Bovine herpesvirus 1 productive infection and immediate early transcription unit 1 promoter are stimulated by the synthetic corticosteroid dexamethasone

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A B S T R A C T
The primary site for life-long latency of bovine herpesvirus 1 (BHV-1) is sensory neurons. The synthetic corticosteroid dexamethasone consistently induces reactivation from latency; however the mechanism by which corticosteroids mediate reactivation is unclear. In this study, we demonstrate for the first time that dexamethasone stimulates productive infection, in part, because the BHV-1 genome contains more than 100 potential glucocorticoid receptor (GR) response elements (GREs). Immediate early transcription unit 1 (IEtu1) promoter activity, but not IEtu2 or VP16 promoter activity, was stimulated by dexamethasone. Two near perfect consensus GREs located within the IEtu1 promoter were necessary for dexamethasone-mediated stimulation. Electrophoretic mobility shift assays and chromatin immunoprecipitation studies demonstrated that the GR interacts with IEtu1 promoter sequences containing the GREs. Although we hypothesize that DEX-mediated stimulation of IEtu1 promoter activity is important during productive infection and perhaps reactivation from latency, stress likely has pleiotropic effects on virus-infected cells.

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Introduction

Acute infection of cattle with bovine herpesvirus 1 (BHV-1) results in clinical disease within the upper respiratory tract, nasal cavity, and ocular cavity. The ability of BHV-1 to immune-suppress infected cattle can lead to secondary bacterial infections and life-threatening pneumonia, reviewed by Jones (1998, 2003, 2009), Jones et al. (2006), Jones and Chowdhury (2007). Consequently, BHV-1 is a significant cofactor of bovine respiratory disease complex, a poly-microbial disorder that is the most important disease in cattle. Following acute infection of calves, BHV-1 establishes latency in sensory neurons. Periodically, reactivation from latency occurs, which is crucial for virus transmission.

During productive infection of cultured cells, BHV-1 gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E), or late (L), reviewed by Jones (1998, 2003). IE gene expression is stimulated by a virion component, VP16 (Misra et al., 1994, 1995). Two BHV-1 IE transcription units exist: IE transcription unit 1 (IEtu1) and IEtu2 (Wirth et al., 1992, 1989, 1991). IEtu1 encodes functional homologues of two herpes simplex virus type 1 (HSV-1) transcriptional regulatory proteins, ICP0 and ICP4 (bICP0 and bICP4, respectively). The IEtu1 promoter regulates IE expression of bICP4 and bICP0. The bICP0 protein is translated from an IE mRNA (E2.6) because both the IEtu1 promoter and E promoter regulate bICP0 RNA expression (Fraelel et al., 1994; Wirth et al., 1992, 1989, 1991). Expression of the bICP4 protein represses IEtu1 promoter activity whereas bICP0 activates its own E promoter and all other viral promoters. IEtu2 expresses a 1.7 kb IE and L transcript that encodes bICP22, which has been reported to repress viral promoters in transient transfection assays (Koppel et al., 1997; Schwyzer et al., 1994).

Stress, due to deprivation of food and water during shipping of cattle, weaning, or dramatic weather changes increases corticosteroid levels and the incidence of BHV-1 reactivation from latency, reviewed by Jones et al. (2011), Jones and Chowdhury (2007), Perng and Jones (2010). A single IV injection of the synthetic corticosteroid dexamethasone (DEX) induces BHV-1 reactivation from latency 100% of the time (Inman et al., 2002a; Jones, 1998, 2003; Jones et al., 2006, 2000; Rock et al., 1992) suggesting this natural host model can enhance our understanding of steps that occur during early stages of reactivation from latency in vivo, which we have coined the escape...
from latency (Frizzo da Silva et al., 2013). DEX also accelerates reactivation from latency in TG neuronal cultures or TG organ cultures prepared from mice latently infected with HSV-1 (Du et al., 2012; Halford et al., 1996). Canine herpesvirus type 1, another α-herpesvirinae subfamily member, consistently reactivates from latently infected beagles following treatment with the synthetic corticosteroid prednisone (Ledbetter et al., 2009). Collectively, these studies indicate that increased corticosteroids levels, as a result of stressful stimuli, can increase the frequency of reactivation from latency.

Corticosteroids enter cells and bind to the glucocorticoid receptor (GR) or mineralocorticoid receptor (MR), reviewed in Oakley and Cidlowski (2013). The MR or GR dimer bound to a corticosteroid molecule enters the nucleus and within minutes stimulates transcription by binding consensus glucocorticoid receptor response elements (GRE; 5′-GGTACANNNTGTTCT-3′) and remodeling chromatin (Giguere et al., 1986; Wang et al., 2004). Corticosteroids also have anti-inflammatory and immune-suppressive effects, in part by inactivating transcription factors (AP-1 and NF-kb) that stimulate expression of inflammatory cytokines, reviewed in Rhen and Cidlowski (2005).

Approximately 50% of TG sensory neurons express the GR (DeLeon et al., 1994) and the MR is also expressed in neurons (Arriza et al., 1988) suggesting an activated GR and/or MR can influence reactivation from latency.

Within 6 h after latently infected calves are treated with DEX, lytic cycle BHV-1 RNA expression is detected in a subset of trigeminal ganglionic neurons (Winkler et al., 2002, 2000). Two BHV-1 viral regulatory proteins, bICP0 and VP16, are expressed in the same neuron within 90 min after DEX treatment of latently infected calves; conversely two other late proteins (gC and gD) are not readily detected until 6 h after DEX treatment (Frizzo da Silva et al., 2013). Many bICP0+ or VP16+ neurons are also GR+ suggesting activation of the GR by DEX and/or DEX inducible transcription factors stimulate viral gene expression. Two transcription factors, promyelocytic leukemia zinc finger (PLZF) and Slug are induced more than 15-fold 3 h after DEX treatment (Winkler et al., 2012). Five additional DEX induced cellular transcription factors were identified in TG, and they stimulate productive infection and certain key viral promoters, including the IEtu1 and bICP0 early promoters (Workman et al., 2012). A subset of these DEX inducible transcription factors also stimulate HSV-1 ICP0 promoter activity and are induced in TG neurons of mice following explant (Sinani et al., 2013a) suggesting certain common stress-induced cellular transcription factors can stimulate HSV-1 and BHV-1 reactivation from latency.

In this study, we provide evidence for the first time that BHV-1 productive infection is directly stimulated by DEX. More than 100 potential GREs are present in the BHV-1 genome, including 15 GREs within the repeat sequences, which encode viral transcriptional regulatory proteins and origin of replication (ORLs). Two GRE-like motifs were present in the IEtu1 promoter, and sequences containing these GREs are crucial for stimulation by DEX. Mutagenesis of individual GRE motifs indicated that GRE#1 was more important than GRE#2; however, both GREs were necessary for optimal transactivation. Additional studies indicated that the GR directly interacts with sequences containing GRE#1 and GRE#2. In contrast to the IEtu1 promoter, the BHV-1 VP16 and IEtu2 promoters were not stimulated by DEX. These studies suggest that activation of the GR by DEX or natural corticosteroids can directly stimulate productive infection because the BHV-1 genome contains multiple GREs.

**Results**

**DEX stimulates productive infection in cultured bovine cells**

Based on previous studies demonstrating that increased corticosteroids consistently induce BHV-1 reactivation from latency, reviewed by Jones (2013), Jones et al. (2011), we hypothesized that corticosteroids directly stimulate viral gene expression and productive infection. To test whether DEX has an effect on productive infection, BHV-1 genomic DNA was cotransfected with a plasmid expressing the mouse GR into primary bovine kidney (BK) cells and the effects of DEX measured. This approach was used instead of infecting cells because we were concerned that VP16, which is part of an infectious viral particle, would over-ride any effect DEX has on stimulating viral gene expression in permissive cells. For these studies, we used the gBblue BHV-1 recombinant that contains the Lac Z gene downstream of the gC promoter. Since the mammalian GR and corticosteroid signaling pathways are highly conserved (Oakley and Cidlowski, 2013; Rhen and Cidlowski, 2005), the use of a mouse GR was appropriate. Twenty-four hours after transfection was used because this time-point minimized fusion of individual β-galactosidase positive cells (Geiser et al., 2002; Geiser et al., 2003; Inman et al., 2001a, 2001b, 2002b; Meyer et al., 2007). DEX stimulated BHV-1 productive infection approximately three-fold, which was significantly different relative to the negative control (Fig. 1). DEX+ the GR expression plasmid stimulated productive infection but not more than DEX alone indicating that the GR signaling pathway is functional in BK cells.

The results in Fig. 1 suggested that GREs located in the BHV-1 genome are important with respect to DEX stimulating productive infection. Consequently, potential GREs in the BHV-1 genome were identified using the TRANSFAC program and manual inspection by comparing to known GREs (Matys et al., 2006). The BHV-1 genome contains 75 potential GREs on the positive strand and negative strand at the same location, which is due to the palindromic nature of the GRE (Supplemental Fig. 1A). Twenty-one and 18 “unique” sites (i.e. not identified on the opposite strand) were identified on the positive and negative strands, respectively. Fifty-five of the total GREs were located in coding sequences. IEtu1 promoter sequences (Misra et al., 1994), which drive expression of bICP0 and bICP4 (Wirth et al., 1992), contain GREs that are referred to as GRE#1 and GRE#2. GRE#2 contains two partially over-lapping GREs (Supplemental Fig. 1B and Fig. 2A).

**IEtu1 promoter activity is stimulated by the activated GR**

The IEtu1 and IEtu2 promoters were examined for their ability to be stimulated by DEX. Three IEtu1 promoter constructs shown...
in Fig. 2A were cotransfected with a plasmid that expresses the human GR fused to GFP and certain cultures treated with DEX. The use of the GR-GFP fusion allowed us to readily confirm that nuclear translocation of GR occurred following DEX treatment (data not shown). Furthermore, mouse neuroblastoma cells (Neuro-2A) do not respond well to DEX stimulation; but they can be readily transfected and following serum withdrawal they differentiate and sprout neurites (Sinani et al., 2013b, 2014) confirming they have certain neuronal features. IEtu1cat, but not IEtu1catΔ831 or IEtu1catΔ1018, was stimulated approximately 8 fold by DEX treatment (Fig. 2B), which was significantly different compared to promoter activity in cultures not treated with DEX (p < 0.001). An IEtu2 promoter construct (genomic coordinates 111,483–111,861) is trans-activated by VP16 (Koppel et al., 1997) and contains 2 putative GR sites. IEtu2 promoter activity was not stimulated by DEX; but promoter activity was consistently reduced 2-fold following DEX treatment (Fig. 2B). The mouse mammary tumor virus (MMTV) LTR was used as a positive control because it contains multiple GREs (Chandler et al., 1986; Kuhnel et al., 1986), and as expected was stimulated approximately 40 fold by DEX (Fig. 2C).

The late viral promoter, VP16, was also examined because this protein is detected at early times during reactivation from latency (Frizzo da Silva et al., 2013). Furthermore, during heat stress-induced reactivation from latency, HSV-1 encoded VP16 has been proposed to initiate reactivation from latency (Kim et al., 2012; Thompson et al., 1993; O’Hare and Goding, 1988; O’Hare and Hayward, 1985), stress-induced stimulation of VP16 promoter activity could stimulate viral gene expression during productive infection or early phases of reactivation from latency. A BHV-1 VP16 promoter construct containing sequences spanning −547 to +207 from the initiating ATG of the ORF was not stimulated by DEX (Fig. 2B).

**Localization of GR responsive sequences in the IEtu1 promoter**

Additional studies were performed to localize the DEX responsive region within the IEtu1 promoter. The 831 base pairs missing from IEtu1catΔ831 contain GRE#1 and GRE#2 and were predicted to contain the DEX responsive region (DRR) (Fig. 3A and B). All nucleotides in GRE#1 match the required or preferred nucleotides in the consensus GRE (Fig. 3C). GRE#2 contains a single mismatch (denoted by the underlined gray nucleotide) compared to the GRE consensus. The SV40 E promoter construct containing the DRR and 3’-DRR construct, but not the 5’-DRR construct, were stimulated approximately 20 fold by DEX (Fig. 3D, black columns) compared to cultures not treated with DEX (white columns), which was statistically significant (p < 0.005).

When GRE#1 was disrupted and an EcoRI site inserted (3’-DRAA#1; see Fig. 3C for wt and mutated sequence), this construct was stimulated by DEX only 2 fold (Fig. 3D, black columns), which was not significantly different than the empty pCAT3-promoter. When GRE#2 was deleted and replaced by an EcoRI site (3’-DRAA#2), this construct was stimulated approximately 6 fold by DEX, which was significantly different than cultures not treated with DEX (p < 0.005). The construct in which both putative GREs were mutated (3’-DRAA#2xGRE) was not stimulated by DEX. As expected, the MMTV LTR was stimulated by DEX more than 40 fold in these studies (Fig. 3E). These studies indicated that: (1) GRE#1 was more important than GRE#2 with respect to stimulation by DEX, and (2) optimal DEX stimulation required GRE#1 and GRE#2.

**Interaction of cellular factors with IEtu1 promoter sequences containing GRE#1 and GRE#2**

Electrophoretic mobility shift assays (EMSA) were performed to test whether cellular factors interact with GRE#1 and GRE#2. Two distinct shifted radioactive bands were detected when a commercially available oligonucleotide containing a consensus GRE was incubated with cell lysate prepared from Neuro-2A cells (Fig. 4A); conversely only one shifted band was present when an oligonucleotide containing a mutated GRE was incubated with cell lysate (Fig. 4A). One shifted band (denoted by the closed circle) was more intense when a mutated GRE was incubated with cell lysate prepared from Neuro-2A cells transfected with the GR encoding plasmid compared to the other samples (Fig. 4A, lane 4). An oligonucleotide spanning GRE#1, but not the GRE#1 mutant oligonucleotide, contained two shifted bands following incubation with cell lysate prepared from Neuro-2A cells (Fig. 4B). Addition of DEX (lanes 3 and 5) did not increase binding to the consensus GRE or GRE#1, which may be due to the finding that when cells are lysed the GR can specifically bind DNA and activate transcription in the absence of corticosteroids (Schmitt and Stunnenberg, 1993). Shifted bands were not readily detected when GRE#2 (wt or mutant) was incubated with cell lysate prepared from Neuro-2A cells (Fig. 4C), which was surprising because there is only one mismatched nucleotide in GRE#2 compared to the consensus (Fig. 3C).

Low levels of non-radioactive competitor GRE#1 (lane 3, 6 pmol for example) reduced binding of nuclear factors to the radioactive GRE#1 probe (Fig. 4D). Conversely, the GRE#1 mutant oligo required at least 20 fold higher concentrations of cold competitor to reduce binding (lane 7, 120 pmol). The consensus GRE oligonucleotide also reduced binding to GRE#1 but was not...
To directly test whether the GR was bound to IEtu1 promoter sequences, chromatin immunoprecipitation (ChIP) studies were performed in Neuro-2A cells transfected with ΔIEtu1Cat. This approach was used because “super-shift” assays did not consistently reveal novel shifted bands following incubation of the commercially available GRE probe or oligonucleotide containing GRE#1 and the GR antibody with extracts from Neuro-2A cells (data not shown). Aliquots of isolated chromatin from Neuro-2A cells were subjected to ChIP using a GR specific antibody. Primer sets that specifically amplify the GREs (GRE1x yields a 224 bp product and GRE2x yields a 543 bp product, Fig. 5A) were used to amplify DNA immunoprecipitated by the GR antibody (Fig. 5B, GR IP panel). We detected the GR associated with a DNA fragment that spans GRE#1 (lanes #1) and a fragment spanning both GREs (lane #2), because primers specifically amplified that region. In contrast, the primer set that amplifies the TATA box region did not yield a PCR product (Fig. 5B, lanes #3) indicating the GR was not bound to this region. In samples transfected with IEtu1Cat promoter that lacks both GREs (IEtu1CatΔAS31), the GR antibody did not immunoprecipitate DNA that was amplified by the respective primer sets. As expected, mock-transfected cells also did not contain amplified products using any of the respective probes following immunoprecipitation with the GR antibody. An isotype control antibody did not specifically immunoprecipitate viral DNA that was amplified by the respective primers (Fig. 5C). PCR performed with non-immunoprecipitated samples (Fig. 5D) yielded the expected size amplicon when chromatin was derived from samples transfected with the IEtu1Cat promoter (GR+IE1 and GR+IE1 DEX samples). In summary, EMSA studies demonstrated that cellular factors specifically bind to GRE#1 sequences and ChIP analysis revealed that GREs present in the IEtu1 promoter were bound by the GR.

**Examination of GR protein expression in Neuro-2A following DEX treatment**

Steady state GR protein levels were measured because increased binding to IEtu1 promoter sequences containing GRE#1 and GRE#2 were not observed following DEX treatment (Figs. 4 and 5). Reduced levels of endogenous GR were detected after Neuro-2A cells were treated with DEX (Fig. 6A), which was consistent with an independent study (Nishimura et al., 2014). The GR-GFP fusion protein (denoted by the closed circle) was not dramatically reduced, in part because the human CMV IE promoter drives GR expression. Regardless of treatment, tubulin levels were similar in all cells. We suggest that GR levels are reduced following DEX treatment to prevent constitutive corticosteroid signaling.

It is also possible that DEX treatment did not lead to enhanced binding to a GRE because low levels of GR were present in a subset of nuclei following stripped serum treatment. To test this prediction, confocal microscopy was performed following treatment of Neuro-2A cells with stripped fetal calf serum. As expected, all Neuro-2A cells grown in the presence of fetal calf serum contained nuclear GR (Fig. 6B). Treatment with 2% stripped fetal calf serum clearly increased the levels of cytoplasmic GR and many nuclei did not contain detectable GR. It was also clear that a low percent of nuclei were still GR positive after incubation with stripped FBS. DEX treatment led to nuclear translocation of GR in all nuclei, which is consistent with hormone activation of the GR, reviewed by Oakley and Cidlowski (2013), Rhen and Cidlowski (2005). These results indicate that steady state levels of endogenous GR decrease following DEX treatment and that a low percent of GR+ nuclei were detected following incubation of cultures with stripped FBS.

**Discussion**

More than 100 GRE like motifs were identified within the BHV-1 genome and the presence of these GREs correlate with DEX-mediated stimulation of productive infection. Two GREs within the IEtu1 promoter were important for stimulation by DEX. Conversely, the GRE like motifs present in the IEtu2 promoter were not trans-activated by the GR suggesting that certain GRE-like motifs in the BHV-1 genome are not functional. It is also possible that GREs located in the IEtu2 promoter are only important in the context of productive
independent experiments. Contained increasing concentrations of the designated non-radioactive oligonucleotides (6, 15, 30, 60, 120, or 300 pmol, respectively). These results are representative of two studies. Lane 1 was only radioactive probe (no cell lysate) and lane 2, radioactive probe (transfected with GR), and Lane 5 is probe (transfected with GR and treated with DEX). Arrows or closed circles denote shifted bands. Competition assays were performed to examine the specificity of binding to the radioactive probe (GRE#1). Increasing concentrations of “cold” GRE#1, GRE#1 mutant (Panel D), or the consensus GRE (Panel E) were used for these studies. Lane 1 was only radioactive probe (no cell lysate) and lane 2, radioactive probe (-treated) cell lysate treated with 10 μM DEX. Lanes 3–8 contained increasing concentrations of the designated non-radioactive oligonucleotides (6, 15, 30, 60, 120, or 300 pmol, respectively). These results are representative of two independent experiments.

Fig. 4. Binding of cellular proteins to GRE-like sequences. Cell lysate was prepared from Neuro-2A cells as described in the materials and methods. Radioactive probes were prepared from a consensus GRE probe (Santa Cruz Biotechnology) or GRE mutant (Santa Cruz Biotechnology; Panel A), GRE#1 or GRE#1 mutant (Panel B) mutant, GRE#2 or GRE#2 mutant (Panel C). For samples in Panels A–C, Lane 1 is probe only, Lane 2 is probe + cell lysate, Lane 3 is probe + cell lysate (DEX treated), Lane 4 is probe + cell lysate (transfected with GR), and Lane 5 is probe + cell lysate (transfected with GR and treated with DEX). Arrows or closed circles denote shifted bands. Competition assays were performed to examine the specificity of binding to the radioactive probe (GRE#1). Increasing concentrations of “cold” GRE#1, GRE#1 mutant (Panel D), or the consensus GRE (Panel E) were used for these studies. Lane 1 was only radioactive probe (no cell lysate) and lane 2, radioactive probe + cell lysate treated with 10 μM DEX. Lanes 3–8 contained increasing concentrations of the designated non-radioactive oligonucleotides (6, 15, 30, 60, 120, or 300 pmol, respectively). These results are representative of two independent experiments.

infection. Although it is well established that increased corticosteroid levels stimulate BHV-1 reactivation from latency (Jones, 1998, 2003; Jones et al., 2011; Jones and Chowdhury, 2007), there are no previous published reports demonstrating that corticosteroids stimulate BHV-1 productive infection or increase virus shedding during acute infection of cattle. Since viral RNA and proteins are expressed a few hours after latently infected calves are treated with DEX (Frizzo da Silva et al., 2013; Winkler et al., 2002), we hypothesize that corticosteroids directly stimulate viral gene expression and productive infection. It will be of interest to identify which viral genes are stimulated by DEX during productive infection and whether their promoters contain GREs.

During HSV-1 and presumably BHV-1 latency, the viral genome primarily exists as “silent” chromatin, reviewed by Knipe and Cliffe (2008), indicating that extensive chromatin remodeling of the viral genome occurs during early stages of reactivation from latency. Cell lysate, Lane 3 is probe + cell lysate (transfected with GR), and Lane 4 is probe + cell lysate (transfected with GR and treated with DEX). Arrows or closed circles denote shifted bands. Competition assays were performed to examine the specificity of binding to the radioactive probe (GRE#1). Increasing concentrations of “cold” GRE#1, GRE#1 mutant (Panel D), or the consensus GRE (Panel E) were used for these studies. Lane 1 was only radioactive probe (no cell lysate) and lane 2, radioactive probe + cell lysate treated with 10 μM DEX. Lanes 3–8 contained increasing concentrations of the designated non-radioactive oligonucleotides (6, 15, 30, 60, 120, or 300 pmol, respectively). These results are representative of two independent experiments.

Although activation of GREs in the IEtu1 promoter is likely to be important for DEX stimulation, GREs located 2–3 kb upstream of the IEtu1 promoter may also influence DEX-mediated stimulation of IEtu1 promoter activity because GREs in cellular chromosomes can be located 5–19 kb pairs upstream of a promoter and still stimulate promoter activity (Polman et al., 2012). The 3′-DRR fragment that contains both GREs also contains 3 potential 1/2 GR binding sites (data not shown). These 1/2 GR binding sites may be relevant in the context of the viral genome because they can positively or negatively regulate transcription when bound by the GR (Oakley and Cidlowski, 2013; Rhen and Cidlowski, 2005).

Although activation of GREs in the IEtu1 promoter is likely to be important in stimulating productive infection and/or reactivation from latency, corticosteroids may also have additional effects on infected cells. For example, DEX-inducible cellular transcription factors identified in TG neurons can also stimulate viral promoters and productive infection (Workman et al., 2012). Second, DEX treatment of latently infected calves induces apoptosis of T cells that persist in TG during latency (Winkler et al., 2002), which may increase reactivation from latency because T cells, in particular CD8+ T cells, maintain latency in small animal models of HSV-1 infection (Decman et al., 2005; Khana et al., 2004; Knickelbein et al., 2008; Liu et al., 2000). Thus, additional studies are needed to completely understand the complex virus-host interactions that regulate stress-induced reactivation from latency.

Materials and methods

Cells and virus

Murine neuroblastoma cells (Neuro-2A) were grown in Eagle’s minimal essential medium (EMEM) supplemented with 10% FCS, penicillin (10 U/ml), and streptomycin (100 μg/ml). The designated
that DEX and expression of the GR had on productive infection is

The effect the number of plaques (Geiser et al., 2002; Inman et al., 2001a, 2001b, 2002b) was set at 1 for each experiment. The effect of productive infection is expressed as fold induction relative to the control. This representation of the data minimizes differences in cell density, variation in Lipofectamine 2000 lots, and transfection efficiency.

Plasmids

pIE1 (IEtu1cat), pIE1Δ831 (IEtu1catΔ831), and pIE1Δ1018 (IEtu1catΔ1018) were obtained from Vickram Misra (University of Saskatchewan) and were described previously (Misra et al., 1994, 1995). The BHV-1 VP16 promoter contains sequences that span –547 to +207 from the initiating ATG of the VP16 ORF. A fragment containing the core IE2 promoter (−348 to +33) was described in an earlier publication (Koppel et al., 1997). VP16 and IE2 promoter fragments were synthesized by Integrated DNA Technology (IDT; Coralville, Iowa) and they contain a unique KpnI at their 5′ terminus as well as a Xhol site at their 3′ terminus. The respective promoters were cloned into the chloramphenicol acetyltransferase (CAT) promoter minus vector (pCAT3-Basic Vector; Promega) at the unique KpnI and XhoI restriction enzyme sites.

The IEtu1 DRR enhancer constructs are summarized in Fig. 3B and were prepared as described below. The DRR contains sequences from the SpI site to the Δ831 5′-terminus. The 5′-DRR contains the 5′-415 base pairs of the DRR. The 3′-DRR contains 416 base pair of the 3′ end of the DRR. The 3′-DRR contains an EcoRI restriction enzyme site in place of GRE#1 and lacks key nucleotides in the consensus GRE (Fig. 4C). The 3′-DRR contains an EcoRI restriction enzyme site in place of GRE#2 and lacks key nucleotides in the consensus GRE (Fig. 3C). The 3′-DRR Δ2 GRE lacks both GRE#1 and GRE#2. All of these constructs were synthesized by IDT, contain unique KpnI and Xhol restriction sites at their 5′ and 3′ termini respectively and were cloned into the same restriction enzyme sites of pCAT3-Promoter.
vector (Promega). The pCAT3-Promoter vector contains a minimal SV40 early promoter linked to CAT and the respective IEdU promoter sequences are cloned upstream of the SV40 early promoter.

A mouse GR expression vector was obtained from Dr. Joseph Cidlowski, NIH. A human GR expression vector was used for certain studies and was obtained from Addgene (pk7-GR-GFP), which was provided by Dr. Ian McKara, U of Vermont. The human GR ORF is fused with GFP, which allowed monitoring of subcellular levels of GR in transfected cells prior to DEX treatment. All plasmids were prepared from bacterial cultures by alkaline lysis and 2 rounds of cesium chloride centrifugation.

Measurement of CAT activity

Neuro-2A cells grown in 60 mm dishes were co-transfected with the designated plasmids as indicated in the respective figure legends using NeuroTransit (MIR2145; Mirus). After 5 h of transfection, cells were incubated in EMEM supplemented with 2% charcoal stripped fetal bovine serum (Gibco). As designated, cultures were treated with 10 μM water-soluble DEX (Sigma) for 24 h prior to harvesting cells. At 48 h after transfection, cell extract was prepared by three freeze/thaw cycles in 0.25 M Tris–HCl, pH 7.4. Cell debris was pelleted by centrifugation, and protein concentrations determined. CAT activity was measured by incubating with 0.1 μCi (14C)-chloramphenicol (CFA754; Amersham Biosciences) and 0.5 mM AcetylCoA (A2181; Sigma). The reaction was incubated at 37 °C for 5 to 30 min. All forms of chloramphenicol were separated by thin-layer chromatography. CAT activity was quantified using a Bio-Rad Molecular Imager FX (Molecular Dynamics, CA) and is expressed as fold induction of CAT relative to no DEX treatment.

Electrophoretic mobility shift assay (EMSA)

Neuro-2A whole-cell lysate was prepared by lysing cells with NP-40 lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40 and protease inhibitor (78430; Thermo scientific)). Oligonucleotides were labeled with γ-32P-ATP using T4 polynucleotide kinase (M0201S; New England Biolabs) and purified using chromatography columns (732–6006; Bio-Rad). Thirty micrograms of protein extract was incubated in 4 μl of 5 × binding buffer (50 mM Tris–HCl, pH 8, 750 mM KCl, 2.5 mM EDTA, 0.5% Triton X-100, 62.5% glycerol and 1 mM DTT) in the presence of 1 μg poly(dI-dC) (A2181; Sigma). The reaction was incubated at 37 °C for 30 min. All forms of chloramphenicol were separated by thin-layer chromatography. CAT activity was quantified using a Bio-Rad Molecular Imager FX (Molecular Dynamics, CA) and is expressed as fold induction of samples containing DEX relative to no DEX treatment.

Chlorocorticoid receptor binding site identification

The genomic sequence for Bovine herpesvirus 1.1 Cooper Strain, GenBank accession number X98982201, was analyzed for GR-binding sites using AliBaba 2.1 software, which is available at www.generegulation.com. This software uses the TRANSFAC database (Matys et al., 2006).

Chromatin immunoprecipitation (ChIP) assay

Neuro-2A cells were washed with phosphate-buffered saline (PBS) and suspended in 50 ml of medium with no serum. A volume of 1.35 ml of 37% formaldehyde was added for cross-linking and the cell suspension was allowed to gently shake at 20 °C for 15 min. Cross-linking was stopped by addition of 2.5 ml of 2.5 M glycine and then incubating at 48 °C for 5 min. Cells were pelleted by centrifuging at 1000 × g followed by two washes with ice-cold PBS that contained 1 mM phenylmethylsulfonfyl fluoride (PMSF). The final pellet was suspended in 3 ml of cell lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 0.5% Nonidet P40 (NP40)) and incubated on ice for 10 min. Cells were vortexed every 20 min to promote lysis. Crude nuclei were pelleted and suspended in 2 ml of nuclear lysis buffer (50 mM Tris–HCl pH 8.1, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)) and incubated on ice for 10 min. The suspension was then sonicated three times for 30 s on ice. Sonicated samples were divided into two tubes and diluted to 10 ml with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, pH 8, 167 mM Tris–HCl, pH 8.1, 167 mM NaCl, 1 mM PMSF). Samples were pre-cleared by adding 75 ml of agarose/salmon sperm DNA protein A beads (Upstate) and incubating for 1 h at 4 °C. Agarose beads were removed by centrifugation and 10 mg of GR antibody (Ab) was added. A tube that contained an isotype control IgG (Bethesda; Sigma) was used as a control for specific binding to the GR antibody. Tubes were incubated overnight at 48 °C, and samples were continuously rotated. Seventy-five microliters of agarose protein A beads were added the next morning and allowed to incubate at 48 °C. Beads were pelleted and washed with 1 ml of each of the following buffers: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, pH 8), and TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). DNA-protein complexes were eluted from beads by incubating with 500 μl of elution buffer (1% SDS, 0.1 M NaHCO3) and vortexing gently for 15 min at room temperature. Agarose beads were centrifuged and the supernatant transferred to another tube. Twenty microliters of 5 M NaCl was added to each tube and placed in
a water bath at 65 °C overnight to de-cross-link proteins from DNA. Samples were then extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform. DNA was precipitated with isomyl alcohol, washed with 70% ethanol, dried in a vacuum microfuge, and suspended in 30 to 50 μl of water. Polymerase chain reaction (PCR) was then performed using primers described below and in Fig. 6.

GR forward: TCCCCCTTTTGTATCG
GR 1x reverse: CCTACTTTTTCCGTTG
GR 2x reverse: GCATTAGTTTGGTGGTGT
TATA forward: CGGCCATGCTCTCAGCGAATGGCGCCGACACC
TATA reverse: AGACCGGCGACGCCAGGTGTCCAGTACCGGCTG
All primers are 5′-3′.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virology.2015.06.010.

References
