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Regulation of Bovine Herpesvirus 1 (BHV-1) Productive Infection by Cellular Transcription Factors

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REGULATION OF BOVINE HERPESVIRUS 1 (BHV-1) PRODUCTIVE INFECTION
BY CELLULAR TRANSCRIPTION FACTORS.

by

Aspen M. Workman

A DISSERTATION

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Regulation of Bovine Herpesvirus 1 (BHV-1) Productive Infection by Cellular Transcription Factors.

Aspen M. Workman, Ph.D.

University of Nebraska, 2011

Advisor: Clinton Jones

Bovine herpesvirus 1 (BHV-1) is a significant viral pathogen of cattle responsible for a variety of disease conditions, including: conjunctivitis, pneumonia, genital disorders, abortions, and shipping fever, a serious upper respiratory tract infection. Following acute infection in mucosal epithelium, BHV-1 establishes a lifelong latent infection in sensory ganglionic neurons. During latency, transcription is restricted to the latency related (LR) gene. Elevated corticosteroid levels due to stress and/or immune suppression can initiate reactivation from latency, resulting in virus shedding and spread to susceptible cattle. Additionally, administration of dexamethasone (Dex), a synthetic corticosteroid, to calves latently infected with BHV-1 reproducibly leads to reactivation from latency. During reactivation, productive viral gene expression is readily detected in sensory neurons, LR gene expression decreases, and infectious virus is secreted. However, as with other alpha-herpesviruses, the molecular mechanisms that occur during successful reactivation from latency are poorly understood. Therefore, this dissertation was aimed at the elucidation of the early events of the latency-reactivation cycle of alpha-herpesviruses. Furthermore, the aim was to reveal the function of cellular transcription factors in the latency-reactivation cycle. Since BHV-1 is the only *alpha*herpesvirinae

subfamily member that can be reproducibly induced to reactivate from latency, we were also interested in examining the effect of these cellular factors on other important members of this virus family.

Collectively, studies presented in this dissertation characterized several cellular transcription factors that are induced in the trigeminal ganglia of latently infected cattle following treatment with Dex to induce reactivation from latency. These cellular transcription factors activate viral transcription and stimulate BHV-1 productive infection in cultured cells, suggesting they may facilitate the exit from latency. A subset of these cellular factors also regulates herpes simplex virus 1 (HSV-1) productive infection and/or promoter activity. Therefore, based on the data presented in this dissertation, we hypothesize that stressful stimuli promotes the exit from latency by activating specific cellular transcription factors, which consequently activate lytic viral gene expression and production of infectious virus.

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Chapter 1
Literature Review

I. Classification

The *Herpesviridae* family encompasses a group of large double-stranded DNA viruses with a broad host range including mammals, birds, reptiles, amphibians and fish (Roizmann et al., 1992). Common to all herpes viruses are basic similarities in their virion structure as well as certain aspects of their lifecycle. Herpes virus viral particles possess an icosahedral nucleocapsid of 100 nm in diameter, a cell derived envelope containing virally encoded glycoproteins, and a proteinaceous matrix of tegument proteins connecting the capsid to the envelope (Armstrong et al., 1961; Jones, 1998; Jones, 2003a; Tikoo et al., 1995). Furthermore, herpes viruses share the ability to establish latent infections that lead to lifelong persistence of the virus. Herpes viruses, however, differ in the composition and architecture of their genome, host range, duration of infection, cytopathology, and characteristics of latent infection (Roizman et al., 1981). Therefore, based on these characteristics, herpesviruses have been divided into three subfamilies designated *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae*. *Alphaherpesvirinae* have a variable host range with a short replication cycle that results in a rapidly spreading infection and cell lysis. *In vivo*, they establish latency primarily, but not exclusively, in ganglionic neurons from which they periodically reactivate producing a means of transmission of the virus to susceptible animals. *Betaherpesvirinae* have a narrow host range limited to a single species or genera. The replication cycle is relatively long, showing a slow developing focus of infection in which the cells become enlarged before lysis. *In vivo*, latency is established in macrophages of the lymphoreticular tissues, the kidney, and secretory glands. *Gammaherpesvirinae* also have a restricted host range *in vivo* and *in vitro* and they primarily infect lymphocytes and

macrophage as well as some types of epithelial and fibroblast cells. The lytic cycle is variable, as is its cytopathology. The predominant outcome of virus infection is lymphocyte transformation {reviewed in (Roizman and Baines, 1991)}.

II. *Alphaherpesvirinae*

Alphaherpesvirinae represent the largest subfamily of the *Herpesviridae* family and comprise many different, but closely related pathogens of both man and animal. Eight herpesviruses have been identified to infect humans, and three belong to the *alphaherpesvirinae* subfamily: (i) herpes simplex virus type 1 (HSV-1), which is a ubiquitous pathogen causing mainly oral or genital lesions, (ii) herpes simplex virus type 2 (HSV-2), which is closely related to HSV-1, and is one of the most common sexually transmitted viruses globally, causing mainly genital lesions, and (iii) varicella zoster virus (VZV), which causes chicken pox and upon reactivation, shingles. Important animal pathogens include: (i) pseudorabies virus (PRV) or Aujeszky's disease virus in pigs, which causes neurological and respiratory symptoms as well as reproductive failure, (ii) equine herpesvirus type 1 in horses, which has a similar manifestation as pseudorabies virus, and (iii) bovine herpesvirus type 1 (BHV-1) in cattle, which causes respiratory and genital diseases. Of particular interest in this dissertation are HSV-1 and BHV-1.

Herpes simplex virus 1 (HSV-1)

HSV-1 belongs to the genus Simplex virus within the *alphaherpesvirinae* subfamily. Its genome is approximately 152 kb with a GC content of 67% (Becker et al., 1968; Kieff et al., 1971; Plummer et al., 1969). The genome architecture consists of two

covalently joined segments designated unique long (U_L) and unique short (U_S). These unique segments are flanked by inverted repeat sequences: terminal repeats (TR) and internal repeats (IR; (Wadsworth et al., 1975). HSV-1 contains at least 84 open reading frames distributed throughout the genome on both strands, with genes located in the inverted repeat regions present in two copies (Figure 1.1). These genes encode a variety of proteins involved in forming the virion nucleocapsid, tegument, and envelope, as well as regulating the replication cycle of the virus.

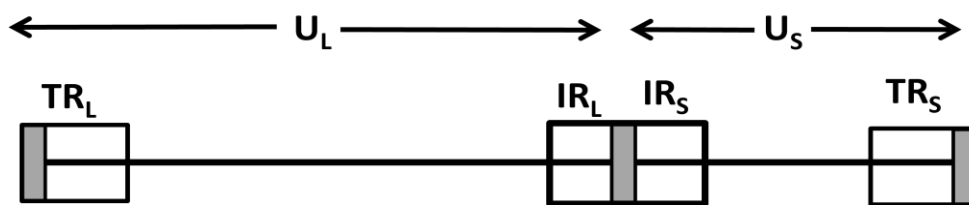


Figure 1.1: Schematic of HSV-1 genome.

HSV-1 possesses a linear, double-stranded DNA genome of 152 kb encoding more than 80 genes. The genome is composed of unique long (U_L) and unique short (U_S) segments, which are flanked by inverted repeats. The inverted repeats are designated as follows: TR_L : terminal repeat of the long segment; IR_L : internal repeat of the long segment; IR_S : internal repeat of the short segment; and TR_S : terminal repeat of the short segment. The genome contains three packaging signals (shown in grey) that assist in packaging the DNA into virions.

Primary infection with HSV-1 usually occurs in childhood, and approximately 90% of adults in the United States are infected (Nahmias and Roizman, 1973). HSV-1 infection can lead to a variety of disorders including recurrent genital and orofacial

lesions, keratitis, and conjunctivitis (Nahmias and Roizman, 1973). In fact, recurrent ocular HSV-1 infections are the major form of infectious corneal blindness in developed nations (Nesburn et al., 1983). HSV-1 infections can also cause gastrointestinal disorders, esophageal disorders, and approximately 25% of genital herpes (Gesser and Koo, 1997; Lohr et al., 1990). HSV-1 infections can also cause sporadic encephalitis; however, this condition is rare compared to other diseases resulting from infection. In addition, the virus establishes an asymptomatic latent infection in sensory neurons which serves as a reservoir for further cycles of peripheral lytic infections. Stress, trauma, and immune suppression can result in reactivation from latency resulting in virus shedding. In general, recurrent diseases caused by HSV-1 are the direct result of reactivation from latency (Asbell et al., 1984; Lewis et al., 1984; Sakaoka et al., 1995). For example, two-thirds of HSV-1 induced infections, corneal blindness, and encephalitis are the result of recurrent disease. To date, there is no HSV vaccine, and two recent genital herpes vaccine trials failed (Cohen, 2010; Stanberry and Rosenthal, 2002).

Bovine herpesvirus 1 (BHV-1)

BHV-1 is classified in the genus *Varcellovirus* within the *alphaherpesvirinae* subfamily and shares a number of biological properties with HSV-1 (Jones, 2003a). The BHV-1 genome is approximately 135 kb in length and contains a GC content of 72% (Tikoo et al., 1995). BHV-1 has at least 73 ORFs. The genome has similar architecture and gene arrangement as described for HSV-1 (See Figure 1.1).

BHV-1 is a significant viral pathogen of cattle that is responsible for a variety of disease conditions. The main clinical manifestations include: infectious bovine

rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV), infectious pustular balanopostitis (IPB), conjunctivitis, abortions, and generalized systemic infections {reviewed in (Muylkens et al., 2007)}. In rare occasions, the virus can cause fatal encephalitis. BHV-1 is also one of the major etiological agents of bovine respiratory disease complex (BRDC), also known as “*shipping fever*”. Other members of the BRDC complex include bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine adenovirus, and bovine parainfluenza virus type 3 (PI-3; Rosenquist, 1983). BHV-1 infection, associated with one or more of these agents, is followed by immune suppression and secondary bacterial infection by opportunistic pathogens such as *Haemophilus somnus*, *Mannheimia haemolytica* or *Mannheimia multocida* (Babiuk et al., 1988; Yates, 1982). Losses associated with BRDC are approximately \$3 billion annually, and approximately 75% of morbidities and over 50% of mortalities in feedlot cattle are attributed to BRDC (Ishmael, 2001; Kapil, 1997; Powell, 2005). BRDC is also recognized as a significant problem for dairy cattle and cow-calf operations. Thus, BRDC is considered to be the most economically important disease affecting the cattle industry. Although modified live vaccines are available, it should be noted that most commercially available vaccines can i) reactivate from latency, ii) induce abortions in cows, and iii) induce systemic and fatal infections in calves. Therefore, there is a need to develop novel modified live vaccines which do not reactivate from latency, yet protect cattle from clinical symptoms induced by virulent field strains.

III. Productive Infection

Overview

Productive infection is initiated by a three step entry process following direct or aerosol contact of the virus with oral, respiratory, ocular or genital mucosal membranes. The first interaction involves low affinity virus attachment between gB and/or gC to cell surface structures such as heparin sulfate sugar moieties. This labile interaction is followed by the stable binding of gD to specific cellular receptors (HveB and HveC), allowing virus penetration by fusion of viral and cellular envelopes (Campadelli-Fiume and Menotti, 2007). Once in the cytosol, the de-enveloped nucleocapsid is then transported toward nuclear pores via dynein motor complexes associated with microtubules in order to permit viral DNA release into the nucleus (Dohner et al., 2002). In the nucleus, the viral genome replicates by three temporally regulated phases of transcription that yield: immediate early (IE) proteins that regulate viral transcription; early (E) proteins that replicate the viral genome; and late (L) proteins which are structural and comprise the virion particle. After the late phase of infection, viral capsids are assembled in the nucleus. Exactly how the mature capsids leave the nucleus to gain secretory vesicles and how they obtain their final envelope is still a matter of debate. The current view of egress and capsid maturation is proposed in a three step model where the capsids bud through the inner nuclear membrane to gain their primary envelope. In order to gain access to the cytoplasm, the virions fuse their primary envelopes with the outer nuclear membrane. Once in the cytoplasm, naked capsids acquire their mature tegument and secondary envelope by budding into the trans- Golgi compartment. The virus would then leave the cell through the secretory pathway. As with all *alphaherpesvirinae*

subfamily members, the cell is killed in the process of productive infection and the virus spreads cell to cell {reviewed in (Muylkens et al., 2007)}.

HSV-1 Productive Infection

HSV-1 IE gene expression does not require protein synthesis and is stimulated by the tegument protein VP16 (O'Hare et al., 1993) and by active cyclin-dependent kinases (Schang et al., 1998; Schang et al., 1999). Five IE genes exist: infected cell protein [ICP]0, ICP4, ICP22, ICP27, and ICP47. ICP4 (Carrozza and DeLuca, 1996; DeLuca et al., 1985; DeLuca and Schaffer, 1985) and ICP27 (McCarthy et al., 1989; McMahan and Schaffer, 1990; Sacks et al., 1985) are required both *in vivo* and *in vitro* for virus growth. ICP4 represses IE gene expression (DeLuca and Schaffer, 1985; Gu et al., 1993; Michael and Roizman, 1993; O'Hare and Hayward, 1985; Roberts et al., 1988) and activates E or L gene expression by interacting with RNA polymerase II basal transcription factors (Gu and DeLuca, 1994; Smith et al., 1993). In the absence of ICP4, infection does not proceed beyond IE gene transcription (DeLuca et al., 1985; Preston, 1979). ICP27 is involved in several functions, including: the switch from early to late gene expression during virus replication, polyadenylation site selection and 3' RNA processing, inhibition of mRNA splicing, and transport of intronless mRNA out of the nucleus {reviewed in (Weir, 2001)}. ICP22 is important for viral growth in some cultured cells but not others (Long et al., 1999; Rice et al., 1995). Although the role of ICP22 in HSV-1 replication has not been elucidated, evidence suggests that ICP22 alters the activities of cellular proteins required for efficient virus replication (Advani et al., 2000a; Bruni et al., 1999; Bruni and

Roizman, 1998; Sears et al., 1985) and late gene expression (Orlando et al., 2006; Poffenberger et al., 1993; Rice et al., 1995; Sears et al., 1985).

ICP0 is a promiscuous activator of gene expression that activates all three classes of viral promoters, but does not directly bind to DNA. ICP0 is not absolutely required for virus growth in cell culture. ICP0 null viruses replicate to nearly wild-type levels when cells are infected with a multiplicity of infection (MOI) greater than one. At MOIs below 0.1, the same ICP0 null viruses establish quiescent infections in 99% of the cells they infect (Everett, 1989; Sacks and Schaffer, 1987). Such observations suggest that ICP0 antagonizes a cellular repression mechanism to silence viral transcription. This repression capacity appears to be saturated with high copy numbers of viral genome (Everett, 1989). The mechanism by which ICP0 counteracts this process is unknown, but critical to its activity is a zinc RING finger domain that mediates E3 ubiquitin ligase activity which targets a number of cellular proteins for proteasome dependent degradation (Enquist et al., 1998; Everett, 2000; Everett et al., 1999b). The list of proteins that may function as ICP0-antagonized repressors of HSV gene expression continues to grow and currently includes: PML (Chee et al., 2003; Everett et al., 2008; Everett et al., 2006; Gu and Roizman, 2009) Sp100 (Chelbi-Alix and de The, 1999), cyclin D3 (Kawaguchi et al., 1997), IRF-3 and IRF-7 (Lin et al., 2004), centromeric proteins CENP-B and CENP-C (Everett et al., 1999a; Lomonte and Morency, 2007), HDAC1/2-CoREST-REST (Gu and Roizman, 2009), DNA-dependent protein kinase (Lees-Miller et al., 1996), and class II histone deacetylases (Lomonte et al., 2004).

ICP47 is the only IE protein that does not play a critical role in the regulation of HSV gene expression. Rather, ICP47 prevents transport of antigenic peptides into the

endoplasmic reticulum (Hill et al., 1995) and is crucial for neurovirulence because it inhibits CD8+ T-cell responses (Goldsmith et al., 1998).

The IE proteins stimulate production of the E genes, whose role is to increase nucleic acid metabolism. These proteins include thymidine kinase, viral DNA polymerase, and other enzymes involved in DNA metabolism. Viral DNA synthesis is detected 3 hours post-infection and continues for another 12 hours (Igarashi et al., 1993; Roizman et al., 1963; Roizman et al., 1965). The optimal expression of L genes requires viral DNA synthesis (Harris-Hamilton and Bachenheimer, 1985; Honess and Roizman, 1974; Honess and Roizman, 1975). The late genes encode structural components of the virion including nucleocapsid proteins, glycoproteins, and tegument proteins. In fully permissive tissue culture cells, the entire process takes approximately 18-20 hours.

BHV-1 Productive Infection

Like HSV-1, BHV-1 gene expression is temporally regulated in three distinct phases. In bovine primary cell cultures, BHV-1 DNA synthesis initiates between 2 and 4 hours post infection (hpi) and peaks at 4 to 6 hpi (Ghram and Minocha, 1986). Viral proteins are detectable at 4 hpi and increase until 6 and 8 hpi (Ghram and Minocha, 1986).

IE gene expression is stimulated by a virion component, bTIF , which is homologous to the HSV-1 encoded VP16. Two immediate early transcription units exist: IE transcription unit 1 (IETu1) and IETu2 (Misra et al., 1994; Misra et al., 1995). IETu1 encodes functional homologs of two HSV-1 proteins, ICP0 and ICP4. *Circ*, a less abundant transcript, encodes a tegument associated protein and arises from the activation

of the IETu1 promoter across the junction of the circularized genome (Fraefel et al., 1993; Fraefel et al., 1994). IETu2 encodes a protein similar to the HSV-1 IE gene, ICP22.

The IE gene products have transcriptional regulatory functions (Jones, 2003a). bICP4 and bICP22 shut off expression of IETu1 and IETu2, while they activate the E promoter of bICP0 as well as other E and L genes (Schwyzer et al., 1993). bICP0 does not specifically bind DNA, yet is a potent activator of viral promoters (Everett, 2000; Wirth et al., 1992). Indeed, bICP0 is considered the major transcriptional regulatory protein encoded by BHV-1 because it activates expression of all three classes of viral promoters (Wirth et al., 1992) and it is expressed at high levels throughout infection (Everett, 2000; Fraefel et al., 1994). bICP0 also functions as a nonspecific enhancer of foreign (non-BHV) genes. This is in contrast to bICP4 which is a specific transactivator of BHV-1 E genes. Although bICP0 is not abundantly expressed during latency in tonsils (Winkler et al., 2000a) or in TG, it must be expressed for reactivation to occur (Jones et al., 2006). Construction of a bICP0 deletion virus, however, showed that similar to ICP0, bICP0 is important, but not required for growth in tissue culture (Geiser and Jones, 2005; Koppel et al., 1996).

IV. Latency-Reactivation Cycle

Overview

A key characteristic of all alpha-herpesviruses is their ability to establish life-long latency within the infected host and periodically reactivate to facilitate transmission of virus to other susceptible hosts. Following infection and local replication at mucosal surfaces, HSV-1 or BHV-1 particles gain access to sensory nerve terminals innervating

the tissue (Enquist et al., 1998). The virus is then transported intra-axonally in a retrograde fashion to neuronal cell bodies in the trigeminal ganglia (TG), where latency is established. Although TG is the main site of latency for BHV-1 and other members of the *alphaherpesvirinae* subfamily, latent infection can also occur in non-neuronal sites, for example tonsils and lymph nodes (Winkler et al., 2000a).

In contrast to acute infection where all viral genes are expressed; latency is characterized by minimal viral gene expression, the absence of lytic viral proteins, and the absence of infectious virus production. Both the restriction of substantial viral gene expression and the maintenance of the viral genome over time during latency allow herpesviral infections to persist for the life of the host, even in the face of intense immune surveillance. Latently infected cells form a reservoir of virus that can periodically be induced to re-enter the lytic replication cycle and reactivate from latency. This latent stage and subsequent reactivations cause recurrent disease at peripheral sites and accounts for the high transmission rates and prevalence of herpesviruses in nature.

Establishment of Latency

The steps of the latency-reactivation cycle have been operationally divided into three major steps: establishment, maintenance, and reactivation. Extensive viral gene expression and replication occur within the TG for approximately a week following infection of animal models with HSV-1 (Knotts et al., 1974; Kramer et al., 1998) or cattle with BHV-1 (Schang and Jones, 1997; Winkler et al., 2000a). Around 7 days after infection, extensive viral gene expression is extinguished. Inhibition of IE gene expression reduces cellular toxicity and productive infection, favoring the establishment

of latency (discussed in more detail in the next section). Surviving neurons harbor latent viral genomes which are retained extra-chromosomally as circular episomes (Rock and Fraser, 1983; Rock and Fraser, 1985). In animal models of HSV-1 infection, the total number of cells harboring latent viral genomes is estimated to be approximately 1-5% of trigeminal ganglion neurons (Ackermann et al., 1982; Kutish et al., 1990a). The genome copy number within the infected neuron can range from <10-1,000 copies (Sawtell, 1997). Increasing the viral input titer results in more neurons with greater numbers of viral genome copies; demonstrating a direct correlation between increasing viral input and the number of neurons in which latency is established. Productive replication within the TG also increases the number of latently infected neurons; however, replication is not required for the establishment of latency. For example, viral mutants that cannot replicate will establish latency, but at reduced levels (Coen et al., 1989; Efstathiou et al., 1989; Katz and Middle, 1990; Margolis et al., 1992; Sedarati et al., 1993; Steiner et al., 1990).

The immune system also contributes to the establishment of latency by suppressing infection and limiting the spread of the virus at synaptic junctions. Following primary HSV-1 infection of mice, T-cells and other inflammatory lymphoid cells infiltrate the TG in large numbers between 7 and 10 days post infection (Erturk et al., 1992; Liu et al., 1996; Shimeld et al., 1995b; Stumpf et al., 2001). During this period an increase in cells expressing interferon-gamma (IFN-gamma) and interleukin 4 (IL-4) is also observed. Chronic inflammatory infiltration of lymphocytes in TG of calves following infection with BHV-1 is also observed (Winkler et al., 2002a). The presence of lymphocytes in close association with latently infected ganglionic neurons suggests that the immune system may promote the establishment of latency, perhaps by inducing a

non-permissive environment. This is supported by the finding that IFN-gamma, tumor necrosis factor-alpha (TNF-alpha), and CD8+ T-cells repress HSV-1 productive infection (Cantin et al., 1999a; Cantin et al., 1999b; Cantin et al., 1995b; Halford et al., 1996b). The cytokine environment may also influence neuronal cell health and promote the maintenance of latency.

Maintenance of Latency

Maintenance of latency is a phase that lasts for the life of the host and is operationally defined as a period where infectious virus is not detected by standard virus isolation procedures. Maintaining a latent infection requires that the viral genome be perpetuated and the cell kept alive. As neurons are non-dividing, genome replication and faithful partitioning to daughter cells upon cell division is not a significant concern for alpha-herpesvirus latency as it is for other subfamily members. Perhaps to avoid cell death by apoptosis or immune recognition, abundant expression of viral genes that are required for productive infection does not occur. However, many *alphaherpesvirinae* subfamily members, including HSV-1 and BHV-1, express abundant levels of a latency associated transcript (LAT) in neurons during this stage of latency (Jones, 1998; Jones, 2003a; Jones et al., 2006).

The HSV-1 LAT and the BHV-1 latency-related (LR) gene have a similar genomic position and are antisense to and partially overlap the ICP0 and bICP0 open reading frame respectively. The BHV-1 LR gene has two well defined ORFs (ORF-1 and ORF-2) and two reading frames lacking the initiating ATG (RF-B and RF-C). As a result of alternative splicing of poly(A)⁺ LR-RNA in the TG of infected calves, this RNA is

translated into more than one protein product (Devireddy and Jones, 1998; Hossain et al., 1995a). LR gene products help to maintain latency by inhibiting apoptosis (Ciacci-Zanella et al., 1999; Henderson et al., 2004a; Henderson et al., 2004b) and viral gene expression (Bratanich et al., 1992; Geiser et al., 2002). A mutant BHV-1 strain with three stop codons at the N terminus of ORF-2 does not express ORF-2 or RF-C and does not reactivate from latency (Inman et al., 2002; Jiang et al., 2004). These findings implied that the LR mutant virus was unable to infect neurons that support reactivation from latency or the LR mutant virus killed neurons that support reactivation from latency. Interestingly, higher rates of neuronal apoptosis occur in TG neurons during the establishment of latency following infection with the LR mutant (Lovato et al., 2003). Furthermore, in situ hybridization revealed that a small percentage of viral genome-positive neurons are present in the TG of calves infected with the wt or LR rescued virus but not the LR mutant virus (Inman et al., 2002). These observations suggest that the anti-apoptosis function of a protein encoded by the LR gene helps to establish and maintain latency in a pool of neurons that are capable of supporting reactivation from latency. (Ciacci-Zanella et al., 1999; Devireddy and Jones, 1999a; Geiser et al., 2008; Lovato et al., 2003; Shen and Jones, 2008; Winkler et al., 1999).

HSV-1 LAT also interferes with apoptosis *in vitro* and *in vivo* by inhibiting both major apoptosis pathways (Ahmed et al., 2002; Henderson et al., 2002; Inman et al., 2001a; Jin et al., 2003; Kang et al., 2003; Perng et al., 2000). Although HSV-1 encodes other anti-apoptosis genes (Us3, ICP27, and glycoproteins Us6 and Us5), LAT is the only anti-apoptosis gene that is abundantly expressed at the end of acute infection when latency is being established. The anti-apoptosis functions of LAT correlate with

promoting spontaneous reactivation because three different anti-apoptosis genes restore wt levels of spontaneous reactivation to a LAT null mutant (Jin et al., 2008; Jin et al., 2005; Perng et al., 2002). Additionally, insertion of the BHV-1 LR gene into the HSV-1 LAT locus restores high levels of spontaneous reactivation in the rabbit eye model and in explant induced reactivation (Mott et al., 2003; Perng et al., 2002). Thus, the anti-apoptosis activities of LAT and LR seem to be important for the latency-reactivation cycle.

Reactivation from Latency

Reactivation from latency is initiated by external stimuli (e.g. stress and immunosuppression) that stimulate viral gene expression. The stress induced by moving cattle from one location to another is an important stressful stimulus that is known to trigger reactivation from latency. During reactivation, three significant events occur: (i) productive viral gene expression is stimulated and can be readily detected in sensory neurons, (ii) LAT/LR gene expression decreases, and (iii) infectious virus is transported in an anterograde fashion to mucosal surfaces {reviewed in (Perng and Jones, 2010)}. The ability of herpes viruses to reactivate from latency results in recurrent disease and virus transmission.

V. Viral Gene Expression During Productive Infection vs. Reactivation from Latency.

Regulation of IE Promoters in Neurons versus Nonneuronal Cell Types

The regulated pattern of gene expression during productive infection has been extensively studied in permissive cell types in culture (Clements et al., 1977; Honess and Roizman, 1974). Unlike other large DNA viruses, herpesvirus IE genes are not controlled by constitutively active promoters within the context of the viral genome. Rather, a tegument protein packaged in the virion serves to activate IE gene expression {reviewed in (Wysocka and Herr, 2003)}. Upon viral envelope fusion with the cellular membrane, tegument proteins are shed in the cytosol and are the first to encounter and interact with the intracellular environment. The tegument protein VP16, also known as bTIF in BHV-1, is responsible for initiating IE viral gene expression. VP16 associates with host cell factor 1 (HCF-1) in the cytoplasm and this complex translocates to the nucleus where it recognizes the cellular transcription factor Oct-1 (Octamer binding protein 1) bound to TAATGARAT (where R is a purine) motifs located in the promoters of all IE genes (Kristie et al., 1989; LaMarco and McKnight, 1989). This brings the potent VP16 acidic activation domain in the vicinity of the transcription start site of these genes in order to recruit and stabilize elements of the basal transcription complex (Goodrich et al., 1993; Ingles et al., 1991; Klemm et al., 1995; Lin et al., 1991; Uesugi et al., 1997).

In addition to recruiting RNA polymerase II, the VP16/HCF/Oct-1 complex also controls the histone occupancy, positioning, and modification at viral IE promoters (Hancock et al., 2010; Herrera and Triezenberg, 2004; Peng et al., 2010). Viral genomes packaged into virions and delivered to the nuclei of infected cells are devoid of cellular

histone proteins (Leinbach and Summers, 1980; Oh and Fraser, 2008). However, chromatinization occurs rapidly upon nuclear entry and is maintained throughout infection. The acidic activation domain of VP16 interacts with cellular chromatin remodeling complexes and decreases histone occupancy on viral IE promoters (Memedula and Belmont, 2003; Neely et al., 1999). Additionally, the VP16/HCF/Oct-1 complex controls the post-translational modifications of the cellular histones bound to the viral genome. VP16 interacts with the cellular histone acetyltransferases (HATs) CBP and p300 to increase the markers of active euchromatin by acetylation of histones associated with viral IE promoters (Barlev et al., 1995; Kraus et al., 1999; Kundu et al., 2000; Memedula and Belmont, 2003; Utley et al., 1998). Therefore, VP16 is required for efficient IE gene expression during the initiation of productive infection.

Viral mutants defective in VP16 or IE gene expression do not readily enter productive replication after infection in tissue culture cells, but are retained in a non-replicating 'quiescent' state. This has led to the hypothesis that establishment of latency is the consequence of a failure to initiate IE gene expression. This view is supported by *in vivo* studies in which an ectopic ICP0 promoter was used to drive the expression of the Cre recombinase. ICP0 promoter activation led to Cre expression and the subsequent Cre-mediated activation of the LacZ reporter from the cellular genome (Proenca et al., 2008). This technique revealed that less than a third of latently infected neurons exhibit detectable levels of ICP0 expression sometime during infection of the neuron and establishment of latency. *In vivo* studies using wild type viruses show that during acute infection, two populations of neurons can be identified, those expressing viral antigens, and those expressing latency associated transcripts (LAT), suggesting an early divergence

of lytic and latent pathways (Lachmann, 2003; Sawtell and Thompson, 1992; Speck and Simmons, 1991; Speck and Simmons, 1992).

This lack of IE gene expression has become an area of intense research: why is there not transcription of IE genes in sensory neurons? As mentioned previously, IE genes are induced by a combination of host and viral factors. It is conceivable that the lack of IE gene expression could be due to i) a lack of the viral VP16, ii) a lack of essential host factors (HCF-1 and Oct-1), and/or iii) direct repression of the IE gene promoter (as opposed to a lack of induction).

Evidence from another *alphaherpesvirinae* subfamily member (PRV) suggests that viral nucleocapsids arrive at the neuronal cell body largely devoid of VP16 (Diefenbach et al., 2008; Luxton et al., 2005). Furthermore, any tegument-derived VP16 must pair with HCF in the cytoplasm and then translocate to the nucleus. HCF is expressed in neurons; however, unlike other cell types where the protein is mainly nuclear, HCF has a strict cytoplasmic localization in neurons (Kristie et al., 1999). HCF is sequestered in the cytoplasm through its association with neuronal proteins Zhangfei (Akhova et al., 2005; Lu and Misra, 2000b) and Luman (Lu et al., 1997; Lu and Misra, 2000a), and is often found associated with the Golgi apparatus (Kolb and Kristie, 2008). This has led to the proposition that there may be sequestration of VP16 in the cytoplasm, which would restrict IE transcription (Kristie et al., 1999).

In addition to the loss of functional VP16 in the nucleus, Oct-1 expression is lower in sensory neurons compared to non-neuronal cells, and a number of proteins related to Oct-1 (e.g. Oct-2; Brn-1-4) that fail to complex with VP16 may repress IE gene activation (Efstathiou and Preston, 2005; Lillycrop et al., 1994; Preston, 2000).

Therefore, it appears that VP16 function is compromised during the establishment of latency, thus decreasing the likelihood of IE gene expression in neurons.

Viral factors are also believed to play a role in the restriction of viral gene expression in sensory neurons. As mentioned previously, LAT and the LR gene overlap and are antisense to ICP0 suggesting it represses IE gene expression (Bratanich et al., 1992; Mador et al., 1998; Stevens, 1987b; Thompson and Sawtell, 1997). It has been demonstrated that BHV-1 LR represses the transactivation of IETu1 (Bratanich et al., 1992). LR also encodes at least two micro-RNAs which inhibit bICP0 expression and reduce viral replication (Jaber et al., 2010). LAT also encodes micro-RNAs that inhibit ICP0 and ICP4 protein expression (Umbach et al., 2009). Other experiments have shown that IE gene expression is repressed and latency is established in LAT-null viruses (Ho and Mocarski, 1989; Javier et al., 1988; Lachmann et al., 1999; Steiner et al., 1989). Therefore, it is reasonable to predict that viral factors, especially LAT/LR, as well as cellular factors cooperate to mediate the block in IE gene expression, aiding in the establishment of latency in sensory neurons.

Viral Gene Expression During Reactivation from Latency

Restriction of IE gene expression that occurs in sensory neurons must be overcome for reactivation from latency to occur. While VP16 is crucial for IE gene expression during lytic infection, studies in the early 1990's led to the dogma that VP16 is not required for reactivation from latency (Ecob-Prince et al., 1993a; Sears et al., 1991; Steiner et al., 1990). This conclusion was based on the observations that a VP16 mutant lacking transactivation potential can efficiently reactivate from latency (Ecob-Prince et

al., 1993a; Steiner et al., 1990). This was further supported by the later observation that viral nucleocapsids arrive at the neuronal cell body largely devoid of VP16 (Diefenbach et al., 2008; Luxton et al., 2005), and VP16 is not expressed during latency. Thus, it was concluded that VP16's function in initiating the lytic cycle is fulfilled by another viral function or a host cell factor during reactivation from latency.

There are two hypotheses regarding the initiation of the lytic cycle during reactivation from latency. The first hypothesis proposes that reactivation of latent virus may be the result of changes in cellular transcriptional regulators induced by systemic stress, which consequently stimulate the expression of the viral IE gene, ICP0. ICP0 then serves to initiate reactivation from latency (Amelio et al., 2006; Cai and Schaffer, 1989; Jordan and Schaffer, 1997; Leib et al., 1989). Exogenous expression of ICP0, independent of other viral gene products, can initiate HSV-1 (Halford et al., 2001) or HSV-2 (Zhu et al., 1990) reactivation from latency using an *in vitro* system. Furthermore, ICP0 mutant viruses clearly fail to reactivate from latency (Cai et al., 1993; Halford and Schaffer, 2001). However, this virus also has severe defects in productive infection and establishes latency at reduced levels, making interpretation of the results more complicated. Using a procedure to equalize ICP0 mutant and wild-type genome numbers, Halford and Schaffer (Halford and Schaffer, 2001) conclude that ICP0 is required for efficient reactivation in explants of latently infected TG as well as primary cultures of latently infected TG cells. The requirement of ICP0 in reactivation from latency remains controversial, however, as differing readouts of reactivation (explant vs *in vivo* studies) give contradictory results into the requirement for ICP0 during reactivation from latency

(Cai et al., 1993; Halford and Schaffer, 2001; Miller et al., 2006; Thompson and Sawtell, 2006).

The second hypothesis for HSV-1 reactivation proposes that viral early gene expression and DNA replication precedes and is necessary for efficient IE gene expression during reactivation from latency (Kosz-Vnenchak et al., 1993; Pesola et al., 2005). This is supported by *in vivo* and *in vitro* studies which report a novel program of gene expression during reactivation from latency (Devi-Rao et al., 1994; Nichol et al., 1996; Tal-Singer et al., 1997). Tal-Singer et al. (Tal-Singer et al., 1997) detected E and leaky L genes prior to IE genes during HSV reactivation from latency in infected TG. Nichol et al. (Nichol et al., 1996) found that an inhibitor of viral replication, ACV, inhibited IE and E gene expression in primary neuronal cells but not in Vero cells. Therefore, which cellular factors play a role in the initiation of reactivation and which viral genes are among the first to be expressed (i.e. the IE bICP0 or E replication genes) is yet unknown and an area of intense research.

Cell culture systems have been developed to study the latency-reactivation cycle; however, most are poor models for *in vivo* latency and reactivation. For example, in 1971, Stevens and Cook co-cultured TG explants with an HSV-1 permissive cell line in a functional assay to verify latent infection. While this technique was useful for evaluating establishment of latency, it could not be adopted as a useful reactivation model, as reactivation was induced without treatment (Stevens and Cook, 1971). Neonatal sensory neuron cultures were later developed to study reactivation. This model was better suited as it maintained latency until administration of a specific stressor (Smith et al., 1992; Wilcox and Johnson, 1988). This model is not without drawbacks, however, as it differs

substantially from adult ganglionic neurons *in vivo*. For example, nearly 100% of the cells in the neonatal culture can become latently infected. In the TG, only 5-10% of the cells are neurons, and only 1-5% of these neurons are latently infected following HSV-1 infection (Ackermann et al., 1982; Kutish et al., 1990a). Other cell culture based systems were described in which primary cell cultures were established from the TG of a mouse latently infected with HSV-1. Explant was performed in the presence of an anti-viral drug (BVDU), and latency was maintained until reactivation stimuli was applied (Halford et al., 1996a; Moriya et al., 1994). However, the state of non-permissivity induced in explanted ganglia in the presence of anti-viral drugs is not equivalent to the non-permissive state of the neuronal cells *in vivo*. Additionally, LAT is not abundantly expressed in any of these *in vitro* latency models. Therefore, these model systems may mimic some, but certainly not all, aspects of *in vivo* latency.

The prevailing hypotheses regarding the initiation of the lytic cycle during reactivation have come almost exclusively from systems using explanted ganglia. Although this technique has been widely used, questions have been raised about the validity of results obtained from these experiments. It has become increasingly clear that the explantation process significantly alters neurons, inducing rapid, widespread changes in neuronal physiology. This complicates the interpretation of results obtained from explanted ganglia. Therefore, Thompson and Sawtell have recently re-examined the two prevailing hypotheses of reactivation using *in vivo* reactivation and single neuron approaches. Using this technique, they provide evidence that the exit from latency does not require a viral DNA pre-amplification stage (Sawtell et al., 2006) or functional ICP0 (Thompson and Sawtell, 2006). Reactivation has traditionally been defined as infectious

virus production. In these studies they divided reactivation into two phases: initiation of lytic viral gene expression and infectious virus production. While ICP0 and viral DNA replication were required for infectious virus production, they did not appear to play a role in the initiation of lytic viral gene expression. If the two protagonists in the reactivation debate (ICP0 and DNA replication) are not responsible for the initial events of reactivation, then what is?

Thompson et al. propose that de novo synthesis of VP16 is responsible for the initial events of reactivation (Thompson et al., 2009). A caveat to this hypothesis is that a virus with a mutation in the VP16 acidic activation domain can efficiently reactivate from latency using explant models for reactivation (Ecob-Prince et al., 1993b; Steiner et al., 1990). However, this same mutant fails to reactivate in heat stressed mice (Thompson et al., 2009), highlighting the differences between explant studies and *in vivo* assays. In this experiment they show that in using two different reporter viruses they detect VP16 promoter activity before IE gene production following heat stress induced reactivation (Thompson et al., 2009). Therefore, while VP16 is expressed as a late protein during productive infection, its promoter appears to be activated prior to IE genes during reactivation. Furthermore, this appears to be specific for the VP16 promoter, as the late promoter which drives expression of VP5 could substitute for the VP16 promoter during productive infection *in vitro* and during acute ocular infections in mice, but was severely impaired for replication in mouse TG. This is interesting as it implies that de novo synthesis of VP16 may be required for the short period of replication observed in the TG before the establishment of latency.

As mentioned previously, VP16 does not act alone in IE gene activation. Interestingly, cellular stress also leads to HCF-1 subcellular relocation from the cytoplasm to the nucleus. This is important, as VP16, synthesized in the cytoplasm, is unable to enter the nucleus if binding to HCF-1 is mutated, or if the nuclear localization signal of HCF is deleted (LaBoissiere and O'Hare, 2000). Under these circumstances, IE gene expression is inhibited. Additionally, Oct1 levels and DNA-binding activity are induced following UV exposure or ganglia explant, two stimuli that result in herpesvirus reactivation. This suggests that VP16 may function similarly during reactivation as during productive infection, with the only difference being the source of VP16 (tegument delivered or newly synthesized).

The dependence on cellular factors for reactivation provides an opportunity for the virus to integrate information about the state of the host cell into the control of its own lytic program. The stress induced cellular factors that regulate HSV-1 VP16 expression during reactivation and the promoter sequences that are important for the regulated expression of VP16 in neurons have not been identified. Furthermore, whether or not these findings extend to other *alphaherpesvirinae* subfamily members (such as BHV-1) is yet to be determined.

VI. Corticosteroid Induced Reactivation from BHV-1 Latency

One particularly intriguing feature of BHV-1 infection is that administration of dexamethasone (Dex), a synthetic corticosteroid, to calves or rabbits latently infected with BHV-1 reproducibly leads to reactivation from latency (Rock et al., 1992). Corticosteroid mediated BHV-1 reactivation is likely a significant mechanism underlying

the survival of BHV-1 in nature as well. Naturally occurring stressful conditions lead to epinephrine release from the sympathetic nervous system and glucocorticoid release from the adrenal gland. This increase in endogenous corticosteroids is known to result in reactivation and viral shedding in latently infected animals (Davies and Carmichael, 1973). Therefore, understanding the molecular mechanisms by which Dex causes reactivation can shed light on how natural stress also leads to reactivation from latency.

Glucocorticoids are known regulators of both cellular and viral gene expression and, in addition, they are known to be potent immunosuppressive agents *in vivo* (Rousseau and Baxter, 1979; Yamamoto, 1985). The manner by which Dex induces BHV-1 reactivation is unknown; however, recent studies in human disease models have shed some light on the molecular mechanisms by which glucocorticoids exert their effects.

Glucocorticoids regulate transcription via glucocorticoid receptors (GR). Glucocorticoids enter the cell and bind to inactive GR in the cytosol. Two active GR molecules then form a dimer that couples to FKBP52 and dynein, which mediate the transport of the GR complex into the nucleus (Smith et al., 2000; Wochnik et al., 2005). In the nucleus, the GR dimer binds to a DNA sequence known as the glucocorticoid response element (GRE) to stimulate or silence gene expression (Buckingham, 2006; Jeffery, 2004). Besides its action as a transcription factor, the GR mediates its effects by binding to and modifying the function of other transcription factors, including: NF- κ B (Heck et al., 1997; McKay and Cidlowski, 1998), activator protein 1 (AP-1) (Heck et al., 1994), Smad or Mad-related proteins, and STAT family members leading to transcriptional repression of target genes. These interactions are referred to as trans-

repression because DNA binding activity of GR is not required. Since NF- κ B, AP-1, and STAT activate expression of many inflammatory cytokines and interferon, glucocorticoids and corticosteroids are anti-inflammatory and immune-suppressive (De Bosscher, 2000; Newton and Holden, 2007; Ohmann et al., 1987; Scheinman et al., 1995).

In cattle treated with Dex to initiate reactivation from latency, Dex represses LR promoter activity (Jones et al., 1990) and reduces LR-RNA levels, which culminates in a reduction of the number of infected cells that express LR-RNA at 18-21 hours after treatment (Rock et al., 1992). A single injection of Dex also leads to lytic cycle transcript expression in neurons and tonsil within 6 hours after treatment of latently infected calves (Winkler et al., 2002a; Winkler et al., 2000a). Dex treatment of latently infected calves also induces apoptosis of T cells that persist in TG after infection (Winkler et al., 2002b). Persistence of T cells in TG of humans or mice latently infected with HSV-1 also occurs (Cantin et al., 1995a; Halford et al., 1996b; Liu et al., 1996; Shimeld et al., 1995a; Shimeld et al., 1996; Shimeld et al., 1997; Theil, 2003), and persistent CD8⁺ T cells in TG produce factors (presumably cytokines) that inhibit reactivation from latency (Khanna et al., 2003; Knickelbein et al., 2008; Liu et al., 2001; Liu et al., 2000b; Prbhakaran, 2005). In summary, Dex may alter the neuronal cell environment by activating cellular genes that are able to promote reactivation from latency or repress transcription of genes which actively suppress viral gene expression. Additionally, activated GR may bind to cellular transcription factors to alter their function. These events may promote viral gene expression and subsequent reactivation. Dex may also

reduce expression of T cell factors that help maintain latency. However, the exact molecular mechanisms leading to reactivation from latency remains to be determined.

Goal of this study

The mechanism by which *alphaherpesvirinae* subfamily members reactivate from latency remains one of the most clinically relevant, yet least understood aspects of herpes virus infection. Cell culture systems for human herpesviruses, in which virus reactivation occur have been described. However, most are poor models of *in vivo* latency and reactivation. BHV-1 offers a unique opportunity to study the latency-reactivation cycle *in vivo*. First, BHV-1 can be studied in its natural host. Second, BHV-1 can be predictably reactivated from all latently infected animals following a single intravenous dose of the synthetic corticosteroid, dexamethasone (Dex) (Inman et al., 2002; Jones, 1998; Jones, 2003a; Jones et al., 2006; Jones et al., 2000; Rock et al., 1992). Thus, the ability of Dex to consistently initiate reactivation from latency makes BHV-1 a useful model to identify cellular factors that stimulate reactivation from latency. Since BHV-1 is the only alpha-herpesvirus member that can be reproducibly induced to undergo reactivation from latency, these findings have impact on other important members of this virus family, including HSV-1. Therefore, the overall goal of the studies in this dissertation was to use BHV-1 as a model to characterize the early phases of the latency-reactivation cycle of *alphaherpesvirinae* subfamily members.

Chapter 2

Dexamethasone Treatment of Calves Latently Infected with Bovine Herpesvirus 1 (BHV-1) Leads to Activation of the bICP0 Early Promoter, in part by the Cellular Transcription Factor C/EBP-alpha.

These studies are included in a manuscript published in 2009 in the Journal of Virology, volume 83, pages 8800-8809.

The authors of the manuscript are **Aspen Workman**, Sandra Perez, Alan Doster, and Clinton Jones

ABSTRACT

Sensory neurons within trigeminal ganglia are the primary site for bovine herpesvirus 1 (BHV-1) latency. During latency, viral gene expression is restricted to the latency related (LR) gene and ORF-E. We previously constructed a LR mutant virus that expresses LR RNA, but not any of the known LR proteins. In contrast to calves latently infected with wt BHV-1 or the LR rescued virus, the LR mutant virus does not reactivate from latency following dexamethasone (DEX) treatment. We also demonstrated that bICP0, but not bICP4, transcripts were consistently detected in TG of calves infected with the LR mutant or LR rescued virus following DEX treatment. Calves latently infected with the LR rescued virus, but not the LR mutant virus, expressed late transcripts, which correlated with shedding of infectious virus following DEX treatment. The bICP4 and bICP0 genes share a common immediate early promoter suggesting this promoter was not consistently activated during DEX induced reactivation from latency. The bICP0 gene also contains a novel early promoter, suggesting this promoter is activated during reactivation from latency. In this study, we found that the bICP0 E promoter was activated by DEX in mouse neuroblastoma cells. Expression of a cellular transcription factor, C/EBP-alpha, was stimulated by DEX, and C/EBP-alpha expression was necessary for DEX induction of bICP0 early promoter activity. C/EBP-alpha directly interacted with bICP0 early promoter sequences that were necessary for trans-activation by C/EBP-alpha. In summary, DEX treatment of latently infected calves induced cellular factors that stimulated bICP0 early promoter activity. Activation of bICP0 early promoter activity does not necessarily lead to late gene expression and virus shedding.

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is a significant viral pathogen of cattle that is responsible for a variety of disease conditions, which include conjunctivitis, pneumonia, genital disorders, or abortions. BHV-1 also causes rhinotracheitis, a serious upper respiratory tract infection, and infection can initiate shipping fever, a potentially fatal polymicrobial disease (Tikoo et al., 1995). Like other members of the alpha-herpesvirinae subfamily, BHV-1 establishes lifelong latency in trigeminal ganglionic neurons following acute replication in mucosal epithelium. Reactivation from latency occurs periodically, resulting in virus shedding and spread to susceptible cattle. Reactivation can occur after stress or corticosteroid treatment, which mimics stress (Rock et al., 1992; Sheffy and Davies, 1972).

Viral gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E), or late (L), reviewed in (Jones, 1998; Jones, 2003b). IE gene expression is stimulated by a virion component, bTIF, which interacts with a cellular transcription factor (Oct-1) to transactivate IE gene expression (Misra et al., 1994; Misra et al., 1995). Two IE transcription units exist: IE transcription unit 1 (IEtu1) and IEtu2 (Wirth et al., 1992; Wirth et al., 1991). IEtu1 encodes functional homologues of two HSV-1 proteins, ICP0 and ICP4. IEtu2 encodes a protein that is similar to the HSV-1 IE gene, ICP22 (Fraefel et al., 1994). BHV-1 encoded ICP0 (bICP0) is translated from an IE (IE2.9) or E mRNA (E2.6) because an IE promoter (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). The IE promoter regulates IE expression of bICP4 and bICP0. Expression of bICP4 leads to repression of IEtu1 promoter activity whereas bICP0

activates its own E promoter and all other viral promoters. Thus bICP0 is considered to be the major regulatory protein that stimulates productive infection (Fraefel et al., 1994; Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991).

The latency related (LR) gene is abundantly transcribed in trigeminal ganglia (TG) of latently infected calves (Kutish et al., 1990a; Rock et al., 1992; Rock et al., 1987b). LR-RNA is anti-sense with respect to the bICP0 gene (Jones, 1998; Jones, 2003a; Jones et al., 2006) and encodes at least three proteins. LR gene products inhibit mammalian cell growth (Geiser and Jones, 2005; Schang et al., 1996), productive infection (Bratanich et al., 1992; Geiser et al., 2002; Schang et al., 1996), and apoptosis (Ciacci-Zanella et al., 1999; Henderson et al., 2004a). While expression of LR proteins is necessary for inhibiting apoptosis (Inman et al., 2002), protein expression is not necessary for inhibiting cell growth or productive infection. The LR gene has two open reading frames (ORF1 and ORF2), and two reading frames that lack an initiating ATG (RF-B and RF-C). A mutant BHV-1 virus with 3 stop codons at the beginning of ORF2 was constructed to test whether LR protein expression regulates the latency-reactivation cycle in cattle (Inman et al., 2002). The LR mutant virus grows to similar titers as wt BHV-1 or the LR rescued virus in cultured bovine cells indicating LR gene products are not required for growth. ORF2 and RF-B are expressed when bovine cells are infected with wt or the LR rescued virus, but not with the LR mutant virus (Hossain et al., 1995b; Jiang et al., 1998; Jiang et al., 2004). ORF1 expression is reduced, but not totally eliminated following infection of cultured cells with the LR mutant virus (Meyer et al., 2007b). Calves infected with the LR mutant virus exhibit diminished clinical symptoms, and reduced shedding of infectious virus in the eye, tonsil, or TG (Inman et al., 2002;

Inman et al., 2001b; Perez et al., 2005). The LR mutant virus prematurely expresses LR-RNA relative to rescued or wt BHV-1, which correlates with an enhanced IFN response in cells infected with the LR mutant virus (Perez et al., 2008).

Stress, in part due to increased corticosteroid levels, and/or immune suppression can initiate reactivation from latency. During reactivation from latency, productive viral gene expression is readily detected in sensory neurons, LR gene expression decreases, and infectious virus can be detected in nasal or ocular swabs (Jones, 1998; Jones, 2003a; Jones et al., 2006). Administration of dexamethasone (DEX), a synthetic corticosteroid, to calves or rabbits latently infected with BHV-1 reproducibly leads to activation of viral gene expression and reactivation from latency (Inman et al., 2002; Jones, 1998; Jones, 2003a; Jones et al., 2006; Jones et al., 2000; Rock et al., 1992). After DEX treatment, many neurons express lytic viral genes, but only a small subset of the neurons that express viral genes appear to produce infectious virus (Rock et al., 1992). Thus, the ability of DEX to consistently initiate reactivation from latency makes BHV-1 a useful model to identify cellular factors that stimulate reactivation from latency.

Calves latently infected with the LR mutant virus do not shed detectable levels of infectious virus after DEX treatment (Inman et al., 2002). At a molecular level, two possibilities seemed feasible to explain this observation. First, TG neurons latently infected with the LR mutant virus may express a subset of lytic viral genes after DEX treatment, but infectious virus is not produced. Secondly, DEX treatment of calves latently infected with the LR mutant virus does not stimulate lytic viral gene expression. Therefore, we tested whether calves latently infected with the LR mutant virus express viral genes in TG following DEX treatment. Expression of bICP0, but not bICP4, RNA

was consistently detected in TG following DEX treatment of calves infected with the LR mutant virus or wt BHV-1. The late gC transcript was detected after infection with the wt BHV-1 but not the LR mutant, and this correlated with reactivation. The presence of bICP0 transcript in the absence of bICP4 transcript suggested the bICP0 E promoter may be activated during reactivation from latency (Workman et al., 2009); see Figure 2.1A). Therefore, in this study we were interested in understanding the transcriptional regulation of the bICP0 E promoter. We found that in transient transfection assays, DEX stimulated bICP0 E promoter activity. Furthermore, the ability of DEX to induce expression of a cellular transcription factor (C/EBP-alpha) was important for stimulating bICP0 E promoter activity.

MATERIALS AND METHODS

Cells and Virus

Murine neuroblastoma 2A (neuro-2A) and rabbit skin (RS) cells were grown in Earle's modified Eagle's medium (EMEM) supplemented with 5% fetal calf serum (FCS). Bovine kidney (CRIB) cells were grown in EMEM supplemented with 10% FCS. All media contained penicillin (10 U/ml) and streptomycin (100 µg/ml).

The Cooper strain of BHV-1 (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa.

Plasmids

The C/EBP-alpha wt plasmid contains the wt sequence of mouse C/EBP-alpha in an adenoviral vector (pAdTrack) that contains mammalian promoter/enhancer sequences (Sood, 2009). The C/EBP-alpha mutant plasmid contains a mutation in the C/EBP-alpha ORF that produces a single amino acid change (R290A) in C/EBP-alpha, which results in the loss of DNA binding activity (Sood, 2009). The empty vector pcDNA3.1 was purchased from Invitrogen.

Six DNA reporter constructs were generated by PCR using wt BHV-1 genome as template and a common 3' primer (5'- ctcgag CCTGCTGGGCGACACAAACAACAGA -3') with 5' primers: EP-943, 5'- ggtaccGCGACGGCGGCAATAAAGACGAGTC -3'; EP-638, 5'- ggtaccGCCCTCGGTCTCGGTCCGAG-3'; EP-172, 5'-gggtaccGCCTTGCGTGGGGGGTTTCG-3'; EP-143, 5'-gggtaccAGCCGGGGGGTGC GGCC-3';

EP-133, 5'-gggtaccTGCGGGCCT TTCGCCG-3'; or

EP-71, 5'-gggtaccGCTCCCGGCGCGTCA-3'.

The promoter fragments were cloned into the promoterless vector pCAT-Basic (E1871; Promega) at the unique XhoI and KpnI sites to generate plasmids EP-943, EP-638, EP-172, EP-143, EP-133, and EP-71 (Figure 2.1). The numbers in the plasmid name refers to the length of the bICP0 early promoter fragment inserted into the CAT vector. Two additional constructs, EP-50 and EP-42, were created using synthesized duplex sequences. Duplex oligonucleotides were digested with XhoI and KpnI and cloned into the promoter-less vector pCAT-Basic. E promoter inserts were confirmed by DNA sequencing (Genomics Core Research Facility-UNL). Plasmids were prepared from bacterial cultures by alkaline lysis and two rounds of cesium chloride centrifugation.

Measurement of chloramphenicol acetyltransferase (CAT) activity

Neuro-2A cells grown in 60 mm dishes were co-transfected with the designated plasmids as indicated in the respective figure legends. Neuro-2A cells were transfected with NeuroTransIt (MIR2145; Mirus), according to the manufacturer's instructions. Where indicated, cells were cultured in the presence of 1 uM water soluble DEX (D2915; Sigma) at the time of transfection. After 48 hours, cell extract was prepared by three freeze/thaw cycles in 0.25 M Tris-HCl, pH 7.4. Cell debris was pelleted by centrifugation, and protein concentrations determined. CAT activity was measured in the presence of 0.1 uCi [¹⁴C]-chloramphenicol (CFA754; Amersham Biosciences) and 0.5 mM AcetylCoA (A2181; Sigma). The reaction was incubated at 37° C for 15 minutes to 2 hours depending on the activator used. All forms of chloramphenicol were separated

by thin-layer chromatography. CAT activity was quantified using a Bio-Rad Molecular Imager FX (Molecular Dynamics, CA). Levels of CAT activity are expressed as fold induction relative to the vector control.

SDS-PAGE and Western Blotting

At 48 hours after transfection, whole cell lysate was prepared. Cells were washed with phosphate-buffered saline 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] per 10 ml). Cell lysate was incubated on ice for 30 min, sonicated, and then clarified by centrifugation at 10,000 x 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 1x 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 a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) and blocked for 4 hours 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. The C/EBP-alpha antibody (14AA; Santa Cruz Biotechnology) was diluted 1:500 in the blocking solution. An antibody directed against β -actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. After 45 minutes of washing with TBS-T, the blots were incubated with donkey anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (Amersham Biosciences), which was diluted 1:1000 in 5% nonfat milk in TBS-T. Blots

were washed 45 minutes with TBS-T and exposed to Amersham ECL reagents, and then autoradiography performed.

siRNA transfection for Western Blotting and CAT Assay

Mouse C/EBP-alpha siRNA containing a pool of 3 target-specific 20-25 oligonucleotide siRNAs designed to reduce gene expression (sc-37048; Santa Cruz Biotechnology) were used in this study. The Block-iT-Fluorescent Oligo was used as a control siRNA (44-2926; Invitrogen). It is a fluorescent conjugated control containing a scrambled sequence that does not degrade any known cellular RNA.

Neuro-2A cells grown in 60 mm dishes were co-transfected with 1 ug of EP-172 with either 100nM mouse C/EBP-alpha siRNA or the fluorescent control siRNA. After 4 hours, the transfection mixture was removed and replaced by fresh media. At 48 hours post transfection, cell extract was collected for Western blot analysis or CAT assays.

Measurement of virus titers in bovine cells.

RS cells grown in 60 mm dishes were transfected with 100nM mouse C/EBP-alpha siRNA, the fluorescent control siRNA, or transfection reagent alone. After 4 hours, the complex was removed and replaced by fresh media. After 24 hours, cells were infected with wt BHV-1 at a moi of 0.1 and incubated for an additional 24 hours. Media and cells were collected and subjected to two freeze/thaw cycles. Cell debris was pelleted and the supernatant titrated on CRIB cells (bovine kidney) that were plated onto 6-well plates 24 h prior to virus infection (90% confluence at the time of infection). After 1 h of adsorption at 37°C, cells were rinsed with phosphate-buffered saline (PBS) and

overlaid with a 50:50 mixture of 1.4% Sea Plaque Agar in PBS and EMEM supplemented with 10% FCS.

Electrophoretic Mobility Shift Assay (EMSA)

Neuro-2A whole cell lysate was prepared by lysing cells with NP40 lysis buffer. Thirty micrograms of protein extract were incubated in a volume of 16 ul of binding buffer (10mM Tris-HCl pH8, 150mM KCl, 0.5mM EDTA, 0.1% Triton X-100, 12.5% glycerol) in the presence of 1 ug poly(dI-dC) (P4929; Sigma), and 0.5pmols of double stranded DNA probe labeled with 10 uCi of γ -³²P-ATP. Incubation was for 1 hour at room temperature. For supershift experiments, 5 ug of antibody (C/EBP-alpha, sc-61X or C/EBP-beta, sc-150X; Santa Cruz Biotechnology) were added to the reaction after 30 minutes and allowed to incubate an additional 30 minutes. The DNA-protein complexes were electrophoresed on a 5% polyacrylamide gel in 0.5X TBE for 3 hours at 160 volts. To improve band resolution, 1.5M sodium acetate pH 5.3 was added to the lower buffer chamber during electrophoresis. The gel was exposed to a phosphorimager plate and analyzed using a Bio-Rad Molecular Imager FX. The bICP0 E probes used for EMSA were:

C1: GCCTTGCGTGGGGGGTTTCGCCTTGGGGCAGC,

C2: TGCGGGCCCTTTCGCCGCCCG,

C3: TCTGTTGTTTGTGTCGCCCA

An oligonucleotide containing 3 consensus C/EBP-alpha binding sites, CGCAATATTGCGCAATATTGCAAT, was used as a positive control for binding to C/EBP-alpha.

RESULTS

DEX activates the bICP0 E promoter

Previous studies demonstrated that during reactivation from latency, bICP0 mRNA is detected while bICP4 mRNA is not readily detected (Workman et al., 2009). The bICP4 and bICP0 genes share a common IE promoter, suggesting this promoter was not consistently activated during DEX induced reactivation. bICP0 also contains a novel E promoter (Fraefel et al., 1994; Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991). This suggested that the bICP0 E promoter may be preferentially activated during reactivation from latency (see Figure 2.1A). We hypothesize that DEX treatment may stimulate expression of cellular factors that subsequently activate the bICP0 E promoter. To test this hypothesis, the bICP0 promoter construct was transfected into mouse neuroblastoma cells (neuro-2A) and treated with DEX after transfection. For these studies, 8 constructs containing upstream sequences of the bICP0 E promoter were used (Figure 2.1B). The basal level of promoter activity for EP-943 was slightly higher than EP-638 and at least 2 times higher than EP-172 or EP-133. Basal promoter activity of EP-71, EP-50, and EP-42 was at least 4 fold less than EP-943.

Two E promoter constructs EP-172 and EP-133 were activated by DEX more than 4 fold in neuro-2A cells (Figure 2.2A). More extensive deletions (constructs EP-71 or EP-40) were not activated by DEX. The larger E promoter constructs, EP-943 and EP-638, were activated less than 2 fold by DEX. As a comparison, three IEtu1 promoter constructs were tested for DEX induction (Figure 2.2B). None of the constructs were activated by DEX more than 2 fold. As expected, the empty vector (pCAT basic) was not activated by DEX (Figure 2.2A and B).

DEX activates a cellular transcription factor, C/EBP-alpha, in neuro-2A cells.

A previous study demonstrated that the cellular transcription factor C/EBP-alpha is stimulated when latently infected calves are treated with DEX (Meyer et al., 2007b). Furthermore, C/EBP-alpha stimulates productive infection when over-expressed (Meyer et al., 2007b), and C/EBP-alpha plus bICP0 or bTIF stimulates IEtu1 promoter activity more efficiently than just the viral trans-activator alone (Meyer and Jones, 2009). To test whether DEX induced C/EBP-alpha protein expression in neuro-2A cells, cells were treated with 1 μ M DEX and Western blots performed. Twenty-four hours after DEX treatment, C/EBP-alpha protein expression was consistently induced (Figure 2.3A). Conversely, β -actin protein levels were similar regardless of DEX treatment.

A plasmid that expresses C/EBP-alpha, but not a DNA binding mutant of C/EBP-alpha, trans-activated three bICP0 E promoter constructs (EP-172, EP-133, and EP-143) 4 to 5 fold (Figure 2.3B). Constructs with additional deletions (EP-71, EP-50, and EP-42) were only trans-activated 2 fold. The larger bICP0 E promoter constructs, EP-943 and EP-638, were trans-activated 2-3 fold by C/EBP-alpha. As expected, pCAT-basic was not trans-activated by C/EBP-alpha.

C/EBP-alpha expression is important for DEX induced activation of the bICP0 E promoter.

To test whether induction of C/EBP-alpha by DEX was important for activating bICP0 E promoter activity, neuro-2A cells were transfected with a siRNA directed against C/EBP-alpha and then the ability of DEX to induce bICP0 E promoter activity was measured. A C/EBP-alpha siRNA (Figure 4A, lane C/EBP), but not a control siRNA

(-Ctl lane), reduced C/EBP-alpha protein levels after DEX treatment. As expected, β -actin controls were similar regardless of the siRNA transfected into neuro-2A cells. bICP0 E promoter activity (EP-172) was not stimulated by DEX when cells were transfected with the C/EBP-alpha siRNA (Figure 2.4B). Conversely, the control siRNA had little or no effect on DEX induction of the bICP0 E promoter.

Over-expression of C/EBP-alpha stimulates plaque formation 3-5 fold (Meyer et al., 2007b) suggesting that silencing C/EBP-alpha expression would reduce the levels of plaquing efficiency. In several independent studies, the C/EBP-alpha siRNA (Figure 2.4C), but not the control siRNA inhibited productive infection approximately 3 fold. In summary, these studies suggested C/EBP-alpha expression was necessary for DEX induced bICP0 E promoter activity and efficient productive infection.

C/EBP-alpha interacts with the bICP0 E promoter.

Electrophoretic mobility shift assays (EMSA) were performed to determine whether C/EBP-alpha directly interacted with sequences in the bICP0 E promoter. Since C/EBP-alpha was expressed at low levels in neuro-2A cells, a C/EBP-alpha expression plasmid was transfected into neuro-2A cells to induce protein levels (Figure 2.5A), and then extracts prepared to test whether C/EBP-alpha interacted with the bICP0 E promoter. As a control, an oligonucleotide that contained 3 consensus C/EBP-alpha binding sites was used. As expected, binding to the C/EBP-alpha consensus oligonucleotide was enhanced when C/EBP-alpha was over-expressed in neuro-2A cells (Figure 2.5B, lane 2). An antibody that recognizes C/EBP-alpha (lane 3), but not C/EBP-

beta (lane 4), “super-shifted” the DNA-protein complex confirming that a stable interaction occurred.

Enhanced binding to the EP-172 promoter was also detected after over-expression of C/EBP-alpha (Figure 2.5C, lane 2 versus 1). Addition of a specific antibody directed against C/EBP-alpha (lane 3), but not C/EBP-beta (lane 4), super-shifted the induced band. Although the C/EBP-alpha antibody super-shifted a band when the EP-133 fragment was used as a probe (EP-133 panel, lane 3), binding was less efficient compared to the EP-172 probe. When the EP-72 fragment was used as a probe, a faint super-shifted band was detected (Figure 2.5C, lane 3) that was readily detected when the gel was exposed for longer times (Figure 2.5D, lane 3).

The bICP0 E promoter contains three C/EBP-alpha like binding sites clustered near the 5' terminus of EP-172 and a putative site downstream of the TATA box (Figure 2.6A). To test whether C/EBP-alpha interacted with these individual domains, oligonucleotides spanning these domains (see Figure 2.6A for sequence of C1-C3 oligonucleotides) were synthesized and EMSA performed (Figure 2.6B). Super-shift assays using a C/EBP-alpha specific antibody (Figure 2.7B, lanes A), but not the C/EBP-beta antibody (lanes B), suggested that C/EBP-alpha interacted with the C1 and C2 oligonucleotides. Prolonged exposure of the autoradiograph revealed that C/EBP-alpha interacted less efficiently with the C3 oligonucleotide (Figure 2.6C). In several independent studies, the band super-shifted by the C/EBP-alpha antibody was more distinct relative to samples not treated with antibody suggesting that the antibody stabilized the interaction between C/EBP-alpha and bICP0 E promoter sequences. As expected, the consensus C/EBP-alpha probe interfered with C/EBP-alpha specific binding

(data not shown). In summary, this study indicated that at least 3 different elements within the bICP0 E promoter interacted with C/EBP-alpha.

DISCUSSION

We previously demonstrated that viral gene expression was stimulated in TG of calves latently infected with the LR mutant virus after DEX treatment (Workman et al., 2009). The inability to detect infectious virus in ocular or nasal swabs after calves latently infected with the LR mutant virus were treated with DEX (Inman et al., 2002) correlated with lack of gC transcription after DEX treatment (Workman et al., 2009). These findings implied that the LR mutant virus was unable to infect neurons that support reactivation from latency, or the LR mutant virus killed neurons that support reactivation from latency. Interestingly, higher rates of neuronal apoptosis occur in TG neurons near the end of acute infection (establishment of latency) following infection with the LR mutant virus (Lovato et al., 2003). Furthermore, in situ hybridization revealed that a small percent of viral genome positive neurons are present in TG of calves infected with wt or the LR rescued virus, but not the LR mutant virus (Inman et al., 2002). These observations suggested that the anti-apoptosis activity of a protein encoded by the LR gene promotes survival of a specific subset of neurons that can yield infectious virus after DEX treatment (Ciacci-Zanella et al., 1999; Devireddy and Jones, 1999a; Geiser et al., 2008; Lovato et al., 2003; Shen and Jones, 2008; Winkler et al., 1999). Neurons that contain few copies of viral genomes do not support extensive viral gene expression during acute infection, and consequently they survive acute infection and permit establishment of viral latency, regardless of whether LR gene products are expressed. The finding that different populations of neurons in TG of mice are more permissive for HSV-1 is consistent with the prediction that a subset of neurons in TG contain specific cellular factors that support reactivation from latency.

Relative to other alpha-herpesvirus members, the organization of the BHV-1 ICP4 and ICP0 genes is unique because a common IE promoter drives expression of bICP0 and bICP4 (Wirth et al., 1992) (Figure 2.1A). bICP0 also contains an E promoter located near the 5' end of the coding exon of bICP0. Although it was not well understood why the bICP0 gene contains two promoters, we assumed this was necessary to ensure that constitutive expression of bICP0 occurred during productive infection.

DEX treatment appeared to stimulate cellular factors, which then activated the bICP0 E promoter in TG of latently infected calves (Workman et al., 2009) and mouse neuroblastoma (neuro-2A) cells (Figure 2.2). Previous studies demonstrated that DEX treatment of calves latently infected with BHV-1 leads to C/EBP-alpha expression (Meyer et al., 2007b), which correlated with reactivation from latency and activation of the bICP0 E promoter. The finding that C/EBP-alpha siRNAs inhibited the ability of DEX to activate the bICP0 E promoter supported the conclusion that C/EBP-alpha was necessary for stimulating bICP0 E promoter activity after DEX treatment. DEX can activate C/EBP-alpha in several distinct cell types (Cram et al., 1998; Hausman, 2000; Ramos et al., 1996), including neuro-2A (Figure 2.3) and TG neurons (Meyer et al., 2007b). Three distinct regions of the bICP0 E promoter interacted with C/EBP-alpha as judged by EMSA and super-shift studies. Based on transient transfection assays, we suggest that at least two of the C/EBP-alpha binding sites were necessary for efficient trans-activation by C/EBP-alpha. The weak binding site in the C3 oligonucleotide (Figure 2.6A) was not sufficient for activation by C/EBP-alpha because EP-71, EP-50, and EP-42 were not trans-activated by C/EBP-alpha (Figure 2.4B). The C/EBP-alpha binding sites in the bICP0 E promoter appeared to be low affinity binding sites compared to the

consensus site because 30 ug of nuclear extract was necessary to observe C/EBP-alpha induced binding with EP-172 whereas only 5 ug of protein in nuclear extracts was necessary to detect inducible binding to an oligonucleotide containing three consensus C/EBP-alpha sites (Figure 2.5). The weak C/EBP-alpha binding sites in the bICP0 E promoter may inhibit activation of the E promoter during latency unless the neuronal environment is conducive for reactivation from latency.

The larger bICP0 E promoter constructs (EP-943 and EP-638) were not efficiently activated by C/EBP-alpha or by DEX treatment. However, EP-943 and EP-638 promoter constructs had 2-3 higher levels of basal promoter activity. We suggest there may be cis-acting negative regulatory elements located in the upstream region of the bICP0 early promoter that interfere with the ability of C/EBP-alpha to activate promoter activity. Furthermore, cellular transcription factors that stimulate the larger promoter constructs may interfere with the ability of C/EBP-alpha to activate E promoter activity. Since the bICP0 E promoter overlaps bICP4 protein coding sequences, these additional bICP4 protein-coding sequences may not be a part of the true bICP0 E promoter, and consequently may interfere with transient transfection reporter assays.

A protein encoded by an alternatively spliced LR-RNA stably interacts with C/EBP-alpha (Meyer et al., 2007b). The finding that C/EBP-alpha stimulates productive infection (Meyer et al., 2007b), the IETu1 promoter (Meyer and Jones, 2009), and the bICP0 E promoter suggests that interactions between the LR protein and C/EBP-alpha help to extinguish viral transcription, thus promoting the establishment and maintenance of latency. DEX represses LR-RNA (Rock et al., 1992) and LR promoter activity (Jones et al., 1990) suggesting that LR gene products do not directly stimulate reactivation from

latency. However, reduced levels of LR proteins following DEX induction of reactivation may allow C/EBP-alpha to stimulate viral gene expression. Although C/EBP-alpha appears to be an important component in the DEX induced signaling cascade that stimulates viral gene expression, it seems clear that additional DEX inducible cellular proteins are required for infectious virus to be produced.

Figure 2.1. Schematic of IEtu1 and bICP0 E promoter constructs used in this study.

Panel A: Positions of bICP4 and bICP0 transcripts are shown. The immediate early transcription unit 1 (IEtu1) encodes bICP4 (IE/4.2) and bICP0 (IE/2.9) (Wirth et al., 1989; Wirth et al., 1991). The IEtu1 promoter activates IE expression of IE/4.2 and IE/2.9 (denoted by the black rectangle). E/2.6 is the early transcript that encodes bICP0 and an early promoter activates expression of this transcript (Wirth et al., 1992). Exon 2 (e2) of bICP0 contains all of the protein coding sequences of bICP0. The dashed lines are intron sequences.

Panel B: bICP0 E promoter constructs were prepared as described in the materials and methods. Position of putative C/EBP-alpha binding sites and TATA box are shown. Basal promoter activity was measured in neuro-2A cells.

Figure 2.1.

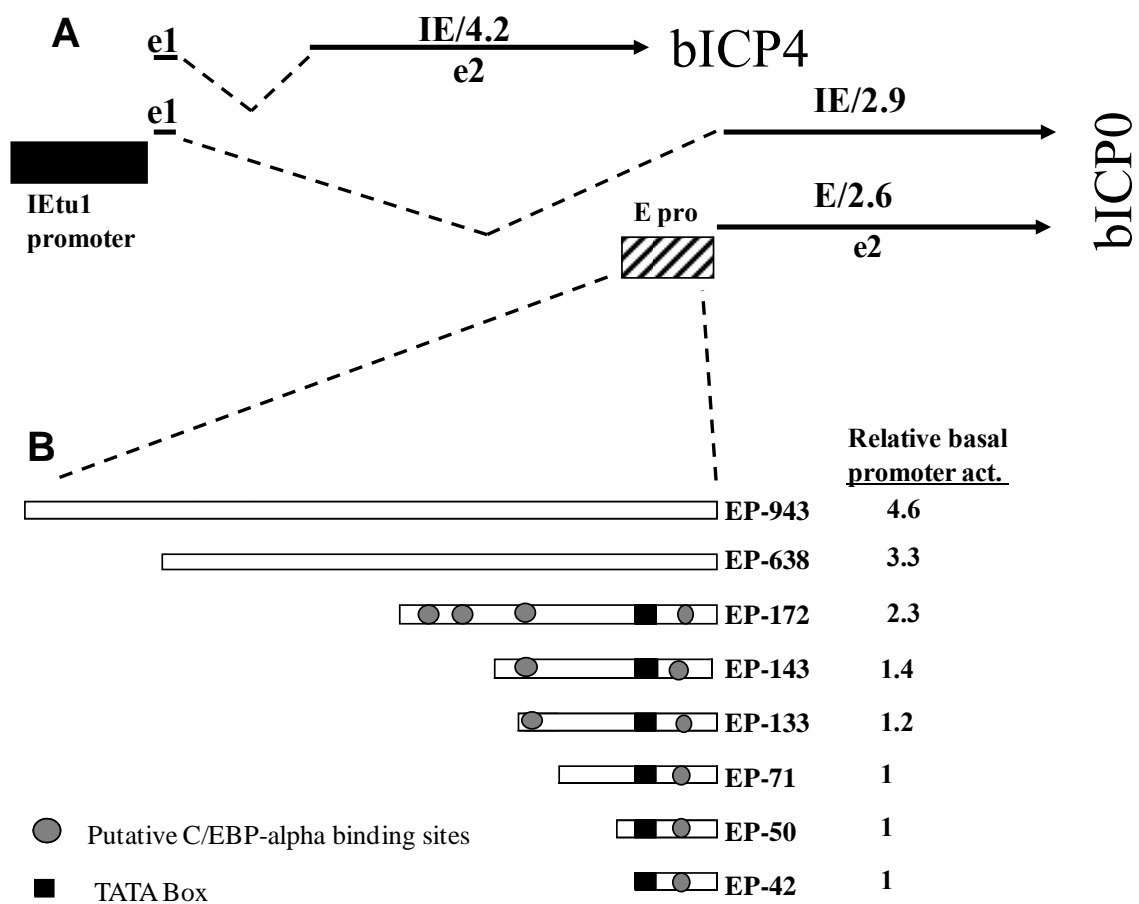


Figure 2.2. DEX induces bICP0 E promoter activity.

Panel A. Neuro-2A cells were transfected with 1 μ g of the designated bICP0 E promoter construct. Cells were cultured in EMEM containing 1% FBS and 1 μ M dexamethasone (DEX) at the time of transfection. At 48 hours post transfection, cells were collected and processed for CAT activity as described in the materials and methods. CAT activity of cells treated with EMEM containing no DEX was set to 1 fold. All other values are expressed as fold activation with respect to their control. The results are the average of three independent experiments.

Panel B: IETu1 promoter constructs were assessed for DEX induction as described in panel A. Description of the IETu1 promoter constructs was described previously (Geiser and Jones, 2003; Meyer and Jones, 2009; Misra et al., 1994). IETu1-CAT contains IETu1 promoter sequences that were cloned upstream of pSV0CAT (a promoter minus CAT expression vector, and this plasmid was provided by V. Misra (Saskatoon, Canada). Two deletion constructs, Δ 1024 IETu1 and Δ 1391 IETu1 have 1024 or 1391 bp removed from the 5' terminus, respectively.

Figure 2.2.

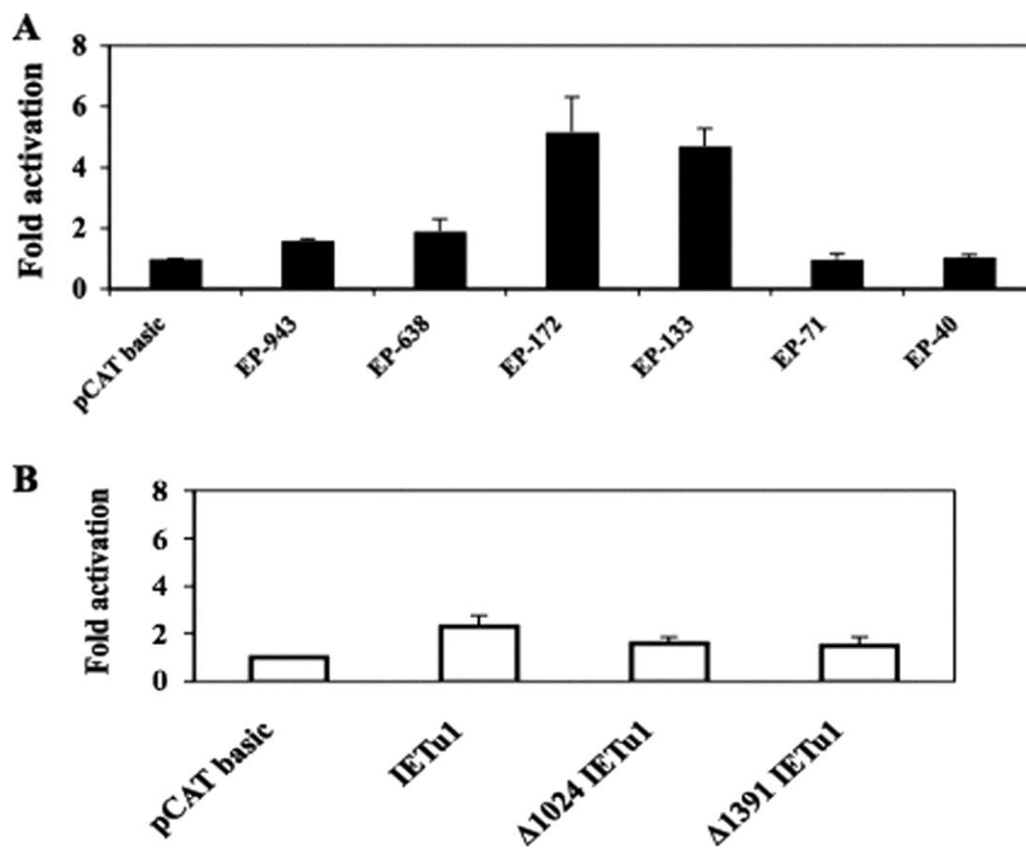


Figure 2.3. DEX induces C/EBP–alpha expression in cultured cells and C/EBP-alpha activates the bICP0 early promoter.

Panel A. Neuro-2A cells were treated with 1 uM DEX and cell lysate collected at various times after treatment (hours). One hundred ug of protein were electrophoresed and Western blot analysis performed using a C/EBP–alpha or β -actin antiserum that was diluted 1:500. The molecular weight marker is in kilodaltons.

Panel B. Neuro-2A cells were co-transfected with 1ug of the designated bICP0 E promoter construct and 0.1 ug of wt C/EBP-alpha (filled columns) or the DNA binding mutant of C/EBP-alpha (mut C/EBP-alpha) (open columns). Constant amounts of DNA were used for all transfections by adding pcDNA3.1, a blank expression vector. At 48 hours post transfection, cells were collected and processed for CAT activity as described in the materials and methods. CAT activity of the cells transfected with the control pCAT basic vector was given a value of one. All other values are expressed as fold activation with respect to the control. The results are the average of four independent experiments.

Figure 2.3.

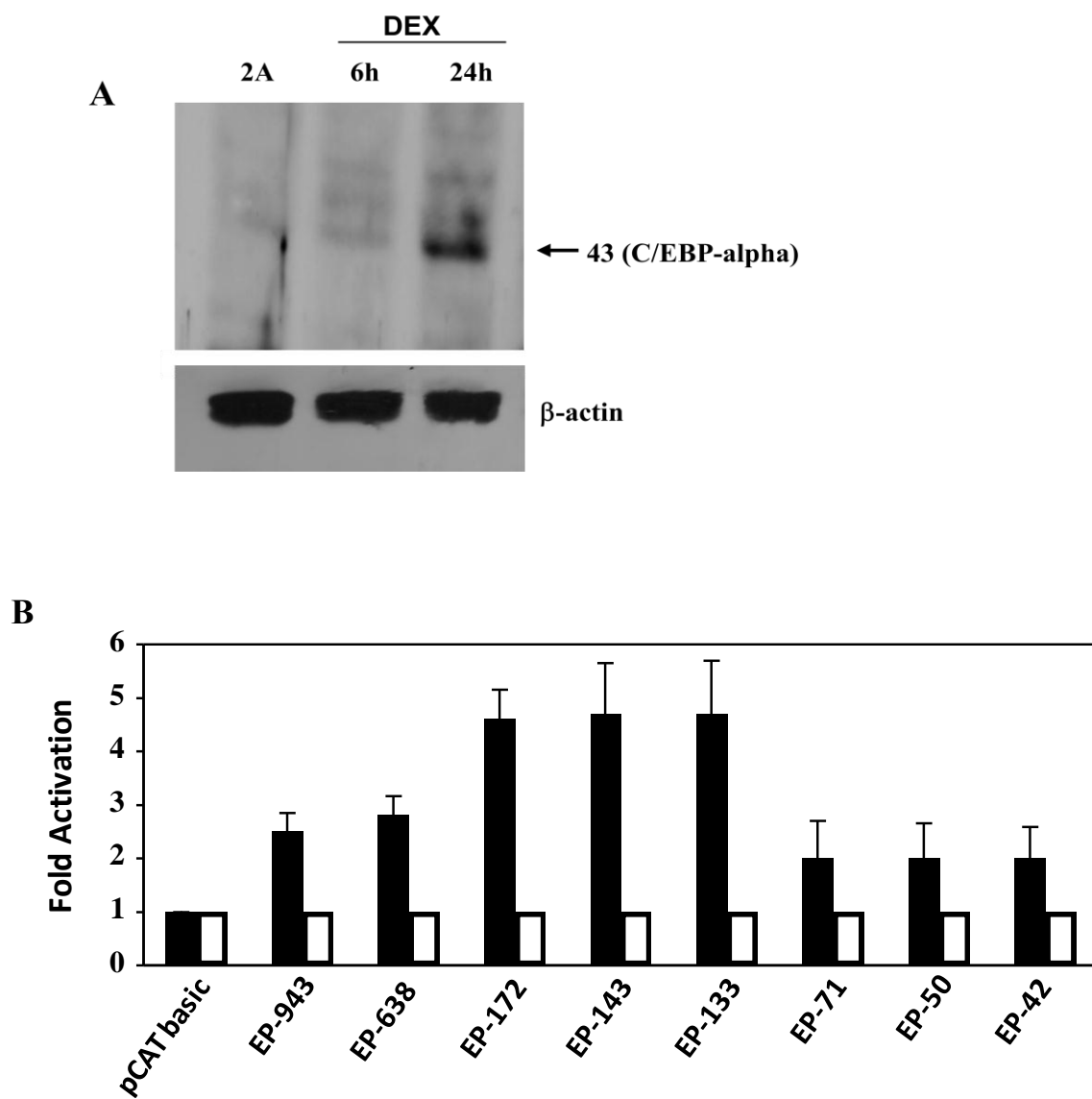


Figure 2.4. The C/EBP-alpha siRNA inhibits the ability of DEX to stimulate bICP0 E promoter activity.

Panel A. Neuro-2A cells were co-transfected with 1ug of C/EBP-alpha and either 100nM C/EBP-alpha siRNA or the control siRNA. At 48 hours post transfection, cells were collected and lysed with NP-40 lysis buffer, and 30 ug protein electrophoresed by 12% SDS-PAGE. Proteins in the gel were transferred onto a PVDV membrane and probed with the C/EBP-alpha antiserum that was diluted 1:500. Molecular weight marker is in kilodaltons.

Panel B. Neuro-2A cells were co-transfected with 1 ug of EP-172 and with 100nM C/EBP α siRNA or a control siRNA as described in the materials and methods. Four hours later the transfection complex was removed and replaced by fresh media containing 1 uM DEX. At 48 hours post transfection, cells were collected and processed for CAT activity. CAT activity of cells transfected with the control pCAT basic vector was given a value of one. All other values are expressed as fold activation with respect to the control. The results are the average of three independent experiments.

Panel C: RS cells were transfected with the C/EBP-alpha siRNA (100 nm), the control siRNA (100 nm), or no siRNA. Twenty-four hours after transfection, cultures were infected with wt BHV-1 at a moi of 0.1. At 24 hours after infection, plaque assays were performed as described in the materials and methods.

Figure 2.4.

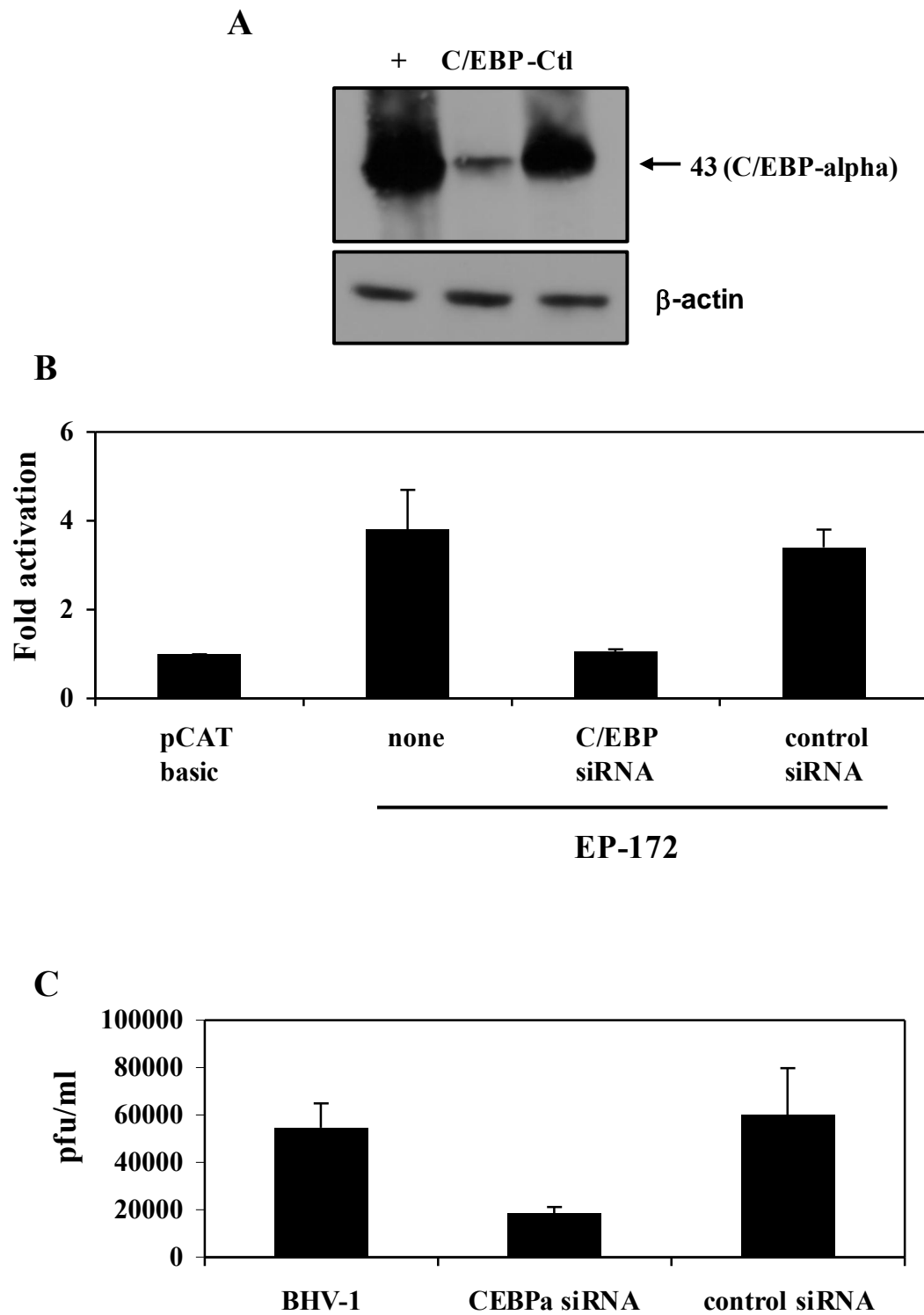


Figure 2.5. C/EBP-alpha directly interacts with the bICP0 E promoter.

Panel A. Neuro-2A cells were transfected with 1ug of C/EBP α . At 48 hours post transfection, cells were collected and lysed with NP-40 lysis buffer. Thirty or 100 ug protein were electrophoresed by 12% SDS-PAGE. Proteins in the gel were transferred onto a PVDV membrane and probed with the C/EBP-alpha antiserum that was diluted 1:500. Molecular weight marker is in kilodaltons.

Panel B. EMSA was performed using a double stranded DNA probe containing three consensus C/EBP-alpha binding sites. The probe was incubated with 5 ug of neuro-2A cell lysate (lane 1) or neuro-2A cell lysate in which C/EBP-alpha was over-expressed (lanes 2-4). The super-shift was visualized by adding C/EBP-alpha (lane 3) or C/EBP-beta antibodies (lane 4) to the binding reaction as described in the materials and methods. The arrow denotes the position of the super-shift while the circle represents the C/EBP-alpha inducible band.

Panel C. EMSA was performed using the bICP0 E promoter fragments derived from EP-172, EP-133 or EP-72. The lanes are the same as described in panel B.

Panel D. To better visualize the super-shift for EP-72, autoradiography was for 24 hours versus 4 as shown in panel C.

Figure 2.5.

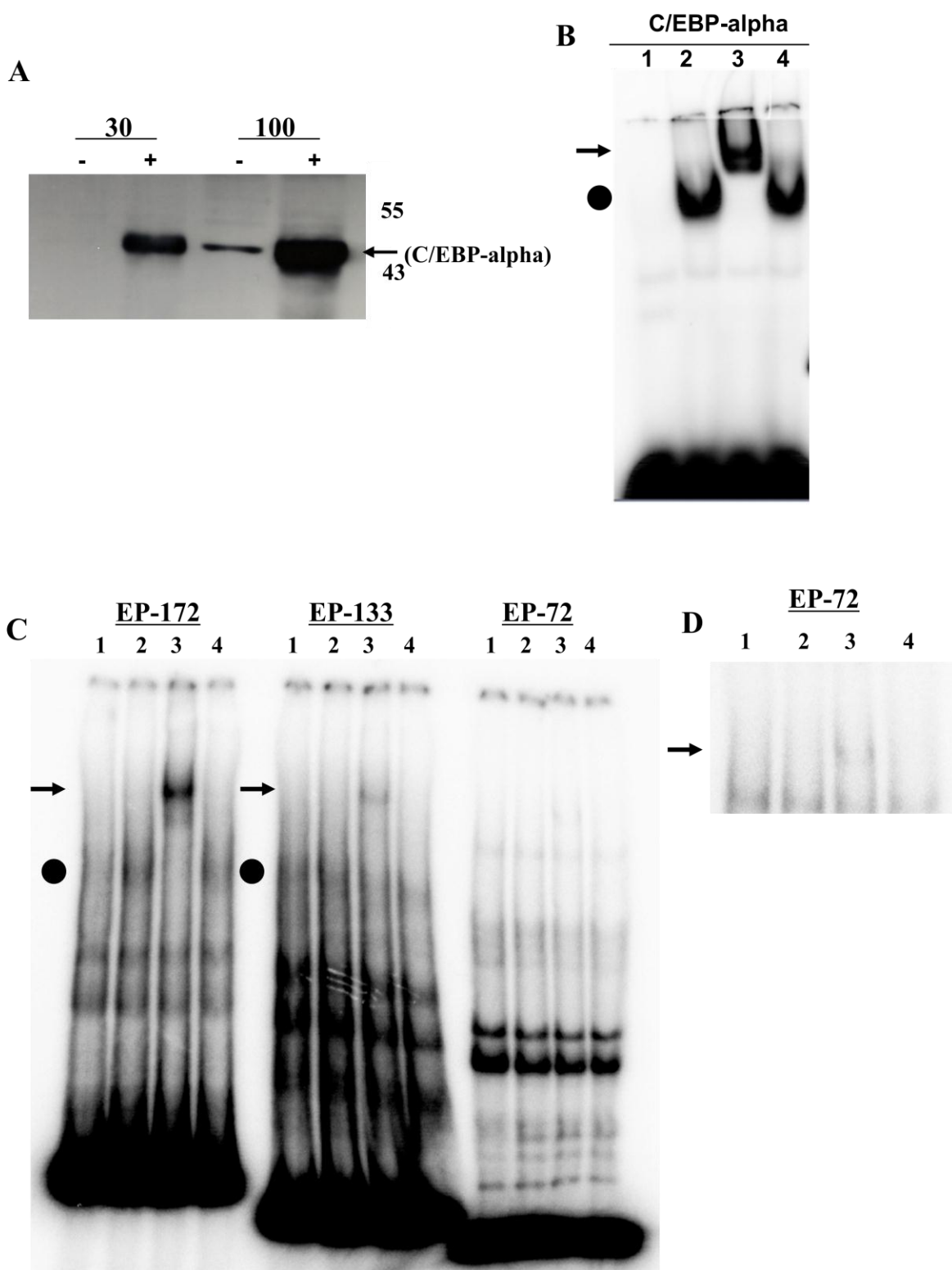


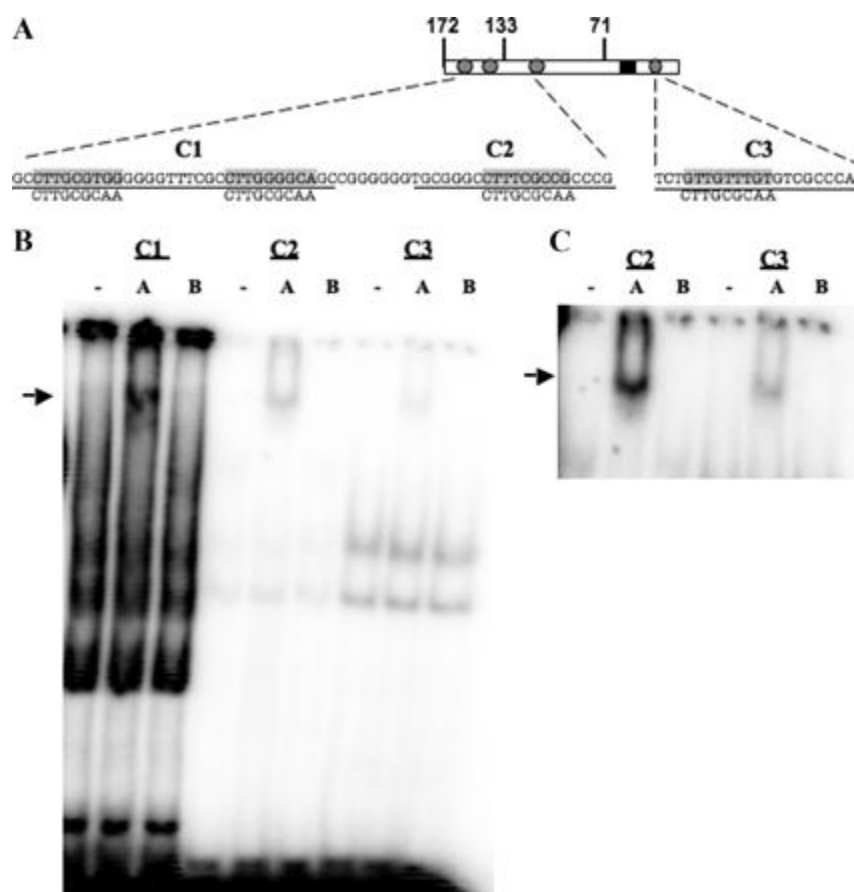
Figure 2.6. Interactions between segments of the bICP0 E promoter and C/EBP-alpha.

Panel A. Schematic of EMSA probes C1, C2, and C3. C1 contains two putative C/EBP-alpha binding sites found within EP-172. C2 contains the other putative C/EBP-alpha binding site found upstream of the TATA box. C3 contains a putative C/EBP-alpha binding site downstream of the TATA box. The consensus C/EBP binding site in the rat CYPD5 promoter (Lees-Miller et al., 1996) is shown as a comparison to the putative sites in the bICP0 E promoter.

Panel B. EMSA using C1, C2, or C3 probes. Probes were incubated with 30 ug of Neuro-2A cell lysate. Antibodies directed against C/EBP-alpha (A) or C/EBP-beta (B) were used in super-shift assays as described in the materials and methods section.

Panel C. To better visualize the super-shift for C2 and C3, autoradiography was performed for 24 hours versus 4 hours as shown in panel B.

Figure 2.6.



Chapter 3

Bovine Herpesvirus 1 (BHV-1) Productive Infection and bICP0 Early Promoter Activity are Stimulated by E2F1.

These studies are included in a manuscript published in 2010 in the Journal of Virology, volume 84, pages 6308-6317.

The authors of the manuscript are **Aspen Workman** and Clinton Jones

ABSTRACT

Bovine herpes virus 1 (BHV-1) is an important viral pathogen of cattle. Like other alpha-herpesvirinae subfamily members, BHV-1 establishes latency in sensory neurons, and has the potential to reactivate from latency. Dexamethasone (DEX) treatment of latently infected calves or rabbits consistently leads to reactivation from latency. The BHV-1 transcript encoding the infected cell protein 0 (bICP0) is consistently detected during reactivation from latency, in part, because the bICP0 early promoter is activated by DEX. During DEX-induced reactivation from latency, cyclin expression is stimulated in infected sensory neurons. Cyclin dependent kinase activity phosphorylates Rb (retinoblastoma tumor suppressor gene product) family proteins and consequently releases the E2F family of transcription factors suggesting that E2F family members stimulate productive infection and/or reactivation from latency. In this study, we provide evidence that repression of E2F1 by a specific siRNA reduced productive infection approximately 5 fold. E2F1 or E2F2 stimulated bICP0 early promoter activity at least 100 fold in transient transfection assays. Two E2F responsive regions (ERR) were identified within the early promoter: one adjacent to the TATA box (ERR1) and one approximately 600 base pairs upstream from the TATA box (ERR2). Mobility shift assays suggested that E2F interacts with ERR1 and ERR2. E2F1 protein levels were increased at late times after infection, which correlated with enhanced binding to a consensus E2F binding site, ERR1, or ERR2. Collectively, these studies suggested that E2F1 stimulates productive infection and bICP0 early promoter activity, in part because E2F family members interact with ERR1 and ERR2.

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is a significant viral pathogen of cattle that can cause conjunctivitis, rhinotracheitis, pneumonia, genital disorders, or abortions. BHV-1 also initiates shipping fever, a potentially fatal polymicrobial disease (Tikoo et al., 1995). Like other members of the alpha-herpesvirinae subfamily, BHV-1 establishes lifelong latency in trigeminal ganglionic neurons following acute replication in mucosal epithelium. Reactivation from latency occurs periodically, resulting in virus shedding and spread to susceptible cattle. Reactivation from latency can occur after stress or corticosteroid treatment, which mimics stress (Rock et al., 1992; Sheffy and Davies, 1972). Dexamethasone (DEX), a synthetic corticosteroid, reproducibly induces expression of BHV-1 lytic cycle genes and reactivation from latency in calves or rabbits (Inman et al., 2002; Jones, 1998; Jones, 2003a; Jones et al., 2006; Jones et al., 2000; Rock et al., 1992).

During productive infection of cultured cells, viral gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E), or late (L), reviewed in (Jones, 1998; Jones, 2003b). IE gene expression is stimulated by a virion component, bTIF, which interacts with a cellular transcription factor (Oct-1) to transactivate IE gene expression (Misra et al., 1994; Misra et al., 1995). Two IE transcription units exist: IE transcription unit 1 (IEtu1) and IEtu2 (Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991). IEtu1 encodes functional homologues of two HSV-1 proteins, ICP0 and ICP4. IEtu2 encodes a protein that is similar to the HSV-1 IE gene, ICP22 (Fraefel et al., 1994). BHV-1 encoded ICP0 (bICP0) is translated from an IE (IE2.9) or E mRNA (E2.6) because an IE promoter (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). The IE promoter regulates IE expression of bICP4 and bICP0. Expression of the bICP4 protein represses IEtu1 promoter activity whereas bICP0 activates its own E promoter and all other viral promoters. A recent study demonstrated that during DEX induced reactivation from latency bICP0 mRNA, but not bICP4, was consistently detected (Workman et al., 2009). In part, this was due to the fact that the bICP0 promoter is activated by DEX induction of the cellular transcription factor CAAT enhancer binding protein alpha (c/EBP-alpha) (Workman et al., 2009). bICP0 transcription appears to be stimulated during reactivation from latency by cellular transcription factors that trans-activate the bICP0 E promoter. Since bICP0 is the major regulatory protein that stimulates BHV-1 productive infection (Fraefel et al., 1994; Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991), identification of cellular factors that stimulate the bICP0 E promoter may help us understand the early stages of reactivation from latency.

The E2F family of transcription factors contain a conserved DNA-binding domain, acidic transcriptional activation domain, and a Rb binding site (Harbour and Dean, 2000). Functional E2F binding sites are present in the promoters of nearly all genes that control cell cycle progression (Nevins et al., 1997; Ohtani et al., 1995a; Schulze et al., 1995; Wells et al., 1997). Several lines of evidence suggest that the E2F family of transcription factors may stimulate BHV-1 productive infection and reactivation from latency. First, during DEX induced reactivation from latency, sensory neurons that express abundant levels of lytic cycle genes also express certain cyclins (cyclin E and cyclin A for example) (Winkler et al., 2000c). Phosphorylation of Rb family members by cyclin dependent kinase/cyclin complexes leads to E2F release, and

consequently certain E2F family members are then able to activate transcription (Attwooll et al., 2004; Harbour and Dean, 2000; Nevins et al., 1997; Weintraub et al., 1992). Furthermore, over-expression of E2F4 stimulates BHV-1 productive infection and E2F1 or E2F2 trans-activates IETu1 promoter activity (Geiser and Jones, 2003). Finally, the HSV-1 TK (thymidine kinase) promoter is activated by E2F1 by virtue of a GC rich motif, not a consensus E2F binding site (Chang et al., 2005).

In this study, we demonstrated that siRNAs directed against E2F1 reduced productive infection. In transient transfection assays, E2F1 or E2F2 activated bICP0 E promoter activity more than 100 fold. Two E2F responsive regions were identified within the bICP0 E promoter. These studies suggest that E2F1 and E2F2 stimulate productive infection, in part, by activating bICP0 E promoter activity.

MATERIALS AND METHODS

Cells and viruses

Murine neuroblastoma 2A (neuro-2A) and rabbit skin (RS) cells were grown in Earle's modified Eagle's medium (EMEM) supplemented with 5% fetal calf serum (FCS). Bovine kidney cells (CRIB) were grown in EMEM supplemented with 10% FCS. All media contained penicillin (10 U/ml) and streptomycin (100 µg/ml).

The Cooper strain of BHV-1 (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa. Stock cultures of BHV-1 were prepared in CRIB cells.

A BHV-1 mutant containing the Lac Z gene in place of the viral gC gene was obtained from S. Chowdury (Baton Rouge, LA) (gCblue virus). The virus grows to similar titers as the wild type parent virus and expresses the Lac Z gene as a true late gene.

Plasmids

Plasmids expressing E2F1 or E2F2, pCMV-E2F1 and pCMV-E2F2 respectively, were obtained from J.R. Nevins (Duke University, Durham, USA). The empty vector pcDNA3.1 was purchased from Invitrogen.

Six bICP0 E promoter constructs were prepared by PCR amplification as previously described (Workman et al., 2009). The promoter fragments were cloned into the promoterless vector pCAT-Basic (E1871; Promega) at the unique XhoI and KpnI sites to generate plasmids EP-943, EP-638, EP-172, EP-143, EP-133, and EP-71 (Figure 2C). For this study, 2 additional constructs were prepared. EP-50 and EP-42 were

synthesized (IDT; Iowa) and contained a XhoI and KpnI restriction site. Duplex oligonucleotides were digested with XhoI and KpnI and cloned into the promoter-less vector pCAT-Basic. The numbers in the plasmid name refer to the length of the bICP0 E promoter fragment inserted into the CAT vector. The deletions were made from the 5' terminus of the bICP0 promoter.

Two additional bICP0 E promoter constructs were generated using the wt BHV-1 genome as a template and a common 3' primer (5'-ctcgagCCTGCTGGGCGACACAAACAACAGA-3') with the following 5' primers: EP-398, 5'-ggggtaccAAGACGCAGAACCCCG-3'; EP-328, 5'-ACCCAGGGGCGGAGC-3'. The promoter fragments were cloned into the promoterless vector pCAT-Basic as described above. The DNA sequence of the E promoter inserts was confirmed by DNA sequencing (Genomics Core Research Facility-UNL). All plasmids were prepared from bacterial cultures by alkaline lysis and two rounds of cesium chloride centrifugation.

To further localize bICP0 E promoter elements that are responsive to E2F, upstream regions of the bICP0 E promoter (E2F responsive regions; ERR) were cloned into a minimal promoter CAT vector (E186A; Promega) containing the SV40 early promoter cloned upstream of CAT.

ERR1 constructs: ERR1/40 was created using synthesized duplex sequences (IDT, Iowa). Duplex oligonucleotides were digested with XhoI and KpnI and cloned into the minimal promoter CAT vector.

ERR2 constructs: ERR2/254 was generated by PCR using the forward primer 5'-GCGACGGCGGCAATAAAGACGAGT-3' with the reverse primer 5'-CGGGGTTCTGCGTCTTGGC-3'. ERR2/180, ERR2/120, ERR2/1-60 and ERR2/61-120

were created using synthesized duplex sequences (IDT, Iowa). Duplex oligonucleotides were digested with KpnI and XhoI and cloned into the minimal promoter CAT vector at the unique KpnI and XhoI sites. The identity of each construct was confirmed by DNA sequencing (Genomics Core Research Facility-UNL). A schematic of ERR1 and ERR2 constructs are shown in Figures 4 and 5 respectively. All plasmids were prepared from bacterial cultures by alkali lysis and two rounds of cesium chloride centrifugation.

Measurement of chloramphenicol acetyltransferase (CAT) activity

Neuro-2A cells grown in 60 mm dishes were co-transfected with the designated plasmids as indicated in the respective figure legends. Neuro-2A cells were transfected with NeuroTransIt (MIR2145; Mirus), according to the manufacture's instructions. After 48 hours, cell extract was prepared by three freeze/thaw cycles in 0.25 M Tris-HCl, pH 7.4. Cell debris was pelleted by centrifugation, and protein concentrations determined. CAT activity was measured by incubating with 0.1 uCi [¹⁴C]-chloramphenicol (CFA754; Amersham Biosciences) and 0.5 mM AcetylCoA (A2181; Sigma). The reaction was incubated at 37° C for 15 to 30 minutes. All forms of chloramphenicol were separated by thin-layer chromatography. CAT activity was quantified using a Bio-Rad Molecular Imager FX (Molecular Dynamics, CA). Levels of CAT activity are expressed as fold induction relative to the vector control.

Electrophoretic Mobility Shift Assay (EMSA)

Neuro-2A cells were transfected with 100 ng of E2F1 using NeuroTransIT (Mirus) according to the manufacturer's instructions. At 48 hours after transfection,

whole cell lysate was prepared. Cells were washed with phosphate-buffered saline 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] per 10 ml). Cell lysate was incubated on ice for 30 min, sonicated, and then clarified by centrifugation at 10,000 x g at 4°C for 15 min.

CRIB cells were infected with wt BHV-1 at a moi of 5 and cultured in EMEM containing 10% FCS. At various times after infection, cells were collected and suspended in NP-40 lysis buffer. Cell lysate was incubated on ice for 30 min, sonicated, and then clarified by centrifugation at 10,000 x g at 4°C for 15 min. Protein concentrations were quantified by the Bradford assay.

Twenty micrograms of protein extract were incubated in a volume of 16 ul of binding buffer (10 mM Tris-HCl pH8, 150 mM KCl, 0.5 mM EDTA, 0.1% Triton X-100, 12.5% glycerol) in the presence of 1 ug poly(dI-dC) (P4929; Sigma), and 0.5 pmols of double stranded DNA probe labeled with 10 uCi of γ -³²P-ATP. Incubation proceeded for 1 hour at room temperature. For competition assays, 500 ng of cold E2F1 consensus probe or a C/EBP-alpha consensus probe were incubated with cell lysate for 20 minutes prior to addition of radiolabeled probe. DNA-protein complexes were run on a 5% polyacrylamide gel in 0.5X TBE for 3 hours at 100 volts. To improve band resolution, 1M sodium acetate pH 5.3 was added to the lower buffer chamber during electrophoresis. The gel was exposed to a phosphorimager plate and analyzed using Bio-Rad Molecular Imager FX. Probes used for EMSA are as follows:

E2F Consensus: ATTTAAGTTTCGCGCCCTTTCTCAA

C/EBP-alpha Consensus: CGCAATATTGCGCAATATTGCAAT

40 bp probe of bICP0 E promoter that was used to construct ERR1/40:
CGGCGCCCTGCCCCGCCCCGCCCCCGCCCTCGCGGCC

1-60 bp probe of bICP0 E promoter that was used to construct ERR2/1-60:
CCGGCGCGCGGCGCGGGGCGGGCCCCGGGGCGCGAAGCCCGGGAGGGAC
GCGGGCGTG.

SDS-PAGE and Western Blotting of E2F expression

RS cells were transfected with 1 ug of pcDNA3.1 empty vector, 100 mM E2F1 siRNA (sc-35247; Santa Cruz Biotechnology) or control siRNA (44-2926; Invitrogen) using Lipofectamine 2000 according to the manufacture's specifications (11668-019; Invitrogen). The Block-iT-Fluorescent Oligo was used as a control siRNA (44-2926; Invitrogen). It is a fluorescent conjugated control containing a scrambled sequence that does not reduce the levels of any known mammalian gene. At 48 hours after transfection, whole cell lysate was prepared as previously described (Workman et al., 2009). Protein concentrations were quantified by the Bradford assay. Standard 12% SDS-gels were prepared and used for these studies. Proteins were transferred to PVDF membrane (miliopore), blocked for 4 hours in 5% nonfat dry milk with Tris-buffered saline-0.1% Tween 20 (TBS-T), and incubated with primary antibody overnight at 4° C. The E2F1 antibody (sc-193X; Santa Cruz Biotechnology) was diluted 1:10,000 in blocking solution. The E2F2 antibody (sc-633X; Santa Cruz Biotechnology) was diluted 1:10,000 in blocking solution. Antisera directed against cleaved caspase 3 (Cell Signaling, 96618), was also used for these studies at a 1: 10,000 dilution in blocking solution. After 45

minutes of washing with TBS-T, blots were incubated with donkey anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (Amersham Biosciences), which was diluted 1:2000 in 5% nonfat milk in TBS-T. Blots were washed 45 minutes with TBS-T and exposed to Amersham ECL reagents, and then autoradiography performed. The β -actin protein was used as a loading control, and this protein was detected using a polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA).

Analysis of Lac Z gene expression in productively infected cells

RS cells grown in 60 mM dishes were transfected with 1 ug of blank pcDNA3.1 vector, 100 nM E2F1 siRNA or 100 nM control siRNA using Lipofectamine 2000 according to the manufacture's specifications (11668-019; Invitrogen). At 24 hours after transfection, cells were transfected with 1 ug of gCBlue virus genome. After 24 hours, cells were fixed (2% Formaldehyde, 0.2% Glutaraldehyde in PBS), stained (1% blue-gal, 5 mM Kferric, 5 mM Kferro, 0.5 M MgCl₂ in PBS), and the number of blue cells was counted as described previously (Geiser et al., 2002; Geiser and Jones, 2003). The number of blue cells in cultures expressing the blank vector was set to 100. To calculate percent plaque formation, the number of blue cells in cultures transfected with the E2F1 or control siRNA were divided by the number of blue cells in cultures transfected with the blank vector. The results are an average of three independent experiments.

Confocal Microscopy

CRIB cells were infected with wt BHV-1 at a moi of 5. At 16 hours after infection, cells were fixed in 4% para-formaldehyde for 10 minutes, followed by three

washes with phosphate-buffered saline (PBS). Cells were permeabilized by incubating with 100% ethanol at -20°C for 5 min. Coverslips were then washed three times and blocked in 3% BSA in PBS for 1 h to reduce non-specific binding. The E2F1 primary antibody (sc-193X; Santa Cruz Biotechnology) was diluted 1:3,000 in PBS with 0.05% Tween 20 and 1% BSA and incubated on coverslips for 2 hours at room temperature. After three washes, coverslips were incubated with Cy5-conjugated donkey anti-rabbit antibody (A-31573; Invitrogen) at a dilution of 1:400 for 1 h in the dark. After slides were washed, DAPI (4',6'-diamidino-2-phenylindole) staining was performed to visualize the nucleus. Coverslips were then mounted on slides using Gelmount aqueous mounting medium (17985-10; Electrom Microscopy Sciences). Images were obtained with a Bio-Rad confocal laser-scanning microscope (MRC-1024ES).

RESULTS

Suppression of E2F1 reduced the levels of productive infection

Our previous studies indicated that over-expression of E2F4, but not E2F1 or E2F2, stimulated BHV-1 productive infection (Geiser and Jones, 2003). In contrast, the same study demonstrated that E2F1 and E2F2, but not E2F4, stimulated the IEtu1 promoter. Since E2F1 and E2F2, but not E2F4, are potent trans-activators (Attwooll et al., 2004; Chang et al., 2005; Harbour and Dean, 2000), this result was surprising. Over-expression of E2F1 can induce apoptosis (Attwooll et al., 2004; Nip et al., 1997) suggesting E2F1 and E2F2 were unable to stimulate productive infection because they were toxic to transfected cells.

To test whether E2F1 specific siRNA affected productive infection, rabbit skin cells (RS) were transfected with E2F1 siRNA and BHV-1 genomic DNA. RS cells were used for these studies because they are permissive for BHV-1 and can be transfected efficiently. For this study, we used the gCblue BHV-1 virus strain, which contains the Lac Z gene inserted downstream of the gC promoter. At 24 h after transfection, cells were fixed and the number of β -Gal⁺ cells identified. This time point was used to minimize the number of virus positive cells that result from virus spread. At later times, many β -Gal⁺ cells lift off the dish making it difficult to count virus positive cells (Geiser et al., 2002; Inman et al., 2001b). The number of β -Gal⁺ cells directly correlates with the number of plaques produced following transfection with the gCblue virus (Geiser et al., 2002; Inman et al., 2001b). Relative to a control siRNA, cotransfection of E2F1 siRNA with BHV-1 genomic DNA reduced the number of β -Gal⁺ cells approximately 4 fold (Figure 3.1A). The control siRNA reduced the number of β -Gal⁺ cells approximately

20% compared to results obtained when RS cells were transfected with just BHV-1 DNA. Western blot analysis demonstrated that the E2F1 siRNA, but not the control siRNA reduced E2F1 steady state protein levels (Figure 3.1B). Conversely, the E2F1 specific siRNA had no obvious effect on E2F2 or β -actin protein levels. The E2F1 siRNA reduced the percent of cells in S phase at 24 hours after transfection, (data not shown) suggesting that in RS cells there is a correlation between reducing E2F1 protein levels and cell cycle progression.

The bICP0 E promoter is trans-activated by E2F

As with HSV-1 or HSV-2, there are two copies of the bICP0 and bICP4 genes in the BHV-1 repeats (Figure 3.2A). However, the organization of the BHV-1 ICP4 and ICP0 genes is unique because a common IE promoter (IEtu1 promoter) drives expression of bICP0 and bICP4 mRNA (Wirth et al., 1992) (Figure 3.2B). bICP0 also contains an E promoter located near the 5' end of the bICP0 coding exon (e2). Recent evidence indicated that the bICP0 E promoter, but not the IEtu1 promoter, is stimulated by DEX treatment (Workman et al., 2009). Expression of the cellular transcription factor C/EBP-alpha is stimulated by DEX, thus stimulating bICP0 E promoter activity. Since repression of E2F1 protein levels by specific siRNAs reduced productive infection, we hypothesized that E2F1 may trans-activate the bICP0 early promoter.

To test this hypothesis, neuro-2A cells were cotransfected with a bICP0 E promoter construct (Figure 3.2C) plus an E2F1 or E2F2 expression plasmid, and CAT activity measured. Neuro-2A cells were use for this study because these cells are neuronal-like and it was of interest to begin to understand whether E2F regulates gene

expression in neurons. E2F1 and E2F2 are potent stimulators of E2F-responsive promoters (Attwooll et al., 2004; Harbour and Dean, 2000; Nevins et al., 1997; Weintraub et al., 1992), and thus were used for these studies. E2F1 trans-activated the EP-943 and EP-638 promoter constructs approximately 200 fold, and E2F2 trans-activated the same promoter constructs more than 100 fold (Figure 3.2C). EP-172, EP-143, and EP-133 were trans-activated by E2F1 more than 40 fold and at least 11 fold by E2F2. EP-72, EP-50, EP-42, and the promoter minus CAT vector (pCATbasic) were not trans-activated by E2F1 or E2F2.

Transfection of neuro-2A cells with increasing concentrations of a plasmid expressing E2F1 led to increased levels of cleaved caspase 3 in neuro-2A cells (Figure 3.2D). Although low levels of cleaved caspase 3 were detected in mock transfected cells, increasing levels of E2F1 correlated with increased levels of cleaved caspase 3, which is considered to be the point of “no return” during apoptosis (Shimeld et al., 1996). We believe that low levels of apoptosis in mock-transfected cells was due to the fact that these cells have a tendency to lift off the plate if they are not sub-cultured every 3-4 days and the stress of transfection. Cultures that were transfected with the highest levels of E2F1 contained cells that were rounded up and appeared to be dead. When neuro-2A cells were cotransfected with 0.1 or 1 ug E2F1, many cells had a similar morphology as mock-transfected cells (data not shown). Although E2F1 can induce apoptosis (Attwooll et al., 2004; Nip et al., 1997), we do not believe that merely inducing apoptosis accounts for trans-activation of the bICP0 E promoter by E2F1 because we used low levels of E2F1 (0.1 ug) for the trans-activation studies and this amount of E2F1 did not alter the morphology of neuro-2A cells or dramatically increase cleaved caspase 3 levels relative

to mock transfected cells (Figure 3.2D). Furthermore, Bax, a known apoptotic gene (Shimeld et al., 1996), did not trans-activate the bICP0 E promoter (data not shown).

The ability of E2F1 or E2F2 to trans-activate the IEtu1 promoter was also examined because a previous study demonstrated that E2F1 trans-activated the IEtu1 promoter 15-20 fold in bovine testicle cells (Geiser and Jones, 2003). However, in neuro-2A cells, E2F1 or E2F2 trans-activated the three IEtu1 promoters (Figure 3.3A) only 3-5 fold (Figure 3.3B). In summary, this study demonstrated that E2F1 or E2F2 efficiently trans-activated the bICP0 E promoter, but not the IEtu1 promoter, in neuro-2A cells.

The bICP0 E promoter contains two E2F responsive regions

The results in Figure 3.2 suggested that the bICP0 E promoter contained two separate E2F responsive regions: 1) E2F responsive region 1 (ERR1) that spanned the 5' terminal 60 bases of EP-133, and 2) E2F responsive region 2 (ERR2) located at or near the 5' terminus of EP-638. To test whether ERR1 conferred E2F responsiveness to a heterologous promoter, 40 bp spanning the 5' terminus of EP-133 to EP-71 were synthesized and cloned upstream of a CAT reporter construct containing a minimal SV40 E promoter construct (see figure 3.4A for a schematic of the predicted ERR1 region). This construct (ERR1/40) was transactivated by E2F1 approximately 5 fold (Figure 3.4B). A 60 base pair fragment between EP-133 and EP-71 was not trans-activated more efficiently than ERR1/40 (data not shown). In contrast, the SV40 E promoter (SV40 min-CAT) was not efficiently trans-activated by E2F1.

Sequences spanning the 5' terminus of EP-638 and adjacent regions within EP-943 (ERR2) were also synthesized and then cloned into the SV40 min-CAT construct (see Figure 3.5A for a schematic of these constructs). The construct containing 254 bp of the bICP0 E promoter (ERR2/254), 180 bp of the E promoter (ERR2/180), or 120 bp of the E promoter (ERR2/120) was trans-activated by E2F1 approximately 20 fold (Figure 3.5B). The 120 bp fragment within ERR2 was further divided into two equal pieces (ERR2/1-60 and ERR2/61-120) and then tested for trans-activation by E2F1. ERR2/1-60, but not ERR2/61-120, was trans-activated by E2F1 more than 15 fold (Figure 3.5B). In summary, these studies identified a 60 bp fragment within ERR2 that was trans-activated by E2F1 when cloned upstream of a minimal SV40 E promoter.

In Figure 3.6A and B, the DNA sequence of ERR1 and ERR2 is shown. ERR1 is located within sequences that are present in EP-133 but lacking in EP-72. Within this region, there are two regions of overlapping Sp1 binding sites (Figure 3.6A). The E2F transcriptional activator is a heterodimer consisting of an E2F family member and a Dp family member (Attwooll et al., 2004; Harbour and Dean, 2000; Nevins et al., 1997). The core DNA binding site of an E2F/Dp heterodimer is (g/cGCGCc/g) (Zheng et al., 1999). Within ERR1, there is one E2F/Dp core consensus sequence. Two LSF (late SV40 transcription factor) binding sites, a transcription factor that interacts with the SV40 21 base repeats and activates late transcription (Kwon et al., 2006), and binding sites for a transcription factor that preferentially interacts with CAC sequences were also identified. Finally, a Yi binding site, which is present in the mouse thymidine kinase promoter was detected (Dou et al., 1991). Proteins that interact with the Yi binding site

exhibit G1/S phase inducible binding. Within sequences of ERR1/40, binding sites for E2F/Dp, Sp1, Y1, LSF, and CAC are clustered.

Within the first 60 bp of ERR2 (Figure 3.6B), 5 Sp1 binding sites were identified: 3 match those of the mouse Erk1 gene (Pages et al., 1995), 1 is present in the SV40 E promoter (Gidoni, 1984), and one is a target for binding to SP1 (Thiesen and Bach, 1990). In addition, 5 core E2F/Dp binding sites were present. Four of these are overlapping and located near the 5' terminus of this fragment. Conversely, nucleotides spanning nucleotides 61-120 of ERR2, which were not efficiently trans-activated by E2F1, contained just 2 E2F/Dp consensus binding sites and one Sp1 binding site present in the human heat shock binding protein 70 promoter (Greene et al., 1987). In summary, these studies suggested that a cluster of E2F/Dp core binding sites and Sp1 binding sites located within the 60 bp of ERR2 may be important for E2F mediated trans-activation.

Cellular factors interact with ERR1 and ERR2

To test whether E2F interacts with ERR1 or ERR2, electrophoretic mobility shift assays (EMSA) were performed with oligonucleotides derived from ERR1, ERR2, or a consensus E2F binding site and whole cell extracts prepared from neuro-2A cells. Shifted bands were readily detected when the respective probes were incubated with neuro-2A cell extracts (Figure 3.7A, shifted bands denoted by the brackets). E2F interacted with the E2F consensus probe as well as ERR1 and ERR2 probes because an oligonucleotide containing a consensus E2F binding site, but not a C/EBP-alpha binding site, competed for binding of nuclear factors (Figure 3.7A).

Additional studies were performed to test whether productive infection stimulated binding to the E2F consensus binding site, ERR1, or ERR2. For these studies, we used bovine kidney cells (CRIB) because they are permissive for BHV-1, whereas neuro-2A cells are not. At 16 or 24 hours after infection, increased binding to the E2F consensus, ERR1, and ERR2 was observed (Figure 3.7B, enhanced binding denoted by the closed circles). A consensus E2F sequence, but not a C/EBP-alpha binding site, reduced the intensity of certain bands when incubated with the respective radioactive probes (Figure 3.7B). Although these studies indicated that E2F interacted with ERR1 or ERR2, this was not readily detectable until late stages of productive infection suggesting E2F family members were not the major activators of bICP0 E promoter activity.

Western blot analysis determined that E2F1 protein levels, but not E2F2, increased during the course of productive infection (Figure 3.8). At 16 and 24 hours after infection, E2F1 protein levels were dramatically higher, which correlated with enhanced binding to the E2F consensus, ERR1, or ERR2 oligonucleotide. Confocal microscopy was performed to determine if E2F1 was detected in the nucleus of infected cells at 16 hours after infection (Figure 3.8B). Higher levels of E2F1 were detected in infected cells, and the E2F1 protein was localized in the nucleus, which was in agreement with the Western blot studies shown in Figure 3.8A. Previous studies have demonstrated that BHV-1 E transcripts (thymidine kinase and ribonucleotide reductase) are readily detected at 2 hours after infection (moi of 0.02) using poly dT as a primer for RT-PCR (Schang and Jones, 1997). Using a moi of 5, most CRIB cells were rounded up and some were beginning to detach from the dish between 16-24 hours after infection (Figure 3.8B).

DISCUSSION

In this study, we demonstrated that the bICP0 E promoter, but not the IEtu1 promoter, was trans-activated by E2F1 or E2F2 more than 100 fold in neuro-2A cells. Silencing of E2F1 reduced virus infection approximately 4 fold indicating E2F1 has the potential to stimulate productive infection. Previous studies also suggested that E2F family members stimulated BHV-1 productive infection (Geiser and Jones, 2003; Winkler et al., 2000a). With respect to HSV-1 productive infection, E2F that is not associated with Rb family members increase following infection of human cells (C33-A) (Hilton et al., 1995). Relocalization of E2F4 to the nucleus occurs in C33-A and U2-OS human cells following HSV-1 infection (Olgiate et al., 1999). Further support for E2F4 playing a role in HSV-1 replication comes from the findings that infection of p107^{-/-}/p130^{-/-} mouse cells leads to reduced infectious virus (Ehmann et al., 2001). In primary human fibroblasts or Hela cells, the subcellular distribution of E2F4 is altered following HSV-1 infection, which is assumed to inactivate E2F-4 activity (Advani et al., 2000a). This same study also concluded that HSV-1 infections leads to post-translational modification of E2F1 and E2F5, translocation of E2F family members from the nucleus to the cytoplasm, and reduced E2F binding to consensus E2F binding sites. In contrast, enhanced binding to a consensus E2F binding site was detected at late times after infection with BHV-1 (Figure 3.7B). Many DNA synthetic genes are activated by E2F family members (Harbour and Dean, 2000) suggesting transient induction of E2F family members may stimulate viral synthesis in highly differentiated cells. It is also possible that induction of E2F binding activity occurs because BHV-1 induces p53 dependent apoptosis during productive infection (Devireddy and Jones, 1999b).

The bICP0 E promoter contained two separate regions that were trans-activated by E2F: an upstream region that was localized to a 60 bp fragment (ERR2) and sequences located near the 5' terminus of the EP-133 construct (ERR1). Since ERR2 was trans-activated by E2F1 approximately 20 fold, but EP-638 was trans-activated approximately 200 fold by E2F1 we suggest that other sequences within the bICP0 E promoter play a role in trans-activation by E2F. EMSA assays suggested that E2F interacted with ERR1 and ERR2 because a consensus E2F sequence, but not a C/EBP-alpha binding site, competed for binding of nuclear factors (Figure 3.7). ERR2 contains 5 core E2F/Dp binding sites (Figure 6B) (Zheng et al., 1999) suggesting these elements may be important for E2F mediated trans-activation. The 5 Sp1 binding sites located in ERR2 may also be crucial for trans-activation because E2F family members can interact with and trans-activate certain promoters containing GC rich motifs that resemble Sp1 binding sites. For example, E2F1 can trans-activate GC rich motifs in the HSV-1 thymidine kinase promoter (Chang et al., 2005) and the human ASK (activator of S phase kinase) gene encoding the regulatory subunit for human cdc7-related kinase (Yamada et al., 2002). In addition, a subunit of the mouse DNA polymerase alpha promoter contains a GC rich element that is crucial for cell cycle regulation (Nishikawa et al., 2000). Finally, 3 GC rich motifs in the human thymidine kinase promoter are bound by E2F, and are crucial for cell cycle dependent expression (Tommasi and Pfeifer, 1997). Not all GC rich promoters are trans-activated by E2F because we previously demonstrated that the BHV-1 I_{Et}2 promoter was not efficiently trans-activated by E2F1 or E2F2 (Geiser and Jones, 2003).

In trigeminal ganglia of latently infected calves, bICP0 transcription is stimulated from the E promoter during DEX induced reactivation from latency regardless of whether infectious virus is detected (Workman et al., 2009). In addition, the bICP0 E promoter is stimulated by DEX, in part because of the cellular transcription factor C/EBP-alpha. Conversely, the IEtu1 promoter does not appear to be as active during reactivation from latency because bICP4 is not consistently detected. This may be important because activating bICP0, but not bICP4, during the early stages of reactivation may allow BHV-1 to “test the waters” and determine whether important cellular factors are present for producing infectious virus without extensive viral gene expression occurring, which could lead to neuronal death. If bICP4 and bICP0 are expressed equally, extensive viral gene expression may occur in too many neurons that cannot support production of infectious virus. The ability of E2F1 or E2F2 to strongly trans-activate the bICP0 E promoter, but not the IEtu1 promoter, may play role in this process. It will be of interest to test whether E2F1 or E2F2 can cooperate with bICP0 or bICP4 to stimulate E or L genes since they also contain GC rich promoters.

Figure 3.1. Suppression of E2F1 reduced the levels of BHV-1 productive infection.

Panel A: RS cells were transfected with pcDNA3.1 empty vector, 100 nM E2F1 siRNA, or 100 nM control siRNA. Twenty-four hours later, cells were transfected with 1 ug of gCBlue BHV-1 DNA. At 24 hours after transfection, cells were fixed and B-gal positive cells were counted. The results constitute the average of four independent experiments.

Panel B. RS cells were transfected with 100 nM E2F1 siRNA or 100 nM control siRNA. At 48 hours after transfection, cells were collected and lysed with NP-40 lysis buffer, and 100 ug of protein was electrophoresed by 12% SDS-PAGE. Proteins in the gel were transferred onto a polyvinylidene difluoride membrane and probed with the E2F1 antiserum that was diluted 1:10,000. As controls, 100 ug cell lysate was probed with antiserum that specifically recognized E2F2 or β -actin.

Figure 3.1

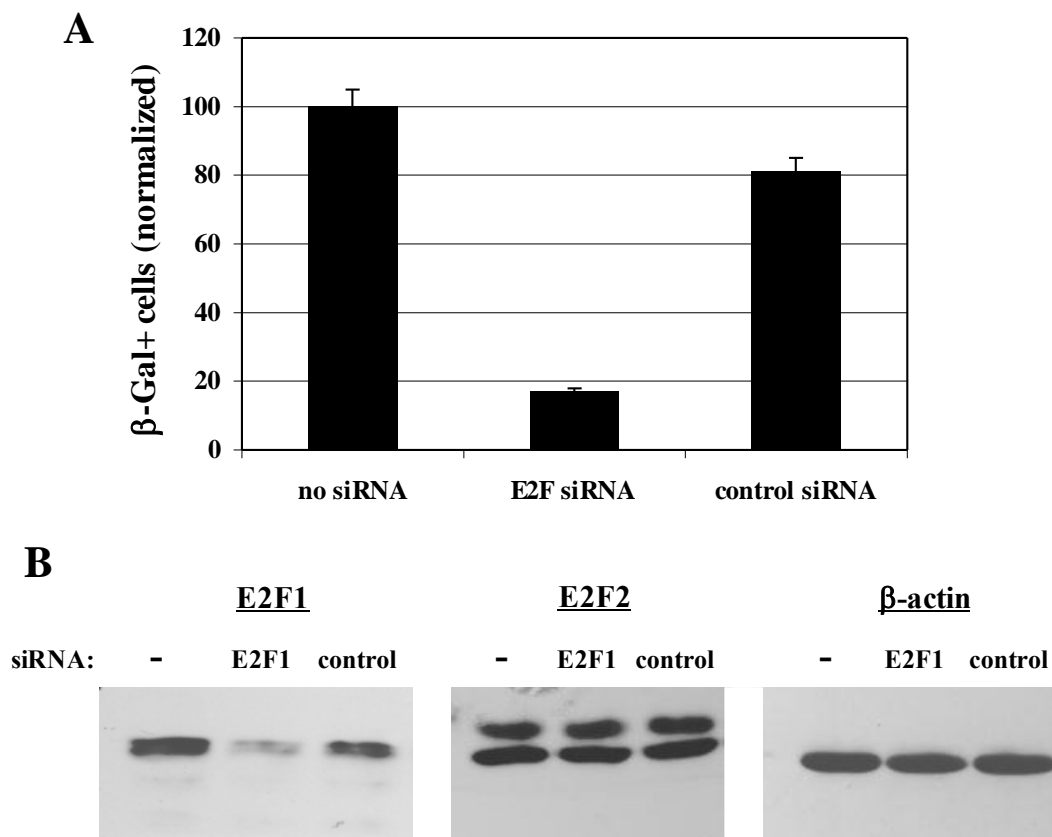


Figure 3.2. Schematic of IEtu1 and bICP0 E promoter constructs used in this study.

Panel A: Location of the unique long (L) and unique short (S) regions of the BHV-1 genome. The repeats are denoted by the open rectangles. Genes encoding bICP0 and bICP4 are present within the repeats. **Panel B:** Positions of bICP4 and bICP0 transcripts are shown. The immediate early transcription unit 1 (IEtu1) encodes bICP4 (IE/4.2) and bICP0 (IE/2.9) (Wirth et al., 1989; Wirth et al., 1991). The IEtu1 promoter activates IE expression of IE/4.2 and IE/2.9 (denoted by the black rectangle). E/2.6 is the early transcript that encodes bICP0 and an early promoter activates expression of this transcript (Wirth et al., 1992). Exon 2 (e2) of bICP0 contains all of the protein coding sequences of bICP0. The dashed lines are intron sequences. **Panel C:** bICP0 E promoter constructs were prepared as described in the materials and methods. Position of putative SP1 binding sites, E2F like sites, and TATA box are shown. Neuro2-A cells were co-transfected with 1 ug of the designated bICP0 E promoter/CAT construct and 0.1 ug of E2F1 or E2F2 expression plasmid. DNA amounts were equalized for all transfections using pcDNA3.1, a blank expression vector. At 48 hours post transfection, cells were collected and processed for CAT activity as described in the Materials and Methods. CAT activity of cells transfected with the control CAT vector was set to one for each experiment. All other values are expressed as fold activation with respect to the control. The results are the average of three independent experiments. **Panel D:** Neuro-2A cells were transfected with 0.1 ug E2F1 (lane 2), 1 ug E2F1 (lane 3), 5 ug E2F1 (lane 4), or 10 ug E2F1 (lane 5). At 48 hours after transfection whole cell lysate was prepared and 200 ug protein/lane was used for Western Blot assays. Antisera directed against E2F1, cleaved caspase 3 (Cell Signaling, 96618), or β -actin was used for these studies.

Figure 3.2.

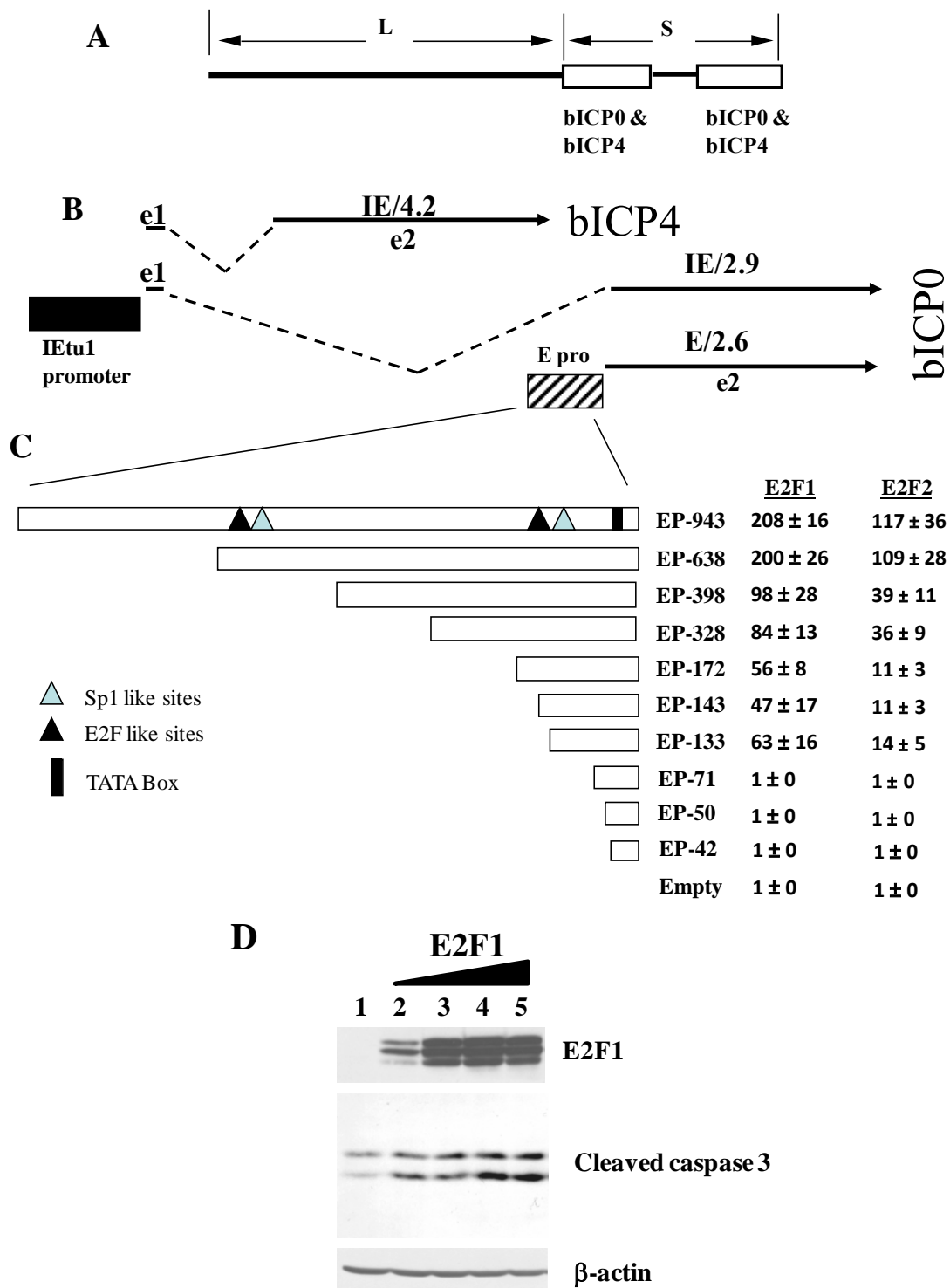


Figure 3.3. E2F trans-activation of IETu1 promoter deletion mutants.

Panel A. Schematic of IETu1cat constructs used to identify regions of the promoter that are responsive to E2F1. IETu1-CAT contains IETu1 promoter sequences cloned upstream of pSV0CAT (a promoter minus CAT expression vector). V. Misra, Saskatoon, Canada, provided the IETu1cat plasmid. Two deletion constructs $\Delta 1024$ and $\Delta 1391$ IETu1 have 1024 or 1391 bp removed from the 5' terminus. The location of cis-acting sequences within IETu1cat and the details of the respective plasmid constructs were described previously (Misra et al., 1994).

Panel B: Neuro-2A cells were cotransfected with an IETu1 promoter construct (1 ug DNA) and the E2F1 or E2F2 (0.1 ug) expression plasmid as described in Figure 1C. CAT activity of cells transfected with the control CAT vector was set to one for each experiment. All other values are expressed as fold activation with respect to the control. The results are the average of three independent experiments.

Figure 3.3.

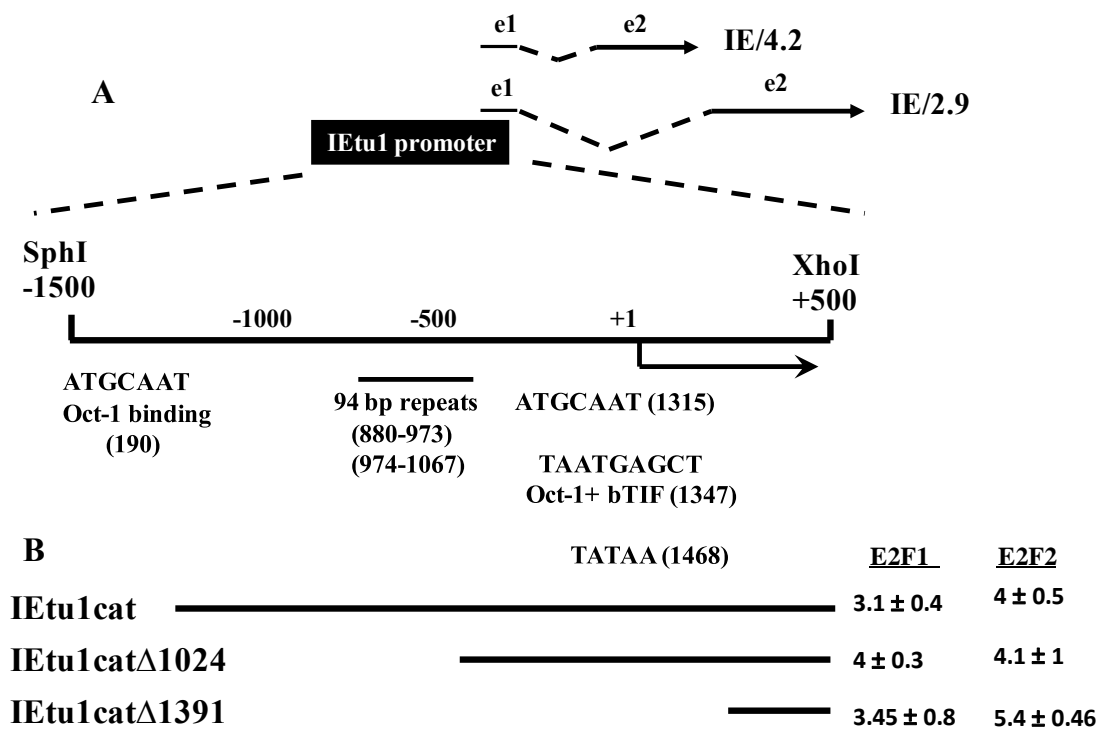


Figure 3.4: Localization of ERR1 within the bICP0 E promoter

Panel A. Schematic of the bICP0 E promoter and the positions of EP-133 and EP-71. A 40 nucleotide fragment that spans EP-133 and EP-71 was synthesized and cloned upstream of the SV40 E promoter (ERR1/40).

Panel B: Neuro-2A cells were co-transfected with 1ug of the designated fragment of the bICP0 E promoter cloned upstream of the SV40 minimal promoter CAT vector (ERR1/40) and 1 ug of the E2F1 expression plasmid. At 48 hours post transfection, cells were collected and processed for CAT activity as described in the Materials and Methods. CAT activity of cells transfected with the control CAT vector was set to one fold. All other values are expressed as fold activation with respect to the control. Experiments constitute the average of three independent experiments.

Figure 3.4.

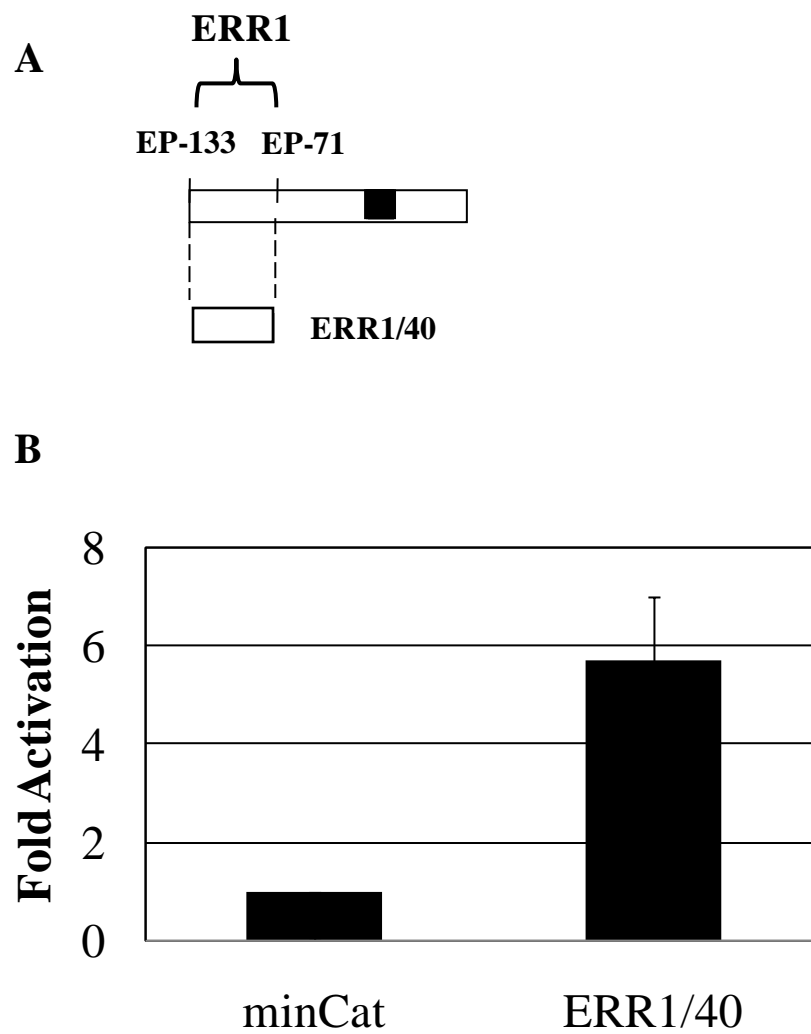


Figure 3.5. Localization of ERR2 within the bICP0 E promoter

Panel A: To localize sequences within ERR2 that are responsive to E2F1, bICP0 E promoter sequences were cloned into a minimal promoter CAT vector containing an SV40 promoter cloned upstream of the CAT gene as described in the Materials and Methods.

Panel B: Neuro-2A cells were co-transfected with 1ug of the designated fragment of the bICP0 E promoter cloned upstream of the SV40 minimal promoter CAT vector and 1 ug of E2F1. At 48 hours post transfection, cells were collected and processed for CAT activity as described in the Materials and Methods. CAT activity of cells transfected with the control CAT vector was set to one fold. All other values are expressed as fold activation with respect to the control. Experiments constitute the average of three independent experiments.

Figure 3.5.

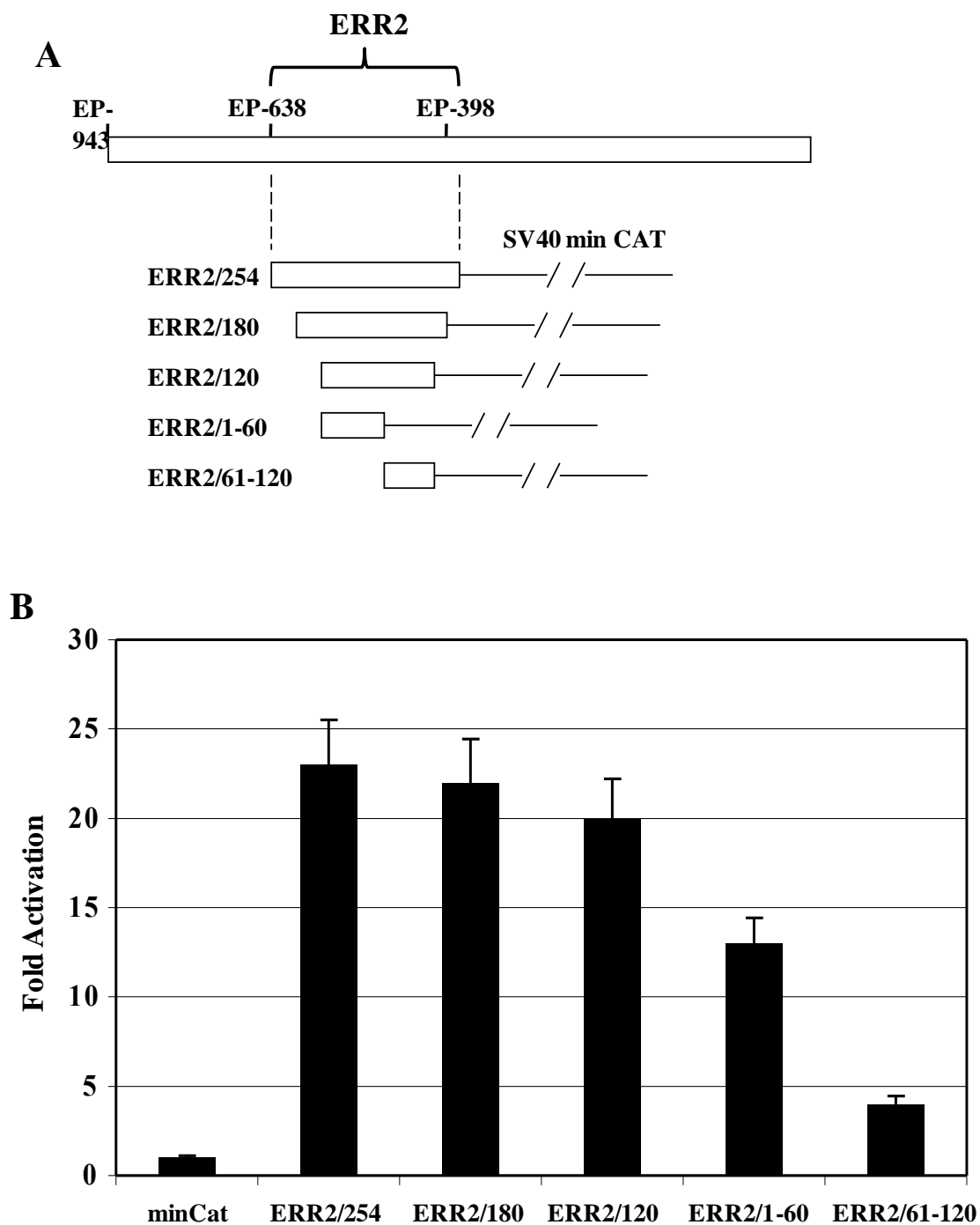


Figure 3.6. Nucleotide sequence of ERR1 and ERR2 within the bICP0 E promoter.

Panel A: Nucleotide sequences spanning sequences from EP-133 to the 5' terminus of the bICP0 E mRNA. The vertical black arrows denote sequences used in the ERR1/40 construct.

Panel B: Nucleotide sequences spanning ERR2. The 120 nucleotides that span ERR2 are shown to allow a comparison between the first 60 nucleotides that comprised the minimal fragment that was efficiently stimulated by E2F1 and the last 60 nucleotides that was not efficiently trans-activated by E2F1. Shown are locations of Sp1 binding sites from various promoters, **C/EBP-alpha binding sites** previously identified within the bICP0 E promoter (Workman, 2009), **Sp1 sites** from the designated promoters, Yi consensus binding sites (CCNCNCCCN), E2F/DP core binding sites, CAC binding sites, and LSF binding sites.

Figure 3.7. Interaction between E2F and ERR1 or ERR2.

Panel A: EMSA was performed using a probe that contains a consensus E2F binding site (E2F consensus), sequences spanning ERR1/40 (ERR1), or ERR2/1-60 (ERR2), which were described in the materials and methods. The respective ³²P-radiolabeled probe was incubated with 20 ug of Neuro-2A cell lysate that was transfected with the E2F1 expression construct (100 ng). For competition assays, 500 ng of the cold E2F consensus probe (lane E2F) or the C/EBP-alpha consensus probe (lane C) was incubated with cell extracts as described in the Materials and Methods. Brackets indicate the region of shifted bands.

Panel B: CRIB cells were infected with wt BHV-1 at a moi of 5 for 8, 16, or 24 hours. The respective ³²P radiolabeled probes, E2F binding sites (E2F consensus), sequences spanning ERR1/40 (ERR1), or ERR2/1-60 (ERR2), were incubated with 20 ug of CRIB cell lysate (lane M) or CRIB cell lysate prepared from cells infected for 8, 16, or 24 hours after infection. For competition assays, 500 ng of the cold E2F consensus probe (lane E2F) or C/EBP-alpha consensus probe (lane C) was incubated with cell extracts as described in the Materials and Methods. Brackets indicate the region of shifted bands, and the closed circles denote shifted bands that were reduced when incubated with the cold E2F consensus probe.

Figure 3.7.

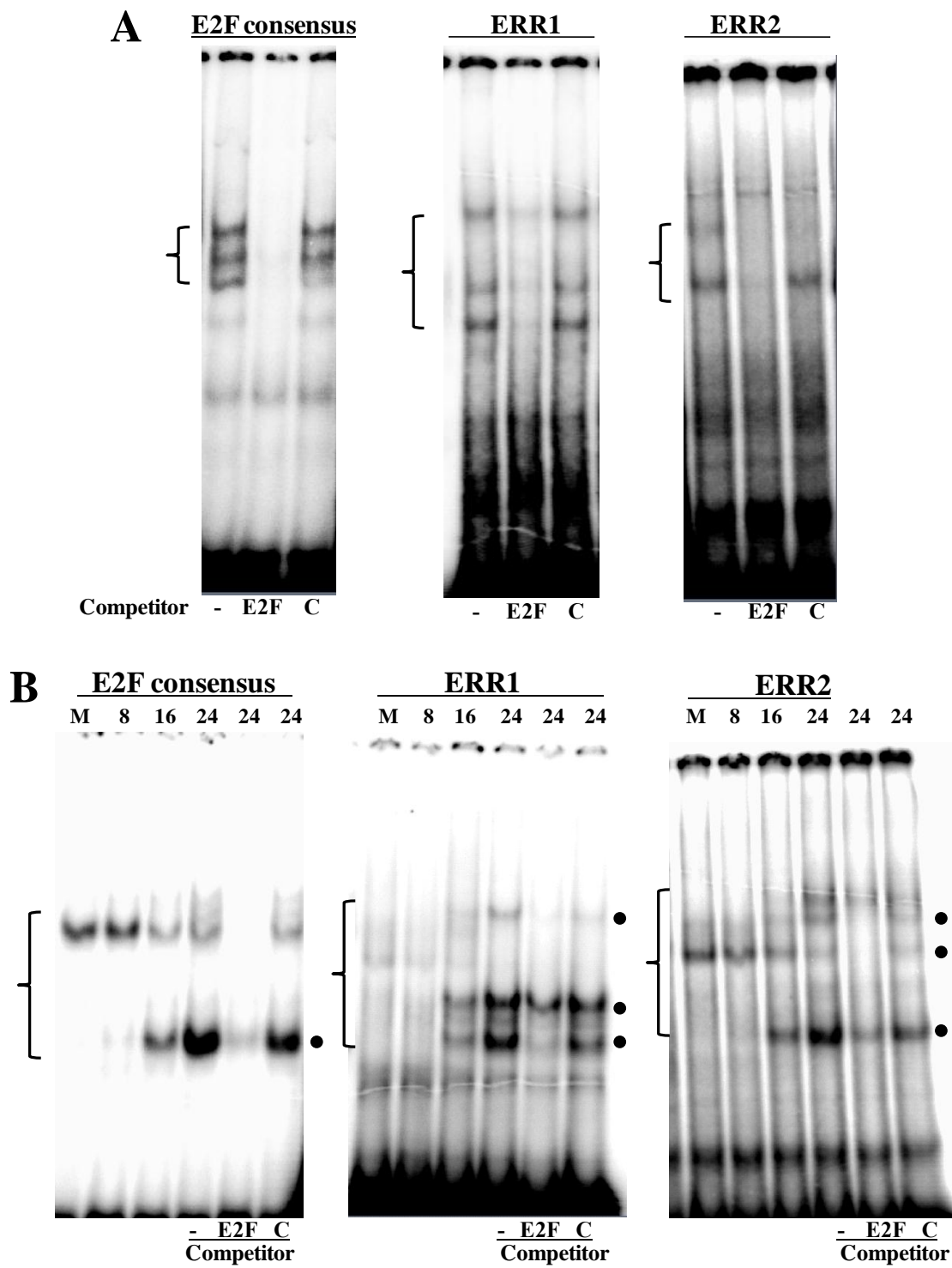
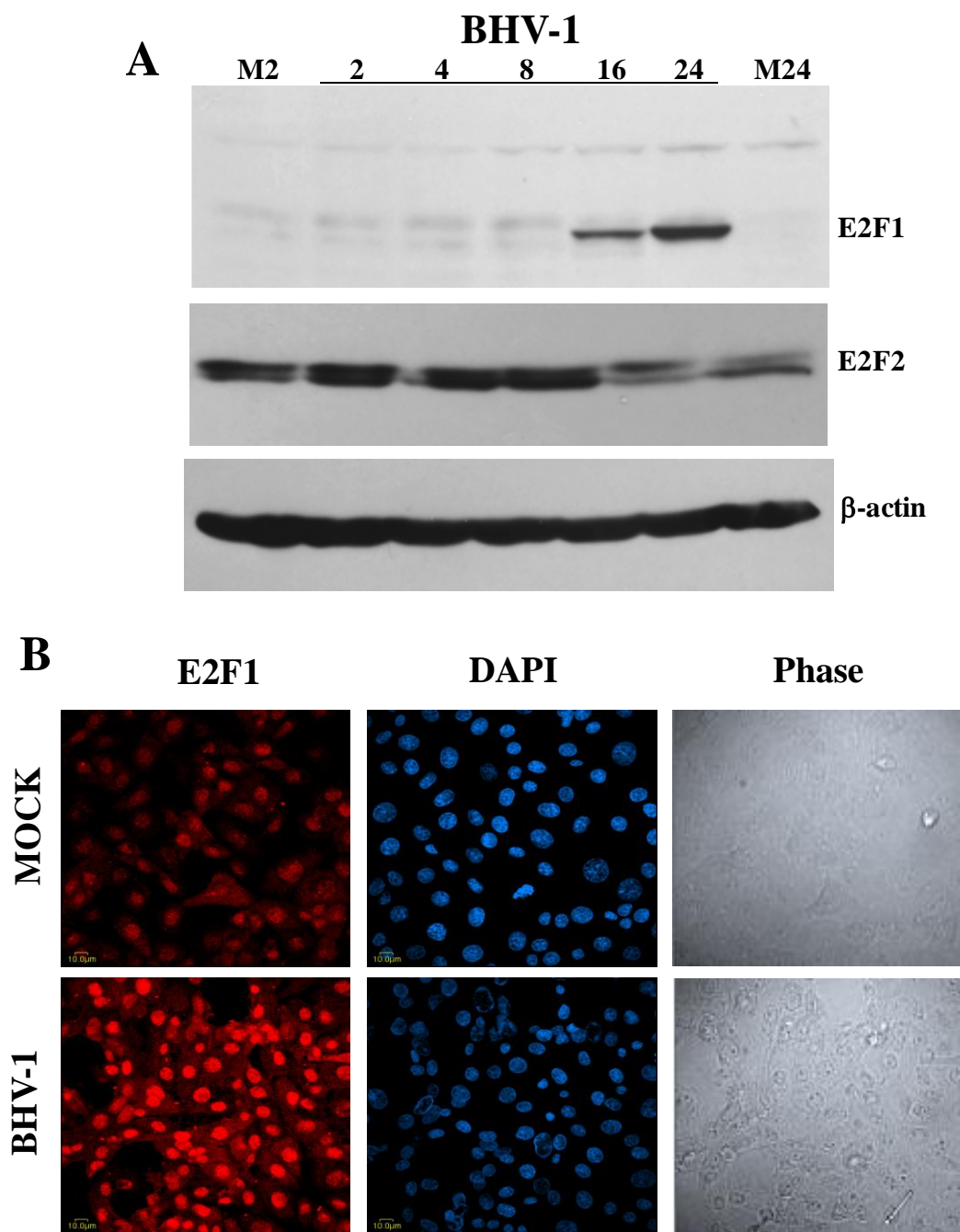


Figure 3.8. Analysis of E2F1 protein levels during productive infection.

Panel A: Western blot analysis of E2F1, E2F2, and β -actin expression following infection with BHV-1. CRIB cells were infected with wt BHV-1 at a moi of 5 for 2, 4, 8, 16, or 24 hours. Lanes M2 and M24 are mock-infected cells that were collected 2 and 24 hours after the other cells were infected with BHV-1. One hundred ug total cell lysate was loaded for each lane.

Panel B: CRIB cells were infected with wt BHV-1 at a moi of 5. At 16 hours after infection, cells were fixed in 4% para-formaldehyde and stained for confocal microscopy as described in the Materials and Methods. E2F1 primary antibody was used at a 1:3,000 dilution and Cy5-conjugated donkey anti-rabbit secondary antibody was used at a 1:400 dilution.

Figure 3.8.



Chapter 4

Analysis of the Cell Cycle Regulatory Protein E2F1 after Infection of Cultured Cells with Bovine Herpesvirus 1 (BHV-1) or Herpes Simplex Virus Type 1 (HSV-1).

This work has recently been accepted for publication in the journal Virus Research.

The authors of this manuscript are **Aspen Workman** and Clinton Jones.

ABSTRACT

The E2F family of transcription factors control the expression of genes involved in cell cycle progression and cell death. During the transition from G1 to S phase in the cell cycle, there is a sequential activation of cyclin-dependent kinases, which phosphorylate the retinoblastoma (Rb) protein, causing the release and activation of E2F. Previous studies indicated that (i) bovine herpes virus 1 (BHV-1) productive infection increases E2F1 protein levels, (ii) viral promoter activity is trans-activated by E2F1 or E2F2, and (iii) silencing E2F1 reduces the efficiency of productive infection. In this study, the effect of herpes simplex virus type 1 (HSV-1) productive infection on E2F protein levels and regulation of E2F dependent transcription was compared to BHV-1 infection in the same permissive cell line, rabbit skin (RS) cells. Silencing E2F1 with a specific siRNA reduces HSV-1 productive infection approximately 10 fold in RS cells. Furthermore, total E2F1 protein levels increased during productive infection. In contrast to RS cells infected with BHV-1, a fraction of total E2F1 protein was localized to the cytoplasm in HSV-1 infected RS cells. Furthermore, E2F1 did not efficiently trans-activate the HSV-1 ICP0 or ICP4 promoter. When RS cells were transfected with an E2F reporter construct or the cyclin D1 promoter and then infected with BHV-1, promoter activity increased after infection. In contrast, HSV-1 infection of RS cells had little effect on E2F dependent transcription and cyclin D1 promoter activity was reduced. In summary, these studies indicated that silencing E2F1 reduced the efficiency of HSV-1 and BHV-1 productive infection, but only BHV-1 infection induced E2F dependent transcription.

INTRODUCTION

Bovine herpes virus 1 (BHV-1) and herpes simplex virus type 1 (HSV-1) are *alphaherpesvirinae* subfamily members that establish latency in sensory neurons. In contrast to small DNA tumor viruses, *alphaherpesvirinae* subfamily members do not appear to promote entry into the S phase of the cell cycle. For example, HSV-1 encodes several genes, ICP0, ICP27, ICP22/U_S1.5, and U_L13, which inhibit cell cycle progression (Advani et al., 2000b; Flemington, 2001; Hobbs and DeLuca, 1999; Lomonte and Everett, 1999; Orlando et al., 2006; Song et al., 2001). However, drugs that interfere with cyclin dependent kinase activity, roscovitine and olomucine, reduce the efficiency of HSV-1 productive infection (Schang et al., 1998; Schang et al., 1999). Therefore, although herpesviruses block cell cycle progression into S phase, it appears that they require certain cell cycle regulatory proteins to facilitate efficient replication.

This is exemplified by recent studies suggesting that the E2F family of transcription factors stimulate BHV-1 productive infection and reactivation from latency. The E2F family of transcription factors contains cell cycle regulatory proteins that interact with retinoblastoma (Rb) family members (Harbour and Dean, 2000). During cell cycle progression from G1 to S phase, phosphorylation of Rb family members by cyclin dependent kinase/cyclin complexes leads to E2F release, and consequently certain E2F family members (E2F1, -2, or -3) activate transcription (Attwooll et al., 2004; Harbour and Dean, 2000; Nevins et al., 1997; Weintraub et al., 1992). Consensus E2F binding sites are present in the promoters of many cellular genes that control cell cycle progression (DeGregori et al., 1995; Nevins et al., 1997; Ohtani et al., 1995b; Schulze et al., 1995; Wells et al., 1997). Despite the herpesvirus induced block in cell cycle

progression, BHV-1 requires E2F family members for efficient productive infection. For example, a siRNA directed against E2F1 inhibits BHV-1 productive infection, E2F1 protein levels and binding activity increase after infection, and E2F1 or E2F2 stimulates the bICP0 early promoter more than 100 fold (Workman and Jones, 2010). Furthermore, over-expression of E2F4 stimulates BHV-1 productive infection and E2F1 or E2F2 transactivates IETu1 (immediate early transcription unit 1) promoter activity (Geiser and Jones, 2003). Furthermore, during dexamethasone induced reactivation from BHV-1 latency, sensory neurons that express abundant levels of lytic cycle genes also express cyclin E and cyclin A (Winkler et al., 2000b). This suggests that transient induction of E2F dependent transcription by *alphaherpesvirinae* subfamily members may enhance productive infection in highly differentiated cells, such as neurons. Although these studies suggest that increased E2F protein levels and binding activity were important for productive infection, BHV-1 may also increase E2F1 protein levels because productive infection leads to p53 dependent apoptosis (Devireddy and Jones, 1999a).

With respect to HSV-1 productive infection, “free” E2F (not associated with Rb family members) increases following infection of a human tumor cell line (C33-A) (Hilton et al., 1995). Re-localization of E2F4 to the nucleus occurs in human tumor cell lines (C33-A and U2-OS) following HSV-1 infection (Olgiate et al., 1999). Further support for E2F4 playing a role in HSV-1 replication comes from the findings that infection of mouse cells that lack p107^{-/-} and p130^{-/-}, two Rb family members that are known to interact with E2F4, leads to reduced infectious virus (Ehmann et al., 2001). In primary human fibroblasts or Hela cells, the subcellular distribution of E2F4 is altered following HSV-1 infection, which is assumed to inactivate E2F4 (Advani et al., 2000b).

This same study also concluded that HSV-1 infection leads to post-translational modification of E2F1 and E2F5, translocation of E2F family members from the nucleus to the cytoplasm, and reduced E2F binding to consensus E2F binding sites. Based on these observations, HSV-1 appears to inactivate E2F dependent signaling.

In this study, we compared the effects of HSV-1 versus BHV-1 infection on E2F1 and E2F dependent transcription in the same cell line. Like BHV-1, an E2F1-specific siRNA reduced HSV-1 productive infection following infection of rabbit skin cells. Although BHV-1 and HSV-1 both increased E2F1 protein levels, a significant amount of E2F1 protein was localized in a cytoplasmic fraction following HSV-1 infection. Conversely, E2F1 was localized to the nucleus following BHV-1 infection. BHV-1, but not HSV-1, infection of rabbit skin cells enhanced E2F dependent transcription.

MATERIAL AND METHODS

Cells and viruses

Murine neuroblastoma 2A (neuro-2A), human embryonic lung (HEL), and rabbit skin (RS) cells were grown in Earle's modified Eagle's medium (EMEM) supplemented with 5% fetal calf serum (FCS). Bovine kidney cells (CRIB) were grown in EMEM supplemented with 10% FCS. All media contained penicillin (10 U/ml) and streptomycin (100 µg/ml).

The Cooper strain of BHV-1 (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, Iowa. Stock cultures of BHV-1 were prepared in CRIB cells.

The HSV-1 McKrae strain was obtained from S. Wechsler (U of California-Irvine). Stock cultures of the McKrae strain were prepared in RS cells.

Plasmids and measurement of promoter activity in transfected cells

Plasmids expressing E2F1 or E2F2, pCMV-E2F1 and pCMV-E2F2 respectively, were obtained from J.R. Nevins (Duke University, Durham, USA). CAT constructs used in this study, HSV-1 ICP0-CAT (pAB5) and ICP4-CAT, were previously described (Devireddy and Jones, 2000). Luciferase promoter constructs used in this study, pGL3basic, VP16-luc, gC-Luc, UL42-Luc and ICP6-Luc, were obtained from P. Schaffer and have been previously described (Kushnir et al., 2010). The empty vector pcDNA3.1 was purchased from Invitrogen.

Measurement of chloramphenicol acetyltransferase (CAT) activity in neuro-2A cells was performed as described previously (Workman and Jones, 2010; Workman et al.,

2009; Workman et al., 2011). Luciferase activity in transfected neuro-2A cells was performed using the Promega Luciferase Assay System as described by the manufacturer (E4030; Promega).

For productive infection assays, RS cells were transfected with an E2F-responsive promoter construct using Lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours later, cells were infected with BHV-1 or HSV-1 at a moi of 0.1, 0.5, or 5 for 24 hours. Cells were then collected and processed as above for luciferase activity. Data for CAT and luciferase activity were averaged from the results of multiple transfections performed in at least three independent experiments.

SDS-polyacrylamide gels and Western Blotting of E2F Expression

RS cells or HEL cells were infected with wt BHV-1 or HSV-1 at the moi indicated in the respective figure legend and cell lysate collected at various times (hours) after infection. Whole cell lysate was prepared as described previously (Workman and Jones, 2010; Workman et al., 2011), and protein concentrations were quantified by the Bradford assay. Standard 8% SDS-polyacrylamide gels were used to analyze E2F1 protein levels as described previously (Workman and Jones, 2010; Workman et al., 2011). The E2F1 antibody (sc-193X; Santa Cruz Biotechnology) was diluted 1:10,000 in blocking solution. An antibody directed against β -actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control.

Nuclear and Cytoplasmic Fractionation

RS cells were infected with BHV-1 or HSV-1 at a moi of 0.1, 0.5, or 5 for 8, 16 or 24 hours. Cells were harvested by centrifugation at 1,000 rpm for 5 min and washed twice in cold phosphate buffered saline (PBS). Cells were then suspended in buffer A (50 mM NaCl, 10 mM HEPES pH8, 500 mM sucrose, 1 mM EDTA, 0.5% NP40), vortexed briefly, and centrifuged at 5,000-x g for 2 minutes. The supernatant was collected and saved as the cytoplasmic fraction. The nuclear pellet was washed twice with low salt buffer B (50 mM NaCl, 10 mM HEPES pH8, 25% glycerol, 0.1 mM EDTA). The pellet was then suspended in high salt lysis buffer C (350 mM NaCl, 10 mM HEPES pH8, 25% glycerol, 0.1 mM EDTA). Lysis was carried out at 4⁰ C for 30 min. Supernatant was cleared by centrifuging at 13,000 rpm for 15 minutes at 4⁰ C. Protein concentrations were quantified by the Bradford assay and standard 8% SDS-polyacrylamide gels were used as described above to analyze E2F1 protein levels. An antibody directed against histone H3 (abcam; ab1191) was diluted 1:500 and used a control for fractionation studies.

Analysis of HSV-1 productive infection

RS cells grown in 60 mM dishes were transfected with 1 µg of blank pcDNA3.1 vector, 100 nM E2F1 siRNA (sc-35247; Santa Cruz Biotechnology) or 100 nM control siRNA (44-2926; Invitrogen) using Lipofectamine 2000 according to the manufacture's specifications. At 24 hours after transfection, cells were transfected with 2 µg of the HSV-1 viral genome. Forty-eight hours after transfection cells were collected and subjected to three rounds of freeze/thaw cycles. Cell debris was pelleted by

centrifugation, and the supernatant was analyzed by plaque assay on RS cells using 10 fold dilutions.

Cell Cycle Analysis by Flow Cytometry using the Telford Method

RS cells grown in 60 mM dishes were transfected with 100 nM E2F1 siRNA or a control siRNA using Lipofectamine-2000 according to the manufacture's specifications. At 24 hours or 48 hours after transfection, an aliquot of 1×10^6 RS cells were pelleted, then suspended in 1 mL of ice-cold 70% ethanol, and rotated at 4 degrees for 30 minutes. Cells were then pelleted and suspended in Telford reagent (100 mM EDTA, 0.1% TritonX-100, 75 mM Propidium Iodide, and 2.5 U/mL RNase A in PBS pH 7.4) and rotated at 4 degrees for 2 hours. Cells were then analyzed using a Becton Dickson FACS caliber flow cytometer. The data was analyzed using what program Cell Quest.

Alkaline Phosphatase (CIAP) treatment

Whole cell lysate was collected as described above. Twenty μg of infected cell lysate was treated with 10 U of alkaline phosphatase (04 898 133 001; Roche) and incubated at 37 degrees for 30 minutes. Reactions were stopped by the addition of SDS-polyacrylamide gel loading buffer. Samples were boiled for 5 minutes and assayed by immunoblotting.

RESULTS

An E2F1 specific siRNA reduced the levels of HSV-1 productive infection.

Silencing E2F1 decreases the levels of BHV-1 productive infection, in part, because E2F1 stimulates the bICP0 early (E) promoter more than 100 fold in transient transfection assays (Workman and Jones, 2010). Since BHV-1 and HSV-1 are both *alphaherpesvirinae* subfamily members, we hypothesized that both viruses require similar transcription factors, including E2F1, for efficient productive infection. To test whether E2F1 influenced HSV-1 productive infection, we examined the effect of an E2F1 specific siRNA on viral growth in rabbit skin (RS) cells, which supports productive infection by BHV-1 and HSV-1. RS cells are not transformed by a known oncogene, they can be readily transfected with siRNA and plasmids (Workman and Jones, 2010), and thus are well suited for these studies. Relative to the control siRNA, co-transfection of the E2F1 siRNA with the HSV-1 genome reduced the number of plaques more than 10-fold (Figure 4.1A). In contrast, the control siRNA slightly increased the number of plaques produced.

Previous studies demonstrated that the E2F1 specific siRNA reduced E2F1 protein levels, but not E2F2 or β -actin protein levels (Workman and Jones, 2010). Additional studies were performed to evaluate the effect of the E2F1 siRNA on the cell cycle in uninfected cells. The E2F1 siRNA reduced the percent of cells in S phase less than 2 fold at 24 hours after transfection (Figure 4.1B). However, silencing of E2F1 did not stimulate apoptosis or cell death, in general. By 48 hours after transfection, there was no difference in the percent of cells in S phase regardless of the treatment. In summary, these studies demonstrated that silencing E2F protein levels reduced HSV-1 productive

infection, which was consistent with previous studies that examined the effect of the same siRNA on BHV-1 productive infection (Workman and Jones, 2010).

Analysis of E2F1 trans-activation of HSV-1 promoter constructs

Previous studies revealed E2F1 trans-activated the BHV-1 bICP0 E promoter more than 100 fold in transient transfection assays (Workman and Jones, 2010). Two E2F1 responsive regions (ERR) were identified within the bICP0 E promoter, one adjacent to the TATA box (ERR1) and one approximately 600 bp upstream of the TATA box (ERR2). When cloned upstream of a simple HSV-1 thymidine kinase promoter, ERR1 stimulated promoter activity 6 fold whereas ERR2 stimulate promoter activity more than 20 fold. Mobility shift assays provided further evidence that E2F1 interacted directly with these sequences (Workman and Jones, 2010). Therefore, we hypothesized that E2F1 may stimulate promoters of important HSV-1 regulatory genes.

To test this hypothesis, neuro-2A cells were cotransfected with an HSV-1 ICP0 or ICP4 promoter construct plus an E2F1 expression plasmid, and CAT activity was measured. Neuro-2A cells were use for this study because these cells are neuronal-like and thus it was of interest to begin to understand whether E2F regulates gene expression in neurons. Furthermore, E2F1 over-expression is not as toxic in neuro-2A cells when compared to RS cells (data not shown). The HSV-1 ICP0 promoter was activated only 3 fold by E2F1 (Figure 4.2A). The ICP4 promoter was consistently lower than basal activity when cotransfected with E2F1 (Figure 4.2A).

We next examined whether reducing E2F1 protein levels by the E2F1 specific siRNA had an effect on ICP0 and ICP4 basal promoter activity. The HSV-1 promoter

constructs had high basal activity in neuro-2A cells compared to BHV-1 promoter constructs. Consequently, we may have underestimated the effects of E2F1 on ICP0 and ICP4 promoter activity. Neuro-2A cells were cotransfected with the designated promoter-CAT construct and the E2F1 siRNA or a control siRNA (cont-si). At 48 hours after transfection, cells were collected and CAT activity measured. The E2F1 specific siRNA reduced ICP0 promoter activity approximately 60%: whereas the control siRNA did not reduce ICP0 promoter activity (Figure 4.2B). The E2F1 siRNA reduced ICP4 promoter activity by approximately 40%. In contrast, the control siRNA reduced ICP4 promoter activity less than 20% (Figure 2B).

Finally, we tested whether E2F1 had an effect on a panel of HSV-1 promoter-luciferase constructs. Of those examined, the early UL42 promoter was activated approximately 2.5 fold (Figure 4.2C). UL42 encodes a protein that stimulates the viral DNA polymerase (Gottlieb and Challberg, 1994). Since E2F1 activates expression of genes that are necessary for DNA replication (DeGregori et al., 1995), this result suggested that under certain conditions E2F1 may promote expression of certain viral genes that are necessary for DNA replication. The late VP16 and gC promoters as well as the early ICP6 promoter were not activated by E2F1. In summary, the HSV-1 promoters we examined were not efficiently trans-activated by E2F1 in neuro-2A cells.

Analysis of E2F1 protein levels during productive infection

Previous studies demonstrated that E2F1 protein levels increased during BHV-1 productive infection in bovine kidney cells (Workman and Jones, 2010). Additional studies were performed to directly compare the effect of BHV-1 or HSV-1 productive

infection on E2F1 protein levels in RS cells. RS cells were infected with BHV-1 or HSV-1 using a moi of 5 and whole cell lysate was collected at the designated times post-infection. Western blot analysis revealed that BHV-1 increased E2F1 protein levels by 16 hours after infection {(Workman and Jones, 2010) and (Figure 4.3A)}. Higher levels of E2F1 were detected in RS cell within 8 hours after infection with HSV-1 using a moi of 5 (Figure 4.3B). When RS cells were infected with HSV-1 at a moi of 0.5 (Figure 4.3C) or 0.1 (data not shown), enhanced E2F1 levels were not readily detected until 16 hours after infection. In RS cells, HSV-1 grows faster than BHV-1 at a moi of 0.5 or lower; however, the end point titers of BHV-1 and HSV-1 are similar when RS cells are infected with a moi of 0.5 or higher (data not shown). We suggest that differences in the growth rate of the two viruses was the main reason why higher levels of E2F1 were detected in HSV-1 infected RS cells at 8 hours after infection.

The E2F1 band detected after HSV-1 infection appeared to migrate differently compared to E2F1 after BHV-1 infection or in mock-infected cells (Figure 4.4A). E2F can be phosphorylated and phosphorylation is crucial for regulating transcriptional activation, localization, and stability (Garcia-Alvarez et al., 2007; Real et al., 2010; Weei-Chin L., 2011). To test whether E2F1 was phosphorylated, whole cell extract from HSV-1 infected cells was treated with calf intestinal alkaline phosphatase (CIAP) and then western blot analysis performed using an anti-E2F1 antibody (Figure 4.4B). CIAP treatment resulted in a decrease in the electrophoretic mobility of E2F1 in RS cells (Figure 4.4B) suggesting the observed shift in E2F1 mobility after HSV-1 infection was the result of phosphorylation. To visualize E2F1 in mock-infected cells, 200 ug of protein in the cell lysate was loaded versus 20 ug protein derived from infected samples.

Similar results were observed when comparing BHV-1 and HSV-1 infection in the human osteosarcoma cell line (U2OS) that is permissive for both viruses (data not shown).

Additional studies were performed to examine E2F1 protein levels in primary human embryonic lung (HEL) fibroblasts after infection with HSV-1. The rationale for this study was to compare the results obtained in RS cells to a low passage human cell type. Confluent cultures of HEL fibroblasts were infected with HSV-1 at a moi of 1 and whole cell lysate collected at the designated times (hours) after infection. In HEL cells, HSV-1 infection increased the steady state E2F1 protein levels at 16 hours after infection (Figure 4.5A). CIAP treatment resulted in a decrease in the electrophoretic mobility of E2F1 in HEL cells (Figure 4.5B). In summary, both BHV-1 and HSV-1 increased E2F1 protein levels, and HSV-1 infection led to novel changes in the phosphorylation status of E2F1 in RS and HEL cells.

Analysis of E2F1 localization following infection.

A previous study revealed that E2F1 remained localized to the nucleus following infection of bovine kidney cells with BHV-1 (Workman and Jones, 2010). Conversely, E2F1 was reported to localize to the cytoplasm following HSV-1 infection of HeLa cells (Advani et al., 2000b). Confocal microscopy suggested that a subset of E2F1 was localized to the cytoplasm of HSV-1 infected cells (data not shown). To confirm these findings and to compare them to BHV-1 productive infection, biochemical fractionation studies were conducted in RS cells. Two low moi, 0.1 or 0.5 pfu/cell, were used for the HSV-1 studies. A moi of 5 pfu/cell was used for BHV-1 because BHV-1 does not grow

as quickly as HSV-1 in RS cells (data not shown). Consistent with previous studies (Advani et al., 2000b), E2F1 was detected in the nuclear extract, but not the cytoplasm, of BHV-1 infected cells at 16 or 24 hours after infection (Figure 4.6A). At lower moi, E2F1 was also detected in the nucleus of RS cells infected with BHV-1 (data not shown). Alternatively, E2F1 was detected in the nuclear and cytoplasmic extract following infection with HSV-1 for 16 hours regardless of whether the moi was 0.1 or 0.5 (Figure 4.6B). Eight hours after infection (moi of 0.1 and 0.5 pfu/ml) with HSV-1, E2F1 was primarily detected in the nucleus of infected cells. As a control for nuclear proteins, we examined histone H3 and found, as expected, that histone H3 was detected only in the nucleus after infection (Figure 4.6A and B).

Analysis of E2F-dependent transcription following infection

Studies in Figures 4.3-4.6 indicated that HSV-1 and BHV-1 increased E2F1 protein levels, but these studies did not reveal whether E2F dependent transcription correlated with increased E2F1 protein levels. Therefore, we examined the effect of BHV-1 or HSV-1 productive infection on E2F dependent transcription. RS cells were initially transfected with a luciferase construct containing a minimal promoter with three consensus E2F binding sites (3xE2F) or the Cyclin D promoter (CycD). Cells were then infected with BHV-1 or HSV-1 at a moi of 0.1, 0.5, or 5 for 24 hours. BHV-1 infection increased 3xE2F promoter activity approximately 5 fold and CycD promoter activity approximately 4 fold in RS cells (Figure 4.7). In contrast, HSV-1 had little or no effect on the 3xE2F promoter construct, and CycD promoter activity was consistently reduced.

These results indicated that E2F dependent transcription was stimulated following infection of RS cells with BHV-1, but not HSV-1.

DISCUSSION

In this study, we compared the effect that BHV-1 had on the cell cycle regulated transcription factor E2F1 versus HSV-1. For these studies, we used a spontaneously immortalized rabbit skin cell line that supports productive infection of both viruses. The studies clearly demonstrated that a siRNA directed against E2F1 reduced the plaque forming efficiency of HSV-1 (Figure 4.1) and BHV-1 (Workman and Jones, 2010). For HSV-1, this was somewhat surprising because E2F dependent transcription was not activated after infection. In this study and a previous study, we demonstrated that BHV-1 infection induced E2F dependent transcription and binding to a probe containing E2F consensus binding sites (Workman and Jones, 2010). We suggest that silencing E2F1 prior to infection reduces the number of cells that are cycling, which consequently reduces the efficiency of productive infection. This conclusion is supported by other studies demonstrating that cyclin dependent kinase inhibitors, roscovitine and olomucine, reduce HSV-1 virus replication and transcription (Schang et al., 1998; Schang et al., 1999). Roscovitine also efficiently reduces BHV-1 productive infection (data not shown).

Following infection with HSV-1, E2F1 appeared to be differentially phosphorylated and a fraction of the total E2F1 protein was localized in the cytoplasmic. In contrast, E2F1 was localized to the nucleus after BHV-1 infection, as judged by biochemical fractionation (Figure 4.6) and confocal microscopy (Workman and Jones, 2010). Since HSV-1 inactivated E2F dependent transcription, we suggest that phosphorylation of E2F1 promotes localization of E2F1 to the cytoplasm, resulting in a loss of E2F dependent transcription. Inactivation of E2F1 by HSV-1 may be important

for efficient productive infection because E2F1 induces apoptosis following DNA damage (Nip et al., 1997). HSV-1 encodes two protein kinases, US3 and UL13 that are packaged in the virion and tegument respectively (Chee et al., 1989; Frame et al., 1987; Purves et al., 1987; Smith and Smith, 1989). Plasmids over-expressing the two viral kinases were cotransfected with E2F1 and there was no obvious changes in the mobility of E2F1 (data not shown) suggesting the viral encoded protein kinases do not directly phosphorylate E2F1. E2F1 is directly phosphorylated by several cellular protein kinases, including glycogen synthase kinase-3 β , ATM (Ataxia telangiectasia mutated), and mitogen activated protein kinases (MAPK) (Garcia-Alvarez et al., 2007; Real et al., 2010; Weei-Chin L., 2011). MAPK and ATM protein kinases are activated by HSV-1 (Boutell and Everett, 2004; Corcoran et al., 2006; Hargett et al., 2005) suggesting that E2F1 may be phosphorylated by cellular protein kinases after infection. It is not presently known whether BHV-1 infection induces MAPK or ATM protein kinases during the course of productive infection.

Transcriptionally competent E2F family members are associated with a family of DP proteins (Helin et al., 1993) suggesting BHV-1 stimulates E2F dependent transcriptional activity by promoting interactions between E2F1 and DP family member. Conversely, HSV-1 may uncouple E2F1 dependent transcription by inactivating DP family members. A recent study identified a novel DP family member (DP-4) in several human cell types that exhibits high levels of expression following DNA damage (Ingram et al., 2011). Interestingly, DP-4 binds to E2F1 resulting in a complex that does not bind DNA. Consequently, E2F1 does not induce apoptosis when associated with DP-4. There is no commercially available DP-4 antibody, thus it is not possible to test whether DP-4 is

induced by HSV-1 infection in RS cells, or whether BHV-1 induces DP-4. It will be of interest to examine the effect that HSV-1 has on DP-4 in human cells in the future.

In summary, it was surprising to find that E2F dependent transcription was activated by BHV-1, but not HSV-1. Activation of E2F dependent transcription appears to be important for BHV-1 productive infection because E2F1 and E2F2 strongly stimulate the bICP0 E promoter and to a lesser extent the IE promoter that controls expression of bICP0 and bICP4 (Geiser and Jones, 2003; Workman and Jones, 2010). We suggest that BHV-1 encodes or induces a protein that maintains E2F1 transcriptional activity during productive infection. Conversely, HSV-1 may encode or induce a factor that prevents activation of E2F dependent transcription during productive infection.

Figure 4.1. Suppression of E2F1 reduced the levels of HSV-1 productive infection.

Panel A: To test whether E2F-specific siRNA affected HSV-1 productive infection, RS cells were transfected with 100 nM E2F1 siRNA or 100 nM control siRNA for 24 hours, followed by transfection of HSV-1 genomic DNA as described in the methods. Forty-eight hours after transfection of genomic DNA cells were collected, subjected to three rounds of freeze/thaw cycles and plaque assays performed on RS cells. The data is an average of three independent studies and the error bars are standard deviations.

Panel B: To examine the effect of the siRNAs on cell cycle, RS cells were transfected with 100 nM E2F1 siRNA or 100 nM control siRNA. At 24 or 48 hours after transfection, cells were collected, fixed, stained and analyzed by flow cytometry as described in the methods section. These studies are representative of at least three independent studies.

Figure 4.1.

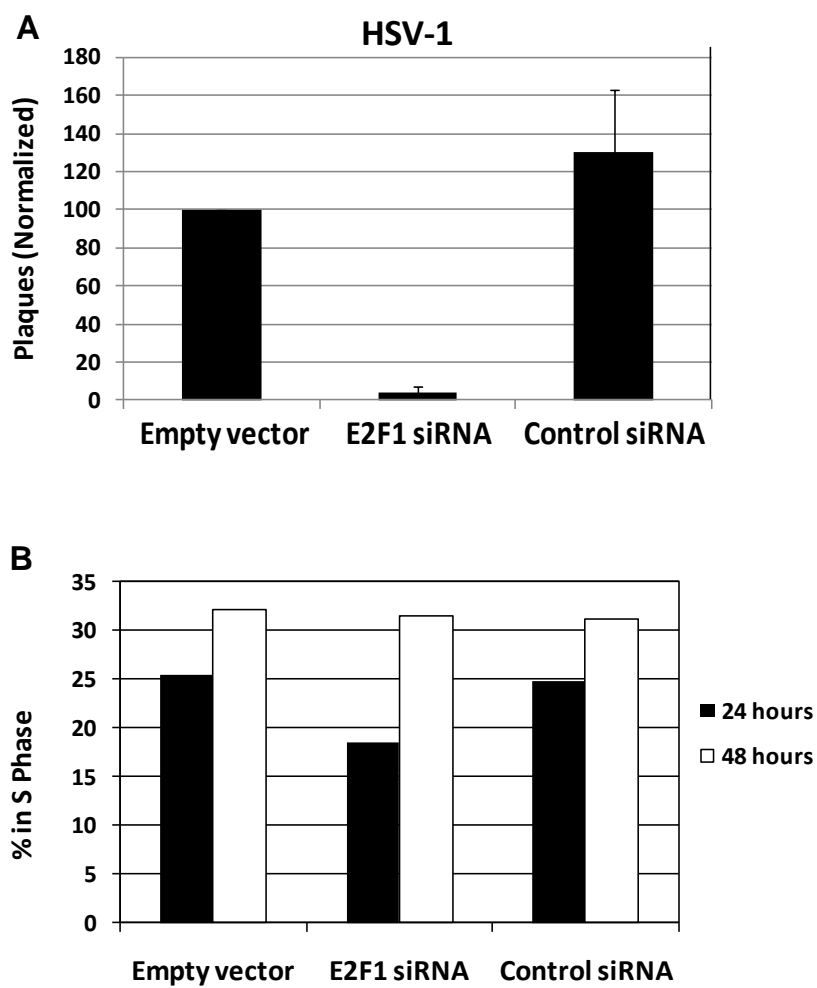


Figure 4.2. Analysis of E2F1 transactivation of HSV-1 promoter constructs.

Panel A: To examine the effect of E2F1 on HSV-1 promoters, neuro-2A cells were cotransfected with 1 μg of the designated promoter-CAT construct and 0.1 μg of the E2F1 expression plasmid. At 48 hours post-transfection, cells were collected and CAT activity measured. The data is an average of three independent studies and the error bars are standard deviations.

Panel B: To test whether silencing E2F1 protein levels reduced basal activity of HSV-1 promoters, neuro-2A cells were cotransfected with 1 μg of the designated promoter-CAT construct and 100 nM E2F1 siRNA or a control siRNA (contsi). At 48 hours post-transfection, cells were collected and processed for CAT activity. The data is an average of three independent studies and the error bars are standard deviations.

Panel C: Neuro-2A cells were cotransfected with 1 μg of the designated promoter-luciferase construct and 0.1 μg of the E2F1 expression plasmid. At 48 hours post-transfection, cells were collected and processed for luciferase expression. The data is an average of three independent studies and the error bars are standard deviations. pGL3basic is the promoterless luciferase construct. UL42 and ICP6 are early promoters while gC and VP16 are late promoters.

Figure 4.2.

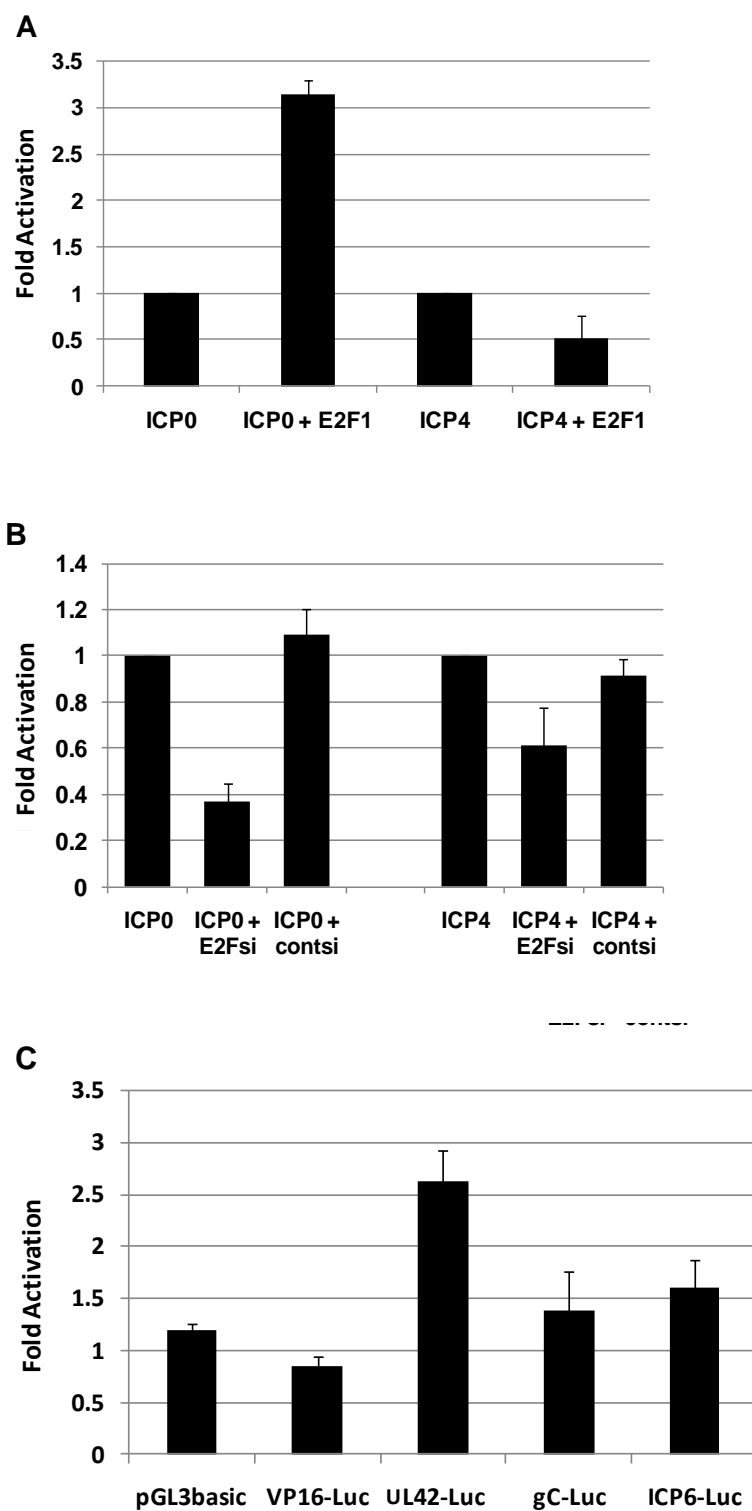


Figure 4.3. Analysis of E2F1 protein levels during productive infection.

The effect of BHV-1 or HSV-1 infection on E2F1 protein levels was measured by western blot analysis. RS cells were infected with BHV-1 at a moi of 5 (**Panel A**), HSV-1 at a moi of 5 (**Panel B**), or HSV-1 at a moi of 0.5 (**Panel C**) for the designated times after infection (hours). Cells were collected, lysed, and 100 μ g of protein was analyzed by western blot using an E2F1 polyclonal antibody diluted 1:10,000. As a control, the blot was also probed with antiserum against β -actin. Lanes M4 or M24 are 4, or 24 hours respectively after mock infection.

Figure 4.3.

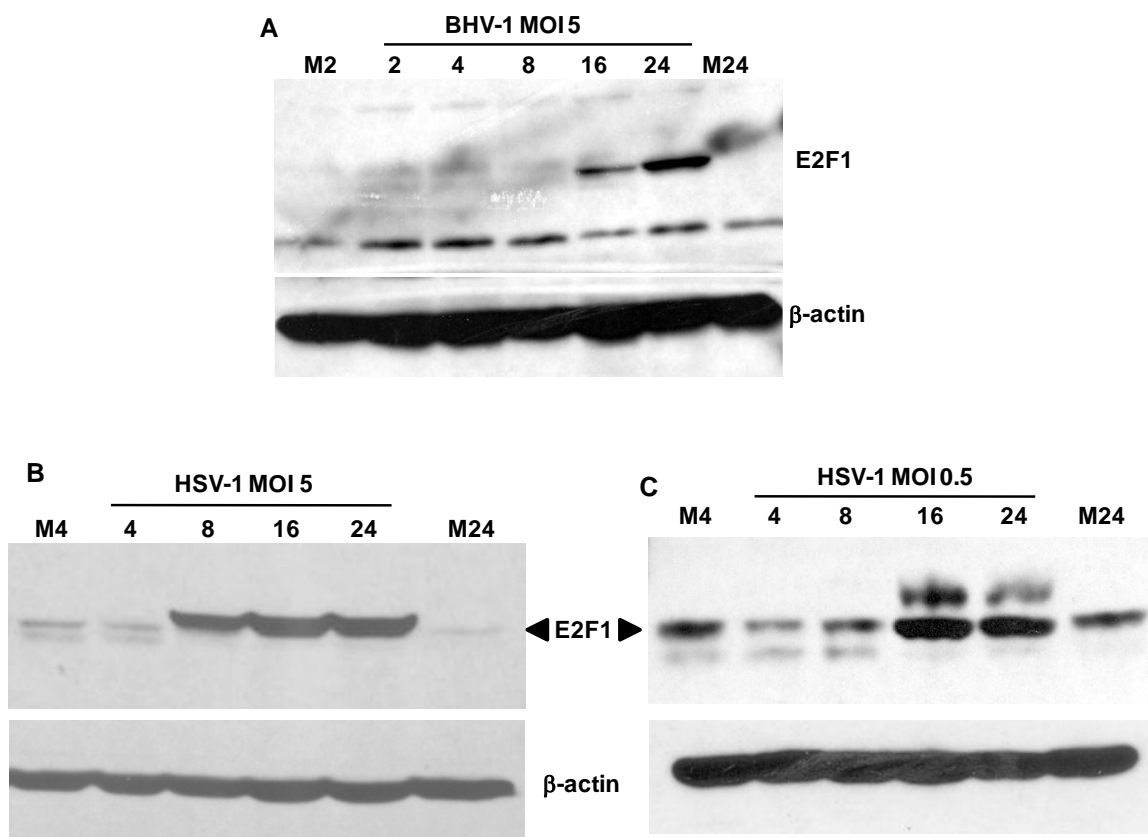


Figure 4.4. Analysis of E2F1 protein levels in infected RS cells.

Panel A: Cell lysate prepared from RS cells infected with BHV-1 (moi of 5) or HSV-1 (moi of 5) for 24 hours were separated on the same SDS-polyacrylamide gel to show mobility differences of E2F1.

Panel B: To determine whether phosphorylation was responsible for the altered mobility of E2F1, 20 μ g of protein derived from cell lysate prepared from RS cells infected with HSV-1 for 24 hours was treated (or not) with calf intestinal alkaline phosphatase (CIAP). As a control, 200 μ g of mock-infected cells was analyzed. More protein was loaded in the mock lane to readily visualize the E2F1-specific bands.

Figure 4.4.

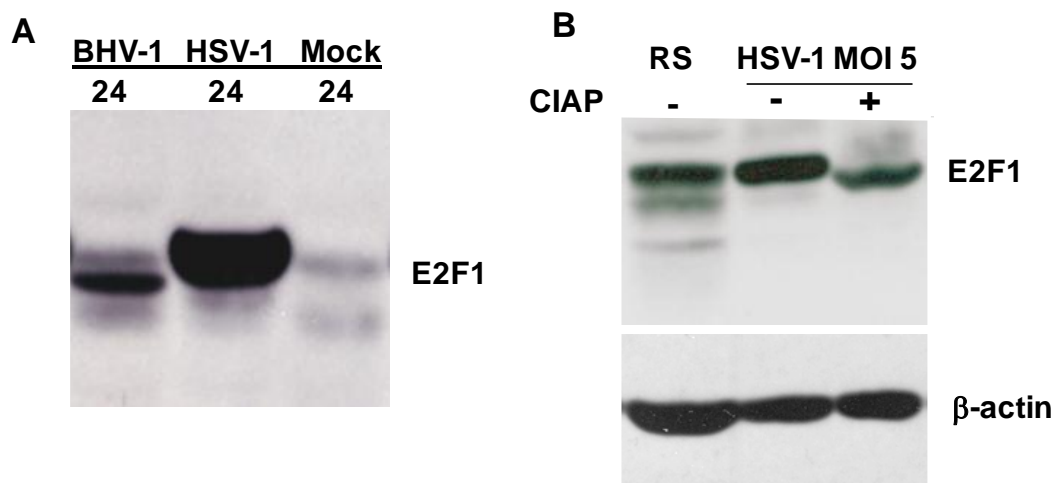


Figure 4.5. Analysis of E2F1 protein levels in primary human lung fibroblasts.

Panel A: Low passage human embryonic lung fibroblasts (HEL) were infected with HSV-1 at a moi of 1 for the designated times after infection (hours). Cells were collected, lysed, and 100 μg of protein analyzed by western blot using an E2F1 polyclonal antibody diluted 1:10,000. As a control, the blot was also probed with antiserum directed against β -actin.

Panel B: Twenty μg of protein from the sample at 24 hours after infection of HEL cells were treated (or not) with calf intestinal alkaline phosphatase (CIAP) and ran next to 200 μg of cell lysate from mock-infected cells. More protein was loaded in the mock-infected lane (lane M) to visualize E2F1.

Figure 4.5.

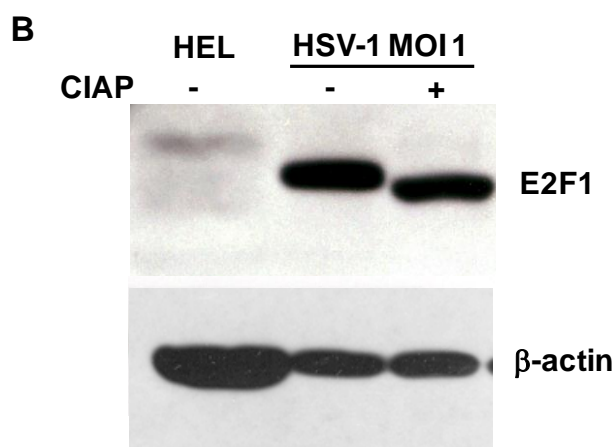
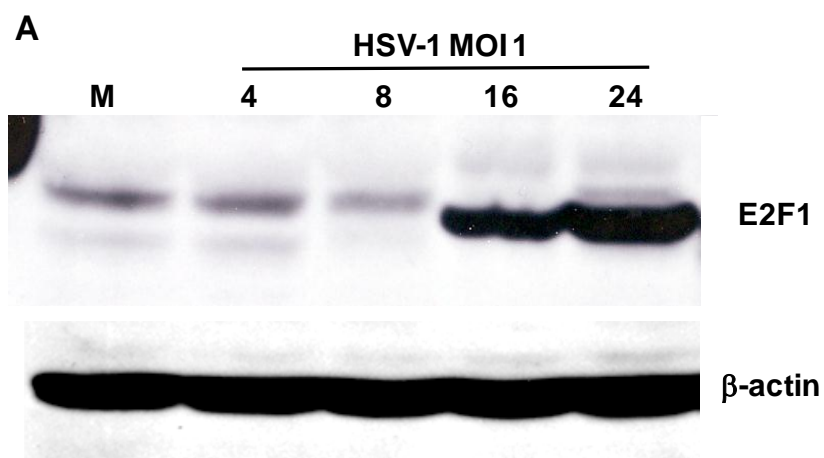


Figure 4.6. Localization of E2F1 after infection with BHV-1 or HSV-1.

Panel A: RS cells were infected with BHV-1 at a moi of 5 for 16 or 24 hours. Cells were then harvested and cell fractionation conducted as described in the methods section. Cytoplasmic and nuclear fractions (100 µg) were analyzed by western blot using an E2F1 polyclonal antibody diluted 1:10,000. As a control for proteins that are in the nucleus, the respective fractions were probed with an antibody directed against Histone H3 (abcam) that was diluted 1:500.

Panel B: RS cells were infected with HSV-1 at a moi of 0.1 or 0.5 for 8 or 16 hours. Cellular fractions were analyzed as described in panel A. Lane M was cell lysate derived from mock-infected cells.

Figure 4.6.

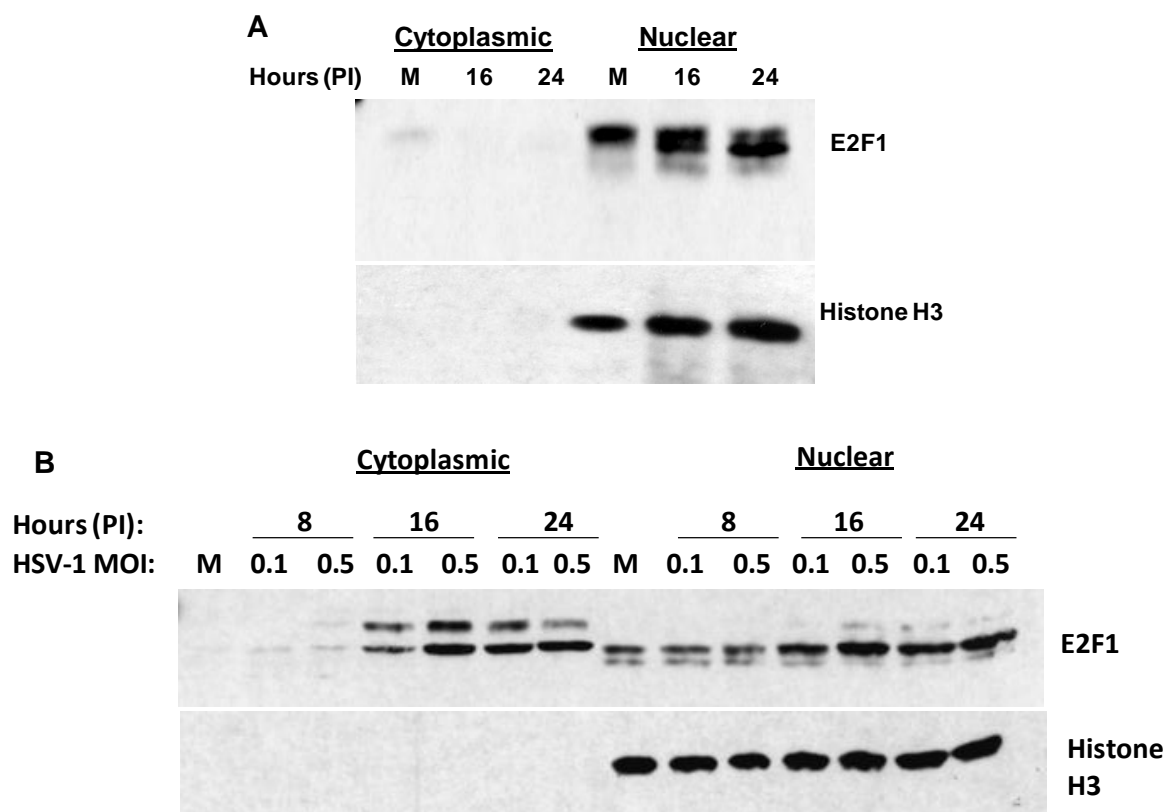
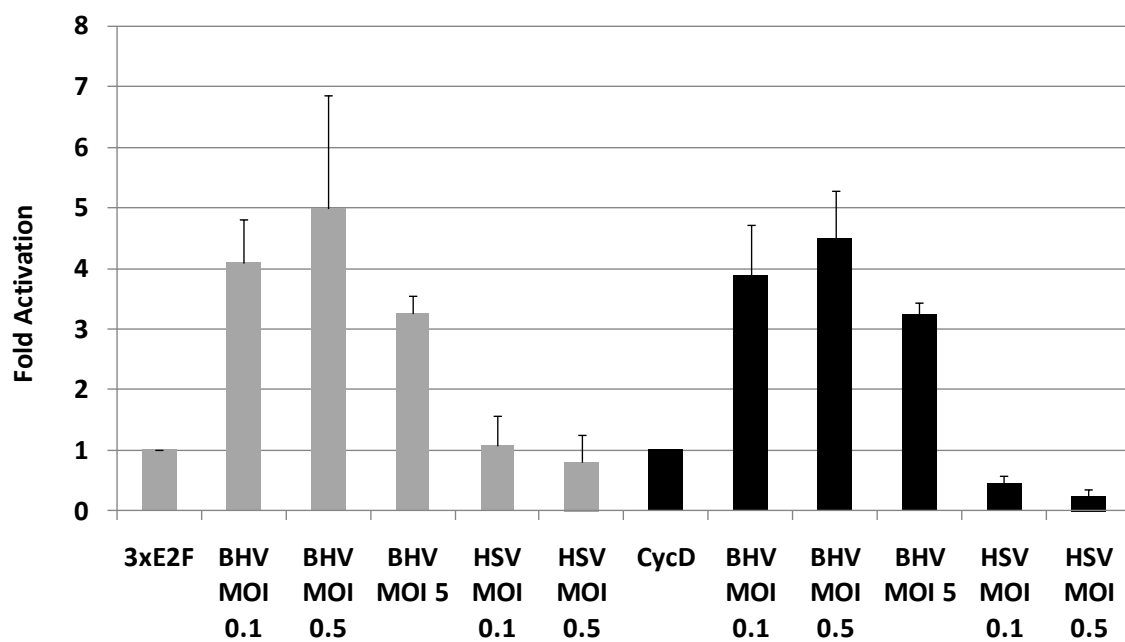


Figure 4.7. Activity of E2F-responsive promoters following BHV-1 or HSV-1 infection.

RS cells were transfected with 1 μg of the designated promoter-luciferase construct (a promoter containing 3 consensus E2F1 binding sites: 3xE2F or the cyclin D promoter: CycD). Twenty-four hours after transfection, cells were infected with BHV-1 or HSV-1 at a moi of 0.1, 0.5 or 5. At 24 hours after infection, cell lysate was collected and luciferase activity measured. The results are the average of 3 independent experiments. The data is an average of three independent studies and the error bars are standard deviations.

Figure 4.7.



Chapter 5

A Protein (ORF2) Encoded by the Latency Related Gene of Bovine Herpesvirus 1 Interacts with Notch1 and Notch3.

These studies are included in a manuscript published in 2011 in the Journal of virology, volume 85, pages 2536-2546.

The authors of this manuscript are **Aspen Workman**, Devis Sinani, Daraporn Pittayakhajonwut, and Clinton Jones

ABSTRACT

Like other alpha-herpesvirinae subfamily members, bovine herpes virus 1 (BHV-1) establishes latency in sensory neurons. The latency-related RNA (LR-RNA) is abundantly expressed in latently infected sensory neurons. A LR mutant virus with stop codons at the amino-terminus of the first ORF in the LR gene (ORF2) does not reactivate from latency, in part, because it induces higher levels of apoptosis in infected neurons. ORF2 is not the only viral product expressed during latency, but it is important for the latency-reactivation cycle because it inhibits apoptosis. In this study, a yeast 2-hybrid screen revealed ORF2 interacted with two cellular transcription factors, Notch1 and Notch3. These interactions were confirmed in mouse neuroblastoma cells by confocal microscopy, and in an *in vitro* “pull down” assay. During reactivation from latency, Notch3 RNA levels in trigeminal ganglia were higher compared to latency suggesting Notch family members promote reactivation from latency or reactivation promotes Notch expression. A plasmid expressing the Notch1 intercellular domain (ICD) stimulated productive infection and promoters that encode the viral transcription factor (bICP0). Notch3 ICD did not stimulate productive infection as efficiently as Notch1 ICD and had no effect on bICP0 promoter activity. Plasmids expressing the Notch1 ICD or Notch3 ICD trans-activated a late promoter encoding glycoprotein C. ORF2 reduced the trans-activation potential of Notch1 and Notch3 suggesting ORF2 interfered with the trans-activation potential of Notch. These studies provide evidence that ORF2, in addition to inhibiting apoptosis, has the potential to promote establishment and maintenance of latency by sequestering cellular transcription factors.

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is an alpha-herpesvirinae subfamily member that causes significant economical losses to the cattle industry (Turin et al., 1999). The ability of BHV-1 to suppress the immune system can result in life-threatening pneumonia due to secondary bacterial infections, and this multi-factorial disorder is known as bovine respiratory disease complex {reviewed in (Jones, 2009; Jones, 2008; Workman, 2010b)}. Like other human alpha-herpesvirinae subfamily members, the primary site for BHV-1 latency is sensory neurons within trigeminal ganglia (TG). Viral gene expression (Schang and Jones, 1997) and infectious virus (Inman, 2002) are detected in TG from 1-6 d after infection, but latency is then established. Stress (due to confinement, transporting cattle, restricting food and water, or weaning) increases corticosteroid levels, and can initiate reactivation from latency (Workman et al., 2009). Administration of a synthetic corticosteroid, dexamethasone (DEX), to calves or rabbits latently infected with BHV-1 reproducibly leads to reactivation from latency, as judged by virus shedding from ocular or nasal cavities and a secondary antibody response (Inman et al., 2002; Jones, 1998; Jones, 2003a; Jones et al., 2006; Jones et al., 2000; Rock et al., 1992). Induction of lytic cycle viral gene expression is also consistently detected in TG neurons of calves latently infected with BHV-1 following DEX treatment.

Many alpha-herpesvirinae subfamily members, including human herpes simplex virus type 1 (HSV-1) and HSV-2, express abundant levels of a latency-associated transcript (Prbhakaran) during latency {reviewed by (Jones, 1998; Jones, 2003a; Perng and Jones, 2010)}. The BHV-1 latency related (LR) gene, which is in a similar genomic position as LAT, expresses an abundant transcript (LR-RNA) in latently infected sensory

neurons (Rock et al., 1987a; Rock and Mayfield, 1987). LAT and the LR gene are antisense with respect to an important viral transcriptional regulator, ICP0 or bICP0 respectively, suggesting they reduce the levels of ICP0 or bICP0. A mutant BHV-1 strain with 3 stop codons at the N-terminus of ORF-2 (LR mutant virus) does not express ORF-2 or RF-C (Jiang et al., 2004) but it expresses reduced levels of ORF1 (Meyer et al., 2007b). The LR mutant virus does not reactivate from latency following DEX treatment (Inman et al., 2002) suggesting that expression of LR proteins regulates the latency-reactivation cycle. Insertion of the LR gene into a HSV-1 LAT null mutant increases the frequency of reactivation from latency (Mott et al., 2003; Perng et al., 2002) indicating that the LR gene can substitute for LAT functions in small animal models of infection. Although LR gene products or LAT are important for the latency-reactivation cycle, it is not clear whether they directly participate in reactivation from latency or if they promote establishment and/or maintenance of latency.

The LR gene encodes more than one product that may be important for the latency-reactivation cycle. For example, the LR gene contains two well defined ORFs (ORF2 and ORF1; Figure 5.1) and two reading frames that lack an initiating methionine (RF-B and RF-C). As a result of alternative splicing of polyA⁺ LR-RNA in TG of infected calves (Devireddy and Jones, 1998; Devireddy et al., 2003), ORF2 can be fused with ORF1 protein coding sequences (7 dpi cDNA) or RF-B (15 dpi cDNA). At 1 day after infection of calves and during latency, splicing occurs in TG such that ORF2 is intact (Figure 1). Two micro-RNAs that are expressed during latency are located upstream of ORF2 (Jiang et al., 2004; Meyer et al., 2007b; Shen et al., 2009). LR gene products inhibit cell proliferation, bICP0 RNA expression (Bratanich and Jones, 1992;

Geiser et al., 2002; Schang et al., 1996; Shen et al., 2009), and apoptosis (Ciacci-Zanella et al., 1999). The LR mutant virus induces higher levels of apoptosis in TG neurons of infected calves during establishment of latency (Lovato et al., 2003), and plasmids with the same stop codon mutations exhibit little or no anti-apoptosis activity (Ciacci-Zanella et al., 1999; Henderson et al., 2004a). ORF2 coding sequences inhibit apoptosis in transiently transfected cells (Shen and Jones, 2008) suggesting ORF2 is the most important function encoded by the LR gene in the context of the latency-reactivation cycle.

Notch receptor family members (Notch1-4) are membrane tethered transcription factors that regulate numerous developmental and physiological processes (Bray, 2006; Ehebauer et al., 2006b). For example, Notch promotes neuronal maintenance, development, and differentiation (Berezovska et al., 1999; Cornell and Eisen, 2005; Justice and Jan, 2002). Notch3 and Notch1 (Nair et al., 2003; Sade et al., 2004) promote cell survival by activating a protein kinase, AKT, that inhibits apoptosis. Other studies have concluded that Notch family members induce apoptosis (Bray, 2006; Ehebauer et al., 2006a) suggesting Notch influences cell survival in a cell-type dependent fashion. When the Notch receptor is engaged by one of its five transmembrane ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, or Delta-like4), the Notch intracellular domain (ICD) is cleaved by specific proteases, and is subsequently translocated to the nucleus. The Notch ICD interacts with members of the CSL family of transcriptional repressors, CBF1, Su(H), or Lag1 (also referred to as RBP κ binding proteins). In general, the Notch ICD-CSL complex binds to specific DNA sequences in certain promoters and subsequently activates genes that regulate growth, cell survival, and differentiation (Bray, 2006;

Ehebauer et al., 2006a).

In this study, we provide evidence that ORF2 interacts with Notch3 and Notch1 proteins. Additional studies suggest that Notch family members promote reactivation from latency or reactivation from latency activates the Notch signaling pathway. For example, the Notch1 ICD stimulates productive infection; Notch1 protein expression increased during the course of productive infection, and Notch1 ICD trans-activated the bICP0 immediate early and early promoters. Notch1 and Notch3 trans-activated a late BHV-1 promoter, glycoprotein C (gC). In addition, ORF2 interfered with the ability of the Notch1 or Notch3 ICD to trans-activate the bICP0 E and gC promoters. Finally, during DEX-induced reactivation from latency, Notch3 RNA levels in TG were higher compared to that during latency.

MATERIALS AND METHODS

Cells and viruses

Murine neuroblastoma cells (neuro-2A), rabbit skin cells (RS), and CRIB (bovine kidney) cells were grown in Earle's modified Eagle's medium (EMEM) supplemented with 5% FCS, penicillin (10 U/ml), and streptomycin (100 µg/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 prepared in CRIB cells. A BHV-1 mutant containing the Lac Z gene in place of the gC gene was obtained from S. Chowdury (Baton Rouge, LA) (gCblue virus). The virus grows to similar titers as the wild type virus and expresses the Lac Z gene as a true late gene.

Plasmids

The construction and characteristics of the bICP0 E promoter-CAT (chloramphenicol acetyltransferase) constructs (EP-172, EP-143, EP-133, EP-71, EP-50, and EP-42) used in this study were described previously (Workman and Jones, 2010; Workman et al., 2009). The numbers in the plasmid name refer to the length of the bICP0 E promoter fragment cloned into the promoterless vector, pCAT-basic.

The mammalian ORF2 construct was described previously (Shen and Jones, 2008). Briefly, sequences derived from cDNA at 1 day post infection (dpi) were cloned into pCMV-Tag-2B (Stratagene, La Jolla, CA) downstream of a Flag epitope using BamHI-HindIII restriction enzymes. ORF2 was also codon-optimized for bacterial expression and synthesized by IDT (Coralville, IA). XhoI and HindIII restriction

enzyme sites were introduced at 5' and 3' ends of the ORF2 gene, respectively. The ORF2 fragment was cleaved with XhoI and HindIII and subsequently ligated in frame into similar sites of a polyhistidine tag within the vector pRSET-A (Invitrogen, USA).

Constructs containing Notch1 ICD or Notch3 ICD were cloned into a human cytomegalovirus expression construct. These constructs were gifts from U. Lendahl, Karolinska Institute, Sweden (Lardelli et al., 1996).

The bICP0 plasmid contains the bICP0 coding sequence under control of the human cytomegalovirus (CMV) promoter and was described previously (Inman et al., 2001b). The empty vector pcDNA3.1 was purchased from Invitrogen.

Yeast 2-hybrid analysis

The yeast 2-hybrid analysis was performed by Hybrigenics (France) using ORF2 as the prey. The screen was performed using a mouse cDNA library from brain as bait.

CAT reporter assays

Neuro-2A cells grown in 60 mm dishes were co-transfected with the designated plasmids as indicated in the respective figure legends using with TransIT Neural according to the manufacturer's instructions. After 48 hours, cell extract was prepared by three freeze/thaw cycles in 0.25 M Tris-HCl, pH 7.4. Cell debris was pelleted by centrifugation, and protein concentration determined. CAT activity was measured in the presence of 0.1 uCi $\{^{14}\text{C}\}$ -chloramphenicol (CFA754; Amersham Biosciences) and 0.5 mM Acetyl-CoA (A2181; Sigma). The reaction was incubated at 37° C for 15 minutes to 1 hour. All forms of chloramphenicol were separated by thin-layer chromatography.

CAT activity in 50-ug cell lysate was quantified using a Bio-Rad Molecular Imager FX (Molecular Dynamics, CA). Levels of CAT activity are expressed as fold induction relative to the vector control.

β -gal assay

The gCblue virus grows to similar titers as wt BHV-1 and was grown in CRIB cells. Procedures for preparing BHV-1 genomic DNA were described previously. RS cells grown in 6 well plates were cotransfected with 0.83 ug of the gCblue viral genome and the designated amounts of plasmid expressing bICP0, Notch1 ICD, or Notch3 ICD using Lipofectamine 2000 (11668-019; Invitrogen). Twenty-four hours after transfection, cells were fixed (2% Formaldehyde, 0.2% Glutaraldehyde in PBS) and the number of β -gal⁺ cells counted as described previously (Workman and Jones, 2010). The number of β -gal⁺ cells in cultures expressing the blank vector was set to 100%. The number of blue cells in cultures transfected with the blank vector to calculate fold difference divided the number of blue cells in cultures transfected with bICP0 or Notch. The results are an average of three independent experiments.

RNA preparation and Reverse Transcription

Neuro-2A cells were cultured in the presence of 1 uM dexamethasone (D2915; Sigma) for the indicated amounts of time. Total RNA was prepared from cells using TRIzol reagent (Life Technologies, USA) as previously described (Workman and Jones, 2010). Three micrograms of RNA were treated with Amplification Grade DNase I (Invitrogen, USA). Reverse Transcription (RT) was performed using SuperScript III

Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's directions. RNA was reverse transcribed using oligo(dT) primers (Invitrogen, USA). A 100 ng of the resulting cDNA were used as a template for PCR using specific primers for Notch. PCR was performed using GoTaq DNA Polymerase (Promega, USA) and initiated at 95° C for 5 min. This was followed by 30 cycles of 95° C for 45 seconds, 55° C (Notch3) or 65° C (Notch1) for 45 seconds, and 72° C for 45 seconds. Final extension was at 72° C for 10 minutes. PCR products were analyzed on a 1.2% agarose gel. The following primer sequences were used for Notch:

Notch1 forward 5'-TCCTACCTCTGCTTATGCCTCAAG-3',

Notch1 reverse 5'-GTATCCAGCGACATCATCAATGC-3',

Notch3 forward 5'-GCTTTGGTCTGCTCAATCCTGTAG-3',

Notch3 reverse 5'-TTGGGGGTAACCTTCTGGTTGG-3'.

GAPDH was used as a control for equivalent sample loading (forward primer; 5' CCATGGAGAAGGCTGGGG-3, reverse primer; 5'CAAAGTTGTCATGGATGACC-3).

TG from BHV-1-infected calves was collected at necropsy at 60 days after infection and at 6 or 24 hours after DEX treatment. TG was stored at -80° C for nucleic acid extraction. TG were minced into small pieces, placed into 3 ml of TRIzol reagent, and processed as stated above.

RESULTS

Notch1 and Notch3 interacted with ORF2 in a yeast two-hybrid screen.

We previously found that a protein encoded by an alternatively spliced LR transcript (7 dpi cDNA) interacts with a cellular transcription factor, c/EBP-alpha (Meyer et al., 2007b). The protein encoded by the 7 dpi cDNA is a fusion between ORF2 and ORF1 (Devireddy and Jones, 1998; Devireddy et al., 2003) (Figure 5.1). Approximately 2/3 of this fusion protein is derived from ORF1. Recent studies have demonstrated that just ORF2 aa sequences (Figure 5.1) reduces cold-shock induced apoptosis in transfected mouse neuroblastoma cells (neuro-2A) (Shen and Jones, 2008). The LR mutant virus does not express LR proteins, including ORF2, (Jiang et al., 2004) and induces higher levels of apoptosis in TG neurons during late stages of acute infection (Lovato et al., 2003) indicating that the anti-apoptosis functions of ORF2 are important for the latency-reactivation cycle. Consequently, we were interested in identifying cellular proteins that interact with just ORF2. To this end, a yeast two-hybrid assay was performed using only ORF2 sequences. Multiple clones of Notch3 and Notch1 were identified in the yeast two-hybrid screen suggesting that ORF2/Notch interactions were stable. Additionally, ORF2 was found to co-localize with Notch 1 and Notch 3 when overexpressed in neuro-2A cells and ORF2 was able to interact with Notch1 and Notch3 using an *in vitro* pull down assay (Workman et al., 2011). Since Notch family members regulate many developmental processes (Berezovska et al., 1999; Cornell and Eisen, 2005; Justice and Jan, 2002), we focused our studies on the interactions between ORF2 and Notch1 or Notch3, and whether Notch family members have the potential to regulate productive infection.

DEX increases steady state levels of Notch RNA in neuro-2A and TG of latently infected calves

We previously found that the protein encoded by the 7 dpi cDNA interacted with C/EBP-alpha, a cellular transcription factor, and C/EBP-alpha expression was stimulated during DEX induced reactivation from latency (Meyer et al., 2007b). To examine Notch1 and Notch3 RNA expression in TG, we performed RT-PCR with RNA samples prepared from TG of latently infected calves or latently infected calves treated with DEX to initiate reactivation from latency. In TG of calves treated with DEX, Notch3 RNA levels were induced at 6 and 24 hours after DEX treatment (Figure 5.2A). Notch1 levels were increased slightly 24 hours after DEX treatment (Figure 5.2B).

C/EBP-alpha RNA levels are also stimulated by DEX treatment in neuro-2A cells (Workman et al., 2009). Thus, it was of interest to compare Notch expression in neuro-2A cells after DEX treatment. Following treatment of neuro-2A cells with DEX, Notch1 levels, but not Notch3, cDNA levels were increased 8 hours after DEX treatment (Figure 5.2C and D). Notch3 RNA levels were elevated slightly at 40 hours after DEX treatment (Figure 5.2C). GAPDH levels remained the same after DEX treatment, which was expected.

Effect of Notch on productive infection

Studies in Figure 2 demonstrated that in TG of calves latently infected with BHV-1 DEX treatment led to increased Notch3 RNA levels and to a lesser extent Notch1. Since DEX stimulates BHV-1 reactivation from latency {reviewed in (Jones, 2003b;

Jones, 2008)}, we speculated that Notch family members regulate certain aspects of productive infection.

To test whether Notch1 or Notch3 regulated productive infection, rabbit skin cells (RS) were cotransfected with increasing concentrations of a plasmid expressing the Notch1 or Notch3 ICD with the BHV-1 gCblue virus and the efficiency of productive infection measured. The gCblue virus contains the Lac Z gene downstream of the gC promoter, which allows one to measure productive infection by counting β -Gal⁺ (beta-galactosidase positive) cells. The number of β -Gal⁺ cells directly correlates with plaque formation (Geiser et al., 2002; Geiser and Jones, 2003; Inman et al., 2001b; Meyer et al., 2007b). The gCblue virus grows to similar titers as wt BHV-1 in bovine cells (Geiser et al., 2002; Geiser and Jones, 2003; Inman et al., 2001b; Meyer et al., 2007b). RS cells were used for these studies because they are permissive for BHV-1 and they do not express high levels of Notch proteins. At 24 hours after transfection, Notch1 (52, 210, 830, or 2000 ng plasmid DNA) significantly increased ($p < 0.05$) the number of β -Gal⁺ cells relative to the empty vector (pcDNA3.1; Figure 5.3A, open columns). In contrast, only the highest concentration of Notch3 (2000 ng plasmid DNA) significantly increased ($p < 0.05$) the number of β -Gal⁺ cells compared to pcDNA3.1 (Figure 5.3A, black columns). As expected, bICP0 (Geiser et al., 2002; Geiser and Jones, 2003; Inman et al., 2001b; Meyer et al., 2007b) increased the number of β -Gal⁺ cells.

During productive infection of RS cells, we observed an increase in Notch1 proteins levels (Figure 5.3B). By 4 or 8 hours after infection, we detected higher levels of Notch1 protein levels when compared to mock infected cells. The size of the Notch1 specific band was the predicted size of Notch1 ICD (Bray, 2006; Ehebauer et al., 2006b)

suggesting productive infection stimulated the Notch1 signaling pathway. Confocal microscopy confirmed that higher levels of Notch1 were detected in the nucleus of infected cells (data not shown), which supported the finding that Notch1 ICD protein levels were higher in RS cell after infection. As expected, β -actin protein levels did not increase after infection. We also examined Notch3 protein levels during the course of productive infection, but the results were inconclusive (data not shown).

Notch1 ICD induces bICP0 promoter activity.

Additional studies were performed to test whether Notch1 or Notch3 trans-activated the immediate early transcription unit 1 (IEtu1) promoter or bICP0 early (E) promoter. Neuro-2A cells were used for these studies because they are neuronal like cells and they do not express detectable levels of Notch1 or Notch3 proteins. The IEtu1 promoter activates immediate early expression of two transcripts, IE2/9 and IE/4.2, which encode the transcriptional regulatory proteins bICP0 and bICP4 respectively (Wirth et al., 1992; Wirth et al., 1991) (see Figure 5.4A for a schematic of the IEtu1 region). Although the largest IEtu1 promoter construct was not trans-activated by Notch1 or Notch3, the two smaller constructs (Δ 1024 IEtu1 or Δ 1391 IEtu1) were trans-activated by Notch1 approximately 3 fold or 5 fold respectively (Figure 5.4B), which was significantly different than the pCATbasic values ($p < 0.05$). Basal promoter activity of IEtu1cat was more than 20 fold higher than IEtu1cat Δ 1391 in neuro-2A cells suggesting that Notch1 activation was only evident when basal levels of the IEtu1 promoter activity were low. Alternatively, upstream sequences may have a negative impact of Notch induced trans-activation. Notch3 did not stimulate any of the IEtu1 promoter constructs, but

consistently reduced the promoter activity of $\Delta 1024$ IETu1 or $\Delta 1391$ IETu1 approximately 2 fold.

The bICP0 E promoter also activates bICP0 expression (Wirth et al., 1992) (see Figure 5.4A for location of the bICP0 E promoter). Recent studies concluded that the bICP0 E promoter is preferentially stimulated during reactivation from latency (Workman et al., 2009). Three bICP0 E promoter constructs (EP-172, EP-143, and EP-133) were stimulated approximately 13 fold by Notch1, but not Notch3 (Figure 5.5A). Conversely, EP-71, EP-50, and EP-42 were not trans-activated more than 4 fold by Notch1 or Notch3. Sequences that are present in EP-133, but lacking in EP-71, were important for Notch1 trans-activation and basal activity. Conversely, the additional sequences in EP-172 played no role in Notch1 trans-activation, but these sequences enhanced basal promoter activity of EP-172 approximately 2 fold relative to EP-133. In summary, these studies demonstrated that Notch1 trans-activated the IETu1 and bICP0 E promoter.

ORF2 inhibits Notch induced trans-activation

Since ORF2 interacted with Notch1 and Notch3 ICD, we tested whether ORF2 affected trans-activation of the bICP0 E promoter by Notch1. The ORF2 plasmid that expresses the ORF2 protein (ORF2 B) reduced the ability of Notch1 to trans-activate EP-172 promoter activity approximately 4 fold (Figure 5.5B), which was significantly different than the trans-activation value of Notch1 ($p < 0.05$). Conversely, the ORF2B construct had no effect on EP-172 promoter activity, in the absence of Notch1. We also examined a construct that was cloned such that the ORF2 protein is not expressed, but

LR-RNA is expressed, because it is not in frame with the FLAG tag (ORF2 C) (Shen and Jones, 2008). Relative to the ORF2 B construct, the ORF2 C construct reduced trans-activation of EP-172 by Notch1 less than 2 fold, which was not significantly different than the trans-activation value of Notch1 alone.

Notch1 and Notch3 ICD stimulate a viral late promoter

To further examine the ability of Notch1 and Notch3 ICD to regulate viral transcription, we tested whether a late promoter, glycoprotein C (gC), could be trans-activated by Notch1 or Notch3 ICD. For these studies, we used three gC promoter constructs, which were previously described (Zhang et al., 2006) (Figure 5.6A). As expected, deletion of the gC promoter sequences led to reduced the basal promoter activity. In neuro-2A cells, a plasmid expressing Notch1 ICD trans-activated the smallest gC promoter construct (gC-PstI-CAT) 10 fold whereas it stimulated the larger constructs less efficiently (Figure 5.6A). The Notch3 ICD expressing plasmid stimulated gC-CAT approximately 8 fold, but lower levels of trans-activation were observed with the two smaller constructs (gC-XhoI-CAT and the gC-PstI-CAT).

Although ORF2 reduced the ability of Notch1 ICD to trans-activate the gC-CAT promoter approximately 2 fold (Figure 5.6B), expression of ORF2 (ORF2B) had little effect on basal promoter activity. The ability of ORF2 to inhibit gC promoter activity was not as efficient as observed with the bICP0 E promoter (Figure 5.5B). The ORF2 construct that was out of frame (ORF2C) had little effect on Notch1 ICD trans-activation (Figure 5.6B). Likewise, ORF2 inhibited the ability of Notch3 ICD to trans-activate gC-PstI-CAT promoter activity approximately 2 fold, but the ORF2B construct had little

effect on basal promoter activity. ORF2B, but not ORF2C, significantly reduced the levels of Notch1 and Notch3 trans-activation ($p < 0.05$). In summary, these studies demonstrated that Notch1 and Notch 3 ICD stimulated gC promoter activity, and that ORF2 protein expression reduced Notch trans-activation.

DISCUSSION

Several lines of evidence presented in this study indicated that ORF2 interacted, directly or indirectly, with Notch1 and Notch3. To the best of our knowledge, this is the first example of a protein encoded by a herpesvirus member that directly interacts with a Notch family member. Notch family members control the developmental pathway of many cell types in mammals and *Drosophila* {reviewed in (Bray, 2006; Ehebauer et al., 2006b)}. A previous study demonstrated that a protein encoded by the alternatively spliced LR transcript (7 dpi cDNA) interacts with C/EBP-alpha, a cellular transcription factor (Meyer et al., 2007b). In the previous two-hybrid screen, we did not detect an interaction between Notch1 or Notch3 and the protein encoded by the 7 dpi cDNA (Devireddy and Jones, 1998; Devireddy et al., 2003). Since the protein encoded by the 7 dpi cDNA contains 2/3 of its aa sequences from ORF1 and the rest from ORF2, the two-hybrid screen likely detected cellular proteins that interacted with ORF1 sequences. These findings also suggested that alternative splicing of LR-RNA in TG of infected cattle leads to expression of a family of LR protein. The LR family of proteins is predicted to interact with different cellular proteins, and perform specific functions during the latency-reactivation cycle.

Eleven known consensus binding sites for CSL (RBP-jk) exist (Persson and Wilson, 2010). The BHV-1 genome contains 82 potential binding sites for CSL, of which 23 are located in non-coding regions (Figure 5.7A). Interestingly, six CSL binding sites are near the 5' terminus of bICP4 and bICP22 (Figure 5.7B). We suggest that CSL binding sites in the BHV-1 genome were partially responsible for the ability of Notch1 to stimulate productive infection. Further support for Notch stimulating productive

infection comes from the finding that the Notch1 ICD trans-activated the bICP0 E promoter, the IETu1 promoter, and certain gC promoter constructs. With respect to the IETu1 promoter, we observed higher levels of trans-activation with the smallest promoter fragment (IETu1cat Δ 1391; Figure 5.4B). All three promoter constructs are efficiently trans-activated by b-TIF, the BHV-1 functional homologue of VP16. The basal activity of IETu1cat was higher than Δ 1024 IETu1 or Δ 1391 IETu1 basal promoter activity (Figure 5.4A), suggesting the high basal activity of IETu1cat masked the trans-activation of Notch 1. Conversely, it is possible that upstream sequences within the IETu1 promoter have a negative impact on Notch1 induced trans-activation. The Notch1 responsive domain within the bICP0 E promoter was localized to 62 bases present in EP-133, but lacking in EP-71 (Figure 5.5A). Within these 62 bases, there are no identical matches to known CSL binding sites. However, there are 8 CSL-like motifs that contain mismatches in 2 bases. This may be significant because a recent report concluded that over-lapping CSL-like binding sites can confer Notch responsiveness dependent on dimerization of Notch family members (Liu et al., 2010). Additional studies will be necessary to identify the cis-acting sequences in the bICP0 E, IETu1, or gC promoter that are necessary for Notch trans-activation and whether activation is by a direct mechanism.

In the absence of Notch family members, CSL binding proteins interact with transcriptional repressors (Borggreffe and Oswald, 2009), including the histone demethylase KDM5A (Liefke et al., 2010). These findings suggest that CSL binding proteins may help maintain latency by recruiting transcriptional repressor complexes to the BHV-1 genome. During the course of DEX-induced reactivation, increased expression levels of Notch family members may result in the displacement of repressors

from the BHV-1 genome. Micro-array analysis revealed that several genes in the Notch signaling pathway, including the proteases that cleave and activate Notch, are stimulated in TG of latently infected calves following DEX treatment for 6 hours (unpublished data) adding further support to the concept that the Notch signaling pathway is activated during early stages of reactivation from latency. Productive infection also activates the Notch1 signaling pathway because we detected higher levels of Notch1 ICD after infection, which correlated with the finding that Notch1 stimulated productive infection. Although we believe the Notch signaling pathway may promote reactivation from latency, it is unlikely that Notch family members are the only cellular transcription factors that stimulate reactivation from latency.

Notch3 had little or no effect on the bICP0 E promoter or the IEtu1 promoter, and stimulated productive infection only two fold. The finding that Notch3 RNA levels were induced within 6 hours after DEX treatment and Notch3 trans-activated a late promoter (gC) suggested that Notch3 has different effects on productive infection relative to Notch1. In keeping with these observations, Notch3 is known to possess novel functions when compared to other Notch family members. For example, the Y box protein-1 is a novel ligand that specifically binds to Notch3 (Rauen et al., 2009). Furthermore, the Notch3 ICD is generally considered to be a poor activator of transcription (Beatus et al., 1999), which partially explains why it did not efficiently stimulate productive infection. Thirdly, mutations in Notch3 are the cause of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a hereditary angiopathy that causes stroke and vascular dementia (Dichgans et al., 2001). Fourth, Notch3 is differentially expressed relative to Notch1 in the developing nervous system (Ichinohe,

2004). Finally, Notch1 and Notch 2 knockout mice die during embryogenesis (Conlon et al., 1995; Swiatek et al., 1994), whereas Notch3 knockout mice are viable and do not apparently have redundant functions compared to the Notch1 gene (Krebs et al., 2003). Consequently, we predict that the novel properties of Notch3 promoted gC promoter activity, and perhaps expression of additional late genes.

The Notch signaling pathway is known to play a role in the latency-reactivation cycle of two human herpesviruses, KSHV (Kaposi's sarcoma-associated herpesvirus) (Liang et al., 2002) and EBV (Epstein Barr virus) (Strobl et al., 1997). In contrast to the finding that Notch itself plays a role in the BHV-1 latency-reactivation cycle, the CSL family of proteins (RBP-Jk) is the key component of the Notch signaling pathway that participates in the latency-reactivation cycle of EBV and KSHV. For example, RBP-Jk is necessary for KSHV reactivation from latency because it interacts with the viral protein RTA (replication and transcriptional activator) (Liang et al., 2002). Conversely, EBNA2 (EBV nuclear antigen 2) interacts with RBP-Jk and trans-activates two EBV promoters that encode 7 proteins necessary for stabilizing the EBV genome, stimulating B cell growth, inhibiting B cell apoptosis, and consequently promoting Latency III (Kieff et al., 1971). Notch ICD cannot entirely replace RTA to reactivate KSHV latency (Carroll et al., 2006; Chang et al., 2005; Liang et al., 2002) or EBNA-2 for establishing and maintaining Latency III (Gordadze et al., 2001; Strobl et al., 1997). Although KSHV and EBV usurp the Notch signaling pathway, they do not appear to encode a protein that physically interacts with Notch family members, and negatively regulates Notch-dependent transcription.

Since LR gene expression, and presumably ORF2 expression, is dramatically reduced during DEX induced reactivation from latency (Rock et al., 1992), ORF2 does not appear to directly stimulate reactivation from latency. We propose that ORF2 increases the pool of latently infected neurons capable of supporting reactivation from latency. Interactions between ORF2 and Notch1 or Notch3 are thus expected to promote the establishment and/or maintenance of latency. Although ORF2 inhibits apoptosis (Shen and Jones, 2008), this study suggested ORF2 also regulates viral transcription by interacting with cellular transcription factors. Two additional studies provide evidence that ORF2 may regulate transcription (Devireddy et al., 2003; Meyer et al., 2007b). Consequently, we suggest that the ability of ORF2 to inhibit apoptosis and interact with cellular transcription factors promote life-long latency. The LR gene encodes other proteins and at least 2 micro-RNAs that reduce bICP0 protein levels (Jiang et al., 2004; Meyer et al., 2007b; Shen et al., 2009) suggesting these functions play a supportive role during the latency-reactivation cycle.

Figure 5.1. Schematic of protein coding regions within the LR genes and ORF2 isoforms encoded by alternatively spliced LR transcripts.

ORF-1 and ORF-2 are the open reading frames present in the LR gene (Kutish et al., 1990b). Reading Frame B and C (RF-B and RF-C) each contain an open reading frame that lacks an initiating methionine. The numbers in parenthesis are the approximate size of ORFs (Kd) that are located in LR gene sequences. The ORF2 isoforms encoded by alternatively spliced LR transcripts detected in TG at 1 dpi and latency, 7 dpi, and 15 dpi, (Devireddy et al., 2003) are shown as a comparison to ORF2 present in the LR gene. Although the LR-RNA is differentially spliced at 1 day after infection in TG of cattle, both transcripts encode an intact ORF2. The (*) denotes the position of stop codons that are in frame with the respective ORF.

Figure 5.1.

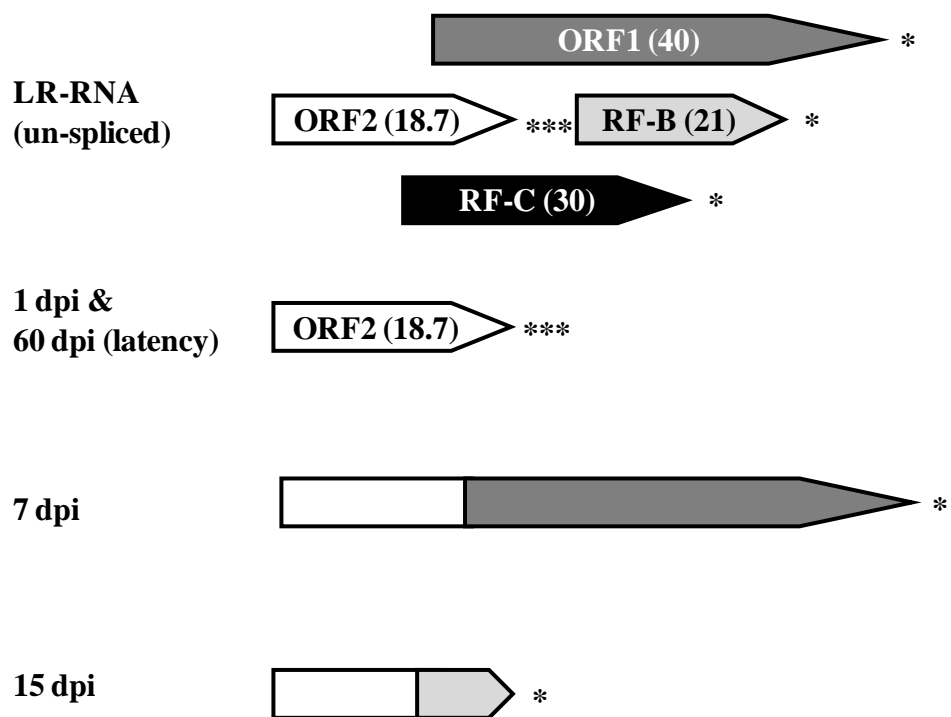


Figure 5.2. DEX treatment induces Notch RNA levels.

Panel A and B. TG from BHV-1-infected calves was collected at 60 days after infection (latency) and at 6 or 24 hours after DEX treatment of latently infected calves. These TG were obtained during the course of other published studies (Inman et al., 2002; Perez et al., 2005). TG were minced into small pieces and then processed as described in the Materials and Methods. Total RNA was collected and RT-PCR performed using oligo(dT) primers. cDNA was used as the template for semi-quantitative PCR using specific primers for Notch1 (panel B) or Notch3 (panel A).

Panel C and D. Neuro-2A cells were cultured in the presence of 1 μ M DEX for the designated times (hours). Total RNA was collected and RT-PCR performed using oligo(dT) primers. cDNA was used as the template for semi-quantitative PCR using specific primers for Notch1 (panel D) or Notch3 (panel C) as described in the Materials and Methods. GAPDH was used as a loading control for all studies.

Figure 5.2.

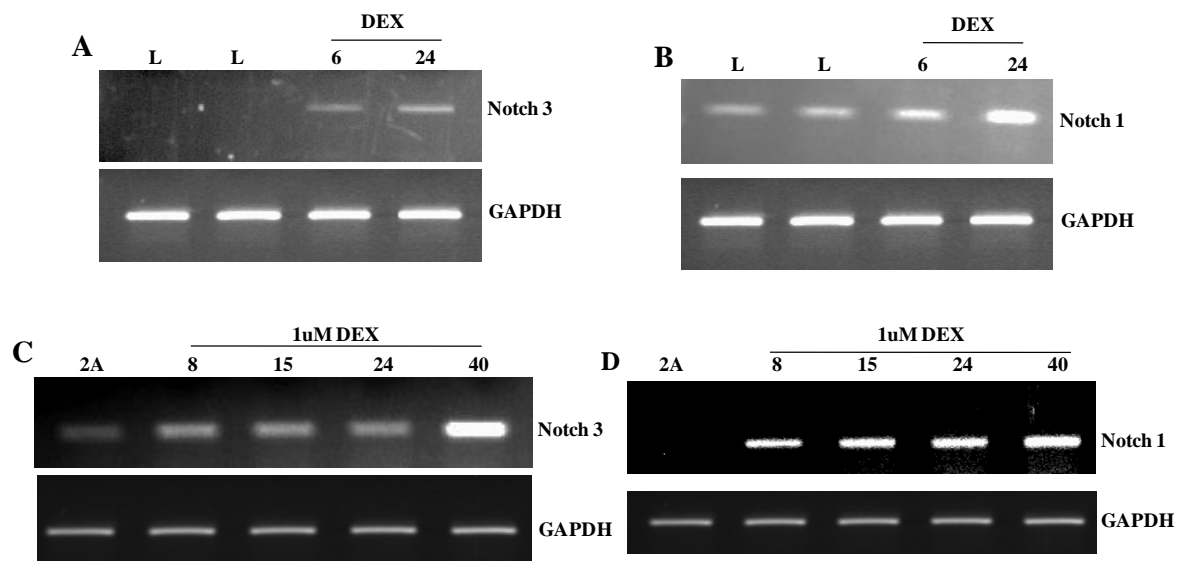


Figure 5.3. Notch1, but not Notch3, stimulated productive infection.

Panel A: Rabbit Skin (RS) cells were co-transfected with increasing amounts of the Notch1 ICD (open columns) or Notch3 ICD plasmid (black columns) (15 ng, 52 ng, 0.21 ug, 0.83 ug, or 2 ug) or bICP0 (52 ng), and the BHV-1 gCblue virus genome (0.83 ug) using Lipofectamine 2000. A blank expression vector (pcDNA 3.1) was used to maintain equivalent amounts of DNA. Twenty-four hours after transfection, cells were fixed, stained, and the number of β -gal+ cells was counted. The number of β -gal+ cells in the vector control was set to 1 and the number of β -gal+ cells in each plate was calculated as the fold difference relative to the vector control. The results are the average of three independent experiments. An asterisk denotes significant differences ($p < 0.05$) from the pcDNA3.1 value as determined by the Student T-test.

Panel B: RS cells were infected with wt BHV-1 ($\text{moi} = 1$) for the designated times after infection (hours). Cell lysate was prepared as described previously and Western blot assays performed. The rabbit anti-Notch1 primary antibody was purchased from Cell Signaling (catalogue #32685). Rabbit IgG was detected using a donkey anti-rabbit IgG (GE Healthcare; catalogue # NA934V). The goat anti-actin antiserum was purchased from Santa Cruz Biotechnology (catalogue # sc-1616). Donkey anti-goat IgG was purchased from Santa Cruz Biotechnology (catalogue # sc-2020).

Figure 5.3.

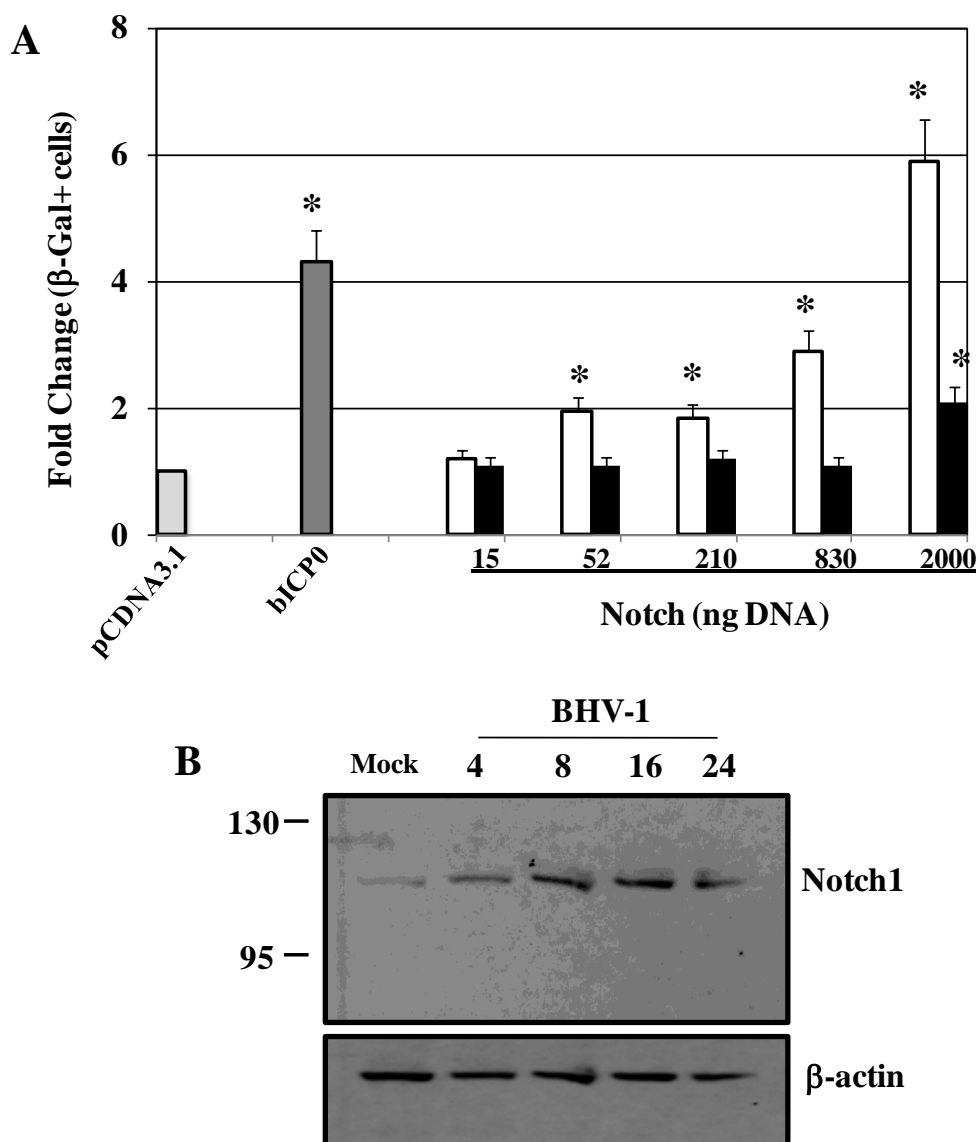


Figure 5.4. Notch1, but not Notch3, stimulates IETu1 promoter activity.

Panel A: Position of transcripts that encode bICP4 and bICP0 are shown. The immediate early transcription unit 1 (IETu1) encodes bICP4 (IE/4.2) and bICP0 (IE/2.9) (Wirth et al., 1989; Wirth et al., 1991). The IETu1 promoter (denoted by the black rectangle) activates IE expression of IE/4.2 and IE/2.9. E/2.6 is the early transcript that encodes bICP0 and an early promoter (denoted by the grey rectangle) activates expression of this early transcript (Wirth et al., 1992). Exon 2 (e2) of bICP0 contains all of the protein coding sequences of bICP0. The dashed lines are intron sequences. IETu1-CAT contains 1.5 kb of upstream sequences cloned at the 5' terminus of pSV0CAT (a promoter minus CAT expression vector). V. Misra, Saskatoon, Canada, provided the IETu1CAT plasmid (Misra et al., 1994). Two deletion constructs $\Delta 1024$ and $\Delta 1391$ IETu1 have 1024 or 1391 bp sequences removed from the 5' terminus. The basal promoter activity of the respective constructs was measured in neuro-2A cells. Basal promoter activity of IETu1cat was normalized to 100% and the other 2 promoters compared to this value. These results were the average of 3 independent experiments.

Panel B: Neuro-2A cells were cotransfected with the designated IETu1 promoter construct (1 μ g DNA) and plasmids expressing Notch1 ICD (open columns) or Notch3 ICD (black columns) using 1 μ g of plasmid DNA. CAT activity of cells transfected with the control CAT vector was set to one. All other values are expressed as fold activation with respect to the control. These studies are the average of three independent experiments. An asterisk denotes significant differences ($p < 0.05$) from the pCAT basic trans-activation value as determined by the Student T-test.

Figure 5.5. Notch1 stimulates bICP0 E promoter activity.

Panel A: Neuro-2A cells were co-transfected with 1 μ g of the designated bICP0 E promoter construct and 1 μ g of a plasmid that expresses Notch1 ICD or Notch3 ICD. DNA amounts were equalized for all transfections using pcDNA3.1, a blank expression vector. For further details of the respective bICP0 E promoter construct, see the materials and methods and (Workman and Jones, 2010; Workman et al., 2009). At 48 hours post transfection, cells were collected and processed for CAT activity as described in the materials and methods. Basal promoter activity of EP-172 was set at 100 and the values of the other constructs were compared to EP-172. CAT activity of cells transfected with the control CAT vector was set to one. All other values are expressed as fold activation with respect to the control. These studies are the average of three independent experiments.

Panel B: Neuro-2A cells were co-transfected with 1 μ g EP-172 and 1 μ g of the plasmid that expresses Notch1 ICD along with 1 μ g of empty vector (pcDNA3.1), ORF2 in the correct reading frame (ORF2-B), or ORF 2 in the incorrect reading frame (ORF2-C). At 48 hours after transfection, cells were collected and processed for CAT activity as described in the Materials and Methods. CAT activity of cells transfected with the control CAT vector was set to one fold. All other values are expressed as fold activation with respect to the control. The results are the average of three independent experiments. An asterisk denotes significant differences ($p < 0.05$) from the Notch1 trans-activation value as determined by the Student T-test.

Figure 5.5.

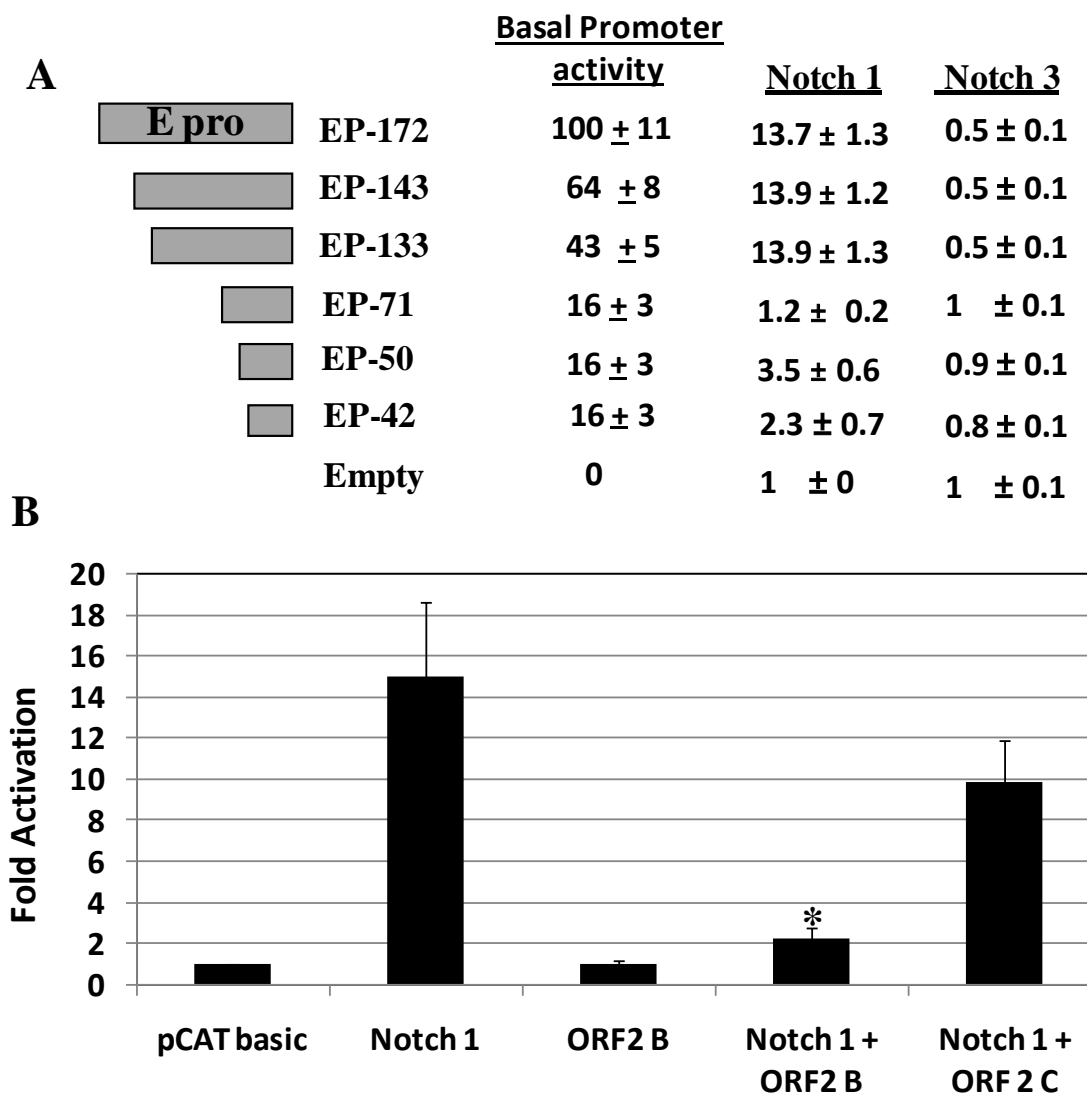


Figure 5.6. Notch stimulates glycoprotein C promoter activity.

Panel A: Neuro-2A cells were co-transfected with 1 μ g of the designated glycoprotein C (gC) promoter construct and 1 μ g of a plasmid expressing Notch1 ICD or Notch3 ICD. DNA amounts were equalized for all transfections using pcDNA3.1. The gC promoter constructs were previously described (Zhang et al., 2006) and the basal promoter activity of gC-Cat was assigned the value of 100.

Panel B: Neuro-2A cells were co-transfected with 1 μ g gC-PstI-CAT or gC-CAT, 1 μ g of Notch1 ICD or Notch3 ICD respectively, and ORF2 in the correct reading frame (ORF2-B), or ORF 2 in the incorrect reading frame (ORF2-C). As a control for basal promoter activity, the respective promoters were cotransfected with ORF2B. Empty vector (pcDNA3.1) was used to keep the same amount of DNA in the transfection mixture. At 48 hours after transfection, cells were collected and processed for CAT activity as described in the Materials and Methods. CAT activity of cells transfected with the control CAT vector was set to one fold. All other values are expressed as fold activation with respect to the control. The results are the average of three independent experiments. An asterisk denotes significant differences ($p < 0.05$) from the Notch alone trans-activation value as determined by the Student T-test.

Figure 5.6.

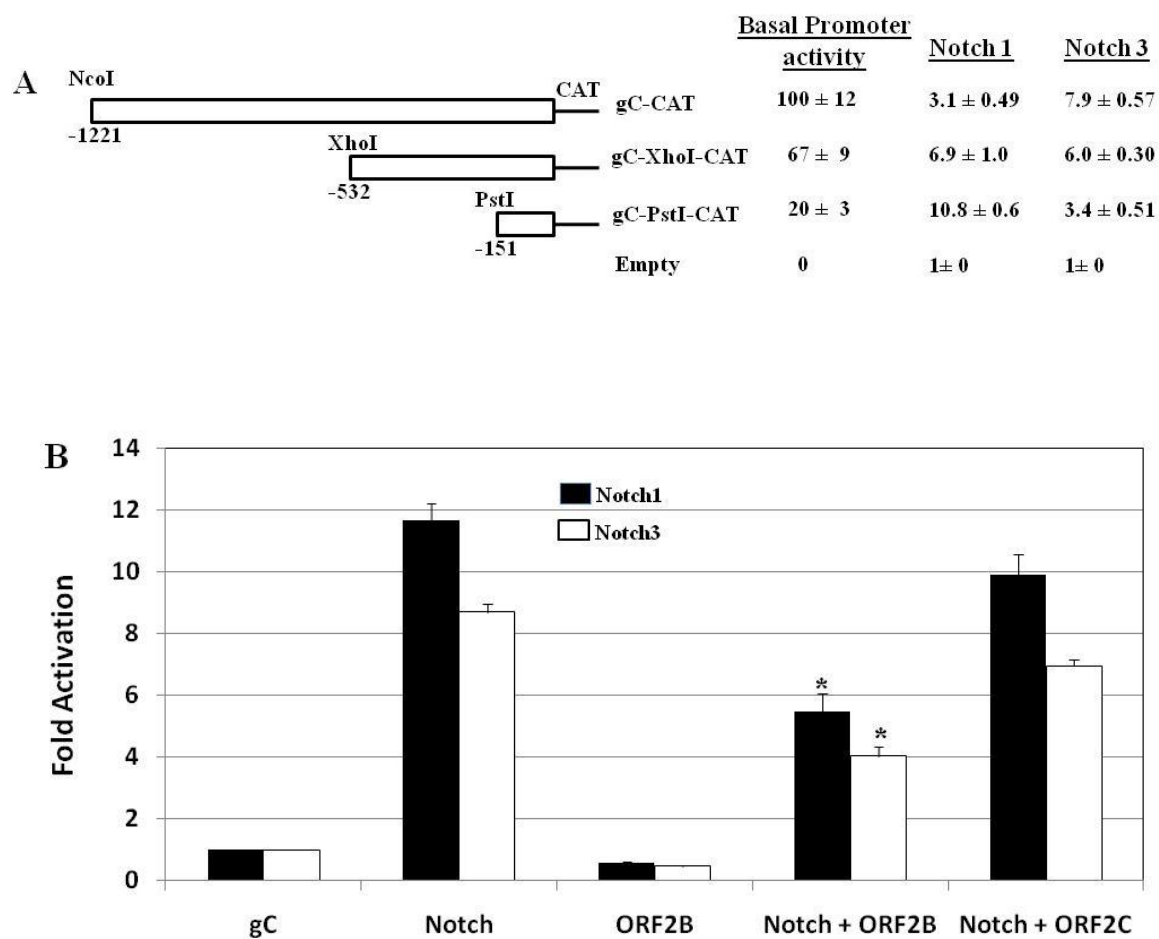
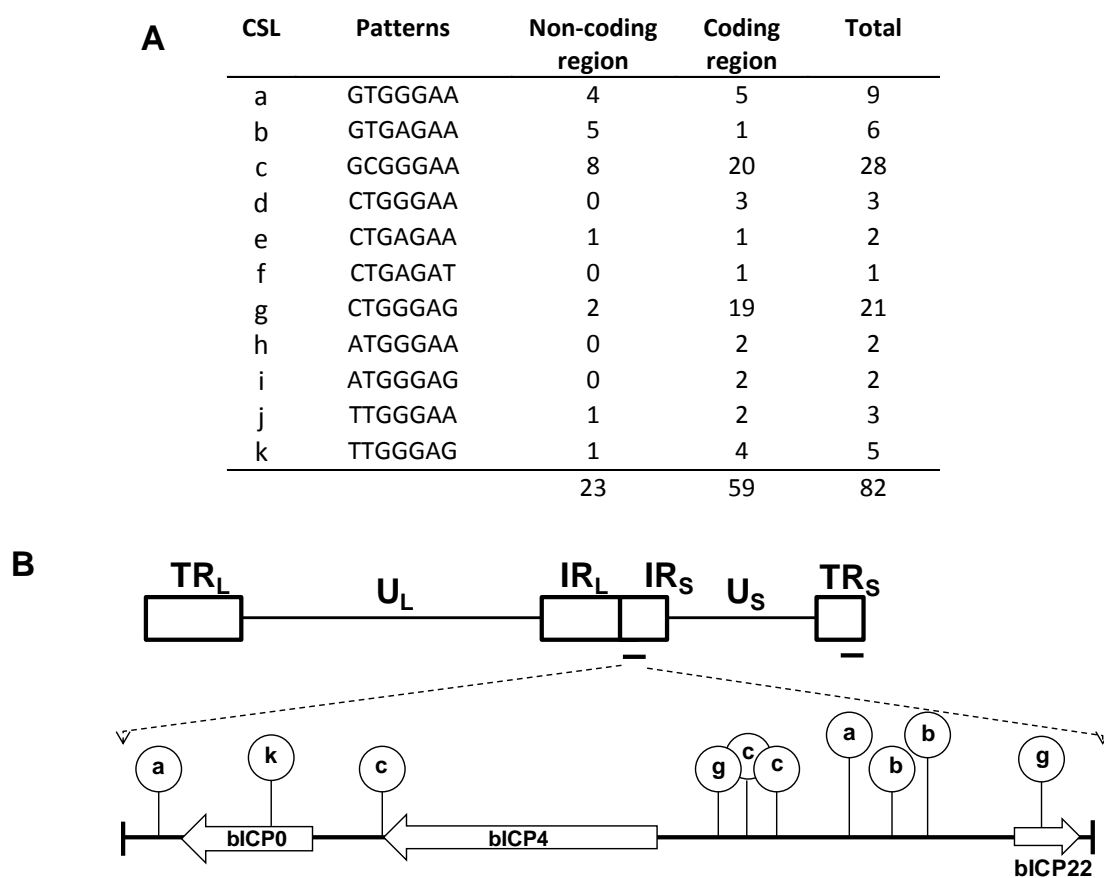


Figure 5.7. Predicted CSL binding sites in the BHV-1 genome.

Panel A: Eleven variants of known canonical CSL binding sites have been identified (Persson and Wilson, 2010). The number of each CSL consensus binding sites identified in the non-coding or coding regions of the BHV-1 genome is denoted.

Panel B: Schematic of the entire BHV-1 genome, which consists of two unique regions; U_L and U_S , terminal repeats; TR_L and TR_S , and internal repeats between the unique regions; IR_L and IR_S . Six potential CSL sites binding sites are located within the intergenic region between bICP4 and bICP22 coding regions. At least one CSL binding site is detected upstream of bICP0.

Figure 5.7.



Chapter 6

Identification and Characterization of Cellular Factors that are Expressed During Dexamethasone Induced Reactivation from Bovine Herpesvirus 1 (BHV-1) Latency in Neurons.

ABSTRACT

Bovine herpesvirus 1 (BHV-1) is an *alphaherpesvirinae* subfamily member that establishes latency in sensory neurons. Elevated corticosteroid levels, due to stress or immune suppression, can initiate reactivation from latency. The BHV-1 latency-reactivation cycle is crucial for virus transmission; however, the molecular events that occur during a successful reactivation from latency are unknown. Our central hypothesis is that latency is established in sensory neurons due to the lack of permissive transcription factors and/or the presence of cellular factors that repress viral transcription. We hypothesize that stress alters cellular gene expression in sensory neurons, which consequently initiates BHV-1 gene expression and reactivation from latency. In this study, we used a Bovine Gene Chip, which contains more than 23,000 genes, to compare cellular gene expression in latently infected calves versus calves treated with Dexamethasone (Dex; a synthetic corticosteroid) to initiate reactivation from latency. Relative to TG prepared from latently infected calves, 97 cellular genes were induced more than 10 fold at 3 hours after Dex treatment. From this subset, 6 cellular transcription factors were induced more than 10 fold. In this study, we show that these cellular transcription factors stimulate BHV-1 productive infection and viral transcription when analyzed in transient transfection assays. This suggests these transcription factors may play a role in the early events of reactivation from BHV-1 latency.

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is an *alphaherpesvirinae* subfamily member that causes significant economical losses to the cattle industry (Turin et al., 1999). The ability of BHV-1 to suppress the immune system can result in life-threatening pneumonia due to secondary bacterial infections, and this multi-factorial disorder is referred to as bovine respiratory disease complex {reviewed in (Jones, 2009; Jones, 2008; Jones, 2010)}. Like other human *alphaherpesvirinae* subfamily members, the primary site for BHV-1 latency is sensory neurons within trigeminal ganglia (TG). Viral gene expression (Schang and Jones, 1997) and infectious virus (Inman, 2002) are detected in TG from 1-6 days after acute infection. Lytic cycle viral gene expression is subsequently extinguished in sensory neurons, and latency is established. The BHV-1 genome is stably maintained in sensory neurons as an episome, but infectious virus is not detected by standard virological methods {reviewed in (Jones, 1998; Jones, 2003b)}. The only viral gene abundantly expressed in latently infected sensory neurons is the latency-related (LR) gene, reviewed in (Jones, 2006). Stress (due to confinement, transportation of cattle, restriction of food and water, or weaning) increases corticosteroid levels and reactivation from latency occurs (Jones, 2010). The latency-reactivation cycle of BHV-1 is crucial for virus transmission and survival in nature.

Administration of a synthetic corticosteroid, dexamethasone (Dex), to latently infected calves or rabbits consistently initiates reactivation from latency (Inman et al., 2002; Jones, 1998; Jones, 2003a; Jones et al., 2000; Rock et al., 1992). Dex represses LR promoter activity (Jones et al., 1990) and reduces LR-RNA levels, which culminates in a reduction of the number of TG neurons that express LR-RNA at 18-21 hours after

treatment (Rock et al., 1992). A single injection of Dex also leads to viral lytic cycle transcript expression in neurons within 6 hours after treatment of latently infected calves (Winkler et al., 2002a; Winkler et al., 2000a). Calves latently infected with wild type BHV-1 and the LR mutant virus frequently express bICP0 mRNA after Dex treatment, but only wild type BHV-1 expresses late genes (Workman et al., 2009), which correlates with virus shedding during reactivation from latency (Inman et al., 2002). These findings are consistent with an earlier study that concluded Dex induces viral gene expression in many latently infected neurons, but only a subset of neurons produce infectious virus (Rock et al., 1992). Dex treatment of latently infected calves also induces apoptosis of T cells that persist in TG after infection (Winkler et al., 2002a). Persistence of T cells in TG of humans or mice latently infected with HSV-1 also occurs (Cantin et al., 1995a; Halford et al., 1996b; Liu et al., 1996; Shimeld et al., 1995a; Shimeld et al., 1996; Shimeld et al., 1997; Theil, 2003), and persistent CD8+ T cells in TG produce factors (presumably cytokines) that inhibit reactivation from latency (Khanna et al., 2003; Knickelbein et al., 2008; Liu et al., 2001; Liu et al., 2000a; Liu et al., 2000b; Prbhakaran, 2005). Although it seems clear that Dex has many effects on TG that leads to reactivation from latency, the mechanism by which Dex stimulates lytic viral gene expression is not understood.

In this study, cellular gene expression was examined in TG during the early stages of Dex induced reactivation from BHV-1 latency. We identified genes that were regulated by Dex in TG of calves latently infected with BHV-1. Cellular transcription factors identified by microarray were then examined for their ability to stimulate productive infection and viral promoter activity using transient transfection assays.

MATERIALS AND METHODS

Cells and Virus

Murine neuroblastoma (neuro-2A), rabbit skin (RS), human osteosarcoma (U2OS) and bovine kidney (CRIB) cells were grown in Earle's modified Eagle's medium (EMEM) supplemented with 10% fetal calf serum, penicillin (10 U/ml), and streptomycin (100 µg/ml).

A BHV-1 recombinant virus (gCblue) containing the Lac Z gene in place of the viral gC gene was obtained from S. Chowdury (Louisiana State University, Baton Rouge, LA). The virus grows to similar titers as the wild type parent virus and expresses the β-Gal gene as a true late gene.

RNA preparation for Microarray Analysis and Reverse Transcription

BHV-1-free crossbred calves (~200 kg) were randomly assigned and housed in isolation rooms to prevent cross-contamination. Calves were inoculated with 10^6 PFU of wtBHV-1 into each nostril and eye, without scarification, for a total of 4×10^6 PFU per animal, as described previously (Inman et al., 2002). TG from BHV-1-infected calves were collected at necropsy at 60 days after infection (latency) and at 1.5, 3, 6 or 24 hours after Dex treatment. TG was stored at -80° C for nucleic acid extraction. TG were minced into small pieces and total RNA was prepared from cells using TRIzol reagent (Life Technologies, USA) as previously described (Workman et al., 2009; Workman et al., 2011). RNA was labeled and changes in cellular gene expression were examined using the Bovine Gene Chip (Affymetrix, Santa Clara, CA), which contains more than

23,000 genes. These studies were performed by Jim Eudy at the University of Nebraska Medical Center (UNMC) in Omaha, Nebraska.

For validation of microarray data, RT-PCR was performed. One microgram of RNA was treated with Amplification Grade DNase I (Invitrogen, USA). Reverse Transcription (RT) was performed using SuperScript III Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's directions. RNA was reverse transcribed using oligo(dT) primers (Invitrogen, USA). One-hundred ng of the resulting cDNA were used as a template for PCR using specific primers for the cellular gene of interest. PCR was performed using GoTaq DNA Polymerase (Promega, USA) and initiated at 95° C for 5 min. This was followed by 30 cycles of 95° C for 45 seconds, annealing (at temperature listed in table below) for 45 seconds, and 72° C for 45 seconds. Final extension was done at 72° C for 10 minutes. PCR products were analyzed on a 1.3% agarose gel. The following primer sequences and PCR conditions were used:

	Forward primer (5'-3')	Reverse Primer (5'-3')	Anneal temp
SPDEF	CAAGAAGGGCATCATCCG	CCCTCTTTCCCAGCTCTG	48
PLZF	TCTTTGGCATATGGGCTCAGTC	AGGCTGACTTCTGTCTCCACA	51
SLUG	GACCCAGAAATGGAACAGC	CAGGATTGCCTAACACACAGC	51
Pentraxin	AAGGAGAGAGTTGAGAT	TTCTCCAGTCTCCCT T	46
GAPDH	CCATGGAGAAGGCTGGGG	CAAAGTTGTCATGGATGACC	55

β-gal Assay

The gCblue virus grows to similar titers as wt BHV-1 and was grown in CRIB cells. Procedures for preparing genomic DNA were described previously. RS cells grown in 6 well plates were co-transfected with 1 µg of the gCblue viral genome and the designated amounts of plasmid expressing bICP0 or the Dex-inducible cellular

transcription factor of interest using Lipofectamine 2000 (11668-019; Invitrogen). Twenty-four hours after transfection, cells were fixed (2% Formaldehyde, 0.2% Glutaraldehyde in PBS), stained (1% blue-gal, 5 mM Kferric, 5 mM Kferro, 0.5 M MgCl₂ in PBS), and the number of β -gal⁺ cells counted as described previously (Workman and Jones, 2010). The number of β -gal⁺ cells in cultures expressing the blank vector was set to 100%. To calculate percent plaque formation, the number of blue cells in cultures transfected with the cellular transcription factor were divided by the number of blue cells in cultures transfected with the blank vector. This representation of the data minimized the differences in cell density, Lipofectamine lot variation, and transfection efficiency. The results are an average of at least three independent experiments.

siRNA knockdown of cellular transcription factors was accomplished by transfection of U2OS cells with 100 nM of specific siRNA or control siRNA using Lipofectamine 2000 according to the manufacture's specifications (11668-019; Invitrogen). The Block-iT-Fluorescent Oligo was used as a control siRNA (44-2926; Invitrogen). It is a fluorescent conjugated control containing a scrambled sequence that does not reduce the levels of any known mammalian gene. Twenty-four hours after transfection of the siRNA, cells were transfected with 1 μ g of gCblue viral genome. Twenty-four hours after transfection of viral genome, cells were fixed, stained, and β -gal positive cells were counted as described above.

siRNA Target	Santa Cruz Biotech Catalog Number
GATA6	sc-37907
SPDEF	sc-45845
PLZF	sc-37149
SLUG	sc-38393
KLF6	sc-38021
KLF4	sc-35480

Western Blot analysis and siRNA knock down of cellular transcription factors

To evaluate the efficiency of siRNA knock down, U2OS cells were co-transfected with 0.5 μ g of the plasmid encoding a Dex-inducible transcription factor and 100 nM of the respective siRNA or control siRNA as described above. At 36 hours after transfection, whole cell lysate was prepared. Cells were washed with phosphate-buffered saline (PBS) and re-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] per 10 ml). Cell lysate was incubated on ice for 30 min, sonicated, and then clarified by centrifugation at 10,000 x 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 1x 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 a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore) and blocked for 4 hours 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 (see table below). The primary antibody was diluted 1:1000 in the blocking solution. An antibody directed against β -actin (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. After 45 minutes of washing with TBS-T, the blots were incubated with donkey anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (Amersham Biosciences), which was diluted 1:2000 in 5% nonfat milk in TBS-T. Blots were washed 45 minutes with TBS-T and exposed to Amersham ECL reagents, and then autoradiography performed.

Primary Antibody	Santa Cruz Biotech Catalog Number
GATA6	sc-9055
SPDEF	sc-67022
PLZF	sc-22839
KLF6	sc-7158
KLF4	sc-20691

RT-PCR analysis of siRNA knock down of Slug

As we were unable to detect Slug by western blot, RT-PCR was performed to evaluate the effectiveness of the Slug siRNA. U2OS cells were transfected with 100 nM Slug siRNA (sc-38393; Santa Cruz Biotechnology) or a fluorescent control siRNA (44-2926; Invitrogen) using Lipofectamine 2000 as described above. At 24 hours after transfection, total RNA was prepared from cells using TRIzol reagent (Life Technologies, USA) as previously described (Workman and Jones, 2010). One microgram of RNA was treated with Amplification Grade DNase I (Invitrogen, USA). Reverse Transcription (RT) was performed using SuperScript III Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's directions. RNA was reverse transcribed using oligo(dT) primers (Invitrogen, USA). A 100 ng aliquot of the resulting cDNA was used as a template for PCR using specific primers for Slug (listed above). PCR products were analyzed on a 1.3% agarose gel.

Plasmids and promoter activity measured in transfected cells

The construction and characteristics of the BHV-1 bICP0 E promoter-CAT constructs (EP-172, EP-143, EP-133, EP-71, EP-50, and EP-42) used in this study were

described previously (Workman, 2009; Workman, 2010a). The numbers in the plasmid name refer to the length of the bICP0 E promoter fragment cloned into the promoterless vector, pCAT-basic (Promega). Truncations to the promoter were made from the 5' terminus. IETu1-CAT contains 1.5 kb of upstream sequences cloned at the 5' terminus of pSV0CAT (a promoter minus CAT expression vector). V. Misra, Saskatoon, Canada, provided the IETu1CAT plasmid (Misra et al., 1994). Two deletion constructs Δ 1024 and Δ 1391 IETu1 have 1024 or 1391 bp sequences removed from the 5' terminus. The gC promoter constructs (gC-CAT, gC-PstI-CAT, and gC-XhoI-CAT) were previously described (Zhang et al., 2006). The empty vector pcDNA3.1 was purchased from Invitrogen. SPDEF was purchased from Origene (Rockville, MD). The remaining Dex-inducible genes were graciously provided to us from labs studying these transcription factors. PLZF: Derek Sant'Angelo (Sloan Kettering Cancer Center). SLUG: Paul Wade (NIH). KLF4: Jonathan Katz (University of Pennsylvania). GATA6: Brian Herring (Indiana University Medical Center) KLF6: Bing Guo (North Dakota State University).

Neuro-2A cells grown in 60 mm dishes were co-transfected with the designated plasmids as indicated in the respective figure legends using with TransIT Neural according to the manufacturer's instructions. Measurement of chloramphenicol acetyltransferase (CAT) activity in Neuro-2A cells was performed as described previously (Workman and Jones, 2010; Workman et al., 2009; Workman et al., 2011).

RESULTS

Microarray analysis of cellular gene expression during BHV-1 reactivation from latency

Our central hypothesis is that latency is established in sensory neurons due to the lack of permissive transcription factors and/or the presence of cellular factors that repress viral transcription. We further hypothesize that reactivation stimuli alter cellular gene expression in sensory neurons, which consequently initiates BHV-1 gene expression and reactivation from latency. To test this hypothesis, we examined cellular gene expression during Dex-induced reactivation from latency in trigeminal ganglia (TG).

A single injection of Dex into the jugular vein of latently infected calves (100 mg) consistently and reproducibly initiates reactivation from latency (Jones, 1998; Jones, 2003a; Jones, 2006). Calves latently infected with BHV-1 were injected with Dex and TG was harvested at 1.5, 3, 6, or 24 hours after Dex treatment. TG were collected and frozen at -80° C. As controls, TG were collected from calves latently infected with BHV-1 (no Dex treatment), or mock infected controls. Total RNA was prepared from the respective TG, RNA was labeled, and changes in cellular gene expression were examined using the Bovine Gene Chip (Affymetrix, Santa Clara, CA), which contains more than 23,000 genes.

We first examined cellular genes stimulated by Dex. Relative to RNA prepared from TG of latently infected calves, 97 cellular genes were induced more than 10 fold at 3 hours after Dex treatment (Table 1 shows a subset of these genes). Two of the top 4 cellular genes stimulated at 3 hours after Dex treatment were transcription factors: 1) zinc Ring finger and BTB domain protein (PLZF) that is a member of the Kruppel

transcription factor family (Bieker, 2001), and 2) Snail Homolog 2 (SLUG) (Nakakura et al., 2001). Two other Kruppel transcription family members (KLF4 and KLF6) were also stimulated more than 10 fold at 3 hours after Dex treatment. Finally, transcription factors SPDEF (Sood et al., 2009) and GATA-6 (Yin and Herring, 2005) were stimulated more than 10 fold at 3 hours after Dex treatment. Although certain genes were stably induced by Dex, others were only stimulated 3 hours after Dex treatment, and then their expression was reduced. For example, Kruppel-like factor 6 (KLF6) and GATA-6 were stimulated more than 10 fold at 3 hours after Dex treatment but were induced less than 4 fold at 6 hours after Dex treatment. In summary, 6 cellular transcription factors were induced more than 10 fold when latently infected calves were treated with Dex to initiate reactivation from latency.

RT-PCR confirmation of Dex-inducible cellular genes

To verify these changes in cellular gene expression by an alternate method, we analyzed the expression of 4 cellular genes using RT-PCR. These included Pentraxin, the gene stimulated most by Dex, and three cellular transcription factors: PLZF, SLUG, and SPDEF. Bovine GAPDH was assayed as a normalization control. PCR products were separated by agarose gel electrophoresis (Figure 6.1A), and the relative amounts of PCR products were evaluated by densitometry using a biomolecular imager (Bio-Rad) (Figure 6.1B). The graph represents the average density of three TG samples from three calves at each time point. As shown in Figure 6.1, Pentraxin expression increased following Dex treatment with a peak at 6 hours after Dex treatment. PLZF and SLUG were expressed with similar kinetics with a peak in gene expression at 3 hours after Dex treatment with

reduced expression at later times. These kinetics were similar to that observed by microarray analysis. Alternatively, SLUG was shown to be stably induced following Dex treatment by microarray. While we observed an increase in SLUG mRNA levels over the latency control, we were unable to detect a significant increase after averaging the levels from three individual TG. In general, we were able to validate changes in expression of specific genes of interest by RT-PCR.

Dex-inducible transcription factors stimulate BHV-1 productive infection

To determine whether Dex-inducible genes activated productive infection, a plasmid expressing the specific cellular gene of interest was co-transfected with genomic DNA from a BHV-1 strain called gCblue virus and efficiency of productive infection was measured. The gCblue virus contains the Lac Z gene downstream of the glycoprotein C (gC) promoter. This allows measurement of productive infection by counting β -Gal⁺ (beta-galactosidase) cells, which directly correlates with plaque formation (Geiser et al., 2002; Geiser and Jones, 2003; Inman et al., 2001b; Meyer et al., 2007a). Previous studies have demonstrated that transfection of BHV-1 genomic DNA yields low levels of infectious virus unless co-transfected with bICP0, a viral transactivator which activates expression of all classes of viral promoters (Inman et al., 2001b). Therefore, cellular transcription factors which activate viral transcription could, in part, substitute for bICP0 functions in stimulating productive infection. We initially focused on the six transcription factors that were stimulated by Dex more than 10 fold at 3 hours after Dex treatment. At 24 hours after transfection, GATA6, SPDEF, SLUG, and KLF6 were all found to increase the number of β -gal⁺ cells approximately 4 fold (Figure 6.2). KLF4 increased

the number of β -gal+ cells 9 fold, while PLZF increased the number of β -gal+ cells 21 fold (Figure 6.2). In summary, all 6 Dex-inducible transcription factors stimulated BHV-1 productive infection in cell culture.

In addition to over expression studies, we also examined the effect of siRNA knock down of Dex-inducible transcription factors on BHV-1 productive infection in cultured cells. These studies were performed in a human osteosarcoma (U2OS) cell line that is permissive to BHV-1 infection so commercially available siRNAs targeting the human transcription factor could be used. Cells were transfected with 100 nM of specific siRNA or a scrambled control siRNA 24 hours prior to transfection with gCblue viral genome. Twenty four hours after transfection with the viral genome, cells were fixed, stained, and β -gal+ cells were counted. With the exception of SPDEF, knock down of Dex-inducible cellular transcription factors reduced the number of β -gal+ cells between 40 and 60 percent (Figure 6.3A). Western blot analysis shows efficiency of siRNA knockdown (Figure 6.3B). The SLUG antibody was unable to recognize a specific band; therefore, RT-PCR was performed to show knock down of endogenous SLUG mRNA (Figure 6.3C).

Dex-inducible cellular transcription factors activate viral gene expression

To further examine the mechanism by which the 6 Dex-inducible cellular transcription factors stimulate productive infection, we examined their ability to transactivate BHV-1 promoters that regulate expression of important viral genes. For these studies, we tested the immediate early transcription unit 1 promoter (IEtu1), bICP0 early (E) promoter, or the gC late (L) promoter. The IEtu1 promoter activates expression

of bICP0 and bICP4, the major transcriptional regulatory proteins encoded by BHV-1 (see Figure 6.5A) (Wirth et al., 1992; Wirth et al., 1991). The bICP0 E promoter also activates bICP0 expression, and recent studies suggest it is preferentially stimulated during reactivation from latency (Workman et al., 2009).

Neuro-2A cells were co-transfected with the designated promoter construct and a plasmid expressing the designated Dex-inducible gene, and CAT activity was measured at 48 hours after transfection. PLZF, which activated productive infection 21 fold, was unable to directly activate viral promoters (Figure 6.4), suggesting an alternative mechanism for stimulating productive infection. KLF4 activated the IE and E promoter constructs 50 fold and 120 fold respectively, but did not activate the L promoter (Figure 6.4). KLF6 was unable to activate the IE or E promoter constructs, but activated the L promoter approximately 3 fold. SLUG activated the IE promoter 5 fold and the L promoter 13 fold. SPDEF was able to activate all three classes of viral promoters between 4 and 10 fold. As GATA6 was able to activate the promoter minus control, no data could be obtained for the remaining CAT constructs. These studies suggest that a subset of Dex-inducible transcription factors enhanced productive infection by trans-activating viral promoter activity.

IETu1 and the bICP0 E promoter contain putative KLF4 binding sites

The results shown in Figure 6.4 demonstrate that KLF4 was able to activate the IETu1 and bICP0 E promoter greater than 50 fold in transient transfection assays. Therefore, we were interested in localizing the KLF4 binding sites within these promoters. We used a panel of 5' promoter deletion mutants which contained consecutive

5' deletions to map the KLF4 responsive regions. Neuro-2A cells were co-transfected with the designated promoter construct along with KLF4 and CAT reporter activity was measured. KLF4 was found to activate the full length IETu1 25 fold and IETu1 Δ 1024 and IETu1 Δ 1391 approximately 50 fold (Figure 6.5B). We next examined the promoter for KLF4 consensus sequences. The KLF4 consensus has been described as CACCC or RCRCCYY. Within the full length IETu1, there were 3 CACCC motifs as well as 4 RCRCCYY motifs. IETu1 Δ 1024 lost 3 RCRCCYY motifs, yet no promoter activity was lost, suggesting these motifs were not important for KLF4 responsiveness. Additionally, IETu1 Δ 1391 lost 1 CACCC motif without losing any activity. The two remaining CACCC motifs are located just upstream of the initiation ATG and are shown in Figure 6.5D. Finally, all three promoter constructs contained the first 500bp upstream of the initiation ATG. This sequence contained an additional RCRCCYY motif. In summary there are three putative KLF4 binding sites in the IETu1 promoter.

We next mapped the bICP0 E promoter to localize the KLF4 responsive domains. EP-638, EP-398 and EP-328 were all activated approximately 100 fold when co-expressed with a KLF4 expression plasmid. EP-172 was activated approximately 50 fold while EP-133 and EP-71 were activated less than 5 fold (Figure 6.6B). When we analyzed the promoter sequence for KLF4 consensus motifs, we found 2 CACCC in the forward orientation as well as 1 in the reverse orientation. Additionally, there was one RCRCCYY motif in the forward orientation. From EP-328 to EP-172, approximately half of the promoter activity is lost. This is accompanied by the loss of one CACCC consensus in the forward orientation as well as the RCRCCYY motif. From EP-172 to EP-133, there is a second dramatic drop in promoter responsiveness to KLF4. This

deletion is accompanied by the loss of one CACCC consensus in the reverse orientation. Maintained in EP-133 and EP-71 is one CACCC consensus; however, this motif does not appear to be important for KLF4 responsiveness in the bICP0 E promoter (see Figure 6.6C). In summary, we were able to identify KLF4 binding sites in the IETu1 promoter as well as the bICP0 E promoter that are important for KLF4 activation of these viral promoters.

DISCUSSION

During latency, the viral genome is in a transcriptionally repressed state, and the latency related (LR) gene is the only abundant transcript. During corticosteroid-induced reactivation, Dex represses LR promoter activity, reducing LR gene expression (Jones et al., 1990; Rock et al., 1992). Thus, LR is not believed to directly play a role in reactivation from latency; although, LR may actively inhibit reactivation from latency. In the absence of viral transactivators, we hypothesize that stress-induced cellular factors act directly on viral promoters to activate viral gene expression, and subsequently induce reactivation from latency.

Several lines of evidence support this hypothesis and suggest that Dex-induced cellular transcription factors stimulate production of infectious virus during reactivation from latency. For example, Dex stimulates the expression of a cellular transcription factor, C/EBP-alpha, which directly activates the bICP0 early (E) promoter (Meyer and Jones, 2009; Meyer et al., 2007a; Meyer, 2008; Workman et al., 2009). Furthermore, Notch 1 and Notch 3 expression is stimulated by Dex during reactivation from latency, and Notch 1 stimulates viral promoters and productive infection (Workman et al., 2011). Therefore, in this study we used microarrays to examine global changes in cellular gene expression in the TG of calves during Dex-induced reactivation to identify additional cellular factors that may regulate the latency-reactivation cycle.

In as little as 3 hours after Dex treatment, 97 cellular genes were activated in the TG. Six of these were cellular transcription factors: 1) promyelocytic leukemia zinc finger (PLZF), a member of the Kruppel like transcription factor (KLF) family, 2) SLUG, a zing finger protein involved in neuronal development, cancer, and apoptosis, 3) KLF4,

4) KLF6, 5) SAM pointed domain containing ets (erythrocyte transformation specific) like transcription factor (SPDEF), which is a zinc finger containing protein that regulates cell lineage specification, proliferation, angiogenesis and apoptosis, and 6) GATA6 which is important for regulating terminal differentiation or proliferation (see Table 6.1). Following RT-PCR validation of these results (Figure 6.1), cellular transcription factors were examined for their ability to stimulate productive infection and viral promoter activity using transient transfection assays (Figures 6.3 and 6.4).

PLZF induced productive infection 21 fold in transient transfection assay (Figure 6.2), but was unable to significantly activate viral promoters (Figure 6.4). PLZF is a member of the ‘‘BTB/POZ’’ (bric a’ brac, tramtrack, broad complex/poxvirus zinc finger) family of transcription factors. The N-terminal BTB/POZ domain mediates interactions with proteins such as transcriptional co-repressors (Melnick et al., 2000) resulting in chromatin remodeling and transcriptional silencing (Chauchereau et al., 2004; David et al., 1998; Guidez et al., 2005; Hong et al., 1997; McLoughlin et al., 2002). The C-terminal 9 C₂H₂ Kruppel-like zinc fingers interact with DNA in a sequence specific manner to confer specificity of the repressor activity. Contrary to the previous designation of PLZF as a repressor of transcription, a recent study showed that PLZF is required for the activation of a subset of interferon stimulated genes which are important in the innate immune response to viruses (Xu et al., 2009). Interferon led to the phosphorylation of PLZF within the BTB domain, which transformed the protein from a transcriptional repressor to an activator. Therefore, it is possible that PLZF acts to activate transcription under certain conditions. For example, viral infection induces interferon expression. Therefore, interferon-induced phosphorylation of PLZF may lead

to the subsequent activation of viral promoters in our productive infection assays. Promoter activity was evaluated using reporter plasmids in the absence of viral infection, and therefore the absence of interferon induction. It is possible that PLZF was not phosphorylated and could not activate viral promoter activity in the absence of interferon. It will be of interest to determine whether interferon stimulation of cells can convert PLZF to an activator of viral promoters in our reporter assay. It is also possible that other signaling cascades lead to PLZF phosphorylation and activation.

Another Kruppel like transcription factor, KLF4, activated productive infection 9 fold, and activated the IETu1 and bICP0 E promoter 50 fold and 120 fold, respectively. This suggests that KLF4 activates productive infection by directly or indirectly activating viral promoters. We hypothesized that KLF4 may directly bind viral promoters, because: i) KLF4 binds GC rich motifs resembling Sp1 binding sites, ii) the BHV-1 genomes is approximately 70% GC rich (Tikoo et al., 1995), and iii) most viral promoters contain multiple Sp1 consensus sequences (Jones and Tjian, 1985). Therefore, we examined the IETu1 and bICP0 E promoter for known KLF4 binding sites. The KLF4 consensus has been described as CACC or RCRCCYY. Using 5' deletions of the bICP0 E promoter, we were able to identify three potential KLF4 binding sites that confer KLF4 responsiveness using reporter assays (Figure 6.6). For the IETu1, we identified two putative KLF4 binding sites upstream of the transcription start site as well as 1 binding sites within 500bp downstream of the transcription start (Figure 6.5).

Recent work by Thompson et al. (Thompson et al., 2009), using an in vivo approach to study HSV-1 reactivation, show that the tegument protein VP16, which is normally expressed with late kinetics, is expressed prior to IE genes during reactivation

from latency. During productive infection, VP16 is delivered in the tegument of the virion and serves to initiate IE gene expression. From these findings, they conclude de novo synthesis of VP16 (due to specific activation of the VP16 promoter) is responsible for the initial events of reactivation from latency. Therefore, we examined the effect of these Dex-inducible transcription factors to regulate bTIF (the BHV-1 homolog of HSV-1 VP16) promoter activity. None of the cellular factors tested were able to trans-activate the bTIF promoter (data not shown).

In summary, we have identified 6 cellular transcription factors that are induced during Dex-induced BHV-1 reactivation from latency. These cellular transcription factors stimulate BHV-1 productive infection and a subset of these activates viral transcription when analyzed in transient transfection assays. This suggests these transcription factors may play a role in the early events of reactivation from BHV-1 latency by activating viral gene expression and infectious virus production. Since BHV-1 is the only *alphaherpesvirinae* subfamily member that can be reproducibly induced to reactivate from latency, these findings have impact on other important members of this virus family.

Table 6.1 Identification of cellular genes induced by Dex within TG of latently infected calves.

Three latently infected calves were treated with Dex for 3 hours, 6 hours, or 24 hours. Cellular gene expression was analyzed using the Affymetrix bovine Gene Chip. The common name of the gene is provided in the first column. Second column (Fold Induction): the numbers are levels of induced gene expression at 3 (1st number), 6 (2nd number), or 24 hours (3rd number) after Dex treatment. The base line of expression was derived from 3 calves latently infected with BHV-1. Known functions of the respective genes are summarized in the third column.

Table 6.1.

Gene Name	Fold Induction	Functions
PTX3: Pentraxin-related gene	65, 101, 5	Innate immunity, neuronal degeneration
SHISA3: Shisha homolog 3 (Xenopus laevis)	37, 23, 3	Wnt signaling
PLZF : Zn finger and BTB domain protein (Kruppel transcription factor)	37, 17, 8	Transcription factor: differentiation, growth control
SNAI2: snail homolog 2 (Drosophila) also called SLUG in mammals	28, 10, 2	Transcription factor: neuronal development, apoptosis, cancer
HBB: hemoglobin, beta	26, 2, 1	
COL1A1: Collagen, type I, alpha 1	25, 4, 1	
LYVE1: Lymphatic vessel endothelial hyaluronan receptor 1	25, 11, 3	Lymph-specific Receptor for Hyaluronan
CYR61: Cysteine-rich, angiogenic inducer, 61	24, 11, 4	Stimulates chemotaxis
DKK1: dickkopf homolog 1 (Xenopus laevis)	23, 8, 2	Inhibits Wnt signaling
ACTG2: actin, gamma 2, smooth muscle, enteric	22, 5, 6	
ASPN: Asporin	20, 13, 4	Leu-rich protein assoc with cartilage
SPOP: speckle-type POZ protein	20, 4, 1	Adaptor for DAXX (Cul-3 Ub system)
KLF4 : Kruppel-like transcription factor 4	12, 9, 3	Transcription factor: GC binding (SP1 like binding activity)
SPDEF : SAM pointed domain containing ets like transcription factor	11, 10, 6	Transcription factor: contains Zinc finger
GATA-6	11, 3, 2	Transcription factor: binds GGA(A/T) sequences
KLF6 : Kruppel-like factor 6	11, 2, 2	Transcription factor: GC binding (SP1 like binding activity)

Figure 6.1. RT-PCR confirmation of Dex-inducible cellular genes.

Panel A: RT-PCR using specific primers was performed to analyze expression of cellular genes identified by microarray analysis. Three latently infected calves were treated with Dex for 1.5 hours, 3 hours, 6 hours, or 24 hours. Additionally, three mock (M) infected calves as well as 3 latently infected calves (no Dex; L) were used as controls for baseline expression values. TG was collected at necropsy and total RNA was isolated. RT-PCR was performed using specific primers as described in the materials and methods. Products were separated by agarose gel electrophoresis and visualized using ethidium bromide.

Panel B: Graphical representation of RT-PCR results. The amount of PCR products were quantified using a biomolecular imager (Bio-Rad) and density values were normalized to a GAPDH control. Values represent the average of 3 TG from 3 calves at each time point.

Figure 6.1.

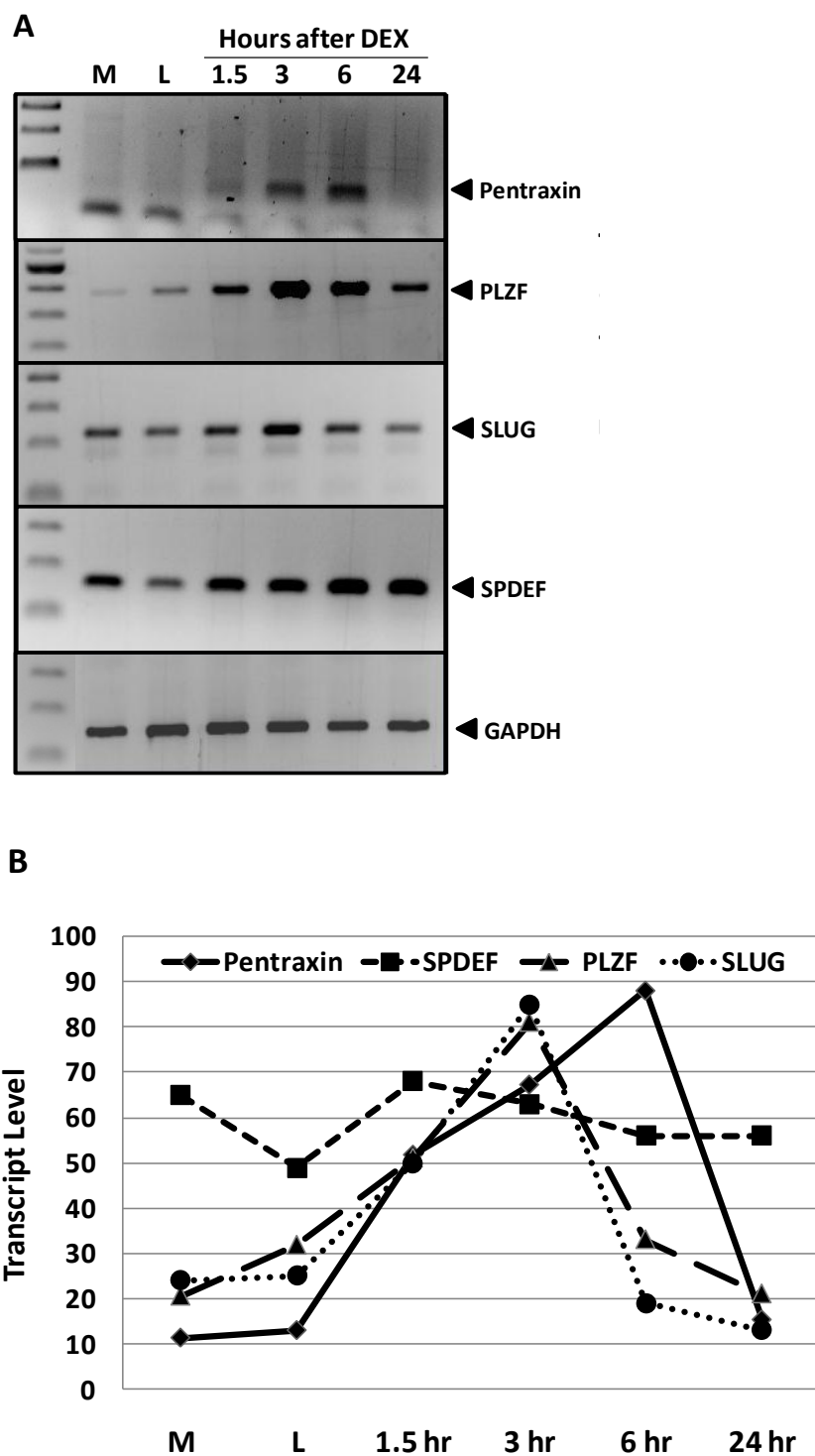


Figure 6.2. Dex-inducible transcription factors stimulate BHV-1 productive infection.

Rabbit skin (RS) cells were cotransfected with 62 ng of a plasmid encoding bICP0 or increasing concentrations of the respective plasmids (62 ng, 250 ng, 1 μ g, or 2 μ g) encoding Dex inducible transcription factors along with 1 μ g of gCblue BHV-1 DNA, which contains the Lac Z gene inserted into the gC locus of BHV-1. At 24 hours after transfection, cells were fixed, stained, and β -gal positive cells were counted. The results are the average of three independent experiments.

Figure 6.2.

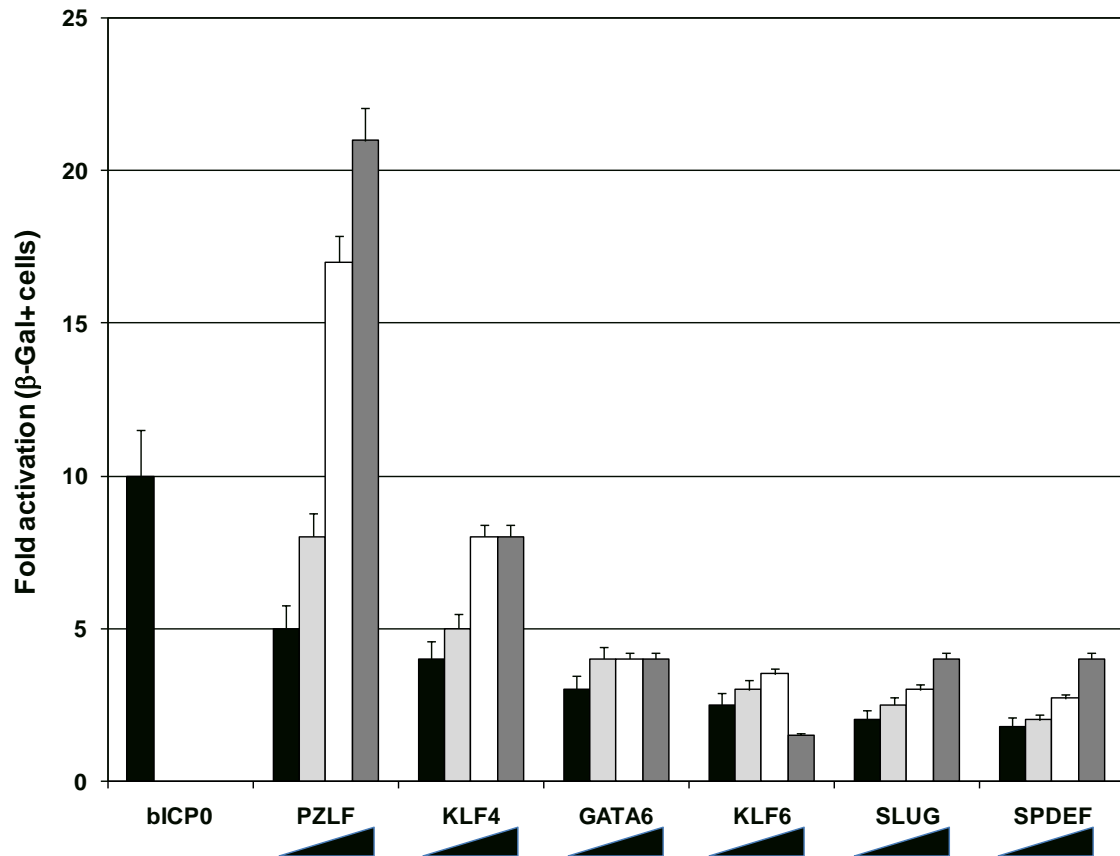


Figure 6.3. Suppression of Dex-induced transcription factors reduced the levels of productive infection.

Panel A: Human osteosarcoma cells (U2OS) were transfected with 100 nM siRNA targeting the designated cellular transcription factor or a control siRNA which does not reduce the expression of any known mammalian gene. Twenty four hours later, cells were transfected with gCblue viral genome and efficiency of productive infection was measured. The results are the average of three independent experiments.

Panel B: To show the efficiency of the siRNAs, human osteosarcoma cells (U2OS) were co-transfected with 100 nM siRNA and 0.5 μ g of the designated plasmid expressing the Dex-inducible transcription factor. Twenty four hours after transfection, cells were collected and processed for western blot. Actin was assayed as a loading control.

Panel C: The slug antibody was unable to recognize a specific protein. Therefore, U2OS cells were transfected with 100 nM Slug siRNA, control siRNA, or no siRNA, and RT-PCR was performed as described in the material and methods. PCR products were run on a 1.3% agarose gel and visualized with ethidium bromide.

Figure 6.3.

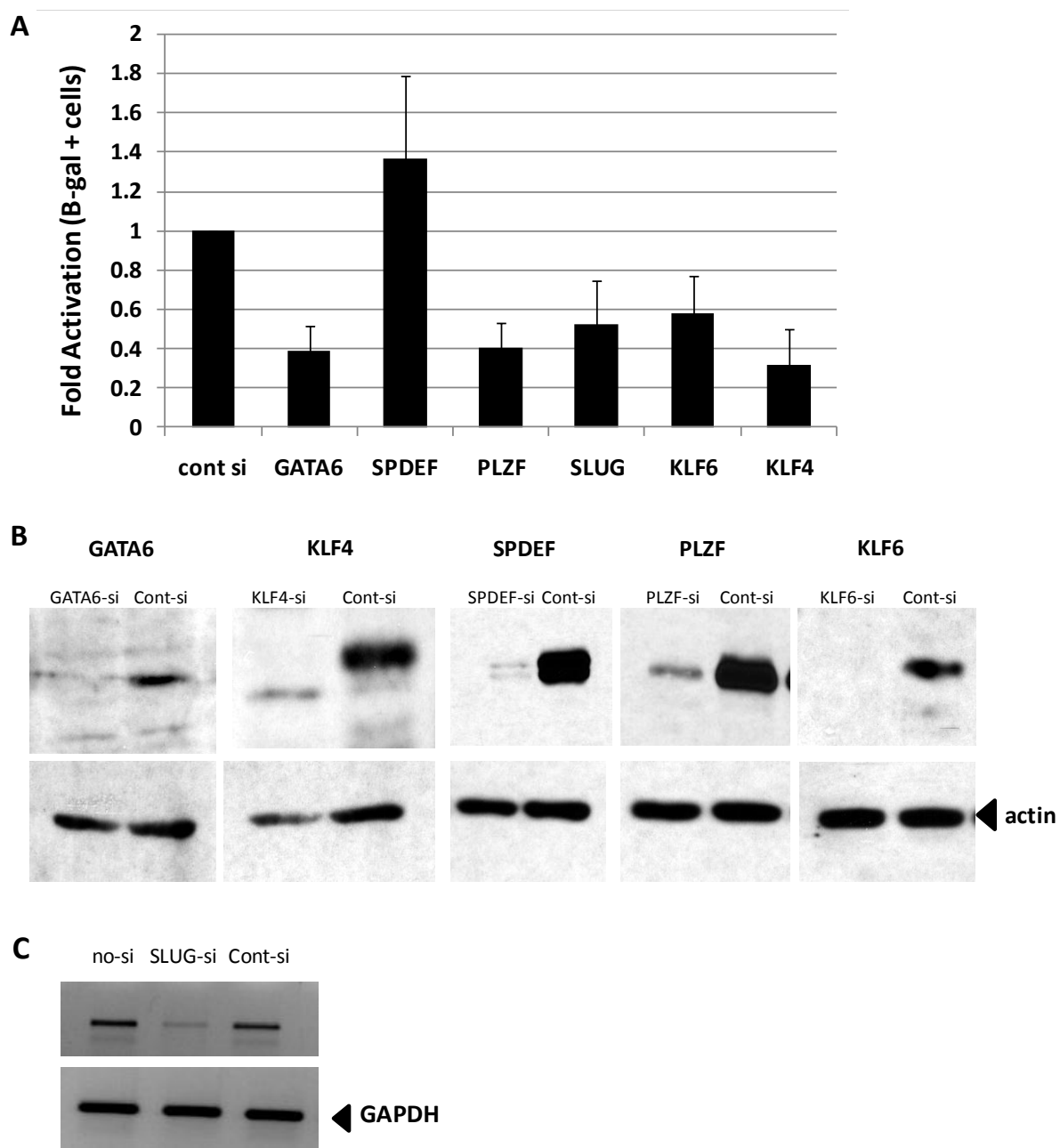


Figure 6.4. Dex-inducible cellular transcription factors activate viral gene expression.

Mouse neuroblastoma cells (neuro-2A) were cotransfected with 1 μ g of a CAT reporter plasmid with the IETu1 promoter (IETu1 Δ 1024), bICP0 E promoter (EP-638), or the gC L promoter and 1 μ g of pcDNA3.1 empty vector or 1 μ g of a plasmid expressing the designated Dex-inducible transcription factor. At 48 hours after transfection, CAT reporter activity was measured. The numbers represent fold induction over the empty vector control. The results are the average of three independent experiments.

Figure 6.4.

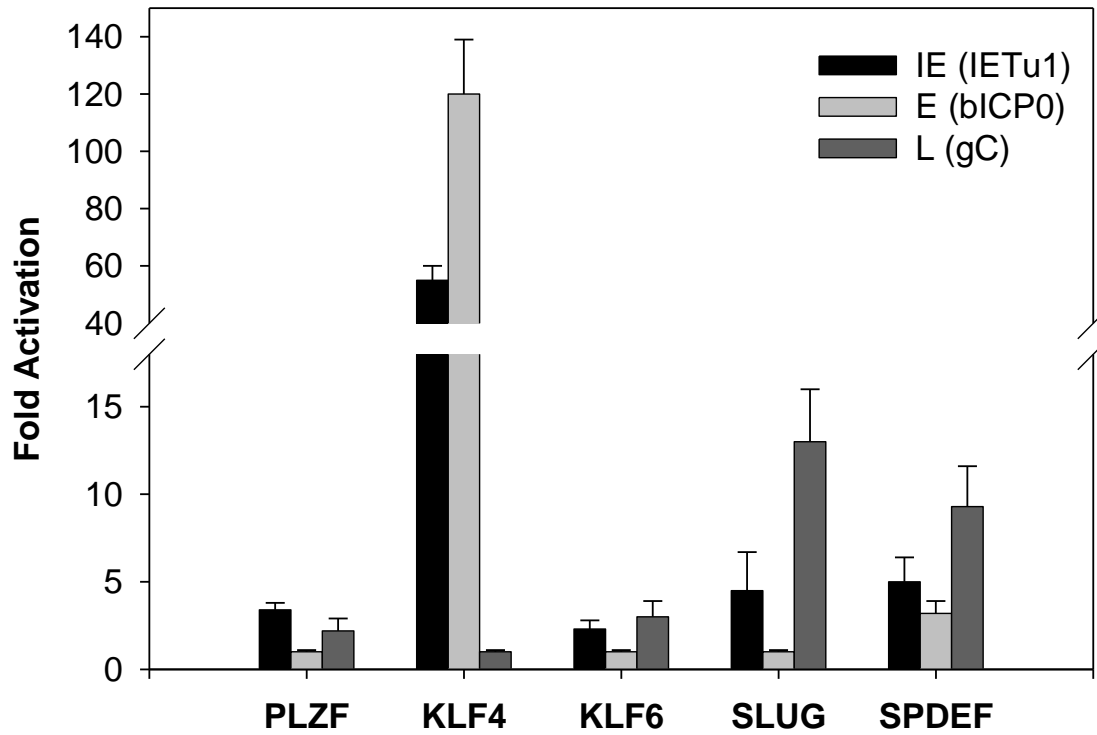


Figure 6.5. Localization of KLF4 binding sites in the IETu1.

Panel A: Position of transcripts that encode bICP4 and bICP0 are shown. The immediate early transcription unit 1 (IETu1) encodes bICP4 (IE/4.2) and bICP0 (IE/2.9) (Wirth et al., 1989; Wirth et al., 1991). The IETu1 promoter (denoted by the black rectangle) activates IE expression of IE/4.2 and IE/2.9. E/2.6 is the early transcript that encodes bICP0 and an early promoter (denoted by the grey rectangle) activates expression of this early transcript (Wirth et al., 1992). Exon 2 (e2) of bICP0 contains all of the protein coding sequences of bICP0. The dashed lines are intron sequences. The basal promoter activity of the respective constructs was measured in neuro-2A cells. Basal promoter activity of IETu1cat was normalized to 100% and the other 2 promoters compared to this value. These results were the average of 3 independent experiments.

Panel B: Mouse neuroblastoma cells (neuro-2A) were cotransfected with the designated IETu1 promoter-CAT reporter plasmid (1 µg) and 1 µg of pcDNA3.1 empty vector or 1 µg of KLF4. At 48 hours after transfection, CAT activity was measured. The numbers represent fold induction over the empty vector control. The results are the average of three independent experiments.

Panel C: KLF4 consensus sequences were located in the IETu1 promoter using the free online program fuzznuc (<http://emboss.open-bio.org/wiki/Appdocs>). The KLF4 consensus has been described as CACC or RCRCCYY. Table includes the number of consensus sequences found without mismatch in the IETu1 promoter and 5' UTR included in CAT constructs.

Panel D: First 56 base pairs in the IETu1. Includes two CACCC consensus sequences.

Figure 6.5.

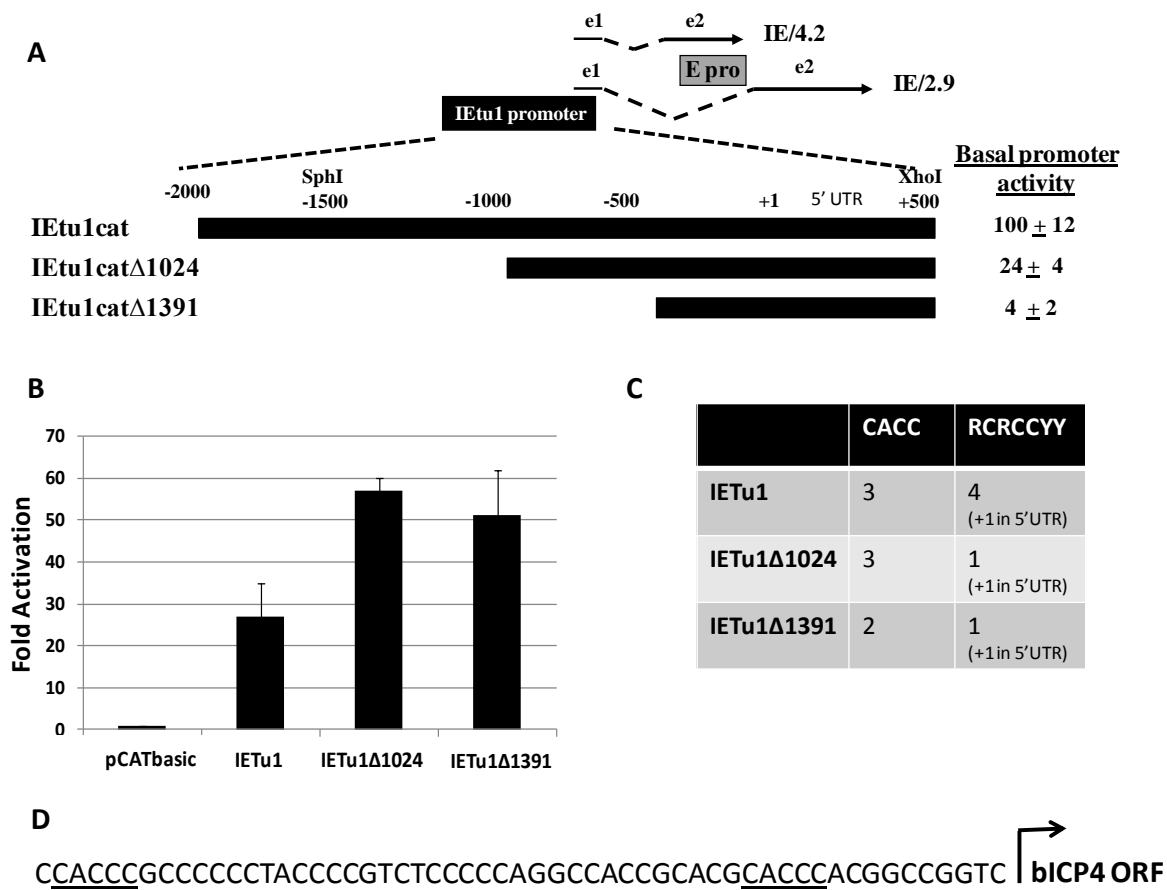


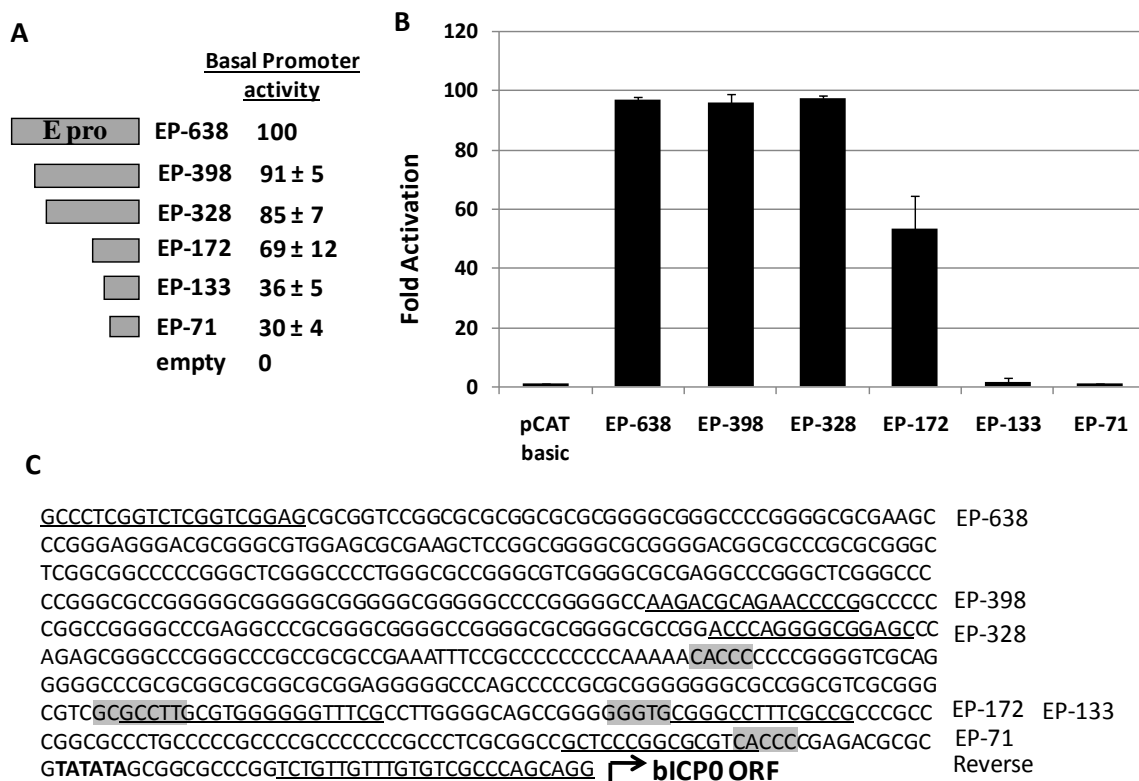
Figure 6.6. Localization of KLF4 binding sites in bICP0 E promoter.

Panel A: Schematic of bICP0 E promoter constructs with corresponding basal promoter activities. Basal promoter activity of EP-638 was set at 100% and the values of the other constructs were compared to EP-638. The results are the average of three independent experiments.

Panel B: Mouse neuroblastoma cells (neuro-2A) were cotransfected with the designated bICP0 E promoter-CAT reporter plasmid (1 μ g) and 1 μ g of pcDNA3.1 empty vector or 1 μ g of KLF4. At 48 hours after transfection, CAT activity was measured. The numbers represent fold induction over the empty vector control. The results are the average of three independent experiments.

Panel C: KLF4 consensus sequences were located in the bICP0 E promoter using the free online program fuzznuc (<http://emboss.open-bio.org/wiki/Appdocs>). The KLF4 consensus has been described as CACC or RCRCCYY. Highlighted are consensus sequences found with no mismatches. Underlined are the primer sequences used for constructing the bICP0 E promoter constructs.

Figure 6.6.



Chapter 7

Certain Dexamethasone Inducible Transcription Factors have the Potential to Regulate Herpes Simplex Virus 1 (HSV-1) Productive Infection and Promoter Activity in Cultured Cells.

ABSTRACT

Bovine herpesvirus 1 (BHV-1) and herpes simplex virus type 1 (HSV-1) are *alphaherpesvirinae* subfamily members that establish latency in sensory neurons. In contrast to acute infection where all viral genes are expressed; latency is characterized by minimal viral gene expression, the absence of lytic viral proteins, and the absence of infectious virus production. These viruses can periodically be induced to reactivate from latency, facilitating the spread of the virus to susceptible hosts. The latency-reactivation cycle is crucial for virus transmission and complicates disease control; however, the steps that occur during a successful reactivation have not been well characterized. We hypothesize that stressful stimuli induce cellular transcription factors which activate viral promoters to increase lytic viral gene expression, culminating in infectious virus production. We have identified 6 cellular transcription factors which are induced greater than 10 fold following dexamethasone (Dex) induced reactivation from BHV-1 latency. These transcription factors activate BHV-1 productive infection and certain viral promoters, suggesting they play a role in the early events of reactivation from BHV-1 latency. In this study, we tested the effect of these Dex-inducible cellular transcription factors on HSV-1 productive infection and viral promoter activity. These studies suggested that certain transcription factors induced during BHV-1 reactivation from latency can also stimulate certain HSV-1 promoters and productive infection in cultured cells.

INTRODUCTION

The lifecycle of herpes simplex virus 1 (HSV-1) can be divided into three phases: i) productive replication of the virus on mucosal surfaces, ii) establishment and maintenance of latency in sensory neurons, and iii) periodic reactivation of the virus from latency. The regulated pattern of gene expression, during the first phase, productive replication, has been extensively studied in permissive cell types in culture. Here it was discovered that viral gene expression is temporally regulated in three distinct phases: immediate early (IE), early (E) and late (L) (Clements et al., 1977; Honess and Roizman, 1974). Unlike other large DNA viruses, herpesvirus IE genes are not controlled by constitutively active promoters within the context of the viral genome. Rather, a tegument protein packaged in the virion serves to activate IE gene expression {reviewed in (Wysocka and Herr, 2003)}. The tegument protein VP16 (virion protein 16) associates with host cell factor 1 (HCF-1) in the cytoplasm and this complex translocates to the nucleus where it binds to the cellular transcription factor Oct-1 (Octamer binding protein 1) bound to specific motifs in the promoters of all IE genes (Kristie et al., 1989; LaMarco and McKnight, 1989). This brings the potent VP16 acidic activation domain in the vicinity of the transcription start site of these genes in order to recruit and stabilize elements of the basal transcription complex (Goodrich et al., 1993; Ingles et al., 1991; Klemm et al., 1995; Lin et al., 1991; Uesugi et al., 1997). During productive infection, all viral genes are expressed, new virus particles are made, and cell death ensues.

The second and third phases of the HSV-1 lifecycle, latency and reactivation, respectively, have been experimentally studied in animal models of HSV-1 infection in mice and rabbits. From these studies, we have learned that during the second phase,

establishment and maintenance of latency, the HSV-1 viral genome is maintained in sensory neurons as an episome (Efstathiou et al., 1986; Rock and Fraser, 1983), but infectious virus is not detected by standard virological methods (Baringer and Swoveland, 1973; Cook et al., 1974; McLennan and Darby, 1980; Stevens and Cook, 1971). In contrast to productive infection, the only viral gene abundantly expressed in latently infected sensory neurons is the latency associated transcript (LAT; (Stevens, 1987a)). Latency is therefore characterized by minimal viral gene expression, the absence of lytic viral proteins, the absence of new virion synthesis, and neuronal viability.

In the third phase, reactivation from latency, various stressors induce lytic viral gene expression and infectious virus production. The latency-reactivation cycle is crucial for virus transmission and survival in nature. However, due to the lack of a suitable cell culture model to study reactivation from latency, the molecular mechanisms that control the exit from latency remain to be elucidated.

Bovine herpesvirus 1 (BHV-1) is an *alphaherpesvirinae* subfamily member that shares a number of biological properties with HSV-1 (Jones, 2003a). Unlike HSV-1, BHV-1 can be studied *in vivo* in its natural host. Furthermore, BHV-1 can be predictably reactivated from all latently infected animals following a single intravenous dose of the synthetic corticosteroid, dexamethasone (Dex) (Inman et al., 2002; Jones, 1998; Jones, 2003a; Jones et al., 2006; Jones et al., 2000; Rock et al., 1992). This makes BHV-1 a suitable model to study alpha-herpesvirus reactivation from latency. In a recent study to identify cellular factors that may stimulate BHV-1 reactivation from latency, cellular gene expression was examined in TG of calves during the early stages of Dex induced reactivation from BHV-1 latency (Chapter 6). A Bovine Gene Chip (Affymetrix)

identified 6 cellular transcription factors that were induced greater than 10 fold at 3 hours after Dex treatment. A subset of these Dex-inducible genes stimulated BHV-1 productive infection, in part, by trans-activating viral promoters. Since BHV-1 and HSV-1 are both *alphaherpesvirinae* subfamily members, we hypothesized that both viruses utilize similar transcription factors to stimulate viral promoters necessary for reactivation from latency. Therefore, in this study, we tested the effect of the Dex-inducible cellular transcription factors on HSV-1 productive infection and viral promoter activity. These studies show that certain transcription factors induced during BHV-1 reactivation from latency can stimulate certain HSV-1 promoters and productive infection.

MATERIALS AND METHODS

Cells and Virus

Murine neuroblastoma (neuro-2A), rabbit skin (RS) and African green monkey kidney cells (Vero) were grown in Earle's modified Eagle's medium (EMEM) supplemented with 10% fetal calf serum, penicillin (10 U/ml), and streptomycin (100 µg/ml).

An HSV-1 recombinant virus (KOS6β) contains the β-Gal gene driven by the viral ICP6 promoter and is expressed as an early gene (David Davido, University of Kansas). The recombinant virus grows to wild type levels when grown in human or mouse cells (Davido, 2002).

β-gal Assay

The KOS6β virus grows to similar titers as the parental strain and was grown in Vero cells. Procedures for preparing genomic DNA were described previously. RS cells grown in 6 well plates were cotransfected with 1 µg of the viral genome and the designated amounts of plasmid expressing bICP0 or the Dex-inducible cellular transcription factor of interest using Lipofectamine 2000 (11668-019; Invitrogen). Twenty-four hours after transfection, cells were fixed (2% Formaldehyde, 0.2% Glutaraldehyde in PBS), stained (1% blue-gal, 5 mM Kferric, 5 mM Kferro, 0.5 M MgCl₂ in PBS), and the number of β-gal⁺ cells counted as described previously (Workman and Jones, 2010; Workman et al., 2009; Workman et al., 2011). The number of β-gal⁺ cells in cultures expressing the blank vector was set to 100%. To calculate percent plaque formation, the number of blue cells in cultures transfected with the

cellular transcription factor were divided by the number of blue cells in cultures transfected with the blank vector. This representation of the data minimized the differences in cell density, Lipofectamine lot variation, and transfection efficiency. The results are an average of at least three independent experiments.

Plasmids

CAT (chloramphenicol acetyltransferase) constructs used in this study, HSV-1 ICP0-CAT (pAB5) and ICP4-CAT were described previously (Devireddy and Jones, 2000). Luciferase promoter constructs used in this study, pGL3basic, VP16-luc, gC-Luc, UL42-Luc, ICP6-Luc, and LAT-Luc were obtained from Priscilla Schaffer and have been previously described (Kushnir et al., 2010). The empty vector pcDNA3.1 was purchased from Invitrogen. SPDEF was purchased from Origene (Rockville, MD). The remaining Dex-inducible genes were graciously provided to us from labs studying these transcription factors. PLZF: Derek Sant'Angelo (Sloan Kettering Cancer Center). SLUG: Paul Wade (NIH). KLF4: Jonathan Katz (University of Pennsylvania). GATA6: Brian Herring (Indiana University Medical Center) KLF6: Bing Guo (North Dakota State University). The Notch1 and Notch 3 intracellular domain (ICD) were cloned into a CMV expression construct, and were provided by Urban Lendahl (Karolinska Institute, Sweeden).

CAT and Luciferase Reporter Assays

Neuro-2A cells grown in 60 mm dishes were co-transfected with the designated plasmids as indicated in the respective figure legends using TransIT Neural according to

the manufacturer's instructions. Measurement of chloramphenicol acetyltransferase (CAT) activity in Neuro-2A cells was performed as described previously (Workman and Jones, 2010; Workman et al., 2009; Workman et al., 2011). Luciferase activity in transfected Neuro-2A cells was performed using the Promega Luciferase Assay System as described by the manufacturer (E4030; Promega).

RESULTS

Analysis of DEX-inducible transcription factors on HSV-1 productive infection

In a previous study (Chapter 6 of this dissertation) we identified 6 Dex-inducible transcription factors which promote BHV-1 productive infection by directly or indirectly activating viral promoters. Since BHV-1 and HSV-1 are both *alphaherpesvirinae* subfamily members, we hypothesized that both viruses may utilize similar cellular transcription factors for activating viral gene expression during reactivation from latency. To test this hypothesis, we examined the effects of the 6 Dex-inducible transcription factors on HSV-1 productive infection (see Table 7.1 for a list of Dex-inducible transcription factors that were tested in this study).

To determine whether Dex-inducible genes activated productive infection, a plasmid expressing the specific cellular gene of interest was co-transfected with genomic DNA from a HSV-1 strain called KOS6 β and efficiency of productive infection was measured. The KOS6 β virus contains the Lac Z gene downstream of the early ICP6 promoter. This allows measurement of productive infection by counting β -Gal+ (beta-galactosidase) cells, which directly correlates with plaque formation. GATA6 and PLZF did not have a significant effect on the number of β -gal positive cells (Figure 7.1). In contrast, SPDEF, KLF4, and KLF6 all reduced the number of β -gal positive cells relative to the empty vector control. SLUG was only transcription factor to increase HSV-1 productive infection, with a 2.5 fold induction (Figure 7.1). In contrast, PLZF was able to activate BHV-1 productive infection 20 fold and KLF4 activated BHV-1 productive infection approximately 9 fold (Chapter 6). The remaining transcription factors each activated BHV-1 productive infection approximately 4 fold. In summary, the Dex-

inducible transcription factors that stimulated BHV-1 productive infection did not have the same effect on HSV-1 productive infection.

Analysis of DEX-inducible transcription factors on viral gene expression

We next examined the ability of the 6-Dex inducible transcription factors to transactivate HSV-1 promoters that regulate expression of important viral genes. For these studies, we tested the immediate early ICP0 and ICP4 promoters as well as the early ICP6 promoter and the late VP16, gC, and UL42 promoters. Finally, the latency associated transcript (LAT) promoter was tested. Neuro-2A cells were co-transfected with the designated promoter construct plus a Dex-inducible gene, and reporter activity was measured at 48 hours after transfection. GATA6 was found to activate the ICP6, gC, UL42, and VP16 promoters greater than 5 fold (Table 7.2). As GATA6 was able to activate the promoter-minus CAT expression vector, no data could be obtained for the ICP0 and ICP4 promoter constructs. SPDEF activated the VP-16, UL42, and ICP0 promoters greater than 5 fold and the ICP4 promoter 4 fold. PLZF activated the ICP0 promoter 6 fold. Slug activated the ICP0 and ICP4 promoters approximately 6 fold. KLF4 activated the ICP0 promoter 21 fold. KLF6 did not activate any of the promoter constructs examined (Table 7.2). None of the Dex-inducible transcription factors activated the LAT promoter. In summary, several of the Dex-inducible genes were able to trans-activate HSV-1 promoters. In particular, the ICP0 promoter was activated more than 17 fold by SPDEF and 21 fold by KLF4.

Analysis of Notch Induction of HSV-1 productive infection and promoter activity

We recently found that the Notch family of transcription factors play a role in the latency-reactivation cycle of BHV-1 ((Workman et al., 2011); Chapter 5). Notch receptor family members (Notch1-4) are membrane tethered transcription factors that regulate numerous developmental and physiological processes (Bray, 2006; Ehebauer, 2006). When the Notch receptor is engaged by its ligand, the intracellular domain (ICD) is cleaved by specific proteases, and is subsequently translocated to the nucleus. In the nucleus, NICD interacts with CSL family members to stimulate target promoter activity.

The BHV-1 latency related (LR) protein ORF2 was found to interact with Notch 1 and Notch 3 proteins in a yeast-two-hybrid assay (Workman et al., 2011). This interaction was further confirmed by pull down assays and co-localization by confocal microscopy. Notch 1 activated all three classes of BHV-1 promoters, while Notch 3 activated the late gC promoter alone. Trans-activation of viral promoters was attenuated by co-expression of ORF2, suggesting ORF2 negatively regulates Notch function. This data further suggests ORF2 promotes the establishment and maintenance of latency by reducing viral transcription (Chapter 5). Microarray analysis also revealed that several genes in the Notch signaling pathway, including the proteases that cleave and activate Notch, are stimulated in TG of latently infected calves following DEX treatment for 6 hours, adding further support to the concept (Chapter 6). Finally, the Notch signaling pathway plays a role in the latency-reactivation cycle of two human herpesviruses, KSHV (Kaposi's sarcoma-associated herpesvirus) (Liang et al., 2002) and EBV (Epstein Barr virus) (Kieff et al., 1971). Therefore, we were interested in analyzing the effect of Notch on HSV-1 productive infection and promoter activity.

Previous studies revealed that the BHV-1 genome contained 82 consensus CSL binding sites (Workman et al., 2011). We hypothesize that these binding sites are important for Notch induced viral promoter activation. Within the HSV-1 genome there are over a hundred CSL binding sites (CBS; Figure 7.2). This suggests that Notch family members have the potential to regulate some aspect of HSV-1 infection.

To determine whether Notch family members activated productive infection, a plasmid expressing Notch was co-transfected with KOS6 β genomic DNA and efficiency of productive infection was measured. Notch 3 increased the number of β -gal positive cells approximately 2.5 fold while Notch 1 consistently reduced the number of β -gal positive cells approximately 50% when compared to the empty vector control (Figure 3). Notch 2 was unable to stimulate productive infection when compared to the empty vector control (Figure 7.3). This is in contrast to BHV-1 productive infection where Notch 1 and Notch 3 stimulated productive infection 6 fold and 2 fold, respectively (Chapter 5; Figure 5.3).

We next examined the ability of Notch family members to trans-activate HSV-1 promoters that regulate expression of important viral genes. For these studies, we tested the immediate early ICP0 and ICP4 promoters as well as the early ICP6 promoter and the late VP16, gC, and UL42 promoters. Additionally, the latency associated transcript (LAT) promoter was tested. Neuro-2A cells were co-transfected with the designated promoter construct and Notch family member, and reporter activity was measured at 48 hours after transfection. Notch 1 stimulated the ICP0 promoter approximately 3 fold (Figure 7.4A), the UL42 promoter 4 fold and the gC promoter 5.5 fold (Figure 7.4B). Notch 2 activated the ICP4 promoter approximately 4.5 fold, but was unable to activate

the other promoters tested. Finally, Notch 3 was unable to activate any of the promoters we tested (Figure 7.4). In summary, these studies provide evidence that Notch 1, but not Notch 3 stimulated several HSV-1 promoters. Notch 2 was the only Notch family member that was able to stimulate the ICP4 promoter.

DISCUSSION

In general, recurrent diseases caused by HSV-1 are the direct result of reactivation from latency (Asbell et al., 1984; Lewis et al., 1984; Sakaoka et al., 1995). For example, two-thirds of HSV-1 induced infections, corneal blindness, and encephalitis are the result of recurrent disease. Stress, trauma, and/or immune suppression can result in reactivation from latency; however, the steps that occur during a successful reactivation have not been well characterized. While many attempts at cell culture models for latency have been made, none give a true representation of the events that occur during an *in vivo* latency-reactivation cycle. Therefore, our lab studies BHV-1 in its natural host in an attempt to identify virus-host interactions important in the latency-reactivation cycle of alpha-herpesviruses.

In a previous study, we analyzed the cellular gene expression in the TG of calves latently infected with BHV-1 following treatment with a synthetic corticosteroid (Dex) to induce reactivation from latency (Chapter 6). In as little as 3 hours after Dex treatment, approximately 100 cellular genes were activated in the TG. Six of these were cellular transcription factors: 1) promyelocytic leukemia zinc finger (PLZF), a member of the Kruppel like transcription factor (KLF) family, 2) Slug, a zing finger protein involved in neuronal development, cancer, and apoptosis, 3) KLF4, 4) KLF6, 5) SAM pointed domain containing ets (erythrocyte transformation specific) like transcription factor (SPDEF), which is a zinc finger containing protein that regulates cell lineage specification, proliferation, differentiation, angiogenesis and apoptosis, and 6) GATA6 which is important in regulating terminal differentiation or proliferation (see Table 7.1). We were initially interested in the KLF family members because they bind GC rich

motifs resembling Sp1 binding sites, and the herpesvirus genomes are >70% GC rich (Tikoo et al., 1995). Additionally, most viral promoters contain multiple Sp1 consensus sequences (Jones and Tjian, 1985). Indeed, KLF family members activated viral transcription and BHV-1 productive infection in cultured cells (Chapter 6).

Further analysis of the microarray dataset using the Ingenuity program further concluded that the Notch signaling pathway was activated in the TG following Dex-induced reactivation. Notch family members were found to regulate viral promoter activity and stimulate BHV-1 productive infection (Chapter 5). Therefore, this technique was successful at identifying host factors that may play a role in the early events during BHV-1 reactivation from latency.

In this study, we wanted to test the hypothesis that other *alphaherpesvirinae* subfamily members utilize similar host cellular factors to regulate lytic gene expression. To this end, we examined the effect of the 6 Dex-inducible transcription factors as well as the Notch family of transcription factors to regulate HSV-1 promoter activity and productive infection. Slug and Notch 3 each activated HSV-1 productive infection 2.5 fold. Slug activated the ICP0 and ICP4 promoters approximately 6 fold, suggesting Slug may activate productive infection by activating the expression of important viral transcription factors. In contrast, Notch 3 did not activate any of the viral promoters tested. Notch 3 may promote productive infection by activating a viral gene which was not analyzed in this study or Notch 3 may enhance productive infection indirectly by creating a cellular environment more suitable for viral replication.

While none of the other transcription factors activated HSV-1 productive infection, several stimulated expression of important viral promoters. Notably, GATA6

activated the VP16 promoter 16 fold. SPDEF also activated the VP16 promoter 5 fold. Recent work by Thompson et al. (Thompson et al., 2009), using an *in vivo* approach to study HSV-1 reactivation, show that VP16 is expressed prior to IE genes during reactivation from latency. Furthermore, they conclude *de novo* synthesis of VP16 (due to specific activation of the VP16 promoter) is responsible for the initial events of reactivation from latency. The stress-induced cellular factors that regulate HSV-1 VP16 expression during reactivation and the promoter sequences that are important for the regulated expression of VP16 in neurons were not identified. These studies suggest GATA6 or SPDEF activation of the VP16 promoter could potentially induce reactivation from HSV-1 latency.

SPDEF and KLF4 activate the ICP0 promoter 17 fold and 21 fold respectively. Exogenous expression of ICP0, independent of other viral gene products, can initiate HSV-1 (Halford et al., 2001) or HSV-2 (Zhu et al., 1990) reactivation from latency using an *in vitro* system. Furthermore, in the absence of VP16, ICP0 enhances the ability of transfected viral DNA to initiate productive infection in Vero cells by 10,000-fold (Cai and Schaffer, 1989). Based on these and other observations, it has been hypothesized that ICP0 has the potential to initiate reactivation from latency *in vivo*. Therefore, it is possible that induction of ICP0 protein expression by SPDEF or KLF4 could initiate reactivation from latency.

While no definitive conclusions can be drawn regarding the relevance of these findings to the events that occur during *in vivo* reactivation in humans, these studies suggest several Dex-inducible transcription factors identified to stimulate BHV-1 viral

gene transcription and production infection, also have the potential to regulate HSV-1 transcription and/or productive infection.

Table 7.1. Dex-induced cellular transcription factors.

Three latently infected calves (60 days post infection with BHV-1) were treated with Dex for 3 hours, 6 hours, or 24 hours. TG were collected and total RNA was isolated as described previously (Workman et al., 2011). Cellular gene expression was analyzed using the Affymetrix bovine Gene Chip. The common name of the gene is provided in the first column. Second column (Fold Induction): the numbers are levels of induced gene expression at 3 (1st number), 6 (2nd number), or 24 hours (3rd number) after Dex treatment. The base line of expression was derived from 3 calves latently infected with BHV-1 (no Dex). Known functions of the respective genes are summarized in the third column.

Table 7.1

GENE NAME	FOLD INDUCTION	FUNCTIONS
PLZF	37,17,8	Transcription Factor: differentiation, growth control
SLUG	28,10,2	Transcription Factor: neural development, apoptosis, cancer
KLF4	12,9,3	Transcription Factor: SP-1 like binding activity, GC binidng
SPDEF	11,10,6	Transcription Factor: contains zinc finger
GATA6	11,3,2	Transcription Factor: binds GGA(A/T) sequence
KLF6	11,2,2	Transcription Factor: SP-1 like binding activity, GC binidng

Figure 7.1. Analysis of Dex-inducible transcription factors on HSV-1 productive infection.

Rabbit skin (RS) cells were cotransfected with 1 ug of the respective plasmids encoding a Dex inducible transcription factors along with 1 ug of KOS6 β HSV-1 DNA, which contains the Lac Z gene inserted into the ICP6 locus of HSV-1. At 24 hours after infection, cells were fixed, stained, and β -gal positive cells were counted as described in the material and methods. The results are the average of at least three independent experiments.

Figure 7.1.

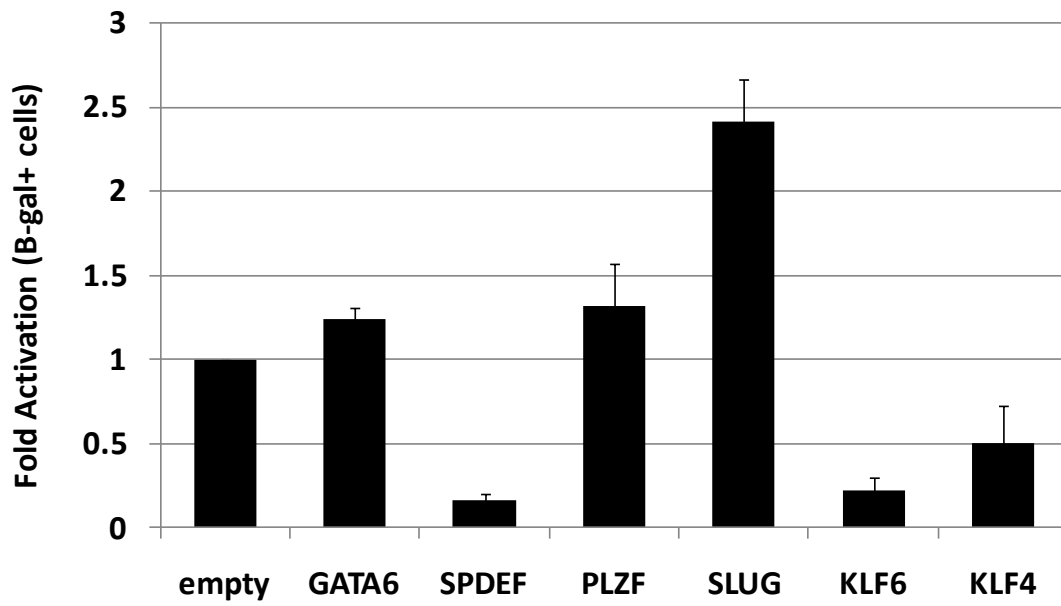


Table 7.2. Analysis of Dex-inducible transcription factors on HSV-1 promoter activity.

Mouse neuroblastoma cells (neuro-2A) were cotransfected with 1 ug of the designated reporter plasmid and 1ug of pcDNA3.1 empty vector or 1 ug of a plasmid expressing the designated Dex-inducible transcription factor. At 48 hours after transfection, reporter activity was measured as described in the material and methods. The numbers represent fold induction over the empty vector control. The results are the average of three independent experiments.

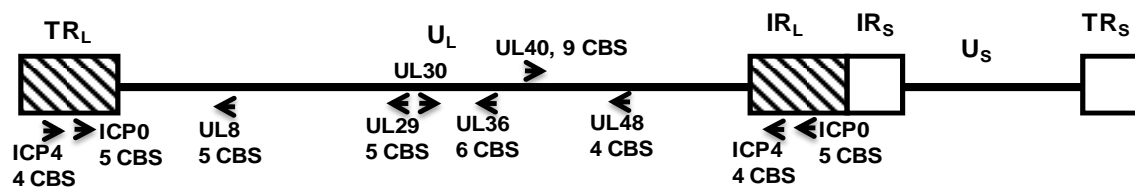
Table 7.2.

	GATA6	SPDEF	PLZF	SLUG	KLF6	KLF4
ICPO-CAT	NA	17±3.4	6±0.2	6.2±1	3.7±1	21.4±3
ICP4-CAT	NA	4±0.2	3±0.4	5.4±0.7	3.4±0.2	3.8±0.2
VP16-Luc	16±3	5.1±0.4	0.5±0.3	0.8±0.1	0.33±0.1	3.5±0.6
UL42-Luc	6.1±1.5	8.2±1.2	0.6±0.1	1.3±0.1	0.3±0.1	0.6±0.1
ICP6-Luc	5.7±0.8	1.8±0.2	0.76±0.1	0.7±0.1	0.5±0.2	3.4±0.6
gC-Luc	5.9±1.1	2.3±0.3	0.7±0.1	0.75±0.1	0.59±0.1	0.65±0.1
LAT-Luc	1±0.3	0.95±0.2	0.5±0.1	1.3±0.3	0.46±0.1	2.4±1

Figure 7.2. Predicted CSL binding sites in the HSV-1 genome.

Eleven variants of known canonical CSL binding sites have been identified (Persson and Wilson, 2010). A schematic of the HSV-1 genome, which consists of two unique regions (U_L and U_S) flanked by terminal repeats (TR_L and TR_S) and internal repeats (IR_L and IR_S), is shown. Listed are the number of CSL binding sites (CBS) located upstream of several important viral genes. Listed below are the functions of some of these important viral genes.

Figure 7.2.



ICP4: transcriptional regulator, specific DNA binding protein
ICP0: transcriptional regulator, modulator of cell state
UL8: component of DNA helicase-primase
UL29: single stranded DNA binding protein
UL30: DNA polymerase, catalytic subunit
UL36: large tegument protein
UL40: small subunit of ribonucleotide reductase
UL48: tegument protein, trans-activator of IE gene expression (VP16)

Figure 7.3. Analysis of Notch family members on HSV-1 productive infection.

Rabbit skin (RS) cells were cotransfected with 1 ug of the respective plasmids encoding Notch along with 1 ug of KOS6 β HSV-1 DNA, which contains the Lac Z gene inserted into the ICP6 locus of HSV-1. At 24 hours after infection, cells were fixed, stained, and β -gal positive cells were counted as described in the material and methods. The results are the average of at least three independent experiments.

Figure 7.3.

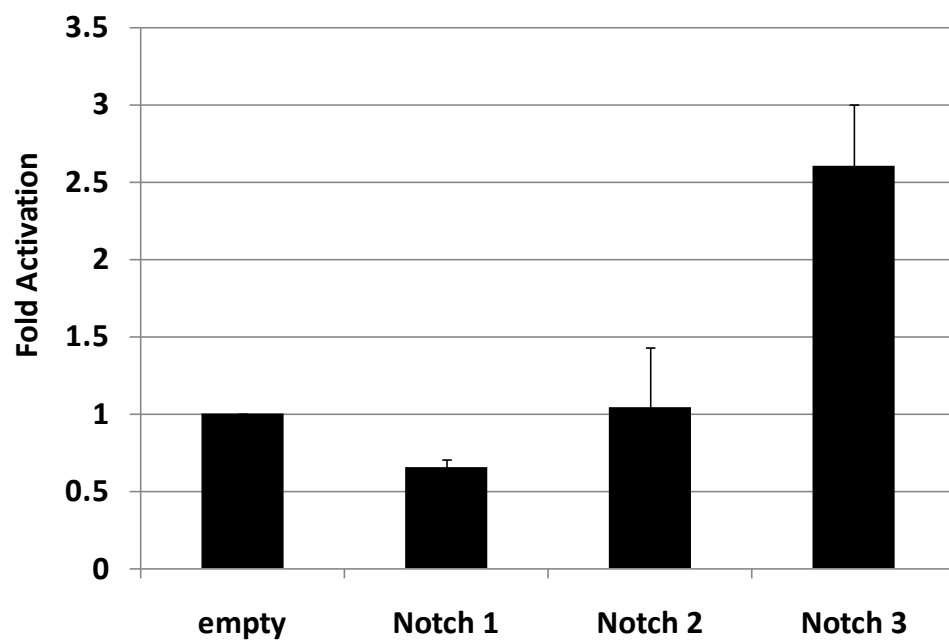


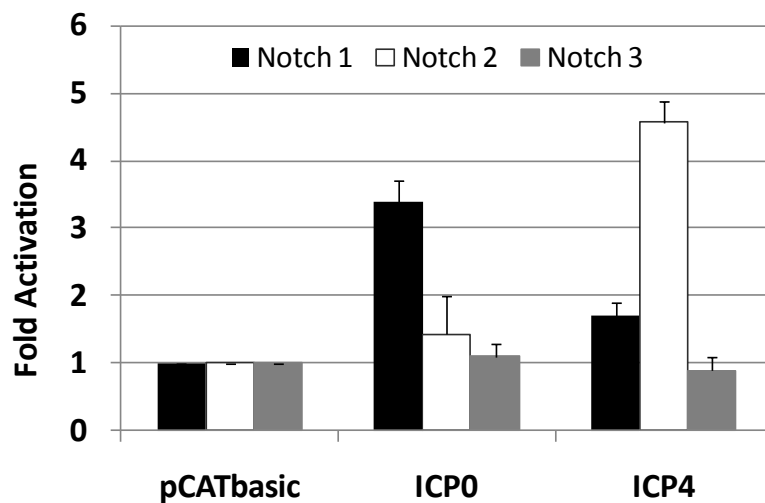
Figure 7.4. Analysis of Notch family members on HSV-1 promoter activity.

Panel A: Mouse neuroblastoma cells (neuro-2A) were cotransfected with 1 ug of the designated CAT plasmid and 1ug of pcDNA3.1 empty vector or 1 ug of a plasmid expressing the designated Notch family member. At 48 hours after transfection, CAT activity was measured. The numbers represent fold induction over the empty vector control. The results are the average of three independent experiments.

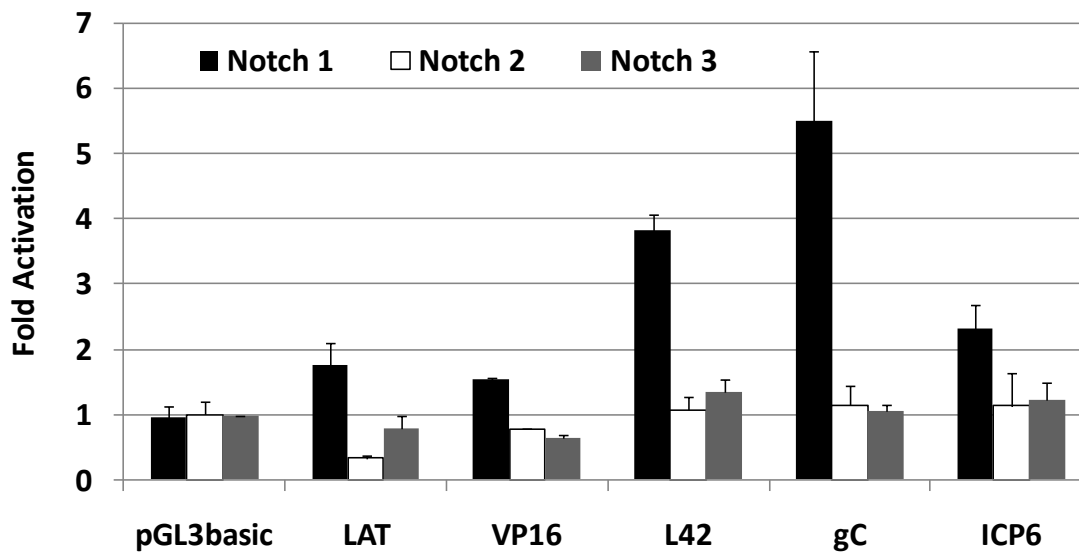
Panel B: Neuro-2A cells were co-transfected with 1 ug of the designated Luciferase construct and 1ug of pcDNA3.1 empty vector or 1 ug of a plasmid expressing the designated Notch family member. At 48 hours after transfection, Luciferase activity was measured. The numbers represent fold induction over the empty vector control. The results are the average of three independent experiments.

Figure 7.4.

A



B



General Conclusions

The work presented in this dissertation was aimed at elucidating the role of cellular factors in the latency-reactivation cycle of alpha-herpesviruses. Although considerable effort has been focused on how alpha-herpesviruses establish, maintain, and reactivate from latency, there remain significant gaps in our knowledge. The molecular mechanisms controlling the exit from latency are of central importance to recurrent disease and transmission, yet the cellular factors that play a role in this process remain unknown. Therefore, our lab studies BHV-1 in its natural host in an attempt to identify virus-host interactions important in the latency-reactivation cycle of alpha-herpesviruses.

A previous study demonstrated that the cellular transcription factor C/EBP-alpha is stimulated when latently infected calves are treated with Dexamethasone (Dex) to induce reactivation from latency (Meyer et al., 2007a). Furthermore, C/EBP-alpha stimulates productive infection when over-expressed (Meyer et al., 2007a), and C/EBP-alpha plus bICP0 stimulate IETu1 promoter activity more efficiently than just the viral trans-activator alone (Meyer and Jones, 2009). Additionally, C/EBP-alpha was unable to stimulate the IETu1 in the absence of bICP0 (Meyer and Jones, 2009). Previous studies also demonstrated that the bICP0 E promoter may be preferentially activated over the IETu1 during reactivation from latency (Workman et al., 2009). Therefore, we hypothesized that C/EBP-alpha may activate the bICP0 E promoter first, and then bICP0 and C/EBP-alpha together activate the IETu1 to stimulate reactivation from latency.

To test this hypothesis, the bICP0 E promoter was cloned into a reporter plasmid and the effect of C/EBP-alpha on reporter gene expression was evaluated. C/EBP-alpha stimulated bICP0 E promoter activity by directly binding to the viral promoter (Workman et al., 2009). However, activation of the bICP0 E promoter does not necessarily lead to

late gene expression and reactivation from latency (Workman et al., 2009). Therefore, although C/EBP-alpha appears to be an important component in the Dex-induced signaling cascade that stimulates viral gene expression, it seems clear that additional Dex-inducible cellular proteins are required for infectious virus to be produced (Chapter 2; Workman et al., 2009).

It was also known that during Dex-induced reactivation from latency, cyclin expression is stimulated in infected sensory neurons (Winkler et al., 2000b). Cyclin dependent kinase activity phosphorylates Rb (retinoblastoma tumor suppressor gene product) family proteins and consequently releases the E2F family of transcription factors. Over-expression of E2F4 stimulates BHV-1 productive infection and E2F1 or E2F2 trans-activates IETu1 promoter activity (Geiser and Jones, 2003). This suggests that E2F family members could have the potential to stimulate productive infection and/or reactivation from latency. Therefore, I silenced E2F1 using a siRNA to examine the effect of E2F1 on BHV-1 productive infection. Silencing E2F1 reduced productive infection approximately 5 fold (Workman and Jones, 2010). Furthermore, E2F1 activated the bICP0 E promoter more than 100 fold in transient transfection assays by directly binding to the promoter, suggesting E2F1 may play a role in the latency-reactivation cycle of BHV-1 by stimulating lytic cycle transcription (Chapter 3; Workman and Jones, 2010).

After characterization of previously identified cellular factors, we set out to identify and characterize a set of new cellular factors that may be involved in the latency-reactivation cycle of BHV-1. Microarrays were performed to identify cellular factors induced in the TG of latently infected calves following Dex treatment to induce

reactivation from latency. Six cellular transcription factors were identified to be induced greater than 10 fold at 3 hours after Dex treatment. Further studies showed that these Dex-inducible transcription factors were able to stimulate BHV-1 productive infection and specific BHV-1 promoters when analyzed in transient transfection assays, suggesting they may play a role in the latency-reactivation cycle of BHV-1 (Chapter 6).

Analysis of the microarray dataset using the Ingenuity program concluded that the Notch signaling pathway was activated in TG following Dex treatment of latently infected calves. This was of interest as the LR protein ORF2 was found to interact with Notch1 and Notch3 using yeast-two-hybrid analysis (Workman et al., 2010). I found that Notch1, but not Notch3, activates BHV-1 productive infection by activating expression of all 3 classes of viral promoters. Notch-induced activation of productive infection or viral gene expression is attenuated by co-expression of ORF2 (Workman et al., 2010). Although ORF2 inhibits apoptosis (Shen and Jones, 2008), this study suggested ORF2 also regulates viral transcription by interacting with cellular transcription factors. Two additional studies provide evidence that ORF2 may regulate transcription (Devireddy et al., 2003; Meyer et al., 2007a). Consequently, we suggest that the ability of ORF2 to inhibit apoptosis and interact with cellular transcription factors promote life-long latency (Chapter 5; Workman et al., 2010).

As BHV-1 and HSV-1 are both *alphaherpesvirinae* subfamily members, we hypothesized that they may require similar cellular factors for productive infection and reactivation from latency. Therefore, I analyzed the effect of Dex-inducible cellular genes on HSV-1 productive infection and viral promoter activity. These studies show that

certain transcription factors induced during BHV-1 reactivation from latency can stimulate certain HSV-1 promoters and productive infection (Chapter 7).

Based on the data presented in this dissertation, we hypothesize that stressful stimuli promotes exit from latency by activating specific cellular transcription factors, which consequently activate lytic viral gene expression and production of infectious virus. We further hypothesize that LR gene products maintain the latent infection by promoting cell survival and preventing lytic gene expression.

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