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REGULATION OF ALPHA-HERPESVIRUS REACTIVATION FROM LATENCY BY STRESS

Insun Kook
University of Nebraska - Lincoln, kismgen@hanmail.net

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REGULATION OF ALPHA-HERPESVIRUS REACTIVATION FROM LATENCY

BY STRESS

by

Insun Kook

A DISSERTATION

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REGULATION OF ALPHA-HERPESVIRUS REACTIVATION FROM LATENCY BY STRESS

Insun Kook, Ph.D.
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Advisor: Clinton Jones

Bovine herpes virus 1 (BHV-1) and Herpes simplex virus 1 (HSV-1) are crucial etiological viral agent of clinical diseases. HSV-1 and BHV-1 establish latent infection in sensory neurons. Periodically, reactivation from latency occurs resulting in virus excretion and transmission. Stress increases corticosteroid levels and the incidence of HSV-1 and BHV-1 reactivation from latency. The synthetic corticosteroid, dexamethasone (DEX) mimics stress and induces BHV-1 and HSV-1 reactivation. However, molecular mechanisms by which corticosteroid mediates viral reactivation are not well understood. My dissertation has focused on elucidating events that induce BHV-1 or HSV-1 reactivation during the early stages of stress-induced escape from latency. The studies presented here revealed that during reactivation from latency, two BHV-1 regulatory proteins, BHV-1 infected cell protein 0 (bICP0) and viral protein 16 (VP16) were detected in trigeminal ganglionic (TG) sensory neurons that express the glucocorticoid receptor (GR). For these studies, reactivation from latency was initiated in latently infected calves with a single IV injection of DEX. These studies suggested that activation of the GR by DEX induces lytic viral gene expression. Furthermore, I discovered that DEX mediated activation of the GR directly stimulated the BHV-1 immediate early transcription unit 1 (IEtu1) promoter that directs expression of bICP0 and bICP4. The GR was bound to IEtu1 sequences that contain two near perfect glucocorticoids response elements (GREs). Additionally, we have shown that Host Cell
Factor 1 (HCF-1) is required for GR-mediated IEtu1 promoter activation.

The serum and glucocorticoid-related protein kinases (SGK1) is another stress-induced cellular factor. Expression of the serine/threonine protein kinase is strongly induced by glucocorticoids and other growth factors. Inhibiting SGK protein kinase activity significantly reduced BHV-1 and HSV-1 replication in cultured cells. The inhibitor also reduced the steady state levels of certain BHV-1 proteins.

Collectively, these studies revealed that the stress induced cellular factor, GR, stimulated BHV-1 IEtu1 promoter and productive infection in concert with HCF-1. We also provided evidence that another stress induced cellular factor, SGK-1, may regulate BHV-1 and HSV-1 replication during stress induced reactivation from latency. The studies suggest that multiple cellular factors play important roles during the early stages of stress-induced escape from latency.
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Chapter 1. Literature review
1.1. Herpes simplex virus type 1 and bovine herpes virus 1

1.1.1. Classification

The Herpesviridae is a family of DNA viruses distinguished by common virion morphology and similar life cycle (Roizmann et al., 1992). Herpesviruses consist of more than 130 species and they can infect mammals, birds and reptiles. Currently, the family *Herpesviridae* is divided into three subfamilies, representing the *Alphaherpesvirinae*, the *Betaherpesvirinae* and the *Gammaherpesvirinae* according to biological properties including tissue tropism and replication characteristic. The *Alphaherpesvirinae* subfamily has a wide host range whereas the *Betaherpesvirinae* and the *Gammaherpesvirinae* subfamilies have a narrow host range (Arvin et al., 2007; Roizmann et al., 1992). The *Alphaherpesvirinae* subfamily has a short replicate cycle but the *Betaherpesvirinae* has a long replicative cycle. All herpesviruses establish latent infection in specific cell types. The sites of latency are sensory neurons for the *Alphaherpesvirinae*, monocytes or macrophage precursors for the *Betaherpesvirinae*, and B and T lymphocytes for the *Gammaherpesvirinae*.

The subfamilies are further grouped into genera based on genome characteristics. Nine human herpes viruses have been identified (A. J. Davison et al., 2009). The subfamily *Alphaherpesvirinae* contains three human herpes viruses such as herpes simplex virus type 1 (HSV-1, also known as Human herpesvirus 1(HHV-1)), HSV-2 (HHV-2), and varicella zoster virus (VZV, HHV-3). The subfamily *Betaherpesvirinae* comprises human cytomegalovirus (hCMV, HHV-5), HHV-6A, HHV-6B and HHV 7. The subfamily *Gammaherpesvirinae* is composed of Epstein-Barr virus (EBV, HHV4) and Kaposi’s sarcoma-associated herpesvirus (KSHV, HHV8).
Eight herpesviruses have been isolated from naturally infected cattle: bovine herpesvirus 1 (BHV-1), BHV-2, BHV-4, BHV-5, bovine lymphotrophic herpesvirus (BLHV), alcelaphine herpesvirus 1 (AlHV-1), ovine herpesvirus 2 (OHV-2) and suid herpesvirus 1 (SuHV-1) (Muylkens, Thiry, Kirten, Schynts, & Thiry, 2007).

1.1.2. Virion

The virion is approximately 200-250nm in diameter depending on viral species and includes an icosahedral nucleocapsid about 125-130nm in diameter (Arvin et al., 2007; Andrew J. Davison, 2002). Herpesviruses contain approximately 125-245bp of linear double-stranded DNA genome, surrounded by viral protein matrix, tegument, which is embedded in lipid envelope containing viral membrane glycoproteins. HSV-1 is approximately 152kb with a GC content of 67% and encodes at least 84 transcripts. HSV-1 contains a unique long (UL) and unique short region (US) bounded by inverted repeat regions. The tegument is an amorphous layer that contains at least 18 proteins including the VP16 and virion host shutoff (VHS) protein. BHV-1 is approximately 135kb with a GC content of 72% and was reported to encode 73 transcripts (Tikoo SK, 1995). In general, BHV-1 has a similar genome structure as HSV-1.

1.1.3. Pathogenesis and epidemiology

The Alphaherpesvirinae consists of Simplexvirus, Varicellovirus and Mardivirus genera, and reptilian herpesvirus (Arvin et al., 2007). HSV-1 and HSV-2 belong to the genus Simplexvirus within the subfamily Alphaherpesvirinae and have highly similarities in genetic properties. HSV-1 infection primarily occurs in the oropharyngeal mucosa whereas HSV-2 is mostly associated with genital infections. Herpes simplex viruses are the most common human pathogen and approximately 90% of worldwide people contain one or both viruses. Immunosuppression is frequently associated with the frequency of
reactivation, extended viral shedding, and recurrent diseases. However, periodic viral reactivation from trigeminal ganglia causes herpes labialis, also known as cold sores. Infectious corneal blindness is primarily caused by recurrent ocular HSV-1 (C. Jones, 2003). HSV-1 rarely invades and replicates in the central nervous system (CNS), but can causes life-threatening encephalitis (Arvin et al., 2007). HSV-1 infection can also cause genital herpes through oral-genital contact. Currently available treatments such as acyclovir or nucleotide analogues target DNA polymerase thereby inhibiting viral replication (Hassan, Masarcikova, & Berchova, 2015). However, these drugs do not eliminate latently infected cells thus the potential for viral reactivation. Higher doses and prolonged use of these drugs are associated with occurrence of drug-resistant strains.

BHV-1 belongs to the genus *Varicellovirus* within the subfamily *Alphaherpesvirinae* (Arvin et al., 2007). BHV-1 infection causes two major infectious diseases of domestic or wild cattle including infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV) (C. Jones, 2003; C. Jones & Chowdhury, 2007, 2010). According to DNA analysis, BHV-1 has been divided into subtypes BHV-1.1, BHV-1.2a and BHV-1.2b. BHV-1.1 has been primarily observed in respiratory tract or aborted fetus and is common form seen in Europe, North American and South America. BHV-1.2a is related to respiratory disease or genital lesions. BHV-1.2 existed in Europe before the 1970s but it is epidemic in Brazil. BHV-1.2b is also the cause of respiratory disease and genital lesions, except abortion. The high prevalence of BHV-1.2b in Australia or Europe has been reported.

BHV-1 induced immune suppression is associated with bovine respiratory disease complex (BRDC), also known as “shipping fever”, and it can also cause secondary
bacterial infection, which can result in pneumonia (C. Jones, 2003; C. Jones & Chowdhury, 2007, 2010). Although modified live vaccines are available, they can cause diseases in young calves or abortions in pregnant cows.

1.1.4. Immune evasion

BHV-1 and HSV-1 inhibit the immune system in several ways. BHV-1 can infect CD4⁺ T cells thus inducing apoptosis (Winkler, Doster, & Jones, 1999). The virion host shutoff (vhs) protein of HSV-1 and homologue encoded by BHV-1 degrade host mRNA to suppress host responses at early time of infection (C. Jones & Chowdhury, 2007; B. Roizman & Whitley, 2013). HSV-1 ICP47 and BHV-1 UL49.5 inhibit MHC class I antigen presentation by blocking the function of the transporter associated with antigen processing (TAP) (Verweij et al., 2015). HSV-1 ICP47 interferes with cytosolic peptide binding to TAP. In contrast, BHV-1 UL49.5 inhibits conformational change of TAP, thus arresting delivery of cytosolic peptides into the endoplasmic reticulum (ER). BHV-1 UL49.5 reduces the levels of TAP via proteasome pathways. BHV-1 encoded bICP0 impedes interferon (IFN) signaling by degrading the levels of IFN regulatory factor 3 protein (IRF3) and interfering with the ability of IRF7 to stimulate IFN-β promoter (Henderson, Zhang, & Jones, 2005; Saira, Zhou, & Jones, 2007). BHV-1 gG binds to chemokines that generally play roles in immune response and inflammation, thereby disabling the action of chemokines (C. Jones & Chowdhury, 2010). Higher levels of interferon β (IFN-β) are detected in BHV-1 latency-related deficient mutant virus infected bovine cells during productive infection or acutely infected tonsil of calves, indicating that the LR mutant directly or indirectly induces IFN-β expression (C. Jones, da Silva, & Sinani, 2011).
1.2. Productive infection

1.2.1. Entry

Viral infection occurs in epithelial cells at a mucosal surface of ocular, respiratory tract, or genital tract, as a result of direct or aerosol contact. Virus infection is initiated by viral attachment and entry through interactions between viral glycoproteins and cellular receptors (G. Campadelli-Fiume, Cocchi, Menotti, & Lopez, 2000; G. Campadelli-Fiume, Menotti, Avitabile, & Gianni, 2012). Interaction of HSV-1 glycoprotein C (gC) and probably gB with glycosaminoglycans (GAGs) of cell surface heparan sulphat mediates viral attachment (Gruenheid S, 1993 ). Four glycoproteins, gB, gD, gH and gL play important roles in the HSV-1 entry process by fusing the viral envelope with cellular membrane (G. Campadelli-Fiume et al., 2000; G. Campadelli-Fiume et al., 2012). The tropism of HSV-1 is determined by alternative receptors, nectin 1, HVEM (herpes virus entry mediator), and modified heparan sulphate and these receptors have important roles during fusion events. Nectin-1 has a wide entry activity of alphaherpesviruses including HSV-1, HSV-2, as well as BHV-1 and it is expressed in neurons (Geraghty RJ, 1998; C. Jones, 2013). Entry process of BHV-1 but not of HSV-1 is mediated by CD155, the poliovirus receptor (Geraghty RJ, 1998). As a result of virus entry, nucleocapsids with tegument proteins are released into cytoplasm, which is then transported along the microtubule using motor protein, dynein to the nuclear pore complex (NPC) (Döhner K, 2002). Subsequently, viral DNA is injected into nucleus.

1.2.2. Herpesvirus gene expression during productive infection

Viral genomes that are not associated with histones enter the nucleus (Oh & Fraser, 2008). After entering the nucleus, viral genomes rapidly circularizes and is
associated with histones, cellular repressors, as well as promyelocytic leukemia (PML) protein (B. Roizman & Whitley, 2013). Therefore, the state of viral genome immediately becomes silenced. During early times of productive infection, histone is associated with chromatin repressive marks, methylation of histone 3H lysine 9 (H3K9) and lysine 27 (H3K27) (T. M. Kristie, 2016). The tegument protein VP16 plays important roles in both transcription and chromatin remodeling. VP16 recognizes cis-acting elements (TAATGARAT) within IE promoter region but does not directly bind to them (B. Roizman & Whitley, 2013). Instead, VP16 forms a transactivation complex with host cell octamer binding protein 1 (Oct-1) and host cell factor (HCF-1), which binds to cis-acting elements and initiates IE gene expression. Chromatin immunoprecipitation (ChIP) assay demonstrated that VP16 recruits general transcription factors (GTFs) including TATA binding protein (TBP) and Pol II to the IE promoter (Herrera & Triezenberg, 2004; Knipe & Cliffe, 2008). In addition, VP16 activates euchromatin modification by recruiting histone acetyltransferases (HATs) including CREB-binding protein (CBP) and p300 as well as ATP-dependent chromatin remodeling SWI/SNF complex proteins such as BRM and BRG1 to the IE promoter, which is required for initiating IE transcription.

HCF-1 is involved in cell cycle progression, transcription, mRNA procession, DNA replication-repair, and signal transduction (Khurana & Kristie, 2004). Numerous studies have demonstrated that HCF-1 plays significant roles in IE gene expression because HCF-1 depletion reduces the levels of IE proteins, ICP0 and ICP4 (Narayanan, Nogueira, Ruyechan, & Kristie, 2005). H3K4 methylation is a canonical mark associated with euchromatin (Knipe & Cliffe, 2008). HCF-1 is included in the Setd1A and MLL histone H3K4 methyltransferase complexes (Vogel & Kristie, 2013). Depletion of
Setd1A reduces HSV-1 transcription and replication (Huang et al., 2006). In addition, HCF-1 also interacts with several acetyltransferases and histone chaperones (Vogel & Kristie, 2013). In summary, HCF-1 can regulate viral gene expression, in particular IE transcription, and replication via interaction with chromatin modification and remodeling.

Expression of HSV-1 and BHV-1 gene is regulated in a temporal manner with immediate early (IE ) first, early (E) next, and finally late (L) gene during productive infection (Honess RW, 1974 ; C. Jones, 2003; Muylkens et al., 2007). HSV-1 transcription depends on host RNA polymerase II (Pol II) since it does not encode its own RNA pol II. IE gene expression does not require de novo protein synthesis. IE proteins encode viral regulatory proteins that activate E, L promoters or its own promoters. E gene proteins generally encode enzymes required for viral DNA synthesis: viral DNA polymerase and thymidine kinase for example. Viral DNA replication generally triggers L gene expression. L genes are further divided into leaky-late (γ1) and true-late (γ2) based on the requirement of viral replication. L genes primarily encode structural proteins such as tegument proteins, glycoproteins, and capsid proteins.

HSV-1 IE proteins contain ICP0, ICP4, ICP22, ICP27 and ICP47. ICP4 facilitates E and L gene expression by enhancing formation of transcription machinery (Arvin et al., 2007). However, it also negatively regulates its own promoter as well as other IE promoters. In addition, ICP4 inhibits expression of the latency-associated transcript by binding to the LAT promoter region during productive infection (Batchelor AH, 1994). ICP22 promotes IE and L gene expression in primary human, rodent and rabbit cells possibly by modification of RNA polymerase II (Pol II) (Arvin et al., 2007). ICP27 functions as a RNA nuclear transporter. ICP27 interferes with splicing process by which
unspliced cellular RNA is transported into cytoplasm at early times after infection. ICP47 blocks the function of transporter associated with antigen processing (TAP) and thus inhibits antigen presentation to immune cells.

BHV-1 IE genes contain two transcription units: IE transcription unit 1(IETu1) and IETu2. IETu1 encodes HSV-1 homologous proteins, bICP0 as well as bICP4, and IETu2 encodes bICP22. Although the genome organization of BHV-1 IE gene is different from that of HSV-1, BHV-1 lytic genes are expressed in the same general cascade as HSV-1 (C. Jones, 2003). bICP4 also negatively regulates IETu1 promoter activity. bICP22 represses different classes of promoters such as IETu1 promoter directing expression of bICP0 and bICP4, E promoter governing the expression of bICP0, and L promoter controlling the expression gC gene (Köppel R1, 1997).

ICP0 and bICP0 are multifunctional proteins that are crucial for productive infection in differentiated cells. ICP0 or bICP0 activates all three classes (IE, E and L) of viral promoters. ICP0 regulates gene expression without binding to DNA, suggesting that ICP0 interacts with cellular transcription machinery or chromatin modifiers to regulate viral gene expression (C. Jones & Chowdhury, 2007). ICP0 and bICP0 harbor C3HC4 zinc RING fingers, which possess E3 ubiquitin ligase activity. These proteins can induce degradation of certain cellular proteins in a proteasome dependent manner. PML protein possesses antiviral activity against HSV-1 in the presence of interferon (IFN) (Chee, Lopez, Pandolfi, & Roizman, 2003). ICP0 degrades PML proteins to inhibit IFN-dependent host immune response. bICP0 also degrades PML (Gaudreault & Jones, 2011). ICP0 interferes with histone deacetylation by releasing histone deacetylases (HDACs) from REST/coREST/HDACs repressor complex to promote the formation of active

1.2.3. Replication, assembly, and egress

Nuclear reorganization of infected cells occurs resulting in formation of replication compartment in which viral DNA is replicated, transcribed and encapsidated (Knipe & Cliffe, 2008). Viral DNA replication occurs via rolling circle mechanism. HSV-1 has three origins of replication, two copies within the inverted repeats of U₅ and one in the middle of U₅ (Everett, 2014). BHV-1 contains two origins of replication located in the inverted repeats of U₅. During lytic replication, virus inhibits host DNA replication and arrests cell growth in the G1 phase, presumably to minimize competition with the host for resources used in DNA synthesis (Arvin et al., 2007). HSV-1 ICP0 protein is also involved in a mitotic block (Lomonte P, 1999). Studies using ICP27 mutant viruses have demonstrated that ICP27 inhibits phosphorylation of the retinoblastoma protein (pRB) thereby inhibiting cell progression into S phase (Song, Yeh, Liu, & Knipe, 2001). The viral capsids gain envelope through budding into perinuclear membrane and lose their envelope during fusion with outer nuclear membrane (Knipe & Cliffe, 2008). Primary enveloped particle lacks some of the tegument and glycoproteins. Through budding into Golgi or the Trans Golgi network (TGN), the capsids acquire the remaining tegument proteins and mature envelope with glycoproteins. Ultimately, mature virions are released from the cell to extracellular compartment or spread to surrounding cells by cell to cell transmission.
1.3. Latent infection and reactivation

1.3.1. Latency-reactivation cycle

The latency-reactivation cycle includes three major phases: establishment, maintenance and reactivation (C. Jones, 2003). During the establishment of latency, viral particles enter the nervous system through the axonal termini of peripheral neurons and travel via retrograde transport to nucleus of neuronal cell body in ganglion. Viral gene expression and replication occur in trigeminal ganglia (TG) for about a week following primary infection. Infectious virus can be detected in TG during acute infection. Replication is not likely to be required for establishment of latency because thymidine kinase (TK) mutants viruses establish latent infection but at lower level (Efstatthiou, Kemp, Darby, & Minson, 1989; C. Jones, 2003; Kosz-Vnenchak, Coen, & Knipe, 1990). Eventually, infectious virus and lytic viral gene expression are extinguished. Only the HSV-1 LAT or BHV-1 LR RNA is abundantly transcribed. The maintenance of latency is characterized by life-long persistence of the genome takes place although infectious virus is not readily detected in TG, lytic cycle viral gene expression is not abundant: but abundant expression of LAT or LR gene products can be detected. Reactivation can be initiated by stimuli that trigger abundant lytic cycle viral gene expression. Successful reactivation leads to production of progeny virus particles that travel back to the surface via anterograde transport to the site of primary infection: occasionally virus particles can also enter the central nerve system (CNS).

1.3.2. Viral gene expression during latent infection

HSV-1 and BHV-1 establish life-long latent infection predominantly in sensory neurons of TG or sacral dorsal root ganglia, in which the viral genome exists as a circular
molecule associated with cellular histones (Deshmane SL, 1989). BHV-1 DNA is also
detectable in non-neuronal cells including tonsils and lymph nodes during latent infection
(Winkler, Doster, & Jones, 2000). During entry into latency, high levels of infectious
virus, viral DNA, and lytic viral gene expression, HSV-1 LAT, small RNAs (sRNAs) and
microRNAs (miRNAs) are detected in TG (C. Jones, 2013; B. Roizman & Whitley,
2013). Eventually infectious virus and lytic viral gene expression is extinguished. HSV-1
LAT and miRNAs are only abundantly expressed in latently infected neurons of mice,
rabbit or human.

Distinct types of neurons in TG have been characterized based on surface markers
and receptors. Up to 40% of sensory neurons can be latently infected (G. C. Perng &
Jones, 2010). The viral DNA copy number in latently infected neurons varies. TG
neurons that contain high copy number of viral genomes express LAT whereas TG
neurons that contain low copy numbers of genome do not express LAT (Catez et al.,
2012). However, single cell analysis has demonstrated that approximately 90% of HSV-1
latently infected neurons express LAT (Ma, Russell, Spelman, Carbone, & Tscharke,
2014).

The molecular mechanisms regulating the transition between lytic and latent
infection have not been well defined. One possible mechanism has been suggested that
inefficient IE gene expression as a result of failure to form a transactivation complex
which consists of VP16, Oct-1 and HCF-1 in neurons triggers establishment of latency.
This model supported by the finding that fibroblasts infected with IE mutant HSV-1 enter
into a quiescent state (Arvin et al., 2007). Lack of VP16 axonal transport into neuronal
nucleus due to either loss of VP16 during long-distance retrograde transport or similar
mechanisms of viral uncoating may also limit IE gene expression (Arvin et al., 2007; B. Roizman & Sears, 1987). The HCF-1 protein is localized in the nucleus of most cells whereas it is uniquely expressed in the cytoplasm of sensory neurons (T. Kristie, Vogel, & Sears, 1999). The cellular transcription factor Luman interacts with HCF-1 resulting in impairing productive infection and sequestering HCF-1 in the cytoplasm of sensory neurons, presumably inhibiting initiation of IE gene expression during latency (R. Lu & V. Misra, 2000). The neuronal transcriptional factor, Zhangfei is another HCF-1 binding protein that interacts with VP16 thus impairing the formation of a VP16 transactivation complex, which consequently inhibits ICP0 expression and viral replication (Akhova, Bainbridge, & Misra, 2005; R. Lu & V. Misra, 2000). Oct-1 contains a POU domain, which has the ability to recognize octamer elements as well as to interact with various cellular factors including VP16, and Oct1 levels are downregulated in neuronal cells (Lakin et al., 1995). Other POU domain transcription factors (N-Oct2, N-Oct3, Brn-2, Brn-3a and Brn-3b) are able to recognize IE sequence: thus repressing IE gene activation (Nicoll, Proenca, & Efstathiou, 2012).

LAT deficient virus reduces HSV-1 genome harboring neurons in mice indicating that LAT enhances establishment of latency (R. Thompson & Sawtell, 1997). LAT can inhibit lytic gene expression. LAT deficient HSV-1 harboring reporter gene induces reporter gene expression as well as the incidence of reactivation from single neurons (Nicoll et al., 2016). LAT deficient virus exhibits increased lytic viral gene expression during ganglionic acute infection in mouse models (Garber, Schaffer, & Knipe, 1997). LAT is located within the long inverted repeat region and is partially antisense to ICP0 coding sequences suggesting LAT inhibit ICP0 expression (C. Jones, 2003). The stable
1.5kb and 2kb LAT species that are alternatively spliced from the primary 8.3kb LAT are abundantly expressed in sensory neurons of latently infected mice, rabbits or humans (C. Jones, 2003, 2013). LAT is predominantly expressed in the nucleus but can also be detected in cytoplasm. Cytoplasmic LAT is associated with ribosome and splicing complexes (Ahmed & Fraser, 2001). LAT encodes eight miRNAs and two sRNAs (Bloom, 2016; Peng, Vitvitskaia, Carpenter, Wechsler, & Jones, 2008). These miRNAs and sRNAs suppress lytic viral gene expression. One of miRNAs, miR-H2-3p inhibits ICP0 expression at the translational level and ICP4 expression is also reduced by miR-H6 (Umbach et al., 2008). In addition, sRNA1 and sRNA2 were detected in latently infected TG of mice and they inhibit productive infection in tissue culture (Shen et al., 2009). Additionally, LAT sRNA2 inhibits ICP4 protein expression. The neurovirulence factor, ICP34.5 expression is inhibited by miR-H4, which is antisense to ICP34.5 mRNA (Flores O, 2013). Furthermore, the neural specific host miR-138 reduces ICP0 expression (Pan et al., 2014).

BHV-1 LR is abundantly expressed in latently infected TG neurons of calves (C. Jones, 2003, 2013). LR has some of functional and genomic characteristics of HSV-1 LAT although their sequences are not highly conserved. LR mutant viruses reduce TG neurons harboring viral genomes indicating that LR plays important roles in establishment of latency. In contrast to the other alpha-herpesviruses, LR encodes functional proteins (Hossain A, 1995 ; Shen & Jones, 2008). An LR-specific antibody recognizes a protein in TG neurons of BHV-1 latently infected calves. LR contains two open reading frames (ORF1 and ORF2) and two reading frames lacking ATG initiation codon (RF-B and RF-C). LR is alternatively spliced and some fraction of spliced
transcript is polyadenylated in latently infected TG neurons of cattle, suggesting LR transcripts can be translated to more than one protein. The intact LR gene and LR mutant gene containing stop codons at the amino terminus of the first ORF reduces bICP0 mediated productive infection, indicating a LR protein is not necessary for repression (Geiser, Inman, Zhang, & Jones, 2002). Intact LR inhibits bICP0 expression at the level of transcription and translation in transiently transfected cells. Two families of small noncoding RNAs (sncRNAs) within LR were identified and they have a potential to be precursors for two mature miRNAs (Jaber, Workman, & Jones, 2010). These LR sncRNAs and miRNAs reduce the levels of bICP0 protein in bovine cells. A LR protein (ORF2) interacts with cellular factors to interfere with productive infection and viral gene expression (C. Jones et al., 2011). For example, ORF2 interacts with Notch family members (Notch1 and Notch3) and another transcription factor (C/EBP-alpha). Notch 1 transactivates BHV-1 immediate early transcription unit 1 (IEtu1) as well as bICP0 early promoters, and Notch 1 and Notch 3 activate late gene, glycoprotein C (gC) promoter (Workman, Sinani, Pittayakh jonwut, & Jones, 2011). ORF2 inhibits Notch mediated viral promoter activation and reduces the levels of Notch 3 through proteasome degradation pathways (Sinani, Frizzo da Silva, & Jones, 2013).

The mechanisms that mediate repression of lytic viral gene expression and activation of LAT transcription during latent infection have not been well characterized. Epigenetic mechanisms including DNA methylation, post translational chromatin modification have been considered to control latent genome. HSV-1 capsid protein VP26 interacts with DNA (cysteine-5) methyltransferase 3A (DNMT3A) (Rowles et al., 2015). DNMT3A knockdown or drug mediated DNMT3A inhibition interferes with HSV-1
replication suggesting that DNA methylases positively regulate viral replication (Lieberman, 2016; Rowles et al., 2015). However, significant DNA methylation within ICP4 and LAT promoter region was not detected during latency suggesting DNA methylation does not play a pivotal role in HSV-1 gene regulation during latent infection (Kubat, Tran, McAnany, & Bloom, 2004).

CCCTC-binding factor (CTCF) is a ubiquitously expressed zinc finger protein in vertebrate and possesses insulator activities (Gaszner & Felsenfeld, 2006). Several roles of insulators have been characterized in eukaryotic cells. The first is “enhancer-blocker” functions that inhibit cooperation between a promoter and enhancer. The second role is barrier function in which insulators act as chromatin boundaries and separate active euchromatin from inactive heterochromatin through recruiting histone modifying enzymes. Finally, the third cellular role for insulator is that they form chromatin loops, which can allow transcription activation between distant loci. Seven CTCF binding element clusters have been identified throughout the HSV-1 genome and two of them flank LAT enhancer/promoter regions (Bloom, Giordani, & Kwiatkowski, 2010). Three CTCF binding sites positioned downstream of the LAT enhancer, upstream of the ICP0 promoter, and upstream of ICP4 promoter are highly occupied by CTCF in HSV-1 latently infected TG (Ertel, Cammarata, Hron, & Neumann, 2012). However, these CTCF binding is diminished during the early times of reactivation. CTCF motifs cluster within the LAT region exhibits enhancer-blocker activity by inhibiting the effect of LAT enhancer to stimulate heterologous promoter activity (SV40) (Amelio, McAnany, & Bloom, 2006). These studies suggest CTCF plays a role in LAT and IE gene expression during latent infection or reactivation from latency.
During latency, increased histone accumulation, high levels of heterochromatin markers (di- or tri-methylation of H3K9me2/3 and H3K27me3), and reduced levels of euchromatin markers on lytic genes are observed whereas reduced levels of heterochromatin markers on LAT locus were detected (Kubat, Amelio, Giordani, & Bloom, 2004; Q. Y. Wang et al., 2005). LAT deficient mutant viruses have decreased heterochromatin markers on viral lytic gene suggesting that LAT suppresses lytic gene expression (Cliffe, Garber, & Knipe, 2009; Q. Y. Wang et al., 2005).

The polycomb repressive complexes (PRCs) are associated with heterochromatin to regulate latent infection. PRCs specifically bind to polycomb response elements resulting in heterochromatonic histone modification and gene silencing (Watson, Dhummakupt, Messer, Phelan, & Bloom, 2013). Two main PRCs have been defined in mammalian cells that are referred to as PRC1 and PRC2 (Simon & Kingston, 2009). PRC2 is involved in H3K27 methylation and PRC1 regulates chromatin structure or nuclear structure. Based on these observations, PRC2 may be recruited to specific loci and trimethylates lysine 27 of histone 3 during latency (Watson et al., 2013). H3K27me3 provides binding sites for PRC1, which compacts nucleosome and silences lytic viral gene expression. Studies examining the kinetics of chromatin modification during establishment of latency have shown that the H3K27me3 was recruited to silenced HSV-1 DNA suggesting PRCs may inhibit gene expression after the initial suppression of lytic gene expression by other factors (Cliffe, Coen, & Knipe, 2013).

1.3.3. Promotion of cell survival and neuronal phenotype

Herpesvirus infection can induce apoptosis and premature cell death can block virus spread (C. Jones, 2003, 2013). HSV-1 U₅1.5 and U₅₁3 induces an apoptosis
mediator, caspase 3 (Hagglund, Munger, Poon, & Roizman, 2002). ICP0 transcription is sufficient to trigger apoptosis without other viral gene expression (Sanfilippo & Blaho, 2006). However, HSV-1 and BHV-1 have evolved to inhibit apoptosis not only for enhancing overall viral loads, but also for promoting life-long latent infection (C. Jones, 2013). HSV-1 encodes numerous anti-apoptotic genes such as ICP27, U₅₃, U₅₅, gJ, gD, and LAT to delay or prevent apoptosis. HSV-1 encoded U₅₃ protein kinase blocks caspase 3 mediated apoptosis through posttranscriptional modification of pro-apoptotic BAD (Munger & Roizman, 2001). However, these viral anti-apoptotic gene products are not expressed during latency, so they cannot block apoptosis (Nicoll et al., 2012).

LAT mutant virus infected TG neurons exhibit enhanced neuronal death (G. Perng et al., 2000; R. L. Thompson & Sawtell, 2001). Many studies have demonstrated anti-apoptotic function of LAT and LR, suggesting LAT and the LR gene promotes neuronal survival by inhibiting apoptosis, which further enhances life-long latency. LAT blocks apoptosis in both in vitro and TG of latently infected mice and rabbits (Ahmed, Lock, Miller, & Fraser, 2002; Inman, Perng, et al., 2001; G. Perng et al., 2000). LAT can inhibit two major apoptotic pathways, the extrinsic pathway and the intrinsic pathway (Carpenter et al., 2007; Henderson G, 2002). LAT decreases caspase 8 and 9 cleavage in the absence of other viral gene. Cold shock induced caspase 3 is inhibited by LAT. LAT sRNA1 and sRNA2 mutually reduce cold shock induced DNA fragmentation and apoptosis (Shen et al., 2009). It has been widely accepted that chronic inflammatory responses enhance maintenance of latency. Granzyme B (GrzB) released by CD8 T cells can activate caspase 3 thereby inducing apoptosis. LAT blocks GrzB activated caspase 3
resulting in inhibition of apoptosis, suggesting LAT defends latently infected neurons from CD8+ T cell lytic granule-mediated apoptosis (Jiang et al., 2011).

The phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathway promotes growth factor induced neuronal survival (Dudek et al., 1997). Following stimulation of receptor tyrosine kinases at the cell membrane, PI3K is activated resulting in Akt activation. Phosphorylated Akt (pAkt) is an active form of Akt kinase and has anti-apoptotic properties (Cooray, 2004). LAT stabilizes the phosphorylation of Akt following cold shock induced apoptosis or serum withdrawal (Carpenter et al., 2015; S. Li, Carpenter, Hsiang, Wechsler, & Jones, 2010).

The BHV-1 LR deficient mutant virus induces higher levels of caspase 3 medicated apoptosis compared to WT or rescued virus during latency: However, the LR mutant virus infected TG of calves contains lower levels of viral DNA (Lovato, Inman, Henderson, Doster, & Jones, 2003). The LR encoded ORF2 fusion protein inhibits both caspase 8 and caspase 9 medicated apoptosis (Shen & Jones, 2008). LR miRNAs also inhibit caspase 3 and caspase 9 medicated apoptosis in neuroblastoma cells (Shen & Jones, 2008; Silva & Jones, 2012). Activated NF-κB by RIG-I can inhibit apoptosis through inducing expression of anti-apoptotic genes. LR miRNAs interact with RIG-I and induce NF-κB mediated transcription in a RIG-I signaling dependent manner, suggesting RIG-I sensing of LR miRNAs promote neuronal survival.

The LAT and LR gene contribute to establishment and maintenance of latent infection by promoting a mature neuronal phenotype such as neurite growth (S. Li et al., 2010; Sinani, Frizzo da Silva, et al., 2013). Notch signaling pathways interfere with neuronal differentiation, thus inducing apoptosis and neuronal degeneration (C. Jones,
LR ORF2 enhances neuronal differentiation of mouse neuroblastoma cells overexpressing Notch 1 and Notch 3 presumably by interrupting with the Notch signaling pathway (Sinani, Frizzo da Silva, et al., 2013).

**1.3.4. Immune System during Latent Infection**

During latency, infiltrating immune cells such as CD8$^+$ T and CD8$\alpha$ dendritic cells (DCs) can be found in close proximity to TG neurons. The infiltrating cells express interferon gamma (IFN-$\gamma$) and interleukin 4 (IL-4) and lytic granules (Freeman, Sheridan, Bonneau, & Hendricks, 2007; Knickelbein JE, 2008; Liu T, 2000; Winkler, Doster, et al., 2000). These infiltrating cells and their secreted cytokines limit viral replication and promote a latent infection. In fact, CD8$^+$ T cells that reside around HSV-1 or BHV-1 latently infected TG neurons apparently promote maintenance of latency and reduce the frequency of reactivation (Bloom, 2016; Clinton Jones, 2014). Studies in *ex vivo* latently HSV-1 infected TG neuron cultures have shown that addition of CD8 antibody exhibits increased lytic viral gene expression (Liu T, 2000). Depletion of CD8$^+$ T cells by CD8 antibody or stress induced immunosuppression triggers HSV-1 reactivation from latency (Freeman et al., 2007). Additionally, studies using knockout mice have demonstrated that CD8$\alpha$ DCs enhances HSV-1 latency (Mott et al., 2014). HSV-1 lytic viral gene expression is suppressed in porcine TG neurons pretreated with IFN-$\alpha$ (De Regge, Van Opdenbosch, Nauwynck, Efstathiou, & Favoreel, 2010). ICP4, which is required for productive infection, is degraded by one of lytic granule components, GrzB (Knickelbein JE, 2008).

**1.3.5. Reactivation from latent infection**
Reactivation can be triggered by physical and psychological stress events. Several approaches including explant, UV radiation, heat shock, nerve growth factor (NGF) depletion and synthetic corticosteroid hormones including DEX have been applied to simulate stress events (Cliffe et al., 2015; Clinton Jones, 2014). Although stress can trigger reactivation, the molecular mechanisms of viral reactivation have not been well understood. Numerous studies suggest that host cell factors possibly initiate lytic viral gene expression during reactivation from latent infection. VP16 promoter is stimulated in latently infected neurons without other viral proteins (Thompson RL, 2009). During reactivation from latency, lytic genes appear to be expressed in two distinct phases, Phase I and Phase II determined by the requirement of de novo protein synthesis, viral replication, and VP16 (Kim, Mandarino, Chao, Mohr, & Wilson, 2012). All three classes of lytic genes are expressed without de novo protein synthesis and VP16 during the early times of reactivation (Phase I). Although it has not been clear which viral genes trigger HSV reactivation, these studies suggest that reactivation may be initiated in a VP16 independent manner. Other studies showed that lytic viral gene expression is not detected in TG neurons infected with VP16 mutant viruses following hyperthermic induced reactivation (Thompson RL, 2009), which support the concept that VP16 is required during reactivation from latency. ICP0 seems to be essential for successful reactivation from latent infection since reactivation occurs inefficiently in ICP0 mutant virus infected neuronal cells following heat stress (W. P. Halford & Schaffer, 2001). The expression of ICP0 mRNA is readily detected compared to other viral transcripts when TG from latently infected mice are induced to reactivate following explant and DEX treatment (Du, Zhou, & Roizman, 2012). Adenoviral vectors expressing just ICP0 are sufficient to
initiate HSV-1 reactivation (W. P. Halford, Kemp, Isler, Davido, & Schaffer, 2001). ICP4 can also promote reactivation from latency in mice TG culture (Clinton Jones, 2014). Although VP16, ICP0 and ICP4 each appear to be capable of triggering reactivation, successful reactivation will eventually, require all three regulatory viral genes.

LAT deficient mutant viruses exhibit relatively lower frequency of reactivation from latency compared to wild type or rescued virus in animal models that support reactivation from latency (Perng GC, 1994). It is unclear whether this is because of its inefficient reactivation or reduced levels of establishment of latency. Approximately 3 fold more TG neurons were latently infected with wild type HSV-1 compared to LAT mutant virus (R. Thompson & Sawtell, 1997). In some studies, LAT is likely to be required for spontaneous or induced reactivation. Periodically, HSV-1 shedding occurs in eyes of infected rabbits due to spontaneous reactivation (Perng GC, 1994). However, LAT deficient mutant recombinant viruses exhibit reduced frequency of spontaneous reactivation even though wild-type and mutant viruses have similar growth properties in tissue culture, rabbit eye and rabbit TG (Perng GC, 1994). The first 1.5kb LAT that does not overlap ICP0 is sufficient to rescue spontaneous reactivation, suggesting suppression of ICP0 is not necessary for spontaneous reactivation (G. C. Perng & Jones, 2010). LAT mutant viruses that encode an anti-apoptotic caspase inhibitor can substitute for the ability of LAT to promote reactivation (Jin et al., 2005). However, other studies suggested that LAT may maintain successful latency by reducing the incidence of reactivation (Nicoll et al., 2016).

A BHV-1 LR deficient mutant virus exhibits reduced symptoms, and sheds less infectious virus in ocular, TG and tonsil compared to WT or rescued virus during acute
infection of calves (Inman, Lovato, Doster, & Jones, 2001; Perez, Inman, Doster, & Jones, 2005). The LR mutant virus that does not express proteins encoded by the LR gene and is unable to reactivate from latency following DEX treatment thus suggesting that an LR protein is pivotal for the latency-reactivation cycle (Inman, Lovato, Doster, & Jones, 2002; Perez et al., 2005). However, the immediate early transcript, bICP0 but not bICP4 or the late transcript gC was detected in TG of calves latently infected with LR mutant virus during DEX mediated reactivation (Workman, Perez, Doster, & Jones, 2009).

In addition to viral genes that regulate the latency-reactivation cycle, there are clearly cellular genes that regulate the latency-reactivation cycle. A brief discussion of certain cellular factors that are known to control the latency-reactivation cycle are mentioned below. First, the phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathway triggered by NGF binding to the TrkA receptor tyrosine kinase is required to maintain HSV-1 latency (Camarena et al., 2010). The mTOR signaling that is regulated by PI3K/Akt signaling is able to control protein synthesis through targeting translation repressors, eIF4E-binding proteins (4E-BPs) (M. Kobayashi, Wilson, Chao, & Mohr, 2012). Interrupting mTOR signaling induces reactivation from latent infection, suggesting mTOR controls expression of proteins, which suppress lytic cycle viral gene expression (M. Kobayashi et al., 2012; Wilson & Mohr, 2012). Secondly, inhibiting demethylase activity decreases HSV-1 reactivation from latency indicating that epigenetic modifications play important roles in reactivation (Cliffe et al., 2015; Messer, Jacobs, Dhummakupt, & Bloom, 2015). Thirdly, heterochromatin protein 1 (HP1) is recruited to H3K9me3 resulting in heterochromatin formation (Fischle et al., 2005). However, phosphorylation of neighboring H3K9me3 is sufficient to evict HP1 in the
absence of H3K9 demethylation. Fourthly, it is well documented that c-Jun N-terminal kinase (JNK) signaling pathway is activated by a wide range of stimuli (Cliffe et al., 2015; Leong, Maiyar, Kim, O'Keeffe, & Firestone, 2003). JUN signaling pathway stimulates HSV-1 reactivation through histone phosphorylation on lytic gene promoter. Finally, HCF-1 that interacts with histone demethylases and methyltransferase appears to play important roles in initiation of reactivation from latency via chromatin modifications since HCF-1 that is located in cytoplasm of sensory neurons translocates into nucleus in response to stresses (T. M. Kristie, 2015).

1.4. Stress and its role during viral infection

1.4.1. Corticosteroid mediated reactivation from latent infection

Corticosteroids including glucocorticoids and mineralocorticoids are a class of steroid hormones (S. Ramamoorthy & Cidlowski, 2016). Natural glucocorticoids designated as cortisol in humans or corticosterone in rodents are the major source of glucocorticoids. Daily release of glucocorticoids is generally controlled by circadian rhythm. Stressors can also activate the hypothalamic-pituitary-adrenal (HPA) axis to release glucocorticoids. Stressors activate the hypothalamus to induce secretion of corticotrophin releasing hormone (CRH), which acts on anterior pituitary to trigger the secretion of adrenocorticotrophin hormone (ACTH). Finally, ACTH activates the adrenal cortex to synthesize and release glucocorticoids into the bloodstream. Once released, the concentration of glucocorticoids is balanced by a negative feedback mechanism in which glucocorticoids target the hypothalamus and pituitary to inhibit excessive release of glucocorticoids. In addition, 11β-hydroxysteroid dehydrogenase (11β-HSD) regulates availability of natural glucocorticoids by interconversion of active and inactive form of
glucocorticoids (Kadmiel & Cidlowski, 2013). However, the synthetic corticosteroid, dexamethasone (DEX) is not inactivated by 11β-HSD (Kadmiel & Cidlowski, 2013; S. Ramamoorthy & Cidlowski, 2016). Glucocorticoids regulate a wide range of cellular functions such as development, homeostasis, metabolism, cognition and inflammation.

Corticosteroids consistently trigger reactivation of α-herpesvirinae subfamily members including HSV-1, BHV-1 and canine herpesvirus type I from latent infection (Clinton Jones, 2014). DEX facilitates HSV-1 lytic viral gene transcription following heat induced reactivation (Halford WP, 1996). Conversely, DEX represses BHV-1 LR promoter activity in bovine cells, and reduces the level of LR transcripts (C. Jones, Delhon, Bratanich, Kutish, & Rock, 1990 ; Rock, Lokensgard, Lewis, & Kutish, 1992). DEX enhances viral DNA replication through glucocorticoid response element (GRE) within a HSV-1 origin of DNA replication (OriL) in neuronal cells (Hardwicke MA, 1997). Notch family members activate certain viral promoters and the levels of Notch 3 transcripts are induced following DEX treatment, indicating that Notch may play important roles in reactivation from latency (Workman et al., 2011). DEX inducible transcription factors have been identified in TG by comparing BHV-1 latently infected calves to BHV-1 latently infected calves following DEX treatment using a Bovine Gene Chip (Workman et al., 2012). These cellular factors promote productive infection and viral promoter activity in both HSV-1 and BHV-1 (Sinani, Cordes, Workman, Thunuguntia, & Jones, 2013; Workman et al., 2012). For example, Kruppel-like factor 4 (KLF4) and KLF 15 promote BHV-1 productive infection, and activate the bICP0 early promoter. In addition, KLF15, Slug and SPDEF induce ICP0 promoter activity. Although ICP0 or bICP0 promoters are activated by DEX, additional neuronal or viral factors are
likely to be required for successful reactivation (C. Jones et al., 2011).

1.4.2. Transcription

The action of glucocorticoid is primarily mediated by glucocorticoid receptor (GR), and to a lesser extent, mineralocorticoid receptor (Mitchell, Cooper, Griffiths, & Barber) (S. Ramamoorthy & Cidlowski, 2016). GR is a member of nuclear receptor subfamilies and a ligand activated transcription factor. In the absence of ligands, GR forms a complex with chaperone proteins (heat shock protein70 (HSP), HSP90, and HSP20) and immunophilins (FKBP51 and FKBP52), and predominantly resides in cytoplasm. Once activated by ligands including glucocorticoids, GR is dissociated from its molecular chaperones and is translocated into the nucleus, where it binds to GREs or negative GRE (nGRE) as a homodimer within promoter region of target gene resulting in activation or repression of gene expression. The consensus imperfect palindromic GRE (GGAACAnnTTGTTCT) consists of two half sites separated by a three-nucleotide spacer whereas the consensus palindromic nGRE (CTCC(n)02GGAG) contains variable spacers ranging from zero to two-nucleotides. Moreover, GR bound to GRE and other cellular factors bound to neighboring sites can cooperatively regulate gene expression. In some cases, GR binding sites does not contain classic GRE sequences in which GR directly binds to other cellular factors such as NFκB, AP1 and Oct-1 instead of binding to DNA. For example, the mouse mammary tumor virus (MMTV) promoter contains octamer motifs that are transcriptionally activated by a physical interactions between GR and Oct-1:However, promoter activity is not activated solely by Oct-1 (Préfontaine GG, 1998). It is also known that GR monomer binds to DNA and can regulate gene expression (Baschant & Tuckermann, 2010).
The serum and glucocorticoid regulated kinase 1 (SGK-1), tristetraproline (TTP), glucocorticoid-induced leucine zipper (GILZ), and mitogen activated protein kinase phosphatase-1 (MKP-1) are typical genes induced by the GR (Kadmiel & Cidlowski, 2013). GR represses expression of genes including β-arrestin, osteocalcin and itself. Interestingly, a repressive complex comprised of GR, NcoR1 and histone deacetylase 3 is recruited to a negative GRE (nGRE) within the GR gene following DEX treatment, which leads to negative autoregulation of GR (S. Ramamoorthy & Cidlowski, 2013).

GR heterogeneity caused by alternative splicing, alternative translation initiation, and posttranslational modification contributes to diversity in glucocorticoid receptor signaling (Kadmiel & Cidlowski, 2013). GRα is a predominant form of GR and GRβ transcripts that are generated by alternative splicing and they are highly homologous variant isoforms (R. Oakley, Sar, & Cidlowski, 1996). GRβ is primarily localized in the nucleus without ligand binding and interferes with the ability of GRα to activate transcription (S. Ramamoorthy & Cidlowski, 2016). Furthermore, GRβ recruits histone deacetylase complex thereby inhibiting transcription of cytokine genes (Kelly et al., 2008). Immune activators or proinflammatory cytokines promote GRβ expression (S. Ramamoorthy & Cidlowski, 2016). The additional isoforms including GRγ, GR-A and GR-P generated by alternative splicing have been identified. Each isoform generated by alternative translational initiation individually regulates unique gene expression. Human GR (hGR) contains at least seven phosphorylation sites (Ser-113, Ser-134, Ser-141, Ser-203, Ser-211, Ser-226 and Ser-404), which are highly conserved in mice and rats. The structure of ligands determines the degree and pattern of phosphorylation. The various kinases such as mitogen activated protein kinase, casein kinase II, glycogen synthesis
kinase 3β, and cyclin-dependent kinases phosphorylate GR. In addition, ubiquitination or sumoylation of GR promotes its degradation and Acetylation of GR reduces its transcriptional activity.

DEX upregulates mRNA expression of Epstein Barr virus (EBV) immediate early gene, BZLF1 through GR mediated pathway (Yang et al., 2010). Prednisolone, another synthetic glucocorticoid triggers human cytomegalovirus (HCMV) reactivation from latently infected primary monocytes (Van Damme et al., 2015). Kaposi’s sarcoma-associated herpesvirus protein, the latency-associated nuclear antigen (LANA) boosts GR-mediated transcriptional activity by directly interacting with GR following DEX treatment (Togi et al., 2015). HCMV major immediate early (MIE) promoter is stimulated following DEX treatment in a GR dependent manner (Inoue-Toyoda, Kato, Nagata, & Yoshikawa, 2015). Human papilloma virus (HPV) 11, HPV16, and HPV 18 contain a promoter denoted as the long control region (LCR) is stimulated by DEX or progesterone treatment (de Villiers, 2003). Three GREs within the HPV16 LCR are important for GR mediated activation of gene expression following DEX treatment. The GR stimulates HPV16 gene expression in combination with a nuclear receptor interaction protein (NRIP) (Chang et al., 2012). The HIV-1 viral protein R (Vpr) induces viral replication in T cells and transactivates HIV long terminal repeat (LTR). However, a GR antagonist RU486 inhibits Vpr mediated LTR transactivation and facilitates virus reactivation from latently infected cells (Schafer, Venkatachari, & Ayyavoo, 2006). The HBV genome contains functional GRE that elevates HBV promoter activity following DEX treatment (Tur-Kaspa et al., 1988). DEX increases the levels of hepatitis B virus (HBV) pregenomic RNA and viral transcripts via GR mediated pathway (Chou , Wang,
Lin, & Chi, 1992). In summary, numerous viruses have evolved to utilize stress responses to promote their replication and transcription.

1.4.3. Chromatin remodeling

It is generally accepted that the GR specifically binds to DNA resulting in local chromatin modification in a hormone dependent manner, indicating that the GR can function as a pioneer factor (Richard-Foy H, 1987). Pioneer factors are known to generate accessible binding site for transcription factors through interaction with closed chromatin. GR, in combination with chromatin modifiers, regulates chromatin architecture. Activated GR is able to recruit chromatin modifiers that regulate assembly of transcription machinery and RNA polymerase II dependent transcription. GR regulates gene expression in a cell type dependent manner and can suppress gene expression through interactions with SMRT/NCoR co-repressor proteins and HDACs (Surjit et al., 2011). In other studies, genome-wide analysis confirmed that most GR occupancy is observed in open chromatin, whereas only a small portion of GR interacts with closed chromatin, thereby inducing chromatin modification (John et al., 2011). A small portion of GRE occupancy and cell type specific occupancy are related to chromatin accessibility and sequence features. These observations suggest that chromatin modifiers or pioneer transcription factors help to establish an accessible chromatin condition prior to GR recruitment. Approximately 62% of total binding sites are occupied by C/EBP-β and abolishing C/EBP-β binding leads to inhibition of GR recruitment in mice liver tissue (Grontved et al., 2013). Activator protein 1(AP1) is often colocalized with GR within GR binding regions and these interactions were found in open chromatin (Biddie et al., 2011). AP1 binding appears to recruit GR into specific binding region
within open chromatin because inhibition of AP1 binding hinders formation of open chromatin and GR binding. However, GR also serves as a binding site for AP1 at a small subset of sites in the genome (Voss et al., 2011). The forkhead box protein 1 (FoxA1) is also known to act as a pioneer factor that attracts steroid receptors such as estrogen receptor (ER), androgen receptor (AR), and GR (Swinstead et al., 2016). At selected sites, the ER or GR promotes FoxA1 binding and these regions are likely to have weaker FoxA1 binding sites which suggests that ER or GR provides FoxA1 with accessible chromatin landscape by recruiting chromatin remodelers. In summary, it seems clear that GR interactions with heterochromatin is complicated and that many chromatin modifying complexes and other transcriptional coactivators play a crucial role in GR mediated gene expression.

1.4.4. Immune Suppression

Glucocorticoids have been widely used to treat inflammatory diseases due to its anti-inflammatory and immunosuppressive properties. For example, glucocorticoid mediated immunomodulation is governed by two mechanisms, genomic and non-genomic mechanisms (Ruiz-Irastorza, Danza, & Khamashta, 2012; Stahn & Buttgereit, 2008). The genomic mechanism is mediated by GR binding to DNA resulting in transactivation or transrepression of target gene expression. Activated GR positively regulates transcription of anti-inflammatory genes such as interleukin-10 (IL-10), annexin A-1 and inhibitor of kappa B (IκB). In addition, expression of pro-inflammatory genes containing IL-1, IL-2, tumor necrosis factor (TNF) and interferon γ (IFN-γ) is transrepressed by GR. With respect to the non-genomic mechanism of immunomodulation, glucocorticoids lower the level of ATP by interacting with cellular
and mitochondrial membrane, thereby impairing the function of immune cells.

Glucocorticoids also promptly diminish T cell receptor (TCR) signaling pathways by interacting with membrane GR. TCR complexes that contain membrane GR, tyrosine kinases LCK, and FYN is dissociated by glucocorticoids resulting in inactivation of LCK and FYN, which consequently blocks phosphorylation of TCR downstream molecules such as mitogen-activated protein kinase (MAPK), Jun kinase N-terminal (JNK) and p38 (Lowenberg et al., 2006; Zen et al., 2011).

A higher frequency of HSV-1 reactivation is observed in immunocompromised individuals, suggesting that immune dysregulation influences reactivation from latency (Webster Marketon & Glaser, 2008). Although restraint stress reduces clinical symptom during HSV-1 productive infection, it inhibits Type I and II IFN expression (Ortiz, Sheridan, & Marucha, 2003). During the early stage of infection, stress or corticosteroids impairs the role of dendritic cells that prime HSV-1 specific CD8\(^+\) T cells in a GR dependent manner, thereby delaying restoration of virus induced lesion and virus clearance (Elftman et al., 2010). Numerous studies have proposed that apoptosis of TG neurons or non-neuronal cells residing in TG facilitate reactivation from latent infection. Restraint stress induces reactivation from latent infection by enhancing the levels of corticosteroids and reducing HSV-1 specific CD8\(^+\) T cells around TG in mice (Freeman et al., 2007). Infiltrating T cells in TG of BHV-1 latently infected calves undergo apoptosis within 6 hours following DEX treatment (Winkler MT, 2002 #78). In addition, TUNEL positive TG neurons were detected at 18 hours after DEX treatment. Glucocorticoids are able to induce apoptosis through GR signaling pathways (Gruver-Yates & Cidlowski, 2013). Activated GR induces expression of pro-apoptotic genes containing Bel2 and Bel-
xL and anti-apoptotic genes such as Bid, Bax, Bim and Bad in a cell type or tissue
dependent manner. High doses of glucocorticoids cause apoptosis in thymocytes, T cells,
B cells and macrophages (Winkler, Doster, Sur, & Jones, 2002).

1.4.5. Additional nuclear receptors can also influence virus replication

The nuclear receptors are ligand-dependent transcription factors that regulate
various cellular responses such as homeostasis, metabolism and development (Rizzo &
Fiorucci, 2006). The nuclear receptor superfamily contains steroid receptors and non-
steroid receptors. Certain studies have clearly provided that the nuclear receptors
implicate in viral oncogenesis, pathogenesis, gene expression and replication. Thyroid
hormone receptor (TR) directly bind to thyroid hormone responsive elements (TRE)
within LAT promoter region and then induces LAT promoter activity through chromatin
remodeling in the presence of thyroid hormone whereas activated TR reduces ICP0
expression in mouse neuroblastoma cells (Bedadala, Pinnoji, Palem, & Hsia, 2010).
Thyroid hormone receptor (TR) inhibits HSV-1 viral replication and promoter activity of
thymidine kinase following thyroid hormone (T3) treatment (F. Chen et al., 2014).
Epidemiological studies support that the levels of circulating estradiol are positively
related to HPV-associated cervical cancers (Arbeit JM, 1996; Rinaldi et al., 2011). The
estrogen receptor α (ER α) containing deficient DNA binding domain (DBD) inhibit
carcinogenesis in the cervix of transgenic mice expressing HPV oncogene E7 (Son, Park,
Lambert, & Chung, 2014). 17β estradiol inhibits HSV-1 reactivation by ER α in mice
models (Vicetti Miguel et al., 2010). In contrast to HPV and HSV-1, the levels of
estrogen are negatively related to HIV-1 and HBV infection. Binding of ER α and β-
catenin to the transcription initiation site of HIV-1 LTR following 17β estradiol
treatment leads to repression of HIV replication and transcription (Szotek, Narasipura, & Al-Harthi, 2013). ER α represses transcriptional activity of HBV enhancer I in concert with HNF-4 α (S. H. Wang et al., 2012). An orphan receptor, estrogen related receptor α (ERR α) confers metabolic environment for efficient HCMV replication (Hwang J, 2014). In summary, it seems clear that other nuclear receptors can influence viral replication, latency, and oncogenesis.
1.5. Hypothesis and goals

*Alpha herpesvirinae* subfamily members including BHV-1 and HSV-1 establish a life-long latent infection in sensory neurons and periodically reactivation from latency occurs. Since currently available treatments do not eliminate latently infected virus or prevent reactivation from latency, understanding the latency-reactivation cycle may provide novel therapeutic strategies.

As a result of stress, corticosteroids increase the frequency of reactivation. The synthetic corticosteroid, DEX mimics stress and accelerates HSV-1 reactivation in TG neuronal or organ cultures (Du et al., 2012; Halford WP, 1996). DEX also consistently initiates BHV-1 reactivation (Inman et al., 2002; C. Jones, 1998, 2003; C. Jones et al., 2006; C. Jones et al., 2000; Rock et al., 1992; Sheffy & Davies, 1972; C. Shimeld, Hill, Blyth, & Easty, 1990). Within 6 hours after DEX treatment in latently infected calves, lytic viral gene expression was detected in a subset of TG neurons (Winkler et al., 2002). DEX-inducible cellular transcription factors in TG have been identified and these factors stimulate BHV-1 productive infection as well as certain viral promoters of BHV-1 and HSV-1 (Sinani, Cordes, et al., 2013; Workman et al., 2012). The action of corticosteroids is primarily mediated by activation of the GR. Based on these observations, we hypothesized that activated GR by DEX initiates lytic cycle viral gene expression and productive infection during early stages of stress-induced escape from latency. **The goal of studies presented in chapter 3** was to examine viral gene expression and investigate whether GR initiates lytic cycle viral gene expression during early stages of stress-induced escape from latency. VP16 or bICP0 was expressed in GR+ neurons during escape from latency. Therefore, **the goal of studies presented in chapter 4** was to test
whether activated GR by DEX stimulates viral promoters through GREs that are within BHV-1 promoters. BHV-1 IEnu1 promoter that directs expression of bICP0 and bICP4 is stimulated by GR and DEX through the GREs. HCF-1 stimulates IE promoter activity of alphaherpesvirinae subfamily members by interacting with cellular transcription factors or chromatin modifiers and the viral protein VP16. Expression of the SGK1 kinase is stimulated by corticosteroids and this cellular factor may mediate certain aspects of reactivation from latency. Therefore, the goal of studies presented in chapter 5 was to test whether the SGK kinase regulates BHV-1 and HSV-1 productive infection. The observations in chapter 4 led us to hypothesize that HCF-1 and the GR cooperate to activate IEnu1 promoter activity. Consequently, the goal of studies presented in chapter 6 was to test the effect that HCF-1 and GR has an IEnu1 promoter activity.
Chapter 2. Materials and methods
**Cells and viruses**

Bovine kidney (CRIB), murine neuroblastoma cells (Neuro 2A), African green monkey kidney (Vero) cells were grown in Earle’s modified Eagle’s medium (EMEM) supplemented with 10% fetal calf serum, penicillin (10 U/ml), and streptomycin (100 g/ml).

The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa. Stock cultures of BHV-1 were grown in CRIB cells and was used for all studies. The KOS strain of HSV-1 was propagated in Vero cells.

**Calf studies**

All TG samples from calves used for this study were previously described (Workman et al., 2012). In brief, BHV-1-free crossbred calves (200 kg) were inoculated with $10^7$ PFU of BHV-1 into each nostril and eye as described previously (Inman, Lovato, et al., 2001; Inman et al., 2002; Lovato et al., 2003; Perez et al., 2005). Calves were housed under strict isolation and given antibiotics before and after BHV-1 infection to prevent secondary bacterial infections. At 60 days postinoculation (dpi), calves were injected intravenously (jugular vein) with 100 mg of DEX. Calves were then transported to the veterinary diagnostic lab. Prior to euthanasia by electrocution, calves were heavily sedated with xylene. After decapitation, TG were collected, and samples from each TG were formalin fixed and then paraffin embedded. The remainder of both TG was minced into small pieces and placed into a single 50-ml conical tube, and the tube was placed in a dry-ice ethanol bath. TG samples were then stored at 80°C. It took approximately 5 min to collect TG, mince the TG, place TG pieces in a 50-ml conical tube, and submerge the
tube in a dry-ice ethanol bath after decapitation. One calf was decapitated at a time to ensure samples were processed in a timely manner. Calves were decapitated in the same order in which they were injected with DEX to ensure that the time points after DEX treatment were as close as possible to the designated time point. Three calves/time point were used for these studies. Experiments were performed in accordance with the American Association of Laboratory Animal Care guidelines and the University of Nebraska IACUC committee.

Plasmids

pIE1 (IEtu1cat), pIE1Δ831 (IEtu1catΔ831), and pIE1Δ1018 (IEtu1catΔ1018) were obtained from Vickram Misra (University of Saskatchewan) and were described previously (V. Misra, Bratanich, Carpenter, & O'Hare, 1994; V1. Misra, Walker, Hayes, & O'Hare, 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 (Köppel, Vogt, & Schwyzser, 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 chloramphenicol acetyltransferase (Catez et al.) promoter minus vector (pCAT3-Basic Vector; Promega) at the unique KpnI and XhoI restriction enzyme sites.

The IEtu1 DEX response region (DRR) 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. The 3′-DRRΔGRE#2 contains an EcoRI restriction enzyme site in place of GRE#2 and lacks key nucleotides in the consensus GRE. 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.

A short hairpin RNA (shRNA) against HCF-1 was ligated into pSilencer 2.1-U6 neo shRNA vector and was provided by Dr. Thomas Kristie, NIH. All plasmids were prepared from bacterial cultures by alkaline lysis and 2 rounds of cesium chloride centrifugation.

**Immunohistochemistry**

Immunohistochemistry was performed essentially as previously described using the ABC kit (Vector Laboratories) (Meyer, Perez, Geiser, et al., 2007; Meyer, Perez, Jiang, et al., 2007; Winkler et al., 2002; Winkler, Schang, Doster, Holt, & Jones, 2000). In brief, TG from calves were fixed in neutral-buffered formalin and then embedded in paraffin. Thin sections (4 to 5 um) were cut and mounted onto slides. Tissue sections were incubated 20 min at 65°C followed by two incubations of 10 min in xylene and
rehydrated in graded alcohols. Tissue sections were then incubated with 0.03% hydrogen peroxide in phosphate-buffered saline (PBS; pH 7.4) for 20 min at room temperature to block endogenous peroxidase. After 3 washes in Tris-buffered saline (TBS; 5 min each) at room temperature, tissue sections were digested with 40 l of a ready-to-use proteinase K solution (catalog no. 53020; Dako) for 20 min at 37°C to enhance antigen retrieval. Tissue sections were then blocked with 5% normal serum diluted in TBS containing 0.25% bovine serum albumin (BSA) for 45 min at room temperature in a humidified chamber. A peptide-specific rabbit antibody was made that was directed against bICP0 (Affinity Bioreagents, Golden, CO), and the antibody was affinity purified. This antibody specifically recognizes bICP0 in infected or transfected cells. A VP16-specific rabbit polyclonal antibody was obtained from Vikram Misra (University of Saskatchewan, Saskatoon, CA). bICP0, VP16 or GR (cell signaling; catalog no. 3660S) antibody was used at a 1:500 dilution and incubated overnight in a humidified chamber at 4°C and were washed in TBS (pH 7.6) the next day. Biotinylated goat anti-rabbit IgG (Vector Laboratories; catalog no. PK-6101) or biotinylated donkey anti-mouse IgG (Vector Laboratories; catalog no. PK-6102) was then incubated with the section for 30 min at room temperature in a humidified chamber. Next, the avidin-biotinylated enzyme complex was added to slides for 30 min at room temperature in a humidified chamber. After 3 washes in TBS, slides were incubated with freshly prepared substrate (Vector Laboratories; catalog no. SK-4800), rinsed with distilled water, and counterstained with hematoxylin. Thin sections from mock infected or latently infected calves were used as a negative control.

**Immunofluorescence to examine GR localization**
CRIB or Neuro-2A cells were incubated in EMEM supplemented with 2% charcoal-stripped fetal bovine serum (FBS) at 37°C for 24 h and then placed on a glass slide for an additional 24 h. After DEX treatment, cells were fixed in 4% paraformaldehyde for 10 min, and confocal microscopy was performed. The GR primary antibody (catalog no. 3660; Cell Signaling) was diluted 1:250 in PBS with 0.05% Tween 20 and 1% BSA and incubated on coverslips for 2 h at room temperature. After three washes, coverslips were incubated with Alexa Fluor 488 goat anti-rabbit IgG (H L) (catalog no. A-11008; Invitrogen) at a dilution of 1:100 for 1 h in the dark. After the slides were washed, DAPI (4′,6-diamidino-2-phenylindole) staining was performed to visualize the nucleus. Coverslips were then mounted on slides by use of Gel Mount aqueous mounting medium (Electron Microscopy Sciences). Images were obtained with a Bio-Rad confocal laser-scanning microscope (MRC-1024ES).

**Western blot analysis**

Cells were washed with PBS and suspended in NP-40 lysis buffer (100 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and one tablet of complete protease inhibitor [Roche Molecular Biochemicals] in 10 ml buffer). Cell lysate was incubated on ice for 30 min, sonicated, and then clarified by centrifugation at 10,000 g at 4°C for 15 min. Protein concentrations were quantified by the Bradford assay. For SDS-PAGE, proteins were mixed with an equal amount of 1 sample loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 50 mM dithiothreitol, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. Proteins were separated in an 8 or 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) and blocked for 4 h
in 5% nonfat dry milk with Tris-buffered saline-0.1% Tween 20 (TBS-T). Membranes were then incubated with primary antibody overnight at 4°C. An antibody directed against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. After 45 min of being washed with TBS-T, blots were incubated with donkey anti-rabbit or anti-mouse horseradish peroxidase-conjugated immunoglobulin G (Amersham Biosciences), which was diluted 1:2,000 in 5% nonfat milk in TBS-T. Blots were washed 45 min with TBS-T and exposed to Amersham ECL reagents, and then autoradiography was performed. Primary antibodies were described above. Primary antibodies were diluted 1:1,000.

**Measurement of CAT activity**

Neuro-2A cells grown in 60 mm dishes were 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 hours prior to harvesting cells. At 48 hours after transfection, cell extract was prepared by three freeze/thaw cycles in 0.25M Tris–HCl, pH 7.4. Cell debris was pelleted by centrifugation, and protein concentrations were determined. CAT activity was measured by incubating with 0.1μCi (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 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 $\gamma^{32}$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 $\gamma^{32}$P-ATP. Incubation was for 1 hour 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, GGCAACTGGGAATTCGGCGTTCTGATCTCGC,
GRE consensus oligonucleotide, GACCCTAGAGGATCTGTACAG-GATGTTCTAGAT, (Santa Cruz, sc-2545), and GRE mutant oligonucleotide, GACCCTAGAGGATCTCAACAGGAT-CATCTAGAT (Santa Cruz, sc-2546).
**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 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 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 hour 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 4°C, and samples were continuously rotated. Seventy-five microliters of agarose protein A beads were added the next morning and allowed to incubate at 4°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 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:

GR forward: TCCCCGCTTTTGTTATCG
GR 1x reverse: CCCTACTTTTGCCTGTGTG
GR 2x reverse: GGCATTTAGTTTTGGTGGTTGG
TATA forward: CGGCCATGCTTTCATGCAAATGAGCCCCGACAGCC
TATA reverse: AGCAGCGGCAGCGGCAGGTGTTGCAGTACGGGTGT
All primers are 5’-3’.

**Analysis of apoptosis in tissue section**

Tissue sections, 4 to 5m thick slides were deparaffinized in xylene for 10min, rehydrate in a graded ethanol series, and washed by immersing the slides in 0.85% NaCl and PBS. TG sections were fixed by immersing the slides in 4% paraformaldehyde
solution for 15min at room temperature and then treated with 20 ug/ml proteinase K in PBS for 15min. Nick end labeling of DNA strand breaks in the tissue was performed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick and labeling (TUNEL) assay, which utilizes alkaline phosphatase (Promega). Slides were counterstained with methyl green (Vector laboratories) and then coverslipped with permanent mounting medium (Fisher).

Dual-luciferase reporter assay

Neuro-2A cells (8 X 10^5) were seeded into 60mm dishes containing EMEM with 10 % FCS at 24 hours prior to transfection. Cells were cotransfected with a plasmid containing the firefly luciferase gene downstream of MMTV LTR, pGL3 DRR or pGL3 empty vector (0.1ug), a plasmid encoding Renilla luciferase under the control of the herpesvirus thymidine kinase (TK) promoter (25ng), GR plasmid (0.5ug), and indicated amount of HCF-1 plasmid. To maintain equal plasmid amounts in the transfection mixtures, the empty expression vector was added as needed. Twenty-four hours after transfection, cells were treated with 10uM DEX. At 48 hours after transfection, cells were harvested and protein extracts were subjected to a dual-luciferase assay by using a commercially available kit (E1960) according to the manufacturer’s instructions. Luminescence was measured by using a GloMax 20/20 luminometer (E5331; Promega).
Chapter 3. Bovine herpesvirus 1 regulatory proteins bICP0 and VP16 are readily detected in trigeminal ganglionic neurons expressing the glucocorticoid receptor during the early stages of reactivation from latency

The studies in this chapter are included in two separate manuscripts that were published in the Journal of Virology and Journal of Neurovirology

Frizzo da Silva L, Kook I, Doster A, Jones C. 2013. Bovine herpesvirus 1 regulatory proteins bICP0 and VP16 are readily detected in trigeminal ganglionic neurons expressing the glucocorticoid receptor during the early stages of reactivation from latency. J Virol 87:11214-11222.

Abstract

Bovine herpesvirus 1 (BHV-1) establishes a lifelong latent infection in sensory neurons following acute infection. Increased corticosteroid levels, due to stress, increases the incidence of reactivation from latency. Within minutes, corticosteroids activate the glucocorticoid receptor and transcription of promoters containing a glucocorticoid receptor element. The synthetic corticosteroid dexamethasone consistently induces reactivation from latency. Cellular transcription factors are induced by dexamethasone in trigeminal ganglionic neurons within 1.5 h after dexamethasone treatment, suggesting they promote viral gene expression during the early phases of reactivation from latency, which we operationally defined as the escape from latency. Within 90 min after latently infected calves are treated with dexamethasone, two BHV-1 regulatory proteins, BHV-1-infected cell protein 0 (bICP0) and viral protein 16 (VP16), are expressed in the same neuron. Two other late proteins, glycoprotein C and D, were not detected until 6 h after dexamethasone treatment and were detected in only a few neurons. These studies provide evidence that VP16 and the promiscuous viral trans-activator (bICP0) are expressed during the escape from latency, suggesting they promote the production of infectious virus in a small subset of latently infected neurons. We now provide evidence that neurons expressing the glucocorticoid receptor expressed bICP0 or VP16 at 1.5 h after dexamethasone treatment. VP16 and bICP0 can also be detected at 22 and 33 min after dexamethasone (DEX) treatment of latently infected calves. However, we were unable to discern whether VP16 or bICP0 was expressed at early times after reactivation. VP16+ neurons consistently express the glucocorticoid receptor suggesting corticosteroid-mediated activation of its receptor rapidly stimulates reactivation from latency.
Introduction


The incidence of BHV-1 reactivation from latency is increased following stressful stimuli that increase corticosteroid levels (C. Jones & Chowdhury, 2007; C. Jones et al., 2011; G. C. Perng & Jones, 2010). Regardless of the reactivation stimulus, lytic cycle viral gene expression, which is nearly undetectable during latency, must be activated. Administration of the synthetic corticosteroid dexamethasone (DEX) to latently infected calves or rabbits initiates BHV-1 reactivation from latency 100% of the time (Inman et al., 2002; C. Jones, 1998, 2003; C. Jones et al., 2006; C. Jones et al., 2000; Rock et al.,
1992). Six hours after DEX treatment, lytic cycle viral RNA expression is readily detected in a subset of trigeminal ganglionic neurons of latently infected calves (Winkler, Doster, et al., 2000; Winkler et al., 2002). DEX treatment of latently infected calves induces apoptosis of T cells that persist in trigeminal ganglia (TG) after infection (Winkler et al., 2002). T cells also persist in TG of humans or mice latently infected with HSV-1 (Cantin, Hinton, Chen, & Openshaw, 1995; W. Halford, Gebhardt, & Carr, 1996; T. Liu, Tang, & Hendricks, 1996; C. Shimeld, Whiteland, Williams, Easty, & Hill, 1996, 1997; C. Shimeld et al., 1995; Theil et al., 2003) and are proposed to promote the maintenance of latency (K. Khanna, Bonneau, Kinchington, & Hendricks, 2003; Knickelbein JE, 2008; T. Liu, Khanna, Carriere, & Hendricks, 2001; Liu T, 2000).

Within 3 h after calves latently infected with BHV-1 are treated with DEX, 11 cellular genes are induced more than 10-fold in TG (Workman et al., 2012). Pentraxin 3, a regulator of innate immunity and neurodegeneration, is stimulated 35- to 63-fold at 3 or 6 h after DEX treatment. Two transcription factors, promyelocytic leukemia zinc finger (PLZF) and Slug, are induced more than 15-fold 3 h after DEX treatment. PLZF or Slug stimulates BHV-1 productive infection 20-fold or 5-fold, respectively, and Slug stimulates the late glycoprotein C promoter more than 10-fold. Additional DEX-induced transcription factors, sterile alpha motif (SAM) pointed domain-containing Ets transcription factor (SPDEF), Kruppel-like transcription factor 15 (KLF15), KLF4, KLF6, and GATA6, stimulate productive infection and certain key viral promoters. The DEX-inducible cellular transcription factors are predicted to mediate certain aspects of the early phases of reactivation from latency, including activation of lytic cycle viral gene expression. Latently infected neurons that express detectable levels of lytic cycle viral
proteins within 6 h after DEX treatment are operationally defined as escaping latency (Workman et al., 2012).

BHV-1-infected cell protein 0 (bICP0) and viral protein 16 (VP16) are expressed in the same neuron within 90 min after DEX administration to latently infected calves; conversely, two late proteins, glycoprotein C (gC) and gD, are not detected until 6 h after DEX treatment (Frizzo da Silva, Kook, Doster, & Jones, 2013). bICP0 and VP16 have functions that stimulate productive infection and consequently may initiate reactivation from latency. For example, HSV-1-encoded VP16 selectively activates immediate early (IE) gene expression (O'Hare & Goding, 1988; O'Hare & Hayward, 1985) and has been reported to be required for efficient reactivation from latency (Camarena et al., 2010; Kim et al., 2012; Thompson RL, 2009). Since the VP16 gene is expressed as a true-late gene during productive infection, one must assume that unknown neuronal specific factors stimulate VP16 promoter activity during the early stages of reactivation from latency if VP16 initiates reactivation from latency. It is also possible that VP16 may be preferentially trans-activated by viral regulatory proteins, and consequently, VP16 enhances the probability that the lytic cycle cascade is efficiently initiated during reactivation from latency. Regardless of how VP16 expression is induced, there is evidence that its expression is crucial for stimulating lytic cycle viral gene expression during reactivation from latency.

ICP0 proteins encoded by alpha-herpesvirinae subfamily members are multi-functional proteins that stimulate viral gene expression, interfere with innate immune responses, and consequently are important for productive infection (Boutell & Everett, 2013). Exogenous expression of ICP0, independent of other viral genes, initiates HSV-1
reactivation from latency using an in vitro neuronal culture system. Furthermore, ICP0 enhances productive infection in cell culture in the absence of VP16 (Cai & Schaffer, 1989). Finally, a bICP0 expression plasmid stimulates plaque-forming efficiency of BHV-1 DNA transfected into cultured cells (Geiser et al., 2002) and bICP0 mutants grow less efficiently than wild-type (wt) virus in cultured cells (Geiser, Zhang, & Jones, 2005; Saira et al., 2008). It is well established that stress-induced neuronal factors can stimulate the HSV-1 (Kushnir, Davido, & Schaffer, 2010; Sinani, Cordes, et al., 2013) or BHV-1 promoters (Workman et al., 2012) that drive ICP0 expression adding support to the hypothesis that ICP0 would be expressed and stimulates lytic cycle viral gene expression during early stages of reactivation from latency. In the context of in vivo reactivation from latency in the natural host, it is reasonable to hypothesize that ICP0 and VP16 play crucial roles in stimulating lytic cycle viral gene expression.

In this study, we demonstrated that bICP0 or VP16 and the glucocorticoid receptor (GR) were detected in the same neuron at 1.5 hour after DEX treatment. We further examined bICP0 or VP16 protein expression at early times after reactivation. bICP0- and VP16- positive TG neurons were detected at 22 and 33min after DEX treatment. VP16+ neurons consistently express the glucocorticoid receptor. These studies suggest that activation of the GR by DEX directly stimulates lytic cycle viral gene expression during the escape from latency.

Results

Sensory neurons that express bICP0 and VP16 frequently express the glucocorticoid receptor.
DEX, like the natural corticosteroids, binds and activates the glucocorticoid receptor (GR) (Funder, 1997; Schoneveld, Gaemers, & Lamers, 2004). Since GR is expressed in rat sensory neurons (DeLeón et al., 1994), we predicted that bICP0 and VP16 expression may be stimulated directly by DEX in neurons that express the GR. Support for this prediction comes from the finding that the bICP0 E promoter is stimulated by DEX in transient-transfection studies (Workman et al., 2009). Thus, it was of interest to determine (i) if the GR is expressed in a subset of bovine TG neurons and (ii) whether VP16 and bICP0 neurons express the GR.

Initial studies tested whether commercially available antiserum recognizes the bovine GR. Antiserum directed against GR (MR-20; Santa Cruz Biotechnology) recognizing mouse, rat, and human proteins was used for this study. Western blots revealed that the GR antiserum specifically recognized a protein with an approximate molecular weight of 90 in bovine kidney cells (CRIB) (Figure 3.1A, B lanes) and mouse cells (M lanes), which is the expected size of the GR (Funder, 1997; Schoneveld et al., 2004). Confocal microscopy demonstrated that the antiserum recognized a nuclear protein in CRIB cells following treatment with DEX (Figure 3.1B), which occurs when the GR binds DEX. Prior to DEX treatment, the GR was localized throughout CRIB cells. The GR antiserum also recognized a subset of TG neurons after treatment with DEX for 3 hour (Figure 3.1C). In general, the signal localized to the nucleus, suggesting the GR was activated after DEX treatment (denoted by arrows). In uninfected bovine TG, the signal was generally disperse and not readily detected compared to TG sections prepared from calves after DEX treatment. The signal appeared to be more concentrated in a few neurons from mock-infected calves (denoted by arrows), which may have been the result
of stress during transportation of calves to the necropsy room prior to euthanasia.

Consecutive sections were prepared from calves treated with DEX, and studies were performed to determine if the GR was expressed in sensory neurons expressing bICP0 or VP16. At 1.5 h after DEX treatment, GR and bICP0 neurons were detected in certain areas of TG sections (Figure 3.2A, double-positive neurons denoted by arrows). We detected 71 GR and bICP0 neurons but only 2 that were GR but not bICP0 and no bICP0 neurons that were GR negative. VP16 and GR neurons were also readily detected at 1.5 h after DEX treatment (Figure 3.2B, double-positive neurons denoted by arrows). GR neurons that were not VP16 were occasionally detected at 1.5 h after DEX treatment (Figure 3.2B, neurons denoted by closed circles). Seventy-two GR and VP16 neurons were detected, but only two were identified in the same field that were just GR. At 1.5 h after DEX treatment, no bICP0 or VP16 neurons were detected that were GR negative.

**Detection of bICP0 and VP16 during the escape from latency.**

Previous studies demonstrated that bICP0 and VP16 are detected in the same neurons at 90 min and 3 h after latently infected calves are treated with DEX to initiate reactivation from latency (Frizzo da Silva et al., 2013). To test whether bICP0 or VP16 could be detected at early times after reactivation, additional studies were performed using calves latently infected with BHV-1 that were given a single IV injection of DEX (100 mg) and TG extracted at 22, 33, or 75 min after DEX treatment (three calves per time-point) as previously described (C. Jones, 1998, 2003; C. Jones et al., 2006; C. Jones et al., 2000; Rock et al., 1992). Samples from latently infected calves or those treated with DEX for 3 or 6 h were from previous studies (Workman et al., 2012). Immunohistochemistry (IHC) studies confirmed that VP16+ neurons are detected at 3 h
after DEX treatment but not in latently infected TG (Figure 3.3A) (Frizzo da Silva et al., 2013). VP16+ neurons were also detected in TG neurons at 22 or 33 min after DEX treatment (Figure 3.3B, C respectively). Staining of VP16+ neurons at 22 or 33 min after DEX treatment was lighter compared to 3 hour, which was to be expected. bICP0+ neurons were weakly detected in certain sections at 22 min (Figure 3.4) and 33 min (data not shown) after DEX treatment. It was also clear that many TG sections at 22 or 33 min after DEX do not contain VP16+ neurons (for example, Figure 3.3B right panel) or bICP0+ neurons (Figure 3.4, 33 min panel for example and data not shown). At 75 min or 3 hour after DEX treatment, bICP0+ neurons were easier to detect in TG neurons.

Although it appeared that VP16 was expressed in more neurons at 22 or 33 min after DEX treatment compared to bICP0, the sensitivity of the antisera may have influenced these results. For example, the VP16 antiserum was prepared from a GST-VP16 fusion protein that was highly purified, whereas the bICP0 antiserum is directed against three peptides within bICP0 and was affinity purified. Thus, the VP16 antiserum would likely be directed against more epitopes than the bICP0 peptide-specific antiserum suggesting it is more sensitive. We have also tested two independent antibodies that recognize bICP4 because ICP4 proteins encoded by alpha-herpesvirinae subfamily members are necessary for productive infection (Smith, Bates, Rivera-Gonzalez, Gu, & DeLuca, 1993; Wu, Watkins, Schaffer, & DeLuca, 1996). However, these antibodies exhibited high background in TG sections making it impossible to discern whether bICP4 is expressed during the escape from latency. In summary, the studies in Figure 3.3 and 3.4 demonstrated that VP16 as well as bICP0 were detected in a subset of sensory neurons within 22 min after latently infected calves were treated with DEX. These studies
also indicated that DEX has a rapid effect on viral gene expression during BHV-1 reactivation from latency.

**VP16+ neurons also express the GR during the escape from latency.**

We further tested whether VP16 is expressed in neurons expressing the GR at 33 min after DEX treatment. VP16+ neurons were examined, as opposed to bICP0+ neurons, because VP16+ neurons were easier to identify at 22 or 33 min after DEX treatment (Figure 3.3 and 3.4). Consecutive sections from TG at were stained with VP16 or GR antiserum. In this section (Figure 3.5; neurons labeled 1–9) and many other sections (data not shown), nearly all VP16+ neurons expressed GR. As expected, GR+ neurons that do not express VP16 were also detected (neurons A–E). DEX-induced cellular transcription factors identified in TG neurons (Workman et al., 2012) or the activated GR (Kook, Henley, Meyer, Hoffmann, & Jones, 2015) do not trans-activate the BHV-1 VP16 promoter suggesting unknown cellular factors or perhaps viral proteins (bICP0 or bICP4) trans-activate the VP16 promoter during the escape from latency. Paradoxically, these same studies demonstrated that bICP0 promoters were trans-activated by DEX-induced cellular transcription factors (Workman et al., 2012) and that hormone-activated GR stimulates the bICP0 and bICP4 promoter (Kook, Henley, et al., 2015). Consequently, we suggest that the bICP0 protein is expressed early during the escape from latency because DEX directly or indirectly stimulates its expression via promoter activation.

**TUNEL analysis of TG from BHV-1 latently infected calves.**

To test whether cell death in TG neurons or non-neuronal cells prior to expression of VP16 and bICP0 during reactivation from latency, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed. The rationale for
these studies is summarized below. Although apoptosis of T cells persisting in TG occurs by 6 h after latently infected calves are treated with DEX (Winkler et al., 2002), earlier time points were not examined. Certain studies have concluded that T cells in TG of humans or mice latently infected with HSV-1 (Cantin et al., 1995; W. Halford et al., 1996; T. Liu et al., 1996; C. Shimeld et al., 1996, 1997; C. Shimeld et al., 1995; Theil et al., 2003) promote maintenance of latency (K. Khanna et al., 2003; Knickelbein JE, 2008; T. Liu et al., 2001; Liu T, 2000) suggesting apoptosis of infiltrating T cells enhances the escape from latency. Conversely, recent studies have suggested that specific populations of dendritic cells, not infiltrating T cells, regulate latency (Matundan, Mott, & Ghiasi, 2014; Mott et al., 2014). Secondly, apoptosis is proposed to stimulate productive infection (Prasad, Remick, & Zeichner, 2013) and reactivation from latency (Du et al., 2012). TG sections from latently infected calves were treated with DNase I and then TUNEL assays performed to discriminate between TUNEL+ cells versus counter-stained cells (Figure 3.6; control panels). Few TUNEL+ cells were identified during latency, at 23 or 33 min (data not shown) or 75 min after DEX treatment (Figure 3.6). TUNEL+ satellite and/or T cells, but not sensory neurons, were sporadically detected in TG sections at 3 h after DEX treatment. As expected (Winkler et al., 2002), TUNEL+ non-neuronal cells were consistently detected at 6 h after DEX treatment (Figure 3.6; arrows denote TUNEL+ cells). We believe these TUNEL+ cells are primarily T cells because TG from uninfected calves did not exhibit TUNEL+ cells 6 h after DEX treatment (Winkler et al., 2002). At 18 h after DEX treatment, we were able to detect TUNEL+ neurons (Winkler et al., 2002); prior to that, they were not detected. Since corticosteroids can cause apoptosis in cells (Chmielewski, Drupt, & Morfin, 2000; Schmidt et al., 1999),
including certain neuronal subtypes (Mitchell et al., 1998) and lymphocytes (Chmielewski et al., 2000), we suggest that signaling pathways induced by apoptotic stimuli, not apoptosis itself, may enhance the escape from latency. The ability of human herpesviruses to adopt an emergency escape alternative replication program when apoptosis signaling pathways are initiated (Prasad et al., 2013) may enhance viral gene expression during the escape from latency. However, apoptosis per se does not appear to directly lead to reactivation from latency during DEX-induced reactivation in calves latently infected with BHV-1.

**Discussion**

In this study, neurons that expressed bICP0 or VP16 also frequently expressed the GR. In general, stress increases corticosteroid levels and activates the GR, which we predict is a molecular switch that can consistently initiate the escape from latency. During the escape from latency, several key events occur that result in lytic cycle viral gene expression. First, specific DEX-induced cellular transcription factors stimulate certain viral promoters and productive infection (Workman et al., 2012). Second, lytic cycle viral gene expression, including that of the bICP0 and VP16 genes, may also be stimulated directly by an activated GR. Third, DEX represses expression of latency-related (Melroe, DeLuca, & Knipe) gene products (Jaber et al., 2010; Rock et al., 1992; Sinani, Frizzo da Silva, et al., 2013), which is important because certain LR gene products can inhibit productive infection (Bratanich, Hanson, & Jones, 1992; Geiser et al., 2002; Jaber et al., 2010; Workman et al., 2011). Finally, apoptosis of T cells in TG that persist during latency occurs following DEX treatment of latently infected calves (Winkler et al., 2002), which may enhance the incidence of reactivation from latency because CD8 T cells are
proposed to maintain latency (Decman, Freeman, Kinchington, & Hendricks, 2005a; K. Khanna et al., 2003; Knickelbein JE, 2008; T. Liu et al., 2001; Liu T, 2000). We predict that the pleiotropic effects of increased corticosteroids levels and activation of the GR lead to the escape from latency.

DEX, as with other natural corticosteroids, specifically bind the GR and mineralocorticoid receptor (Mitchell et al.) (Funder, 1997), resulting in nuclear localization of the GR or MR (Pratt & Toft, 1997). Nuclear GR or MR dimers stimulate transcription by binding consensus glucocorticoid response elements (GRE; 5′-GGTACANN NTGTCTCT-3′) (Giguère, Hollenberg, Rosenfeld, & Evans, 1986; J. Wang et al., 2004). A GR or MR monomer can also stimulate transcription by binding to a GR (1/2) binding site, of which the consensus 1/2 binding site is TGTTTCT or GGTACA, (Schoneveld et al., 2004). Although it is possible that MR TG neurons promote reactivation from latency, we were unable to identify a commercially available antibody that recognizes the MR receptor in bovine cells (data not shown). Within 5 min of glucocorticoid treatment, GR enters the nucleus, binds to glucocorticoid response elements, and induces rapid changes in chromatin conformation and transcriptional activation (J. Chen, Kinyamu, & Archer, 2006; Deroo & Archer, 2001). The BHV-1 genome contains 58 GR (1/2) binding sites in 24 BHV-1 promoters (data not shown), and a previous study demonstrated that DEX stimulates the bICP0 early promoter in transient-transfection assays (Workman et al., 2009). It will be of interest to directly test whether viral promoters are actually bound by a GR during the escape from latency.

This study also found new and important observations that add to our understanding of the early steps during DEX-induced escape from latency. VP16 as well
as bICP0 were detected within 30 min in TG neurons after latently infected calves were treated with DEX. Compared to mouse models of HSV-1 reactivation from latency, BHV-1 reactivation is very rapid and more efficient highlighting the importance of using a natural host model to study reactivation from latency in vivo, reviewed in (Clinton Jones, 2014). Viral protein expression was detected in TG neurons prior to detection of TUNEL+ neurons or non-neuronal cells. It is well established that the abundant viral genes expressed in neurons latently infected with BHV-1 (LR; latency-related gene) or HSV-1 (LAT; latency-associated transcript) interfere with neuronal apoptosis (Ciacci-Zanella, Stone, Henderson, & Jones, 1999; G. Perng et al., 2000). Consequently, the inability to identify neuronal apoptosis during the early stages of reactivation from latency may be due to the inability of DEX to induce apoptosis in sensory neurons as well as the anti-apoptotic functions of the LR gene.
Figure 3.1.
Figure 3.1. Detection of GR in bovine kidney cells (CRIB) and TG neurons.

Panel A. Bovine cells (CRIB) or mouse neuroblastoma cells (Neuro-2A) (30 ug protein/lane) were electrophoresed in an SDS-PAGE gel, and Western blot analysis was performed using the commercially available GR antibody (Santa Cruz; H-300). Lanes denoted B are cell lysate prepared from bovine kidney cells (CRIB), and lanes denoted M are cell lysate from mouse neuroblastoma cells (Neuro-2A).

Panel B: CRIB cells were treated with DEX, and immunofluorescence was performed as described in Materials and Methods. As controls, certain cultures were not treated with DEX. Localization of the GR was examined by immunofluorescence. Nuclear DNA was identified by DAPI staining.

Panel C: IHC was performed with TG samples from uninfected calves and 3 hour after DEX treatment using procedures described in Materials and Methods. Arrows denote the GR neurons. Magnification, 400.
Figure 3.2.
Figure 3.2. GR neurons frequently express bICP0 or VP16 during the escape from latency.

Consecutive sections were cut from formalin-fixed paraffin-embedded TG from calves latently infected with BHV-1 that were treated with DEX for 1.5 h. Arrows denote neurons that were recognized by the respective antibody. Closed circles denote GR neurons that were VP16 negative. Magnification is 400.

Panel A. IHC was performed using the bICP0 antibody on one section and the GR antibody on the adjacent section.

Panel B. IHC was performed using the VP16 antibody on one section and the GR antibody on the adjacent section.
Figure 3.3.
Figure 3.3. VP16 is detected in sensory neurons during the escape from latency.

IHC was performed using the VP16-specific antibody to stain TG sections from latently infected calves (latency), 22min, 33min, 75min, or 3 hour after DEX treatment. The VP16 antibody was diluted 1:500 and the secondary antibody. Arrows denote neurons recognized by the VP16 antibody. Magnification is approximately ×400
Figure 3.4.
Figure 3.4. The viral regulatory protein bICP0 is detected in sensory neurons during the escape from latency.

IHC was performed using the bICP0 antibody to stain TG sections from latently infected calves (latency), 22 min, 33 min, 75 min, or 3 h after DEX treatment. The bICP0 antibody was diluted 1:1000. TG sections were counter-stained lightly with methyl green. Arrows denote neurons recognized by the bICP0 antibody. Magnification is ×200
Figure 3.5. VP16+ neurons also express the GR during the escape from latency.

Consecutive sections were cut from formalin-fixed paraffin-embedded TG from calves latently infected with BHV-1 treated with DEX for 33 min. IHC was performed using the VP16 antibody, which was diluted 1:500, on one section. The adjacent section was stained with a GR antibody that was diluted 1:250. Neurons numbered 1–9 and GR+ and VP16+ neurons whereas neurons A–E are only GR+. TG sections were counter-stained lightly with methyl green. Magnification is ×400
Figure 3.6.
Figure 3.6. Identification of TUNEL+ cells in TG sections following DEX treatment of latently infected calves.

TG sections prepared from latently infected calves or calves treated with DEX for 75 min, 3 h, or 6 h were examined for TUNEL reaction to detect cells undergoing cell death. Arrows denote TUNEL+ cells. The different panels from the same time after DEX treatments are from different calve
Chapter 4. Bovine herpesvirus 1 productive infection and immediate early transcription unit 1 promoter are stimulated by the synthetic corticosteroid dexamethasone.

The studies in this chapter are included in a manuscript recently published in Virology

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 in (C. Jones, 1998, 2003, 2009; C. Jones & Chowdhury, 2007; C. Jones et al., 2006). 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 in C. Jones, 1998, 2003). IE gene expression is stimulated by a virion component, VP16 (V. Misra et al., 1994; V1. Misra et al., 1995). Two BHV-1 IE transcription units exist: IE transcription unit 1 (IEtu1) and IEtu2 (Wirth et al., 1992; Wirth, Gunkel, Engels, & Schwyzer, 1989; Wirth, Vogt, & Schwyzer, 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; Wirth et al., 1989; Wirth et al., 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 (Köppel et al., 1997; Schwyzer, Wirth, B., & Fraefel, 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 (C. Jones & Chowdhury, 2007, 2010; C. Jones et al., 2011). A single IV injection of the synthetic corticosteroid dexamethasone (DEX) induces BHV-1 reactivation from latency 100% of the time (Inman et al., 2002; C. Jones, 1998, 2003; C.
Jones et al., 2006; C. Jones et al., 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; W. 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, Kim, Dubovi, & Bicalho, 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 (Mitchell et al.) (reviewed in (R. H. Oakley & 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′-GGTACANNNNTGTTCT-3′) and remodeling chromatin (Giguère et al., 1986; J. 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 & Cidlowski, 2005). Approximately 50% of TG sensory neurons express the GR (DeLeón et al., 1994) and the MR is also expressed in neurons (Arriza, Simerly, Swanson, & Evans, 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, Doster, et al., 2000; Winkler et al., 2002). 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, Cordes, et al., 2013) 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. 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 trans-
activation. 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

The IEtu1 and IEtu2 promoters contain GRE-like motifs

Based on previous studies demonstrating that increased corticosteroids consistently induce BHV-1 reactivation from latency (reviewed in C. Jones, 2013; C. Jones et al., 2011), we hypothesized that corticosteroids directly stimulate viral gene expression. DEX stimulates productive infection in cultured bovine cells, suggesting 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 (Figure 4.1A). 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 (V. 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 (Figure 4.1B and Figure 4.2A).

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 Figure 4.2A 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, Frizzo da Silva, et al., 2013; Sinani, Liu, & Jones, 2014) confirming they have certain neuronal features. IEtu1cat, but not IEtu1catΔ831 or IEtu1catΔ1018, was stimulated approximately 8 fold by DEX treatment (Figure 4.2B), 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 (Köppel 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 (Figure 4.2B). The mouse mammary tumor virus (MMTV) LTR was used as a positive control because it contains multiple GREs (Chandler, Maler, & Yamamoto, 1983; Kühnel, Buetti, & Diggelmann, 1986), and as expected was stimulated approximately 40 fold by DEX (Figure 4.2C).

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 RL, 2009). Since VP16 selectively activates IE gene expression (O'Hare & Goding, 1988; O'Hare &
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 (Figure 4.2B).

**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) (Figure 4.3A and B). All nucleotides in GRE#1 match the required or preferred nucleotides in the consensus GRE (Figure 4.3C). 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 (Figure 4.3D, 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 Figure 4.3C for wt and mutated sequence), this construct was stimulated by DEX only 2 fold (Figure 4.3D, 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.
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 (Figure 4.4A); conversely only one shifted band was present when an oligonucleotide containing a mutated GRE was incubated with cell lysate (Figure 4.4A). 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 (Figure 4.4A, 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 (Figure 4.4B). 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 & Stunnenberg, 1993). Shifted bands were not readily detected when GRE#2 (wt or mutant) was incubated with cell lysate from Neuro-2A cells (Figure 4.4C), which was surprising because there is only one mismatched nucleotide in GRE#2 compared to the consensus (Figure 4.3C).

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 (Figure 4.4D).

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 quite as efficient as the cold competitor GRE#1 (Figure 4.4E). 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, Figure 4.5A) were used to amplify DNA immunoprecipitated by the GR antibody (Figure 4.5B, 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 (Figure 4.5B, lanes # 3) indicating the GR was not bound to this region. In samples transfected with IEtu1cat promoter that lacks both GREs (IEtu1cat∆831), 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 immuno-precipitation with the GR antibody. An isotype control antibody did not specifically immunoprecipitate viral DNA that was amplified by the respective primers (Figure 4.5C). PCR performed with non-immunoprecipitated samples (Figure 4.5D), 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 (Figure 4.4 and 4.5). Reduced levels of endogenous GR were detected after Neuro-2A cells were treated with DEX (Figure 4.6A), which was consistent with an independent study (Nishimura et al., 2001). 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 (Figure 4.6B).
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 (R. H. Oakley & Cidlowski, 2013; Rhen & 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 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 infection. Although it is well established that increased corticosteroid levels stimulate BHV-1 reactivation from latency (C. Jones, 1998, 2003; C. Jones & Chowdhury, 2007; C. Jones et al., 2011), 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 in Knipe & 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 (Q. Li & Wrange, 1993; Perlmann, 1992) and initiate formation of a nuclease-hypersensitive site and transcription (Richard-Foy & Hager, 1987; K. Zaret & 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 & Zaret, 2014; K. S. Zaret & Carroll, 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, Astrand, & Wrange, 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 (R. H. Oakley & Cidlowski, 2013; Rhen & 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, Freeman, Kinchington, & Hendricks, 2005b; K. M. Khanna, Lepisto, Decman, & Hendricks, 2004; Knickelbein JE, 2008; Liu T, 2000). Thus, additional studies are needed to completely understand the complex virus-host interactions that regulate stress-induced reactivation from latency.
Figure 4.1.
Figure 4.1. The IEtu1 and IEtu2 promoters contain GRE-like motifs.

Panel A. Diagram of linear BHV1.1 genome (horizontal line) with *in-silico* predicted GR responsive elements (GREs) denoted by vertical lines. Lines above the genome represent GREs present on the positive/forward DNA strand, while lines below indicate GREs on the negative strand. The terminal repeats are denoted by the grey rectangles. Numbers denote the genomic coordinates.

Panel B. Expanded genomic region that includes bICP0, bICP4 and bICP22 ORFs. The genomic interval between bCIP4 and bICP22 contains the origin of replication (ORIs) (denoted by the black diamond) and IEtu1 and IEtu2 to the left and right of the ORIs respectively (not highlighted). Black boxes denote enlarged GREs on positive (above) or negative strands (below). GRE#1 and GRE#2 are highlighted and genomic locations provided. Numbers indicate genomic coordinates for the beginning and end of coding sequences. This figure was generated by the co-authors at Mississippi State University and is included to allow readers to understand where GREs are located on the BHV-1 genome.
Figure 4.2.
Figure 4.2. 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 (Burgon et al.), TATA box, binding site for VP16/Oct1 denoted as TAATGARAT (V. 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., 2012; Workman et al., 2009). 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.
Figure 4.3.

A

B

C

Consensus GRE:  
GRE#1:  
ΔGRE#1:  
GRE#2:  
ΔGRE#2:  

tat t a ctc  
gGaACANNNTGTtCT  
GGAACACTGTGTCC  
GGAATTCTCGCATAA  
GGTACACTGTGtGC  
gGAATTCCGCGATCT

D

E

Promoter activity

PCAT3-promoter  
DRR  
5'-DRR  
3'-DRR  
3'-DRR<GRE#1  
3'-DRR<GRE#2  
3'-DRR<2xGRE  

MMTV
**Figure 4.3. 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.
Figure 4.4.
Figure 4.4. 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.
Figure 4.5.

A

CAT

255 bp product; TATA

GRE1x; 244 bp product

GRE2x; 543 bp product

B

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<th>DEX</th>
<th>GR+Δ831</th>
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**Figure 4.5. The GR interacts with the IEtu1 promoter in transfected cells.**

Panel A. Schematic of IEtu1 promoter shown in Figure 4.2A. 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 & Jones, 2009).

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.
Figure 4.6.
Figure 4.6. 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.
Chapter 5. The serum and glucocorticoid-regulated protein kinases (SGK) stimulate bovine herpesvirus 1 and herpes simplex virus 1 productive infection

The studies in this chapter are included in a manuscript that was recently published in Virus Research

Abstract

Serum and glucocorticoid-regulated protein kinases (SGK) are serine/threonine protein kinases that contain a catalytic domain resembling other protein kinases: AKT/protein kinase B, protein kinase A, and protein kinase C-Zeta for example. Unlike these constitutively expressed protein kinases, SGK1 RNA and protein levels are increased by growth factors and corticosteroids. Stress can directly stimulate SGK1 levels as well as stimulate bovine herpesvirus 1 (BoHV-1) and herpes simplex virus 1 (HSV-1) productive infection and reactivation from latency suggesting SGK1 can stimulate productive infection. For the first time, we provide evidence that a specific SGK inhibitor (GSK650394) significantly reduced BoHV-1 and HSV-1 replication in cultured cells. Proteins encoded by the three BoHV-1 immediate early genes (bICP0, bICP4, and bICP22) and two late proteins (VP16 and gE) were consistently reduced by GSK650394 during early stages of productive infection. In summary, these studies suggest SGK may stimulate viral replication following stressful stimuli.

Introduction

Alphaherpesvirinae subfamily members, including herpes simplex virus 1 (HSV-1) and bovine herpesvirus 1 (BoHV-1), initiate infection on mucosal layers (reviewed in C. Jones, 1998, 2003, 2009; C. Jones & Chowdhury, 2007; C. Jones et al., 2006). Although immune responses clear infection, life-long latency is established in sensory neurons. Periodically, reactivation from latency occurs, which is crucial for virus transmission. In general, increased “stress” correlates with a higher incidence of reactivation from latency in humans (Cassidy, Meadows, Catalán, & Barton, 1997; Glaser, Kiecolt-Glaser, Speicher, & Holliday, 1985; Padgett et al., 1998). In keeping with these observations, the synthetic corticosteroid dexamethasone accelerates reactivation from latency in TG
neuronal cultures or TG organ cultures prepared from mice latently infected with HSV-1 (Du et al., 2012; W. Halford et al., 1996). Stress, as a result of food and water deprivation during shipping of cattle, weaning, and/or dramatic weather changes increases corticosteroid levels and the incidence of BoHV-1 reactivation from latency (reviewed in C. Jones, 2013; Clinton Jones, 2014). Furthermore, the synthetic corticosteroid dexamethasone initiates BoHV-1 reactivation from latency 100% of the time (Inman et al., 2002; C. Jones, 1998, 2003; C. Jones et al., 2006; C. Jones et al., 2000; Rock et al., 1992; Sheffy & Davies, 1972; C. Shimeld et al., 1990). Stressful stimuli generally lead to increased corticosteroids levels, which readily diffuse into a cell, and activate the glucocorticoid receptor (GR) or mineralocorticoid receptor (Mitchell et al.) by binding to these receptors, reviewed in (Oakley and Cidlowski, 2013). Activation of the GR and/or MR may stimulate reactivation from latency by several distinct mechanisms.

The family of serum and glucocorticoid-regulated protein kinases (SGK) contains three members (SGK1, SGK2, and SGK3) (reviewed in Pearce, Komander, & Alessi, 2010). SGKs are serine/threonine protein kinases that have a catalytic domain similar to the Akt/protein kinase B, protein kinase A, protein kinase C-zeta, and rat p70S6 K/p85S6 K kinases (reviewed in Pearce et al., 2010). SGK1, but not SGK2, mRNA and protein levels are rapidly stimulated by corticosteroids, mitogenic, and cellular stress signals in a cell type and stimulus-dependent manner (Bell et al., 2000; Brennan & Fuller, 2000; Buse et al., 1999; Imaizumi, Tsuda, Wanaka, Tohyama, & Takagi, 1994; Maiyar, Huang, Phu, Cha, & Firestone, 1996; Mizuno & Nishida, 2001; Webster, Goya, Ge, Maiyar, & Firestone, 1993). All SGK family members can transduce signals that are vital for cell survival and proliferative responses (Brunet et al., 2001; Mikosz, Brickley, Sharkey,
In this study, we tested whether inhibiting SGK kinase activity influenced BoHV-1 and HSV-1 productive infection. Our results demonstrated that a specific SGK inhibitor (GSK650394) consistently and significantly reduced viral replication. Additional studies revealed GSK650394 interfered with BoHV-1 protein expression during early stages of productive infection. However, GSK650394 did not interfere with dexamethasone-mediated activation of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter or the BoHV-1 immediate early transcription unit 1 (IEtu1) promoter. In summary, this study suggested SGK family members might stimulate viral replication following stressful stimuli.

Results

**GSK650394 inhibits viral replication in a dose-dependent manner**

To test whether SGK kinases regulate productive infection, the effects of the only commercially available inhibitor of SGK enzymes (GSK650394) were examined. GSK650394 has 50% inhibitory concentration (IC$_{50}$) values of 62nM and 103nM for SGK1 and SGK2 respectively (Sherk et al., 2008) and (Selleckchem.com). Since SGK3 shares extensive similarity to SGK1 and SGK2, it is assumed GSK650394 inhibits SGK3 activity. Cells were pretreated with the designated levels of GSK650394 for 3h and then infected with BHV-1 at an MOI of 0.01. Although DMSO (solvent control) had no effect on viral replication when cultures were grown in 10% FBS, it slightly reduced BHV-1 titers when CRIB cells were incubated with stripped FBS after infection (Figure 5.1A). BHV-1 replication was reduced by GSK650394 in a dose dependent manner in cells containing normal FBS or stripped FBS. Viral replication in cells incubated with stripped
FBS cell culture was affected more than normal FBS when treated with 10 or 25 nM GSK650394. Furthermore, plaque formation was not observed at 50nM and 100nM when cells were incubated with stripped FBS prior to GSK650394 and infection.

Additional studies assessed whether GSK650394 inhibited HSV-1 replication. As described for BHV-1 studies, Vero cells (pre-treated with GSK650394 for 3 hour) were infected with HSV-1 at an MOI of 0.01. HSV-1 replication was significantly reduced when 50 or 100nM levels of GSK650394 were used (Figure 5.1B). As with BHV-1, the effect of GSK650394 on viral replication was more profound when cells were incubated with stripped FBS after infection because no plaques were observed when 50 or 100 nM GSK650394 were used. At 1 nM GSK650394 concentration, viral replication was not significantly inhibited. If a moi of 1 was used for BHV-1 and HSV-1, GSK650394 had a strong inhibitory effect on viral replication (data not shown).

The effects of GSK650394 on CRIB and Vero cell viability were examined by measuring ATP content in cells, which is a reliable measure of metabolically active cells. Cell viability was not significantly reduced by GSK650394 in CRIB or Vero cells using drug concentrations ranging from 1 to 100nM for 24 hour (Figure 5.1C). In summary, these studies demonstrated that the SGK inhibitor GSK650394 reduced BHV-1 and HSV-1 replication in CRIB or Vero cells respectively.

Analysis of SGK1 protein expression following infection

We examined the effects of BoHV-1 productive infection on SGK1 protein levels because SGK1, but not SGK2 or SGK3, is stimulated by stressful stimuli (Webster et al., 1993). Infection consistently led to a slight increase of SGK1 protein levels at 4 h, but not 8 h after infection (Figure 5.2). SGK1 expression was reduced at 16 h after infection,
which may reflect a function of the virus-host shutoff function or depletion of growth factors in media. At 8 and 16 h after infection, a faint high molecular weight band was recognized by the antibody, which was readily detected upon prolonged exposure (Figure 5.2, middle panel). Since SGK1 isoforms exist as a result of alternative splicing and the protein can be phosphorylated (Burgon et al., 2014; Prince et al., 2004; Webster et al., 1993), this band may represent a phosphorylated band or a novel SGK1 isoform. In summary, these studies demonstrated that BoHV-1 infection did not dramatically alter SGK1 protein levels during early stages of productive infection.

**GSK650394 effects on viral protein synthesis**

We next examined whether GSK650394 influenced steady state levels of certain BHV-1 proteins during productive infection. Since we were interested in comparing viral protein levels during early stages of infection, we increased the MOI to 2. Steady state protein levels of the three BoHV-1 immediate early genes (bICP0, bICP4, and bICP22) were consistently reduced by 25 nM GSK650394 at 4 and 8 h after infection. In particular, bICP22 protein expression was reduced 10 fold at 4 h after infection in the presence of GSK650394. At 16 h after infection, similar levels of bICP0 and bICP22 proteins were detected in DMSO control treated and GSK650394 treated cells whereas a slight reduction of bICP4 protein was still observed in the presence of GSK650493. GSK650394 inhibited steady state protein levels of a true late gene (VP16) by approximately 100 fold at 8 h after infection. In contrast to VP16, steady state protein levels of a leaky late gene (gE) was detected at 4, 8, and 16 h after infection: however gE protein levels were only reduced 3-fold by GSK650394. The gE protein is a glycosylated protein: however cultures treated with GSK650394 did not contain obvious differences in
gE isoforms. In summary, these data demonstrated GSK650394 reduced steady state viral protein levels, which is consistent with its ability to interfere with productive infection (Figure 5.3).

**Effects of GSK650394 on viral promoter activity**

To determine whether reduction of viral protein expression following GSK650394 treatment was the result of transcriptional repression, the IEtu1 and E promoters were examined. Three IEtu1 promoter constructs (IEtu1cat, IEtu1catΔ831 and IEtu1catΔ1018) and two E promoter constructs (EP943 and EP172) that contain upstream sequences of respective promoters were transfected into mouse neuroblastoma (Neuro2A) cells. At 48 hours after transfection, CAT activity was assayed following GSK650394 treatment for 24 hours. None of IEtu1 promoter constructs showed significant change in promoter activity following GSK650394 treatment. EP943, but not EP172 was stimulated approximately 4 fold by GSK650394 treatment compared to DMSO treated control.

Taken together, these results suggested that inhibitory effect of GSK650394 on viral protein synthesis is mediated at a post-transcriptional level. It is also possible that GSK-650394 interferes with entry.

**Effects of GSK650394 on DEX mediated viral promoter activation**

Previous studies demonstrated that the synthetic corticosteroid dexamethasone stimulated the immediate early transcription unit 1 (IEtu1) promoter via glucocorticoid receptor elements (GREs) located in the promoter (Kook, Doster, & Jones, 2015; Kook, Henley, et al., 2015). For these studies, we utilized mouse neuroblastoma cells (Neuro-2A) because they can be readily transfected and we know DEX stimulates IEtu1 promoter activity in these cell lines (Kook, Henley, et al., 2015). Conversely, CRIB cells
are not readily transfected. The mouse mammary tumor virus long terminal repeat (MMTV LTR) was also tested because it contains GREs, which are activated by dexamethasone (Funder, 1997; R. H. Oakley & Cidlowski, 2013; Richard-Foy & Hager, 1987). At 24 hour after transfection, 10 nM dexamethasone was added to cultures in the presence of DMSO or different concentrations of GSK650394. As expected, IEtu1 (Figure 5.5A) and MMTV LTR promoter (Figure 5.5B) activation were induced by dexamethasone treatment and dexamethasone stimulated the MMTV LTR more than the IEtu1 promoter (Kook, Henley, et al., 2015). However, 25 or 100 nM GSK650394 did not significantly decrease basal levels of MMTV LTR or IEtu1 promoter activity.

**Discussion**

In this study, new evidence is provided demonstrating that GSK650394 interferes with BoHV-1 and HSV-1 replication. Three SGK genes have been identified in humans, SGK1, SGK2, and SGK3, and each has at least 80% similarity in their catalytic domains (T. Kobayashi, Deak, Morrice, & Cohen, 1999). SGK1 and SGK3 are expressed in every tissue, whereas SGK2 was reported to be expressed primarily in the liver, kidney, pancreas, and brain (T. Kobayashi et al., 1999). There are at least four alternatively spliced isoforms for each gene in human neutrophils (Burgon et al., 2014; Prince et al., 2004). Since GSK650394 has an effect on each SGK family member, additional studies will be necessary to identify which SGK isoform(s) are important for inhibiting viral replication. In particular, it will be of interest to determine which isoforms are expressed in sensory neurons and whether SGK influences reactivation from latency. The increased antiviral effect GSK650394 had when cells were incubated with stripped serum implicated GSK1 as being more important than the other family members because its
mRNA and protein levels are regulated by serum and corticosteroids (Webster et al., 1993). Nearly all cells contain nuclear GR when grown in the presence of FBS (Kook, Henley, et al., 2015), which is indicative of an activated GR (reviewed in R. H. Oakley & Cidlowski, 2013; Schoneveld et al., 2004). Conversely, the GR is localized to the cytoplasm in most cells following incubation with stripped FBS (Kook, Henley, et al., 2015).

The finding that GSK650394 had no effect on the ability of DEX and the GR to stimulate the MMTV LTR and the BHV-1 IEtu1 promoter was somewhat expected because the ability of the GR to stimulate transcription does not require protein synthesis (reviewed in R. H. Oakley & Cidlowski, 2013; Schoneveld et al., 2004). However, SGK1 gene expression is induced by growth factors and glucocorticoids (Webster et al., 1993): consequently de novo SGK1 protein synthesis is necessary. Based on these observations, it appears that activation of the MMTV LTR and IEtu1 promoter by dexamethasone is upstream of SGK kinase activity.

Although SGK protein kinase activity may regulate certain aspects of productive infection and conceivably reactivation from latency, corticosteroids are likely to influence reactivation by additional means. For example, HSV-1 productive infection is stimulated when cells are pre-treated with dexamethasone (Erlandsson et al., 2002). This stimulatory effect may occur because a functional GR binding site is present in a HSV-1 origin of replication (Hardwicke MA, 1997). BHV-1 productive infection is also stimulated by dexamethasone, in part because an activated GR directly stimulates the BHV-1 IEtu1 promoter (Kook, Henley, et al., 2015). The IEtu1 promoter drives expression of two viral regulatory proteins, bICP0 and bICP4 (Wirth et al., 1992; Wirth
et al., 1989; Wirth et al., 1991). Two BoHV-1 regulatory proteins (bICP0 and VP16) are induced during reactivation from latency and their expression is primarily detected in GR+ sensory neurons (Frizzo da Silva et al., 2013; Kook, Doster, et al., 2015) underscoring the impact that an activated GR has on stimulating viral gene expression during reactivation. In addition to the direct effects that the GR may have on viral transcription, dexamethasone rapidly stimulates expression of several cellular transcription factors in sensory neurons that can activate the ICP0 promoters of BHV-1 (Workman et al., 2012) and HSV-1 (Sinani, Cordes, et al., 2013).

Increased corticosteroid levels can also interfere with immune responses, (reviewed in Barnes, 1998; Funder, 1997; Schoneveld et al., 2004), which we suggest will indirectly promote reactivation from latency. For example, T cells undergo apoptosis following corticosteroid treatment (Barnes, 1998; Funder, 1997; Migliorati et al., 1994; Schoneveld et al., 2004), and soluble factors produced by T cells can interfere with reactivation from latency (Decman et al., 2005b; Knickelbein JE, 2008). Furthermore, the activated GR can interact with two cellular transcription factors, activating protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- B) (reviewed in R. H. Oakley & Cidlowski, 2013; Rhen & Cidlowski, 2005; Smoak & Cidlowski, 2004). Interactions between the GR and AP-1 and NF- B would repress expression of tumor necrosis factor-alpha (TNF-alpha), interleukin 1-beta, as well as other inflammatory cytokines because AP-1 and/or NF- B trans-activate the promoters of these respective genes. AP-1 and NF- B are also required for activating the beta-interferon promoter/enhancer following virus infection (Goodbourn, Zinn, & Maniatis, 1985), which is significant because beta-interferon has anti-herpesvirus activity, including the
ability to reactivate from latency (Hendricks et al., 1991; Katze, He, & Gale, 2002; Leib et al., 1999; T. Liu et al., 2001; Melroe et al., 2004; Mikloska & Cunningham, 2001). In summary, the ability of corticosteroids to interfere with immune responses would likely increase herpesvirus replication and virus shedding during the course of reactivation from latency.
Figure 5.1.
Figure 5.1. The SGK inhibitor inhibits BoHV-1 replication in a dose-dependent manner.

Panel A. CRIB cells were incubated in 10% fetal calf serum and then sub-cultured 24 h prior to infection. Three hours prior to infection, CRIB cells were pretreated with the designated concentration of GSK650394 (nM) or untreated (U) and then infected with BoHV-1 at an MOI of 0.01. Medium containing 10% fetal calf serum (black columns) or 2% stripped fetal calf serum (hatched columns) was then added. Infectious virus was collected at 24 h after infection by three rounds of freeze-thawing and then plaque assays performed. The number of plaques present in cells infected in the presence of DMSO (solvent control) was set at 100% and the values in other samples normalized to this value.

Panel B. Vero cells were incubated in 10% fetal calf serum and then sub-cultured 24 h prior to infection. Three hours prior to infection, Vero cells were treated with the designated concentration of GSK650394 (nM) and then infected with HSV-1 (KOS strain) at an MOI of 0.01. Medium containing 10% fetal calf serum (black columns) or 2% stripped fetal calf serum (hatched columns) was then added. Infectious virus was collected at 24 h after infection by three rounds of freeze thawing, and plaque assays performed. The number of plaques present in cells infected in the presence of DMSO (solvent control) was set at 100% and the values in the other samples were normalized to this value. The studies in Panel A and B were repeated 3 times and an asterisk denotes significant differences between the DMSO control (P < 0.05) as determined by the Student t test.

Panel C. The designated cell line was incubated with the DMSO or the designated
concentration of GSK650394 (nM) for 24 h. The number of viable cells was quantified based on ATP present in culture using the CellTiter-Glo Luminescent Cell Viability Assay (Sigma; G7572) according to manufacturer’s instruction as a measure of cell viability. These studies are the average of three independent studies.
Figure 5.2.
Figure 5.2. Analysis of SGK-1 protein levels during productive infection.

CRIB cells were mock infected or infected with BoHV-1 at an MOI of 2. Cells were incubated in 2% “stripped” fetal bovine serum as described in Figure 5.1 after infection. At the designated times after infection (hours), whole-cell lysate was prepared. Equivalent amounts of protein for each sample (50 ug protein) were electrophoresed in a SDS-PAGE gel, and western blot analysis performed using a commercially available SGK1 antibody (Abcam; ab59337). The closed circle denotes the location of a novel SGK1 band detected in infected cells (middle panel is a longer exposure of the top panel). Tubulin protein levels were analyzed in the respective samples as a loading control. The relative levels of the SGK bands were estimated by comparing the intensity of these bands to tubulin using a BioRad ChemiDoc MP and PMI System. The SGK value was set at 100 and the other values normalized to this value. These results are representative of three independent experiments.
Figure 5.3.
Figure 5.3. GSK650394 treatment reduces steady state levels of BHV-1 proteins.

Cells were grown as described in Figure 5.1 prior to infection. Three hours prior to infection 25 nM of GSK650394 was added to the designated cultures. CRIB cells were infected with BoHV-1 (moi = 2) and then stripped FBS added. At the designated times after infection (hours), whole cell lysate (50 ug protein) was prepared and Western Blots performed using the designated antibodies directed against a viral protein. Arrows denote the position of bICP0 (97 kD), bICP4 (178 kD), bICP22 (45 kD), VP16 (65 kD), and the bracket denotes various gE forms. Tubulin was used as a loading control. The lane marked M are molecular weight markers: numbers to left are the approximate size of the respective bands. These results are representative of 4 independent experiments. The relative levels of the respective BoHV-1 proteins were estimated by comparing the intensity of the bands to tubulin using a BioRad ChemiDoc MP and PMI System. The value of the respective viral protein that was first detected after infection was set at 10 and the other values normalized to this value. ND refers to those lanes in which the viral...
protein was not detected. These results are representative of three independent experiments.

Figure 5.4.
Figure 5.4. GSK650394 stimulated the bICP0 early promoter, but not other BoHV-1 promoters.

Reporter constructs containing the immediate early 1 transcription unit (IEtu1), two IEtu1 deletion mutants (Δ831 and Δ1018), the full length bICP0 early promoter (EP943), and a bICP0 early promoter containing a 771 deletion (EP172) was used to examine the effect that GSK-650394 had on promoter activity. Neuro-2A cells were transfected with the designated construct and at 24 hours after transfection 1 uM GSK-650394 was added to certain cultures. At 48 hours after transfection, promoter activity was measured. Black columns denote cultures treated with GSK-650394 and hatched columns were treated with DMSO, which was the vehicle. These studies are representative of 3 independent experiments.
Figure 5.5.
Figure 5.5. GSK650394 does not inhibit the ability of the synthetic corticosteroid dexamethasone to stimulate IEtu1 or MMTV LTR promoter activity.

Neuro-2A cells were transfected with 2 ug DNA of a reporter construct containing the IEtu1 promoter (Panel A) or the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (Panel B). Five hours post-transfection, cells were incubated with stripped serum containing media. At 24 h post-transfection, the designated cultures were treated with 10 nM dexamethasone (DEX) and 25 or 100 nM GSK650394. At 48 h after transfection, promoter activity was measured as previously described (Kook, Henley, et al., 2015). These studies are the average of 3 independent experiments. An asterisk denotes significant differences between the DMSO control without DEX (P < 0.05) as determined by the Student t test.
Chapter 6. Stress mediated trans-activation of the BHV-1 immediate early transcription unit 1 promoter is enhanced by the transcriptional coactivator, Host Cell Factor-1.
Abstract

The primary site for life-long latency of bovine herpesvirus 1 (BHV-1) is sensory neurons. Reactivation from latency and productive infection is consistently induced by the synthetic corticosteroid dexamethasone. The BHV-1 genome contains more than 100 potential glucocorticoid receptor (GR) response elements (GREs) including two near perfect consensus GREs in the Immediate Early transcription unit 1 (IEtu1) promoter. These elements are bound by the GR and are required for transcriptional stimulation of the Ietu1 promoter-transcriptional unit by dexamethasone. The Ietu1 promoter drives immediate early expression of bICP0 and bICP4 and thus stimulation of this promoter promotes productive infection.

Host cell factor 1 (HCF-1) is a cellular transcriptional coactivator that forms a complex with the viral IE transactivator VP16 and the host cell transcription factor Oct 1. This complex occupies IE enhancers and stimulates IE promoter activity, including the Ietu1 promoter. Since it has been demonstrated that HCF-1 occupies HSV-1 IE promoters during reactivation from latency, we tested whether HCF-1 cooperates with the GR to stimulate Ietu1 promoter activity.

In transfected mouse neuroblastoma cells, silencing of HCF-1 interfered with GR mediated activation of Ietu1 promoter activity, suggesting that HCF-1 may be a critical component of GR-mediated induction of the Ietu1. Importantly, an Ietu1 construct that contains the two GREs, but lacks the OCT-1/VP16-responsive TAATGARAT motif, was still activated by the GR and this stimulation was inhibited by depletion of HCF-1. Conversely, GR mediated activation of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter was not impacted by HCF-1 depletion. Thus, these
studies suggest that HCF-1 functions with the activated GR to stimulate IEtu1 promoter activity in a VP16-independent manner.

Introduction

Host cell factor 1 (HCF-1) is a cellular transcriptional coactivator that is essential for induced expression of the immediate-early (IE) genes of several alphaherpesviruses [herpes simplex virus 1 (HSV-1), varicella-zoster virus (VZV), bovine herpesvirus (BHV-1)] during initiation of lytic infection (C. Jones, 2003; Narayanan et al., 2005). The protein is recruited to the IE enhancer domains via interactions with the viral IE activators (VP16 for HSV-1 and BHV-1), where it forms stable multiprotein complexes on the IE enhancer core elements. The viral IE activators containing VP16, Oct-1 and HCF-1 bind to the TAATGARAT motif in the IEtu1 promoter and transactivate IE promoter (V. Misra et al., 1994). Interestingly, HCF-1 may also be recruited to IE promoters and enhancers through interactions with other cellular transcription factors [Sp1 and GA-binding protein (GABP)] that can mediate expression of the IE genes (Narayanan et al., 2005). HCF-1 is a component of multiple chromatin modification complexes, including histone methyltransferases (SETd1A and MLL1-5) and histone demethylases (LSD1 and JMJD2s) (Vogel & Kristie, 2013). During HSV-1 reactivation from latency, HCF-1 is transported from its sequestered location in the cytoplasm to the nucleus. During the early stages of explant-induced reactivation from latency, HCF-1 is found associated with viral IE promoter domains, suggesting that it plays a role in initiating lytic cycle viral gene expression.

The synthetic corticosteroid dexamethasone consistently initiates BHV-1 reactivation from latency (Clinton Jones, 2014). Two viral regulatory proteins (bICP0
and VP16) can be readily detected in sensory neurons of trigeminal ganglia (TG) within an hour after latently infected calves are injected IV with dexamethasone (Frizzo da Silva et al., 2013; Kook, Doster, et al., 2015). The IE promoter that drives expression of bICP0 and bICP4 (IEtu1) is trans-activated by the glucocorticoid receptor (GR) when transfected cells are treated with dexamethasone (Kook, Henley, et al., 2015). GR mediated activation of the IEtu1 promoter requires two functional GR binding sites that are more than 700 bases upstream of the single TAATGARAT motif in the IEtu1 promoter. Since HCF-1 interacts with multiple cellular transcription factors and promotes viral gene expression during reactivation, we asked whether HCF-1 was important for mediating GR activation of the IEtu1 promoter activity in the absence of other viral genes.

**Results and discussion**

**Effect of HCF-1 knockdown on DEX mediated viral promoter activity**

Previous studies demonstrated that DEX stimulates IEtu1 promoter activities in Neuro-2A cells (Kook, Henley, et al., 2015). We investigated whether HCF-1 knockdown by a specific shRNA affected DEX mediated induction of the IEtu1. MMTV LTR containing GREs is stimulated by DEX so it was also tested as a positive control (Funder, 1997; Richard-Foy & Hager, 1987). Neuro-2A cells were transfected with a plasmid that expresses the HCF-1shRNA and then the ability of DEX to stimulate IEtu1 promoter activity was measured. We first examined the expression of HCF-1 protein levels upon shRNA interference. As shown in Figure 6.1A, HCF-1 shRNA significantly reduces HCF-1 protein levels (lane 2), while scrambled shRNA had no effect when compared with mock-transfected cells (lanes 1 and 3). As expected, IEtu1(Figure 6.1B) and MMTV (Figure 6.1C) promoter activities were induced by DEX treatment when cells were
transfected with mock or scrambled siRNA. IEtu1 but not MMTV LTR promoter activity was significantly reduced by silencing HCF-1 (Figure 6.1 B and C). These studies indicate that the transcriptional co-activator HCF-1 is required for the corticosteroid DEX to trans-activate the BHV-1 IEtu1 promoter. Conversely, HCF-1 was not required for DEX mediated stimulation of the MMTV LTR in Neuro-2A cells.

A DEX response region (DRR) that contains two GREs located within the IEtu1 promoter was previously shown to be important for stimulation by DEX and the GR (Figure 6.2A; Kook, Henley, et al., 2015). The SV40E promoter construct that directs expression of the DRR does not contain the TAATGARAT motif (Figure 6.2A; Kook, Henley, et al., 2015). Additional studies were performed using IEtu1 DRR to further understand the effect of HCF-1 on GR medicated IEtu1 promoter activity. DRR and the 3’-DRR construct but not the 5’-DRR were stimulated by DEX when cells were transfected with mock or scrambled siRNA (Figure 6.2B). HCF-1 inhibits the ability of DEX to stimulate DRR and 3’-DRR (Figure 6.2B). In summary, the region of the IEtu1 promoter that contains two GREs (3’-DRR) was important for DEX mediated induction and the ability of HCF-1 to mediate DEX induction. The TAATGARAT motif in the IEtu1 promoter was not required for the ability of HCF-1 to mediate the DEX response. These studies further suggest that the GR and HCF-1 may cooperate to stimulate IEtu1 promoter activity during stress induced reactivation from latency in the absence of other viral factors.
Figure 6.1.
Figure 6.1. Silencing HCF-1 interferes with the ability of DEX to stimulate IEtu1 but not MMTV LTR promoter activity.

Panel A. Mouse neuroblastoma cells (Neuro-2A) were transfected with a plasmid that expresses an HCF1-specific shRNA (shRNAc504; lane 2) or a plasmid that expresses a scrambled shRNA (lane 3). Additional control Neuro-2A cells were not transfected with a shRNA (lane 1). At 24 hours after transfection, total cell lysate was prepared and 100 ug protein electrophoresed in a SDS-PAGE and Western Blots performed to detect HCF-1. Tubulin was used as a loading control.

Panel B-C. Neuro-2A cells were transfected with either control scrambled or a HCF-1 shRNA construct (HCF-1 shRNAc504). At 24 hours after transfection, cells were cotransfected with the IEtu1 promoter reporter construct (Panel B) or the Mouse Mammary Tumor Virus LTR (Panel C) and a plasmid that expresses the human GR (0.5 ug DNA). Reporter promoter activity is shown in the presence or absence of DEX treatment for 3 hours. Gray columns indicate basal promoter activity derived from cultures not treated with DEX and black columns those treated with DEX. 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 untreated cells, as determined by the Student $t$ test.
Figure 6.2.

A diagram showing various regulatory elements and their positions.

B: A bar chart illustrating the DEX inducible promoter activity with different conditions.
Figure 6.2. HCF-1 is important for DEX stimulation of the DEX-responsive region of the IEtu1 promoter.

Panel A. Schematic of IEtu1 promoter and location of putative DEX responsive region (DRR). The locations of GRE#1 and GRE#2 are denoted by black or gray rectangles respectively in the IEtu1 promoter fragments used to localize sequences mediating DEX stimulation.

Panel B. Neuro-2A cells were transfected with the indicated promoter plasmids, a plasmid expressing GR, and HCF-1 shRNA (shRNA c504) or scrambled shRNA control. At 24 hours after transfection, cells were cotransfected with the DRR, 3’-DRR or 5’-DRR and a plasmid that expresses the human GR (0.5 ug DNA). Reporter promoter activity is shown in the presence or absence of DEX treatment for 3 hours. Promoter activity reflects the basal promoter activity derived from cultures not treated with DEX. 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.
General discussions and conclusions

The research presented in this dissertation was focused on understanding the molecular mechanisms by which stress induces reactivation from latency of two members of the \textit{alphaherpesvirinae} subfamily members, BHV-1 and HSV-1. Although it is well established that stress triggers viral reactivation from latency, the mechanisms of action are not well understood. Since a latent infection is defined by restricted lytic cycle viral gene expression, elucidating the mechanism by which stress induced cellular factors or viral proteins can trigger lytic cycle viral gene expression during reactivation from latency is important for understanding how these viruses escape from a latent infection.

As a result of stress, increased corticosteroids can trigger reactivation of HSV-1 and BHV-1 (Clinton Jones, 2014). The synthetic corticosteroid DEX mimics stress and can induce reactivation by 1) stimulating lytic viral gene expression, 2) inducing apoptosis of infiltrating immune cells in TG, and/or 3) repressing expression of LR gene products (C. Jones et al., 1990; Rock et al., 1992; Winkler et al., 2002; Workman et al., 2012). Previous studies demonstrated that DEX-inducible transcription factors identified in bovine TG promote productive infection and certain viral promoters of both BHV-1 and HSV-1 (Sinani, Cordes, et al., 2013; Workman et al., 2012). The action of corticosteroids is primarily mediated by GR. Based on these observations, we hypothesized that activated GR by DEX may activate certain viral promoters to stimulate reactivation from latency. Immunohistochemistry studies using consecutive sections prepared from latently infected calves following DEX treatment revealed that two regulatory viral proteins VP16 and bICP0 were detected during the early stages of stress-induced escape from latency. Interestingly, these two proteins were expressed in GR+
neurons. This led us to further hypothesize that activated GR by DEX may stimulate expression of VP16 or bICP0 during escape from latency. To test this hypothesis, we examined GREs and identified more than 100 GREs in the BHV-1 genome. Of particular interest was identification of GREs within the IEtu1 promoter region because IEtu1 directs expression of two regulatory proteins, bICP0 and bICP4. We found that GR directly binds to the GREs within the IEtu1 promoter and stimulates promoter activity.

HCF-1 stimulates IE gene expression of BHV-1 and HSV-1 by interacting with cellular transcription factors or chromatin modifiers. Thus, HCF-1 was silenced to examine the effect of HCF-1 on GR mediated IEtu1 promoter activation. A region of the IEtu1 promoter that contains GREs but not the binding site for the VP16 transactivation complex (Narayanan et al., 2005) was stimulated by DEX. However, silencing HCF-1 reduces this stimulation. This suggests that HCF-1 may play an important role in GR mediated IEtu1 promoter activation in a VP16 independent manner.

Since SGK-1 mRNA and protein levels are activated by stress, we hypothesized that SGK may stimulate BHV-1 and HSV-1 productive infection. Therefore, we inhibited SGK kinase activity using a SGK inhibitor, GSK650394 and then examined viral gene expression and replication. GSK650394 inhibits replication of BHV-1 and HSV-1. Expression of certain BHV-1 viral proteins was also reduced by GSK650394 treatment of infected cells. This suggests that SGK-1 has the potential to stimulate BHV-1 and HSV-1 replication.
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