hormone dimer complex specifically binds GREs in the IEtu1 promoter and stimulates bICP0 and bICP4 expression. (C) Additional stress-induced cellular transcription factors, KLF15 and E2F2 for example, are subsequently recruited to the IEtu1 promoter, which cooperatively activates the IEtu1 promoter and subsequently induces viral gene expression.

E2F3b, cooperatively transactivated IEtu1 promoter suggests E2F2 has novel functions important for cooperating with GR. For example, E2F2 over-expression can drive cells into the S phase without inducing apoptosis, whereas E2F1 will induce apoptosis (19). E2F2 has also been reported to regulate the PI3K/Akt/NF-kB signaling pathways culminating in expression of pro-inflammatory mediators (48). Interestingly, the PI3K/Akt signaling axis is linked to the HSV-1 and BoHV-1 latency-reactivation cycle, reviewed in reference (49).

BoHV-1, including modified live vaccines, causes more abortions in catle than any other infectious agent (3). Furthermore, it is well established that BoHV-1 replication readily occurs in rapidly dividing cells within the fetus, and hematogenous viral spread occurs within the fetus. We suggest E2F family members, including E2F2, stimulate viral gene expression and replication in rapidly dividing cells in the reproductive tract. This prediction is supported by previous studies demonstrating that an E2F1-specific siRNA reduced BoHV-1 replication in bovine cells and infection leads to increased E2F-dependent transcription (22, 23). In conclusion, the ability of BoHV-1 to co-opt E2Ffamily members and stimulate IEtu1 or bICP0 E promoter activity will enhance viral spread in rapidly growing cells in reproducitve tissue.

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MATERIALS AND METHODS

Cells and virus

Murine neuroblastoma (Neuro-2A) and monkey kidney cells (CV-1) cells were grown in MEM supplemented with 10% fetal bovine serum. All media contained penicillin (10 U/mL) and streptomycin (100 µg/mL).

The BoHV-1 Cooper strain is a virulent WT virus and the North American prototype strain. The virus was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, IA, USA. BoHV-1 stock cultures were prepared in Madin-Darby bovine kidney cells and used for the infection of calves.

Infection of calves and immunohistochemistry studies

All TG samples from calves used for this study were previously described (15, 26, 50, 51). In brief, BoHV-1-free crossbred calves (~200 kg) were inoculated with 10^7 PFU of BoHV-1 into ocular and nasal cavities as described previously (15, 26, 50–52). At 60 dpi, calves were not shedding the virus and were operationally defined as being latently infected. Certain latently infected calves were injected intravenously in their jugular vein with 100 mg of water-soluble DEX (D2915, Sigma) to initiate reactivation from latency.

Immunohistochemistry studies were performed using an ABC kit (Vector Laboratories) according to the specifications of the manufacturer as previously described (15, 26, 50, 51). Thin sections (4–5 µm) of TG were cut and mounted on glass slides. Slides were incubated at 65°C for ~25 min before deparaffinization in xylene and serial rehydration using decreasing concentrations of ethanol. Endogenous peroxidases were blocked by incubating slides in 0.03% H_2O_2 for 20 min. Antigen retrieval was performed using Proteinase K (S3020; Dako), and slides were blocked using Animal-Free Blocking Solution (15019S; Cell Signaling) for 45 min at room temperature. Slides were stained with anti-E2F2 antibody (PA5-41473; Invitrogen) using a 1:200 antibody dilution and then incubated overnight in a humidified chamber at 4°C. The next day, slides were washed in 1× TBST and incubated in biotinylated goat anti-rabbit IgG (H + L) (BA-1000; Vector Laboratories) for 45 min at room temperature in a humidified chamber. Avidin-biotinylated enzyme complex was added to slides for 30 min of incubation at room temperature. After three washes in 1× TBS, slides were incubated with freshly prepared substrate (SK-4800; Vector Laboratories), rinsed with distilled water, and counterstained with hematoxylin (1.05174.1000; Sigma-Aldrich).

Plasmids

Plasmids expressing E2F1 and E2F2 (pCMV-E2F1 and pCMVE2F2, respectively) were obtained from Dr. J. R. Nevins (Duke University, Durham, NC, USA). E2F3a (Plasmid 3790), E2F3a, and E2F3b (Plasmid 37975) were obtained from Addgene via Dr. Jacqueline Lees (53).

IEtu1 constructs shown in Fig. 2 and 5 were inserted into the pGL4.23[luc2/minP] Vector (Promega; Madison, WI, USA) between the unique SacI and HindIII restriction enzyme sites. The pGL4.23[luc2/minP] vector contains a minimal TATA-box promoter element immediately upstream of the luciferase reporter gene and immediately downstream of the multiple cloning region. Hence, all IEtu1 *cis*-regulatory modules were cloned immediately upstream of the TATA box. It should be noted that the 3′-CRM begins two nucleotides upstream of the IEtu1 TATA box. All IEtu1 fragments were synthesized by GenScript (Piscataway, NJ, USA).

A mouse GR-α expression vector was obtained from Dr. Joseph Cidlowski, NIEHS, Research Triangle Park, NC, USA. All plasmids were prepared from bacterial cultures by alkaline lysis and two rounds of cesium chloride centrifugation.

Transfection and dual-luciferase reporter assay

Neuro-2A or CV-1 cells (6×10^5) were seeded into 60 mm dishes containing MEM with 10% FCS at 24 h prior to transfection. Cells were cotransfected with the designated IEtu1CRM constructs (0.5 µg plasmid DNA) and a plasmid encoding *Renilla* luciferase under the control of a minimal herpesvirus TK promoter (50 ng DNA). To maintain equal plasmid amounts in the transfection mixtures, empty expression vector (pCDNA3.1) was added as needed. Neuro-2A or CV-1 cells were incubated in MEM containing 2% charcoal-stripped FBS after transfection. At 24 h after transfection, cell cultures were treated with water-soluble DEX (10 µM; D2915; Sigma). At 48 h after transfection, cells were harvested, and protein extracts were subjected to a dual-luciferase assay using a commercially available kit (E1910; Promega). Luminescence was measured by using a GloMax 20/20 luminometer (E5331; Promega).

Indirect immunofluorescence analysis

Immunofluorescence was performed as previously described in references (6, 26, 27, 54). Neuro-2A or CV-1 cells were seeded into 2-well chamber slides (177380, Nunc. Inc.) and incubated in MEM supplemented with 10% FBS at 37°C, 5% CO_2 for 24 h. The cells were transfected with GR-α or HA-tagged E2F2 expression plasmids for 36 h. Cells were treated with solvent or 10 µM DEX for 1 h and then fixed in 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature, and permeabilized with 0.25% Triton X-100 in PBS pH 7.4 for 10 min at room temperature. Blocking was performed using 1% BSA in PBST (PBS + 0.1% Tween 20) for 30 min and incubated with anti-GR antibody (3660S; Cell Signaling) and anti-HA antibody (26183; Invitrogen) at a concentration of 10 µg/mL in 1% BSA in PBST overnight at 4°C. After three washes with PBS, cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG (H + L) (A11034; Invitrogen) and Alexa Fluor 633 goat anti-mouse IgG (H + L) (A21050; Invitrogen) at 1:500 dilution for 1 h in the dark. After three washes with PBS, 4′,6-diamidino-2-phenylindole staining was performed to visualize the nucleus. Slides were covered with coverslips by using a Gel Mount aqueous mounting medium. Images were obtained by Cytation 5 Cell Imaging Multimode Reader (Gen5 software, BioTek).

Chromatin immunoprecipitation assay

In brief, ChIP studies were performed as previously described (15, 26, 50, 51). In brief, Neuro-2A or CV-1 cells were grown in 100 mm dishes and cotransfected with the IEtu1GREs construct or BoHV-1 genome (1.5 µg DNA) and plasmids that express GR-α (3 µg) and/or HA-tagged E2F2 (2 µg). For these studies, cells were transfected with the indicated plasmids using Lipofectamine 3000 (L3000015; Invitrogen) according to the manufacturer's instructions. After transfection, cells were cultured in MEM containing 2% charcoal-stripped FBS. Designated cultures were treated with DEX (10 µM; Sigma) for 2 h. Formaldehyde cross-linked cells were lysed in ChIP lysis buffer containing protease inhibitors. Following sonication, cell lysate containing sheared DNA was precleared using Protein G Agarose/Salmon Sperm DNA (16-201; Millipore). Cleared lysate was incubated with 2 µg of anti-GR antibody (3660S; Cell Signaling), anti-HA antibody (26183; Invitrogen), or control rabbit IgG isotype (ab171870; Abcam) overnight at 4°C. Immunoprecipitates containing sheared DNA fixed to the designated transcription factor were collected using Dynabeads protein A beads (10002D; Invitrogen) and washed extensively with washing buffers. Samples were extracted twice with phenol-chloroform-isoamyl alcohol to remove the proteins associated with sheared DNA bound to a specific transcription factor. This DNA was then subjected to PCR using primers that amplify the IEtu1GREs region within the IEtu1 promoter (F: 5′-TTTGCGGTTAGAACAGGTC-3′) and (R: 5′-GCCTAT TTGCGCGGCATTTA-3′), which yielded a 290-bp 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 .

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HHS | NIH | NIH Office of the Director (OD)	P20GM103648	Clinton Jones

ETHICS APPROVAL

This study was approved by the IACUC committee at Oklahoma State University and the University of Nebraska.

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