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

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ABSTRACT

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.

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 (IE2.9) or an E mRNA (E2.6) because both the IEtu1 promoter and E promoter regulate bICP0 RNA expression (Fraefel 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

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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-κb) 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 and stimulate BHV-1 productive infection (Workman 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 (ORI_S). 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 gCblue 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

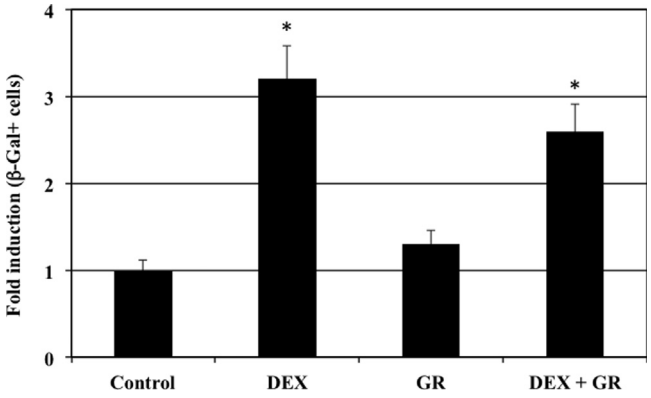

Fig. 1. DEX stimulates productive infection. Bovine kidney (BK) cells were incubated in 2% "stripped" fetal bovine serum for 24 h to reduce cortisol levels present in serum. BK cells were transfected with 0.5 μg BHV-1 gC-Blue and where indicated a plasmid that expresses the mouse GR protein. To maintain the same amount of DNA in each sample, empty vector was included in samples not transfected with the GR plasmid. Designated cultures were then treated with water soluble DEX (10 μM; Sigma). At 24 h after transfection, the number of β-Gal+ cells was counted. The value for the Control (gC-Blue virus treated with PBS after transfection) was set at 1. The results from DEX treated cultures were compared to the Control and are an average of three independent studies. The asterisk denotes a significant difference between the Control ($p < 0.05$) using the Students t test.

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., 2009). Since VP16 selectively activates IE gene expression (O'Hare, 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).

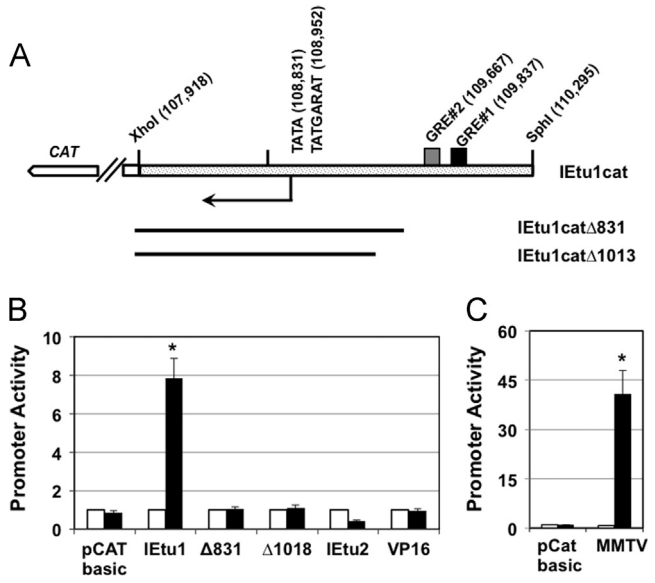

Fig. 2. IEtu1 promoter activity is stimulated by GR following DEX treatment. Panel A: The full length IEtu1 promoter is designated IEtu1cat and was cloned as an XhoI-SphI restriction site. Start site of transcription (arrow), TATA box, binding site for VP16/Oct1 denoted as TAATGARAT (Misra et al., 1994), and location of putative GRE#1 and GRE#2 (black and grey rectangles respectively) are shown. The numbers are genomic coordinates of the first nucleotide of each respective motif or restriction enzyme site. Panel B: Neuro-2A cells were cultured in 2% charcoal stripped fetal calf serum after transfection with the designated IEtu1 promoter constructs (2 μg DNA), IEtu2 promoter (2 μg DNA), or a VP16 promoter (2 μg DNA) fused to CAT and an expression plasmid that expresses the human GR (0.5 μg DNA). Panel C: Neuro-2A cells were cotransfected with a MMTV LTR cat construct and the human GR construct. CAT activity was measured using standard CAT-assays at 48 h after transfection (Workman et al., 2009, 2012). For panels B and C, 24 h after transfection the designated Neuro-2A cultures were treated with water-soluble DEX (10 μM; Sigma) (black columns). White columns indicate basal promoter activity derived from cultures not treated with DEX (see below). The results are the average of 3 independent experiments. An asterisk denotes significant differences ($p < 0.05$) in promoter activity of cells treated with DEX compared to cells not treated with DEX, as determined by the Student *t* test.

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′-DRRΔGRE#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′-DRRΔGRE#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′-DRRΔ2xGRE) 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 cell lysate was 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 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

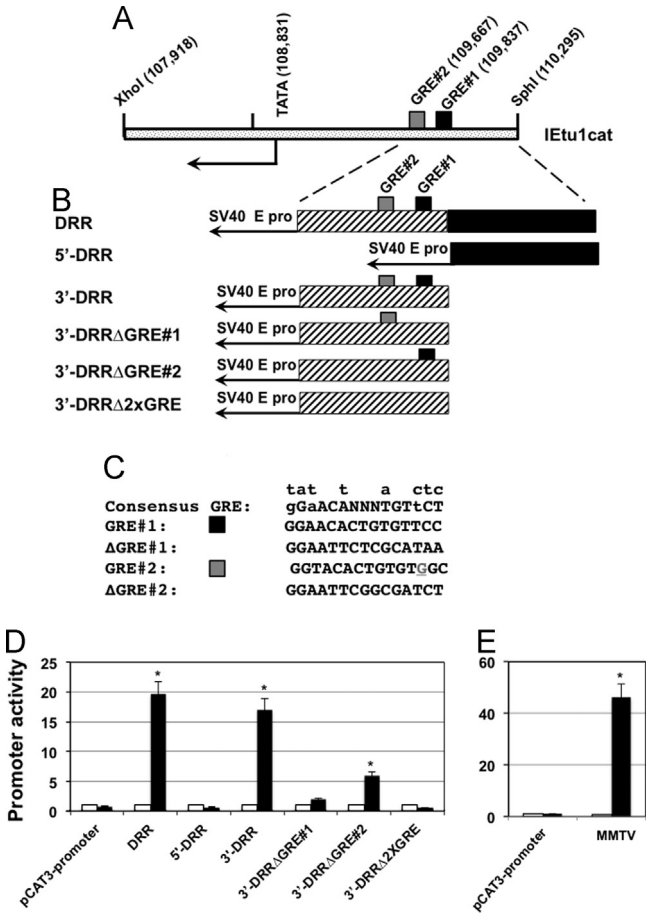

Fig. 3. Localization of IEtu1 sequences necessary for DEX-mediated stimulation. Panel A: Schematic of IEtu1 promoter and location of putative DEX responsive region (DRR). Panel B: DRR fragments used to localize sequences that are stimulated by DEX. Location of GRE#1 and GRE#2 are denoted by black or gray rectangles, respectively. The 3′-DRRΔGRE#1 contains a mutation in GRE#1 described in Panel C. The 3′-DDRΔGRE#2 contains a mutation in GRE#2 described in Panel C. The 3′-DRRΔ2xGRE contains mutations in GRE#1 and GRE#2. Panel C: Sequence of consensus GRE. Small letters above a capital letter denotes 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 (Taniguchi-Yanai et al., 2010). The underlined gray nucleotide in GRE#2 is a mismatch from the consensus GRE. ΔGRE#1 and ΔGRE#2 denote mutations introduced into GRE#1 and GRE#2. Panel D: Neuro-2A cells were cotransfected with 2 μg of the designated plasmid construct and 0.5 μg DNA of the plasmid encoding the human GR. Panel E: Neuro-2A cells were cotransfected with the pCAT3-promoter or the MMTV LTR (2 μg DNA) construct and the plasmid encoding the GR (0.5 μg DNA). For these studies, Neuro-2A cells were cultured in 2% charcoal stripped fetal calf serum after transfection. Twenty-four hours after transfection the designated Neuro-2A cultures were treated with water soluble DEX (10 μM; Sigma). CAT activity was measured using standard procedures at 48 h after transfection. White and black columns indicate the absence and presence of 1.0 μM water-soluble DEX added to cultures, respectively. The results are the average of 3 independent experiments. An asterisk denotes significant differences ($p < 0.05$) in cells transfected with the designated construct in the presence of DEX compared to the same construct without DEX treatment, as determined by the Student *t* test.

quite as efficient as the cold competitor GRE#1 (Fig. 4E). However, the consensus GRE was more efficient than the GRE#1 mutant oligonucleotide.

To directly test whether the GR was bound to IEtu1 promoter sequences, chromatin immunoprecipitation (ChIP) studies were performed in Neuro-2A cells transfected with IEtu1Cat. 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 (IEtu1catΔ831), the GR antibody did not immunprecipitate 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 indicated 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 transactivated 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

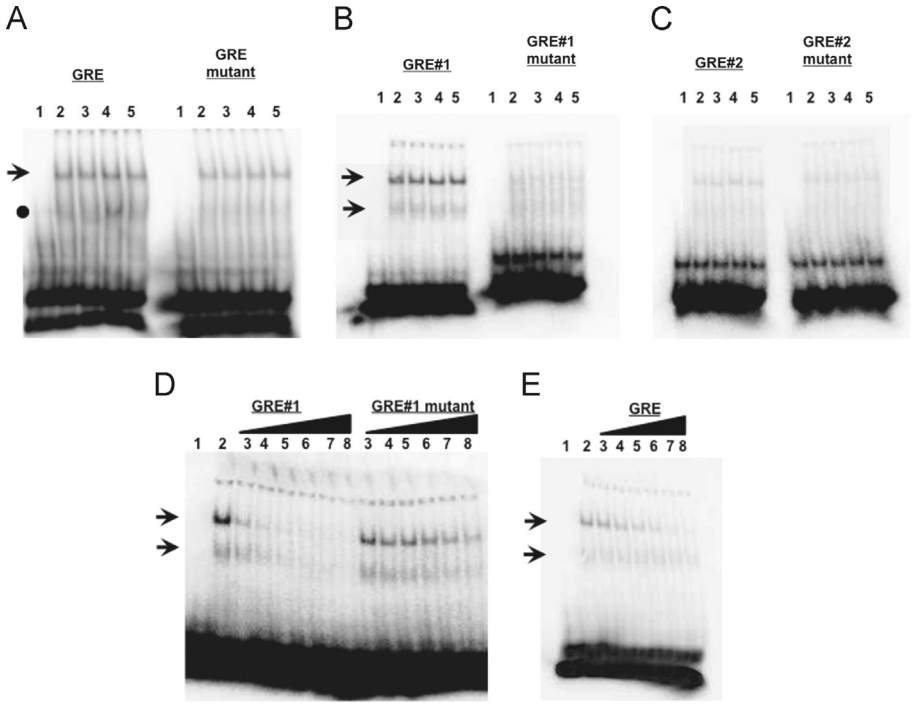

Fig. 4. Binding of cellular proteins to GRE-like sequences. Cell lysate was prepared from Neuro-2A cells as described in the materials and methods. Radioactive probes were prepared from a consensus GRE probe (Santa Cruz Biotechnology) or GRE mutant (Santa Cruz Biotechnology; Panel A), GRE#1 or GRE#1 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. In contrast to many transcription factors, the activated GR can specifically bind transcriptionally silent chromatin (Lin and Wrange, 1993; Perlman, 1992; Perlman and Wrange, 1988) and initiate formation of a nuclease-hypersensitive site and transcription (Foy and Horz, 2003; Zaret and Yamamoto, 1984). Interestingly, the activated GR only binds a subset of GREs in silent chromatin (John et al., 2011; Voss et al., 2011). These novel properties are consistent with the GR being coined as a "pioneer transcription factor", reviewed in Iwafuchi-Doi and Zaret (2014), Zaret and Carrol (2011). FoxA1, a member of the fork-head family of DNA binding proteins, is also a pioneer transcription factor that can target the GR to certain GREs in silent chromatin (Belikov et al., 2009). The ability of an activated GR to specifically bind chromatinized GREs may be important for stimulating productive infection or reactivation from latency.

Based on our results, we hypothesize that stimulation of IEtu1 promoter activity by an activated GR in latently infected neurons increases the incidence of reactivation from latency because it drives expression of bICP0 and bICP4. In addition to GRE#1 and GRE#2 being 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 to stimulate 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

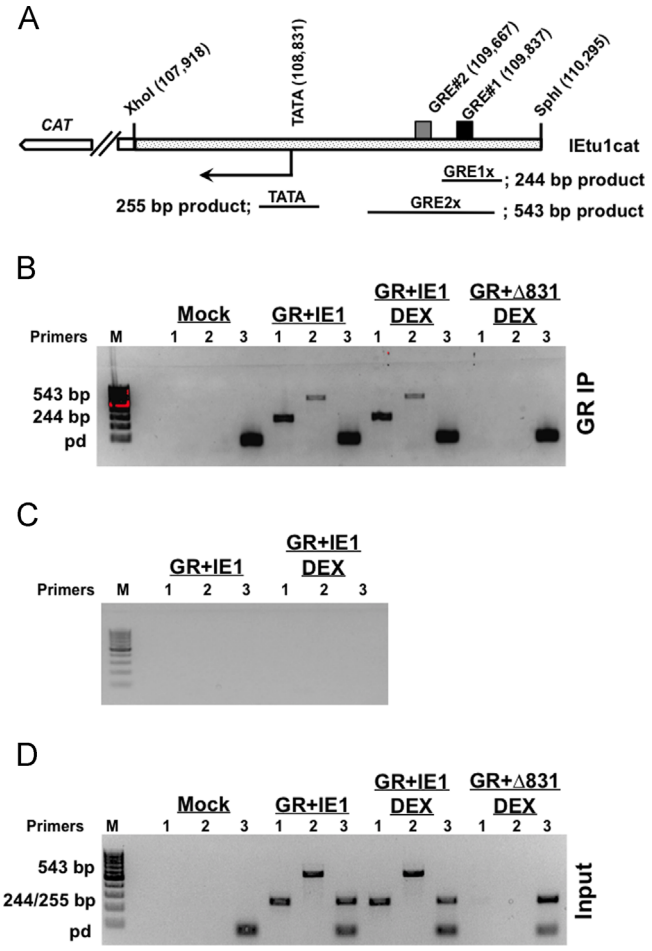

Fig. 5. The GR interacts with the IEtu1 promoter in transfected cells. Panel A: Schematic of IEtu1 promoter shown in Fig. 2A. Primer pairs used for ChIP assays are denoted. GR1x and GR2x have a common forward primer but a unique reverse primer. The TATA primer was previously described (Meyer and Jones, 2008). Panel B: As described in materials and methods, Neuro-2A cells were cotransfected with the IEtu1cat plasmid or IEtu1catΔ831 construct lacking GREs as a negative control. The GR expression plasmid was included in the transfection and designated cultures were treated with DEX as described above. Neuro-2A cells transfected with no plasmid is designated as mock. Transfected cells were processed for ChIP as described in the materials and methods using the GR specific antibody from Cell Signaling. Lane 1 was amplified with GRE1x primers, lane 2 was amplified with GRE2x primers, and lane 3 was amplified with TATA primers. Panel C: GRE+IE1 and GRE+IE1 DEX samples were immunoprecipitated with an isotype control antibody and then ChIP using the same primers described in Panel B. Panel D: Input denotes 10% of the total DNA: protein complexes used for IP was used for PCR using the designated PCR primers. Location of respective PCR products (244, 255, 543) and primer dimer (pd) are denoted on the left side of the gel. These results are representative of three independent studies.

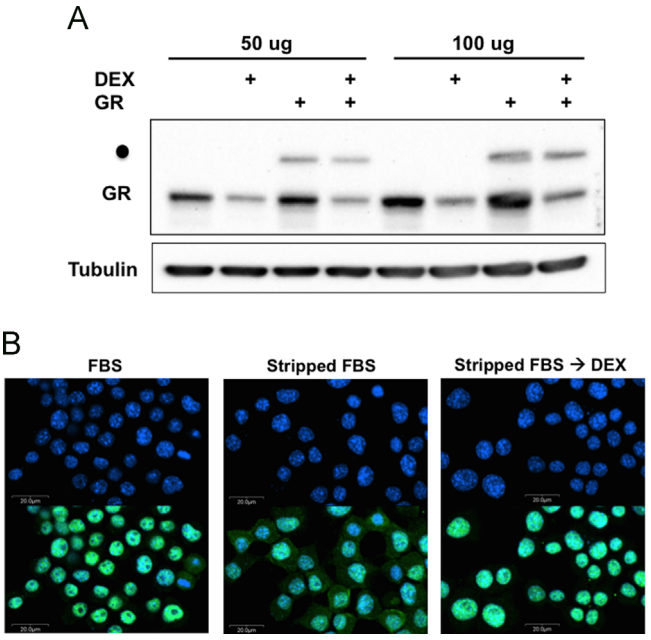

Fig. 6. Examination of GR in Neuro-2A cells. Panel A: Neuro-2A cells or Neuro-2A cells transfected with 1.5 μg DNA of the plasmid encoding the human GR were treated with water-soluble DEX 24 h after transfection. Forty-eight hours later, total cell lysate was prepared and GR levels measured using a specific GR antibody (Cell Signaling; 3660). The designated concentrations of protein in cell lysate were used for Western blot analysis. Western blots were cut and then probed with a tubulin antibody as a loading control. Migration of endogenous GR and GR-GFP fusion protein (closed circle) are denoted. Panel B: Confocal analysis of GR localization in Neuro-2A cells following treatment with stripped FBS and DEX treatment. Confocal microscopy was performed as previously described (Frizzo da Silva et al., 2013). Nuclear DNA was stained with DAPI (top panel). Bottom panel is merge of DAPI stained nucleus and GR staining (green). Brackets denote size in microns.

plasmids were transfected into Neuro-2A cells using TransIT-Neural (Mirus, Madison, WI), according to manufacturer's instructions.

A BHV-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). This virus grows to similar titers as the wild type parent virus and expresses the Lac Z gene. Primary bovine kidney (BK) cells were cotransfected with gCblue viral DNA using Lipofectamine 2000 (Invitrogen). β-Galactosidase (β-Gal) positive cells were counted at 24 h after transfection as described previously (Geiser et al., 2002; Inman et al., 2001a, 2001b, 2002b). The number of β-Gal+ cells in cultures transfected with gCblue genomic DNA reflect the number of plaques (Geiser et al., 2002; Inman et al., 2001a, 2001b, 2002b); and this value was set at 1 for each experiment. The effect that DEX and expression of the GR had on 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 IEtu2 promoter (−348 to +33) was described in an earlier publication (Koppel et al., 1997). VP16 and IEtu2 promoter fragments were synthesized by Integrated DNA Technology (IDT; Coralville, Iowa) and they contain a unique KpnI at the their 5′ terminus as well as a XhoI site at their 3′ terminus. The respective promoters were cloned into the chloramephenicol 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 SphI 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′-DRRΔGRE#1 contains an EcoRI restriction enzyme site in place of GRE#1 and lacks key nucleotides in the consensus GRE (Fig. 4C). The 3′-DRRΔGRE#2 contains an EcoRI restriction enzyme site in place of GRE#2 and lacks key nucleotides in the consensus GRE (Fig. 3C). The 3′-DRRΔ2xGRE lacks both GRE#1 and GRE#2. All of these constructs were synthesized by IDT, contain unique KpnI and XhoI 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 IEtu1 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 (^{14}C)-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 samples containing DEX 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-^{32}P-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) (MB788003; Thermo scientific) and double-stranded DNA probe labeled at its 5′-termini using γ-^{32}P-ATP. Incubation was for 1 h at room temperature. For competition assays, unlabeled double stranded oligonucleotides were incubated with the reaction mixture at room temperature for 20 min prior to additions to radiolabeled probe. DNA-protein complexes were electrophoresed on a 5% polyacrylamide gel in 0.5 × Tris-borate-EDTA buffer for 3 h at 100 V. Radioactive bands on the gel were analyzed using a Bio-Rad Molecular Imager FX. GRE probes used for EMSA are listed below:

GRE#1, GGCTTGAAGGAACACTGTGTTCCTCGCATA,
GRE#1 mutant, GGCTTGAAGGAATTCTCGCATTATCGCATA,
GRE#2, GGCAACTGGTACACTGTGTGGCGATCTCGC,
GRE#2 mutant, GGCAACTGGAATTCGGCGTTCTGATCTCGC,
GRE consensus oligonucleotide, GACCCTAGAGGATCTGTACAGGATGTTCTAGAT, (Santa Cruz, sc-2545), and
GRE mutant oligonucleotide, GACCCTAGAGGATCTCAACAGGATCATCTAGAT (Santa Cruz, sc-2546).

Western blot analysis

Neuro-2A cells were scraped from dishes, cells washed with PBS, and lysed with NP-40 lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40 and protease inhibitor {78430; Thermo scientific}). For SDS-PAGE, proteins were mixed with 5 × sample loading buffer (0.2 M Tris–HCl {pH 6.8}, 10% sodium dodecyl sulfate, 10 mM merchaptoethanol, 0.05% bromophenol blue, 20% glycerol) and boiled for 5 min. Proteins were separated in a 10% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad) and blocked for 2 h at room temperature in 5% nonfat dry milk with Tris-buffered saline-0.1% Tween 20 (TBS-T). Membranes were then incubated with primary antibody for 2 h at 4 °C. An antibody directed against β-tubulin (Sigma) was used as a loading control. After 45 min washing with TBS-T, blots were incubated with donkey anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (Amersham Biosciences), which was diluted 1:2000 in 5% nonfat milk in TBS-T. Blots were washed 45 min with TBS-T, exposed to Amersham ECL reagents, and then autoradiography performed. Primary antibodies are described above. The GR primary antibody (Cell Signaling; 3660) was diluted 1:1000. The secondary donkey anti-rabbit and anti-mouse antibodies were purchased from GE Healthcare.

Glucocorticoid receptor binding site identification

The genomic sequence for Bovine herpesvirus 1.1 Cooper Strain, GenBank accession number JX898220.1, 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 phenylmethylsulfonyl sluoride (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 2 min to promote lysis. Crude nuclei were pelleted and suspended in 3 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, 1.1% Triton X-100, 1.2 mM EDTA, pH 8, 16.7 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 (I8140; 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 $NaHCO_3$) 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:chlorophorm:isoamyl alcohol and once with chloroform. DNA was precipitated with isoamyl 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: TCCCCGCTTTTGTTATCG
GR 1x reverse: CCCTACTTTTGCCTGTGTG
GR 2x reverse: GGCATTTAGTTTTGGTGGTTGG
TATA forward: CGGCCATGCTTTCATGCAAATGAGCCCCGACAGCC
TATA reverse: AGCAGCGGCAGCGGCAGGTGTTGCAGTACGGGTGT
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.virol.2015.06.010.

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