ROLE OF NUCLEAR HORMONE RECEPTORS IN GENE EXPRESSION OF ALPHA-HERPES VIRUSES AND IMPORTANT FUNCTIONS FOR MAINTAINING LATENCY

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Abstract: Bovine alphaherpesvirus 1 (BoHV-1) and human alphaherpesvirus 1 (HSV-1) establish life-long latency following acute infection. BoHV-1 can cause abortion in cattle and reactivation from latency frequently occurs during latter stages of pregnancy. During pregnancy, progesterone (P4) levels increase: consequently, I hypothesized P4 stimulates BoHV-1 reactivation from latency. My studies demonstrated, P4 stimulates productive infection in the presence of the progesterone receptor (PR). Since steroid receptors can increase expression of Krüppel like factor (KLF) family members, the cooperative effects of PR with KLF members were examined. My studies revealed BoHV-1 infected cell protein 0 (bICP0) early promoter is cooperatively transactivated by PR and KLF4. For reactivation to occur, a pool of neurons must be maintained at the site of latency i.e., trigeminal ganglia (TG). Latency related transcript (LRT) is abundantly expressed during latency and encodes a protein (ORF2). Surprisingly, ORF2 mRNA suppresses glucocorticoid receptor (GR) mediated transactivation of immediate early transcription unit 1 (IEtu1) promoter. Since IE gene expression is a key event in productive infection, I hypothesized ORF2 mRNA could inhibit productive infection as well. My studies revealed ORF2 RNA also inhibits the GR, KLF15 and DEX mediated productive infection. Furthermore, a host signaling pathway, Akt supports latency. Since Akt family members inhibit GR or/and KLF15 mediated transactivation of the BoHV-1 IE promoters I hypothesized that Akt does this by influencing GR protein expression. My study demonstrated, Akt-1 reduced GR expression in mouse neuronal cells.

HSV-1 reactivation from latency can cause recurrent ocular disease, oral-facial lesions (cold sores, and life-threatening encephalitis). The ICP0 protein is expressed under immediate early conditions and is a crucial transcriptional regulatory protein. The ICP0 full length promoter is cooperatively transactivated by GR, KLF15 and DEX. Consequently, I hypothesized that the ICP0 promoter contains independent cis-regulatory modules (CRMs) that are transactivated by GR, KLF15 and DEX. These studies revealed three independent CRMs within the ICP0 promoter were transactivated by GR, KLF15 and DEX and consensus specificity protein 1 (Sp1) were required for transactivation. Collectively, these studies demonstrate that nuclear hormone receptors and KLF members increase the incidence of BoHV-1 and HSV-1 reactivation from latency.

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CHAPTER I

REVIEW OF LITERATURE

1. Herpesviridae family

Herpesviridae is a family of DNA viruses that contains three subfamilies. There are approximately 115 members in this family of viruses [1]. They infect a wide variety of hosts ranging from amphibians to mammals [2].

1.1 Subfamilies

Currently there are three main subfamilies within the Herpesviridae family: alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. The genomic structure, speed of viral gene replication and the host range play a major role in deciding what subfamily a virus belongs to. Alphaherpesvirinae subfamily members have a wide host range and rapid viral DNA replication. The most common site of latency is the sensory ganglia [3]. Simplexvirus, Varicellovirus, Scutavirus, Mardivirus and Iltovirus are the genera in this subfamily, as shown in table 1.

Betaherpesvirinae subfamily members have a slow reproductive cycle and a restricted range of hosts. The infected cells usually fuse and give rise to cytomegaly. Furthermore, the site of latency

is lymphoreticular cells and certain secretory glands. The two main genera of this subfamily are cytomegalovirus and Muromegalovirus because they share a similar genetic structure [3, 4].

Gammaherpesvirinae members have a limited host range and the rate of viral DNA replication can be variable. The site of latency is lymphoid tissue. There are 7 genera in this subfamily as mentioned in Table 1[3].

Subfamily	**Genus**	**Species**
Alphaherpesvirinae	Simplexvirus	Human alphaherpesvirus-1 Human alphaherpesvirus-2 Bovine alphaherpesvirus-2
	Varicellovirus	Human alphaherpesvirus 3 Bovine alphaherpesvirus-1 Bovine alphaherpesvirus-5 Canid alphaherpesvirus 1 Caprine alphaherpesvirus 1 Cercopithecine alphaherpesvirus 9 Cervid alphaherpesvirus 1 Cervid alphaherpesvirus 2 Cervid alphaherpesvirus 3 Equid alphaherpesvirus 1 Equid alphaherpesvirus 3 Equid alphaherpesvirus 4 Felid alphaherpesvirus 1
	Scutavirus	*Chelonid alphaherpesvirus 5* *Testudinid alphaherpesvirus 3*

	Mardivirus	*Gallid alphaherpesvirus 2* *Gallid alphaherpesvirus 3* *Meleagrid alphaherpesvirus 1*
	Iltovirus	Cacatuid alphaherpesvirus 2 Gallid alphaherpesvirus 1 Psittacid alphaherpesvirus 1 Psittacid alphaherpesvirus 5
Betaherpesvirinae	Cytomegalovirus	Human cytomegalovirus (HCMV)
	Muromegalovirus	Murine cytomegalovirus
Gammaherpesvirinae	Lymphocryptovirus	Epstein Barr virus Kaposis sarcoma virus (KSHV/HHV-8)
	Rhadinovirus	Ateline gammaherpesvirus 2 Ateline gammaherpesvirus 3 Bovine gammaherpesvirus 4 Colobine gammaherpesvirus 1 Cricetid gammaherpesvirus 2 Human gammaherpesvirus 8 Macacine gammaherpesvirus 5 Macacine gammaherpesvirus 8 Macacine gammaherpesvirus 11 Macacine gammaherpesvirus 12 Murid gammaherpesvirus 4 Murid gammaherpesvirus 7 Saimiriine gammaherpesvirus 2
	Bossavirus	Delphinid gammaherpesvirus 1

	Macavirus	Alcelaphine gammaherpesvirus 1 Alcelaphine gammaherpesvirus 2 Bovine gammaherpesvirus 6 Caprine gammaherpesvirus 2 Hippotragine gammaherpesvirus 1 Ovine gammaherpesvirus 2 Suid gammaherpesvirus 3 Suid gammaherpesvirus 4 Suid gammaherpesvirus 5
	Manticavirus	Phascolarctid gammaherpesvirus 1 Vombatid gammaherpesvirus 1
	Patagivirus	Vespertilionid gammaherpesvirus 3
	Percavirus	Equid gammaherpesvirus 2 Equid gammaherpesvirus 5 Felid gammaherpesvirus 1 Mustelid gammaherpesvirus 1 Phocid gammaherpesvirus 3 Rhinolophid gammaherpesvirus 1

Table-1 Classification of herpesviruses

1.2 Structure

The herpes virion is one of the largest among animal viruses and is 120-300nm in diameter. The structure of the virus contains 4 different components. The inner core contains the linear double

stranded DNA which is surrounded by an icosahedral capsid. The capsid contains approximately 160 capsomeres. Moreover, the capsid is approximately ~100nm in diameter [3]. The proteinaceous layer surrounding the capsid is denoted as the tegument and contains various proteins including virion protein 16 (VP16) and the infected cell protein 0 (ICP0). Tegument is known to have a negative staining in electron microscopy and is not uniform in distribution inside the infected cell [3]. The outermost covering is the envelope which contains lipids coming from the cellular membranes. It also contains spikes made up of glycoproteins that are important for virus attachment to host cells, viral entry and immune responses [5]. Three glycoproteins are conserved among Herpesviridae family members which help in fusion with the cell membrane, named glycoprotein B (gB) and gH-gL [6]. The similarity of gH between gamma herpesviruses are higher than that of between alphaherpesviruses. [7]

1.3 DNA structure and composition

The size of the genome varies between 120-230 kbp. One special feature about herpes viruses is the presence of repeated sequences throughout the genome. These reiterations can occur in various patterns. The most common reiteration pattern is terminal repeats, when one terminal sequence is repeated exactly as it is in the other end of the DNA strand. Some of these terminal repeats can repeat in the middle of the strand which divides the DNA strand into two sections and these repeats are known as direct repeats. Usually, direct repeats give rise to a unique shorter (S) and a unique long (L) region. Another occurrence is when a copy of the terminal repeat is itself repeated in an inverted orientation thus known as inverted repeats [3].

1.4 Viral replication cycle

All members of the Herpesviridae have two phases during their life cycle [8]. Phase1 is the acute lytic viral gene expression and phase 2 is the latency. During acute infection all viral genes are expressed in a temporal fashion: for example, Immediate early (IE/α), early (E/β) and late (L/γ) [9].

There is a sequence of steps to produce progeny virions once a cell is infected with an infectious virus. Such as viral entry, gene expression, genome replication, virion assembly and egress [9].

During the first step in adsorption, the BoHV-1 gD binds heparan sulfate on the host cell membrane [10, 11]. Then the viral envelop fuses with the cell membrane leading to entry of the viral nucleocapsid into the cytoplasm. Then, the virus travels into the nucleus via dynein-microtubule mediated mechanism [12]. Viral tegument proteins (VPs) are also released at the same time. A virion protein, VP16 is responsible for initiating viral gene transcription of α / IE genes. IE gene expression does not require de-novo protein synthesis, which means immediately after the nucleic acid is released into the nucleus IE gene transcription is initiated. The protein machinery which is necessary to initiate IE gene expression is supplied by host cellular factors and viral proteins like VP16. HCF-1 and Oct-1 are host transcription factors that are crucial for binding with enhancer core motifs or TAATGARAT sequences on IE promoters. Availability of VP16 in the viral tegument and HCF-1 and Oct-1 in the host cells makes IE gene expression more efficient [13].

IE gene products (ICP0, ICP4, ICP22, ICP27) will stimulate E gene expression and E genes are involved in viral genome replication [14]. E genes then activate late genes which produce structural proteins. Capsid proteins synthesized by late gene expression initiate virion assembly in the nucleus [15]. Then, the nucleocapsid egresses from the nuclear pore or nuclear membrane to the cytoplasm. The tegument is acquired by the nucleocapsid in the cytoplasm. Finally, the virion buds off/egress from the cell membrane acquiring the envelope [16].

2.Bovine alphaherpes viruses

Bovine alphaherpesviruses infect the Bovidae family including large ruminants i.e., cattle and buffalo, small ruminants, and pigs, but the clinical disease is mainly evident in cattle and buffalo. There are several bovine alphaherpesviruses that infect these hosts. For example, bovine alphaherpesvirus-1 (BoHV-1), BoHV-2 and BoHV-5 are the most clinically significant members of

this group. BoHV-1 is also known as bovine rhino tracheitis virus (BRTV) because of the respiratory symptoms it causes. This BoHV-1 is also one of the causative agents in bovine respiratory disease complex (BRDC) which leads to at least a billion-dollar economic losses (directly and indirectly) to the beef cattle industry in the United States annually. In addition to respiratory disease, BoHV-1 can cause genital disease in both male and female cattle. Infection of the genital tract leads to infectious pustular-vulvovaginitis (IPV) in females and infectious pustular balanoposthitis (IPB) in male animals. This is a highly contagious disease among cattle farming industry due to the close housing of animals because close proximity of animals makes it easier to spread [17].

2.1 Acute infection of BoHV-1

Upon exposure to BoHV-1, animals undergo a series of non-life-threatening to life-threatening clinical signs, as discussed below. The incubation period ranges from 10-20 days and depends on the animal's immunity. There can be different forms of disease in cattle, such as respiratory, genital, conjunctival and encephalomyelitic. The respiratory form, or Infectious bovine rhinotracheitis (IBR) can reach up to 100% morbidity but only 10% mortality. It starts with bilateral serous nasal discharge with *hyperemic* nasal *mucosae*. This discharge may become purulent later due to secondary bacterial infections. Other clinical symptoms include deep bronchial cough, dyspnea, depression and reduced milk yield in lactating cows. Usually, the respiratory form ends up in pneumonia however the uncomplicated cases resolve within 10 days. BoHV-1 also can cause abortion by crossing the placenta in pregnant animals. Even after animals recover, they remain carriers for their entire life due to establishment of latency and reactivation from latency [17].

2.1.1 Bovine respiratory disease complex (BRDC)

BRD is a polymicrobial syndrome that occurs in calves in part due to transportation stress, hence this disease is also called shipping fever. The prominent clinical sign is the breathing difficulty and is the

number one mortality among beef cattle worldwide [18]. It is caused by contribution of several pathogens including viruses and bacteria, viruses being the predisposing factor. BoHV-1, bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus, parainfluenza 3 virus are the main viral pathogens involved in BRD. The ability to suppress immunity by attacking immune cells by BoHV-1 and BVDV predispose the animal to opportunistic bacterial infections such as *Manheimia hemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycolplasma bovis* [19, 20]**.**

2.2.2 Conjunctival form

Conjunctival form may occur concurrently with respiratory form. Extensive tear production is the most common sign. Due to secondary bacterial infections the lacrimation can start to become purulent and can damage cornea by permanent scarring [17].

2.2.3 Encephalitic form

Encephalitic form is caused by BoHV-1.3/BoHV-5 strain which is the neuro-invasive form. This form is mainly isolated from South American countries [20, 21]. BoHV-1.3 is genetically and antigenically similar to BoHV-1 however the former has the ability to extensively replicate in central nervous system [22]. Animals develop ataxia following incoordination. Tremors and opisthotonos are also among common clinical signs. These animals finally end up in coma and die within two weeks of contracting the infection [17].

2.2.4 Genital form

Genital forms of BoHV-1 infection can result in IPV and IPB as mentioned before. Both IPV and IPB have <5days of incubation period. Further, IPV is characterized by frequent micturition due to irritation of the vulva. Secondary bacterial infections may lead to formation of pustules in the labia, which gives rise to purulent vaginal discharge [17].

IPB leads to inflammation of the male genitalia, which is manifested by pustules in the penis and prepuce. Inflammation may lead to adhesions and distortion of the penis. These clinical signs usually resolve within 2 weeks. Moreover, if breeding continues during the disease, it might infect all the animals who are inseminated with infected semen [17].

2.2.4 BoHV-1 induced abortions

Abortions in dairy cattle lead to huge economic losses due to reproductive failure and reduced milk yield [23]. Furthermore, abortions caused by BoHV-1 can occur by natural infection or vaccination with a modified live vaccine (MLV). Infection of naïve unvaccinated herds leads to 25-60% abortion rates [24]. A respiratory infection can cross the placenta of pregnant dams and may develop fetal lose due to retention of placenta [25]. Moreover, under natural conditions, the abortions caused by BoHV-1 are mostly late term abortions. Infection/exposure at the time of breeding gives rise to infertility and infection at early gestation leads to early embryonic death [24]. Whether this is due to pregnancy related hormones or maternal stress caused by growing fetus towards the later part of the pregnancy is not clear. Finally, if an infection occurs during estrus period, reduced fertility rates are observed [26].

Experimental intravenous, intrauterine or intramuscular virus inoculation right before estrous leads to lesions in the corpus luteum similar to lesions induced by vaccinating cows by MLV [27]. Interestingly, BoHV-1 replicates efficiently in ovary and the corpus luteum [28]. Furthermore, in most aborted fetuses, focal necrosis in histopathologic sections is found in various tissues, for example liver, kidney, thymus, lymph nodes even though gross lesions are not evident. In the dam, focal necrosis can be seen in endometrium and in placenta [26].

2.3 Gene expression

A virion protein, VP16 is responsible for initiating viral gene transcription of α / IE genes. Immediate early transcription unit 1 (IEtu1) and IEtu2 of BoHV-1 are transactivated by binding of VP16 to TAATGARAT enhancer motifs via a complex formed by host cellular factor-1 (HCF-1) and a

cellular transcription factor Oct-1. The genes encoded by IEtu1 are bICP0 and bICP4. bICP4 is crucial for productive infection and bICP0 can activate all classes of viral genes and interfere with IFN response thus are crucial for virus replication [29]. Furthermore, there is an early promoter to transcribe ICP0 as well in BoHV-1 which is known as ICP0 early promoter [30]. IEtu2 encodes bICP22, which is also important for early gene expression [31].

In contrast, HSV-1 has separate IE genes for ICP0, ICP4, ICP22, ICP27 and ICP47 that are transcribed immediately after viral entry via the complex formed by VP16, HCF-1 and Oct-1.

2.4 BoHV-1 establishment of latency

The only abundant transcript found during latency is latency related transcript (LRT) in BoHV-1. Neural factors might be transactivating the LRT promoter specifically [32].

LRT has crucial functions in maintaining latency: for example, inhibiting apoptosis of neurons, blocking cell cycle progression and inhibiting bICP0 expression. LRT has 4 open reading frames (ORFs) named ORF1, ORF2, ORF B and ORF C. However only ORF2 encodes for a protein which is abundantly expressed during latency [29]. ORF2 mutant sequence, which cannot encode a protein also inhibits IEtu1 of BoHV-1 supporting the concept ORF2 regulates gene expression without protein translation [33].

2.5 BoHV-1 vaccines

There have been several types of vaccines produced against BoHV-1 over the years, such as killed vaccines, Modified live vaccines (MLVs), subunit vaccines and replication impaired vaccines. All the US based MLVs are extensively passaged in cell culture to attenuate. Thus, they can reactivate under stress. However, vaccines based in Europe, lack a gene encoding a glycoprotein or an enzyme important for replication. The majority of European based killed and MLVs have a deletion in

glycoprotein E (gE). The main importance of gE is it mediates anterograde axonal transport of the virus [34].

Modern European MLVs contain a deletion in a viral encoded thymidine kinase (TK) enzyme in addition to glycoproteins because TK enhances the propagation of viruses to the nervous system, thus acting as a link for establishment of latency. The viral TK is also important because it can phosphorylate nucleoside analogues such as acyclovir, and thus is a crucial target for developing therapeutics [34]. However, TK is not deleted in many MLVs developed in the United States (US).

MLVs provide robust protection against respiratory disease compared to killed vaccines. However, administration of MLVs always leads to establishment of latency. The most common problem is reactivation of this virus in pregnant cows because it leads to abortion. On the other hand, the killed vaccines offer more protection against abortions, but the immune response is weaker for IBR. Thus, there is growing evidence that factors involved in pregnancy also may reactivate the latent virus [31].

3. Human alphaherpesvirus 1 (HSV-1)

3.1 Epidemiology and acute infection

HSV-1 is a highly contagious pathogen that is spread throughout the world. It is reported that 100% of human cadavers above 60 years of age are positive for HSV-1. The main mode of HSV-1 transmission is through contact with infected body secretions [35].

Acute HSV-1 infection can cause orofacial symptoms or genital symptoms; however, the majority of the cases are asymptomatic. Orofacial clinical signs include herpes labialis (cold sores) around the mouth (lips) and eye disease (keratitis). Infection of the eye can lead to conjunctivitis, epithelial keratitis, stromal keratitis, iridocyclitis and blindness. Cold sores are the most frequent lesion that are painful ulcers in lips, buccal mucosae, gingivae, tongue, and palate. Although the primary genital infection is uncommon, secondary genital infection can occur through oral-genital transmission and is

manifested by painful blisters in the genital region with fever. The most severe complication of HSV-1 infection is viral encephalitis which can leave permanent neurological impairments. In fact, HSV-1 is the number one cause of viral encephalitis in the world [36].

3.1.1 HSV encephalitis

Herpes simplex encephalitis (HSE) can be caused by either HSV-1 or HSV-2, but the majority is due to HSV-1. HSE does not have a sex predisposition or a seasonal variation. However, it is more common in people below 20years of age and above 50 years of age and also common in immunocompromised individuals i.e. AIDS, bone marrow transplantation etc. [37].

HSE is mostly caused during reactivation from latency. The route of infection of the central nervous system (CNS) can be via olfactory nerve and trigeminal nerve since the primary site of latency is trigeminal ganglia. However, the hematogenous spread to CNS has also been reported.

How the virus evades host immune system during HSE is under discussion. A viral protein encoded by HSV-1, ICPγ34.5, is known to block autophagy in neurons by binding to a host cellular protein called Beclin-1 which is a major mediator in autophagy. Thus, making ICPγ34.5 a neurovirulence factor for HSE [37, 38].

Furthermore, HSE can cause permanent neurological impairments such as personality changes, cognitive changes, changes in consciousness etc. Diagnosis of HSE is primarily by clinical signs such as fever, headache, and neck stiffness. Confirmatory diagnosis requires analysis of cerebrospinal fluid (CSF), which shows clear pleocytosis, brain biopsy (gold standard) or PCR to detect HSV DNA in blood [37]. However, MRI is also considered as a method of gold standard confirmatory diagnosis for HSE.

The recommended therapy for HSE is acyclovir 10mg/kg intravenously every 8hrs for 2-3 weeks. Acyclovir only acts on actively virus replicating cells by inhibiting viral DNA polymerase by

competitive inhibition [37]. Many HSV-1 isolates that are resistant for Acyclovir adopt this mechanism via deficiency in viral TK enzyme activity because to phosphorylate Acyclovir i.e., activate, viral TK is essential. There have been mutant viruses which have low/deficient TK activity reported such as TK Negative (TK^N), TK partial (TK^P), TK altered (TK^A) contain no TK, reduced levels of TK activity and does not phosphorylate acyclovir respectively [39].

3.1.2. Herpes simplex keratitis (HSK)

HSK is known to be the number one cause of corneal blindness in the United States and worldwide [40]. In developed countries HSK is prevalent among the young population (i.e. early childhood). During reactivation the latent virus in trigeminal ganglia (TG) can travel anterogradely in the ophthalmic branch of trigeminal nerve leading to eye infection [40].

Moreover, the clinical signs of HSK can be blepharitis, conjunctivitis, corneal epithelial keratitis, and stromal keratitis. Stromal keratitis causes severe infection of the deeper layers of the cornea which can lead to permanent blindness as well. Usually, the recurrent disease is characterized by ulcerative/stromal keratitis [41].

3.1.3 Congenital /neonatal herpes

Neonatal herpes is a less frequent consequence of HSV-1 compared to genital infection in adult population. Further, the incidence can vary between 1:3000 to 1:20000 live births[42].

Congenital HSV can be caused by either HSV-1 or HSV-2. There are two different routes of infection of the fetus i.e., through the placenta and via ascending infections. The risk of neonatal herpes is higher in newborns of mothers' who contracted the infection during the last trimester of pregnancy than mothers who have episodes of recurrent disease [42].

Most of the neonatal herpes is due to contracting the virus during birth. Furthermore, congenital herpes can give rise to hydranencephaly, eye infection, skin scarring and death [35]. Thus, delivery via cesarean section is preferred in mothers who have an active infection [42].

4. Establishment of latency

After mucosal infection of oral, ocular, or nasal cavities, the virus is transmitted to nerve endings/axons innervating those regions and transported retrogradely to peripheral nervous system i.e., trigeminal ganglia (TG). Although TG is a primary site for latency, other neurons can support a latent infection.

Neurons lack cellular factors that support high levels of lytic viral gene expression and viral replication. Hence, latency is established.

During establishment of latency, lytic viral gene replication is extinguished. The only abundant transcript found during latency of HSV-1 is latency associated transcript (LAT). LAT is a complicated locus which encodes numerous non-coding RNAs, and it helps to establish and maintain a latent infection [43].

4.1 Molecular pathways involved in BoHV-1 and HSV-1 latency-reactivation cycle

To maintain latency, host and viral factors must prevent neuronal death and lytic viral gene expression and promote neuronal functions. In support of this hypothesis, previous studies demonstrated that canonical Wnt signaling/Beta-catenin pathway is upregulated during latency [44].

The Wnt signaling pathway is comprised of Wnt receptors and co-receptors, which can bind Wnt agonists/antagonists [45]. This pathway enhances neuronal differentiation and survival, thus supporting maintenance of latency [46]. In an unstimulated cell, the Wnt pathway is turned off and thus there is no beta catenin found in the nucleus because the cytoplasmic destruction complex phosphorylates and degrades beta catenin in a proteasomal dependent manner. However, when Wnt

agonists occupy Wnt receptors, the destruction complex is inactive, beta-catenin is stabilized and translocated into the nucleus to initiate T cell factor (TCF) mediated transcription [47]. Interestingly, previous studies demonstrated Wnt antagonists inhibit HSV-1 productive infection [48].

Conversely, in TG of calves latently infected with BoHV-1, beta-catenin was detected whereas during DEX induced reactivation from latency, fewer beta-catenin positive neurons were detected. ORF2, encoded by the BoHV-1 LR gene, interacts with beta-catenin and stimulates beta-catenin mediated transcription [49].

The phosphatidylinositol 3-kinase (PI3K) /Akt pathway is also predicted to support neuronal differentiation and survival. [50]. There are 3 members of Akt family: Akt1, Akt2 and Akt3. Akt3 is expressed extensively in TG in calves latently infected with BoHV-1 but reduced following DEX treatment and reactivation from latency [51].

Finally, the NF-kB signaling pathway is also predicted to maintain latency by promoting neuronal survival [52].

5.Reactivation from latency

5.1 BoHV-1 reactivation from latency induced by the synthetic corticosteroid dexamethasone.

The latent virus in the nervous system is activated by some form of stress: physical, emotional, or following corticosteroid treatment. With respect to cattle, food and water deprivation, transport stress or extreme weather changes are important stressors. Activation of BoHV-1 leads to transportation of virus via axons anterogradely back to mucosal surfaces leading to virus shedding. This process is defined as reactivation from latency. However, reactivation and virus shedding are two different phenomena. Due to stressful stimuli, viral gene expression and limited virus production can occur. In this scenario, not all reactivation episodes lead to virus shedding from mucosal surfaces. If the

animals are vaccinated or exposed to natural infection, they may have sufficient antibody titers to impair virus shedding and transmission[29].

Stress can be mimicked by administering exogenous glucocorticoids, including the synthetic corticosteroid dexamethasone (DEX) [53]. Six hours, after administering DEX to calves or rabbits-latently infected with BoHV-1, triggers lytic viral replication in TG and virus shedding [54]. DEX, like all corticosteroid derivatives, binds to the glucocorticoid receptor (GR) or mineralocorticoid receptor (MR) which in turn activates GR/MR. Activated GR (aGR) dimerizes and translocates to the nucleus where it binds GR response elements (GREs) in the genome (GGAACAnnnTGTTCT) [55] [56]. Interestingly, BoHV-1 and HSV-1 genomes contain many GREs, which can bind activated aGR [57]. Consequently, stress can directly stimulate viral gene expression, culminating in reactivation. It is predicted that only a subset of neurons eventually undergoes lytic viral replication and virus production. This leads to anterograde transport of virus particles to mucosal surfaces in craniofacial region if the latency is established in TGs. Conversely, reactivated virus can occasionally enter the CNS resulting in infection of other neurons or cause viral encephalitis [58].

In studies where a BoHV-1 LR gene mutant was examined, the level of virus progeny shedding from tonsils, eyes or TG are reduced compared to wt-BoHV-1 [59].

The LR gene mutant does not shed detectable levels of infectious virus during DEX induced reactivation. Furthermore, the LR gene mutant induces high levels of neuronal apoptosis in TG, which correlates with undetectable levels of virus shedding during DEX-induced reactivation [29]

5.2 HSV-1 reactivation from latency

In general, HSV-1 reactivation in humans occurs due to exposure to UV light, emotional stress, high fever or diseases that cause immunosuppression. Reactivation occurs in a sequential manner. First the relaxation of the viral chromatin occurs which is otherwise densely packed with histones. Next,

transcription of lytic viral genes occurs, and viral proteins are synthesized leading to production of infectious virion. However, not all neurons complete each step thus full reactivation occurs only in a subset of neurons [60]. To mimic HSV-1 reactivation, in vivo and in vitro models have been used. Nerve growth factor (NGF) deprivation causes HSV-1 reactivation and can occur in humans during adolescence. Furthermore, interruption of cellular signaling pathways also lead to reactivation such as inhibition of PI3K pathway [61].

The expression of lytic viral genes during reactivation occurs in two phases. During the initial phase 1, the IE, E and L genes are expressed by phosphorylation by cellular JNK in the absence of tegument protein VP16 [62]. However, the Phase 2 is dependent on tegument protein VP16 and leads to production of infectious virions [63].

Reactivation from latency has been well documented in patients that undergo trigeminal root surgery where the axotomy occurs [64]. Axotomy turns on cellular pathways that supports reactivation and this mechanism is adopted in ex vivo reactivation such as explant induced reactivation [65]. During explant induced reactivation, HCF-1is translocated in to the nucleus and occupies IE gene enhancer regions leading to IE gene transcription [66].

Moreover, high emotional stress leads to release of epinephrin and activation of hypothalamic pituitary adrenal axis [67]. The downstream effects of activation of these two pathways trigger reactivation. Importantly, a synthetic corticosteroid dexamethasone can reactivate the HSV-1 in TG explants and primary neuronal cultures [62].

6.Nuclear hormone receptors regulate reactivation from latency.

Glucocorticoids are steroid hormones which are secreted from adrenal cortex in response to stress. The glucocorticoid secretion is regulated by the hypothalamic pituitary axis. In response to stress, the hypothalamus secretes adrenocorticotrophic hormone (ACTH) which acts on the anterior pituitary. The pituitary gland will synthesize and secrete corticotrophin releasing hormone (CRH), which enters

the blood stream to reach the adrenal gland. Once CRH reaches its receptor organ i.e., the adrenal gland, it stimulates the adrenal cortex to produce and secrete corticosteroids such as glucocorticoids, mineralocorticoids etc. These hormones can enter a cell by penetrating the cell membrane thus will start to contribute to cellular signaling pathways initiated by binding to its receptor, GR which ultimately leads to changes in GR dependent gene expression [68].

Finally, synthetic glucocorticoids are used to treat inflammatory diseases and to combat autoimmune diseases at immune suppressive doses [68].

6.1 Glucocorticoid receptor (GR)

GR is a nuclear hormone receptor which belongs to the subfamily 3 of steroid hormone receptors (SR). As shown in Figure 1, the GR protein has 4 different regions: an immunogenic domain or AF-1 at the amino terminal, DNA binding domain (DBD), and a ligand binding domain (LBD) at the carboxy terminal. DBD and LBD are separated by a hinge region (HR). Alternative splicing of GR RNA gives rise to two isoforms named GR alpha (GRα) and GR beta (GRβ). In addition to the shared sequences, GRα contains an additional 50 amino acids while GRβ contains only 15 additional amino acids thus the molecular weights are 97kDa and 94kDa respectively. GRα is abundantly present in all cells and positively regulated by glucocorticoids. However, GRβ negatively regulates GRα and does not contain ligand binding activity [69].

Once GR is activated, the DBD of GR binds GREs in DNA. The mineralocorticoid receptor also can bind GREs [69].

Inactive GRα in cytoplasm is bound to hsp90 and other proteins. Upon binding to ligand, nuclear localization signals of GR are unmasked and hsp90 detached. Consequently, GR enters the nucleus through nuclear pores to bind with GREs in certain promoters. Moreover, activated GRα recruits more GRα to the nucleus, which is known as assisted loading. GRα also improves accessibility of

other transcription factors to chromatin as well. Once GRα dependent transcription has occurred, GRα is either returned to cytoplasm and associates with hsp or degraded via ubiquitination [69].

There are two types of DNA sequences to which GRα binds. Palindromic sequences separated by 3 nucleotides i.e., PuGNACANNNTGTNCPy (Pu-Purine, N-any nucleotide, Py-Pyrimidine) bind two GRα molecules and transactivate GR responsive genes. Binding of GR to negative GREs or nGREs, which are quadrimeric palindromes, causes trans-repression [69].

GRα also interacts with other transcription factors to modulate transcription. Usually when promoters lack GREs, GR binds DNA indirectly by binding to other transcription factors to either transactivate or trans-repress the gene activity. Moreover, SR family also contain other nuclear hormone receptors with similar properties such as estrogen receptor, progesterone receptor and androgen receptor as well [69].

GRα phosphorylation is essential for transcriptional activation. Moreover, only phosphorylated GRα can bind with its ligand. There are different sites of phosphorylation of GR protein, and they differ between human, rat, and mouse. The most important phosphorylation site of GR in human is Ser211 and its mouse counterpart is Ser229.

Furthermore, GRα can be phosphorylated by different kinases: for example, glycogen synthase kinase-3 (GSK-3), c-Jun N terminal kinases (JNK), Cyclin dependent kinase-2 (CDK-2) bound to cyclin A or cyclin E [70].

Figure-1 structure of glucocorticoid receptor (GR)

7. Progesterone and its role during reactivation from latency

Progesterone is a steroid hormone which is responsible for female reproduction. It is secreted mainly by corpus luteum of the ovary. The release of progesterone from the ovary is stimulated by luteinizing hormone (LH) in response to follicle stimulating hormone (FSH) released from the anterior pituitary. P4 suppresses the myometrial contractions and maintains pregnancy. Interestingly, P4 also secreted in the nervous system and is responsible for the regulation of sexual behavior [71].

P4 binds to the progesterone receptor (PR) and this binding translocates this complex into the nucleus. However, estrogen, another female reproductive hormone also can bind PR thus making it difficult to identify functions of P4 alone [72].

7.1 Progesterone receptor (PR)

Progesterone receptor belongs to the same superfamily as GR does. PR binds to a steroid hormone called Progesterone (P4). Unbound PR solely lies in the cytoplasm. Gene encoding for PR gives rise to two PR isoforms named PR A and PR B using two different promoters. PR B is the full form of PR, and PR A has a truncated nuclear localization signal sequence thus has a lower molecular weight compared to PR B. Once bound to progesterone, PR-P4 complex translocates into the nucleus and binds consensus PR responsive sequences in DNA. Interestingly, GR and PR share the same hormone response element. Previous studies have shown that the most common DNA binding sequence for PR is progesterone response element or PREs. However, the majority of PREs are in nucleosome rich regions of DNA which raises the question how PR gets access to PREs? There is evidence to support the fact that PR itself act as a pioneer transcription factor thus remodels nucleosomes in order to achieve optimal binding to PREs enriched with nucleosomes [73].

8.Stress induced transcription factors identified in TG

8.1 Krüppel like factor (KLF) family members

KLF members are C2H2 containing zinc finger transcription factors that binds DNA and regulate transcription. There are 17 KLF family members that have been identified. The regulatory sequences at amino terminal of different KLF family members varies such that their interaction with co activators or corepressors also varies [74]. Specific KLF family members regulate cell differentiation, proliferation, and cell death. Further, KLF family members are related to specificity proteins (Sp) and Krox zinc finger proteins. KLF members bind to either GC-rich or consensus CACCC sequences in DNA because of the zinc finger domains that are highly conserved [74]. Certain KLF family members are upregulated in TG of calves during DEX-induced reactivation: for example, KLF4, KLF15, KLF6 and promyelocytic leukemia zinc finger (PLZF) [54].

KLF4 is one of three transcription factors that can induce pluripotent stem cell differentiation. KLF4 has a transactivation domain (TAD) at its amino terminus, a trans repressor domain (TRD) in the middle and three Zn fingers at the carboxy terminal. Effects of KLF4 relies on the availability of DNA sequences which binds either TAD or TRD [71]. KLF4 negatively affects cell proliferation by up or downregulating genes that mediates cell cycle. Others have shown that proliferation of cells that lack the tumor suppressor, p53, is reduced in the presence of KLF4. These observations support the concept that KLF4 inhibits cancer cell progression [75, 76]. For example, KLF4 expression is lost during colon cancer, squamous cell carcinoma and gastric cancer. In contrast, KLF4 is extensively expressed in skin epithelium and protects the skin from UV light exposure. Finally, KLF4 is also a transcription factor in nerve growth factor gene expression [75].

KLF15 is highly expressed in kidney, pancreas, heart, and skeletal muscle. Its structure differs from the other KLF members by having N terminal Serine and a proline. Furthermore, KLF15 has a high affinity to bind with CACCC and GC box sequences of DNA and known to have an essential role in cardiac disease prevention [77]. KLF15 is also required in glucose and lipid metabolism. It regulates

expression of genes associated with lipid metabolism. Cardiac myocytes have crucial metabolic demands, thus imbalances of KLF15 affects cardiac muscle functions leading to cardiac disease [78].

8.2 Sp1 and KLF belong to a superfamily of transcription factors.

Specificity protein (Sp) family members are zinc finger transcription factors and there are 8 members; Sp1-Sp9 [74]. They have a high affinity to bind with GC boxes. Sp1 has a similarity in its carboxy terminal DNA binding domain to KLF family members. Sp family members bind to 'Sp1 sites', which are abundant in many Sp/KLF responsive promoters of housekeeping genes. Sp1 activity is highly regulated in cells due to post translational modifications. Further, Sp1 sites in promoters of vascular endothelial growth factor (VEGF), thymidine kinase (TK) and insulin like growth factor (IGF) binding protein-2 have shown to induce cellular growth. Sp1 can either cooperate with other KLF/Sp family members or antagonize activity of specific promoters [76]. Interestingly, Sp1 interacts with other transcription factors including KLF4. Finally, Sp1 sites are abundant in promoters of pro and antiapoptotic genes such as Bak, Bax, Bcl2 and Bcl3. Hence, Sp family members can regulate apoptosis [76].

9.GR and stress-induced transcription factors stimulate key viral promoters

Previous studies demonstrated GR and additional stress-related transcription factor expression is enhanced during DEX induced reactivation of BoHV-1 [54]. These cellular transcription factors (TFs) include Krüppel like factor (KLF) family members such as KLF15, KLF4, KLF6 and PLZF (Promyelocytic leukemia zinc finger factor). Interestingly, HSV-1 promoters/enhancers also contain GC rich motifs that can be bound by Sp1 and/or KLF family members. Several KLFs and GR have corporative effects on BoHV-1 regulatory promoters [79-82]. Studies have demonstrated that aGR/activated GR cooperates with KLF4 to transactivate bICP0 E promoter. Furthermore, the BoHV-1 IEtu1 promoter is transactivated significantly by KLF15 and GR and other stress induced transcription factors. [79, 81, 83, 84]. Also, aGR and KLF15 cooperatively transactivate HSV-1 ICP0

full length promoter and cis regulatory modules (CRMs) [85, 86] and ICP4 CRMs [87]. Sp1 sites are important for GR mediated transcriptional activity of ICP0 and ICP4. Furthermore, studies have shown that ICP0 CRMs are being transactivated by Sp1 in cooperation with aGR consistently (Chapter 6).

CHAPTER II

MATERIALS AND METHODS

2.1 Cells

Murine neuroblastoma cells (Neuro-2A; CCL-131) were obtained from American Type Culture Collection ATCC (Manassas, VA USA) and Rabbit Skin (RS) cells were obtained from Steve Wechsler (UC-Irvine, USA).

Mouse neuroblastoma cells (Neuro-2A; ATCC CCL131), RS cells, Monkey kidney cells (Vero, ATCC, CCL-81), African green monkey kidney cells (CV-1, ATCC, CCL-70™), NIH-3T3 cells (ATCC, CRL-1658™) and bovine kidney cells (CRIB) were grown in minimum essential medium (EMEM) with 2 mM L-glutamine, 100 mg/mL Streptomycin, 10 U/mL penicillin, and 10% fetal bovine serum (FBS).

When cells are treated with the synthetic corticosteroid Dexamethasone (DEX) or Progesterone cultures were incubated in 2% charcoal stripped FBS. Activated charcoal selectively removes lipophilic molecules, including corticosteroids that drive cellular stress responses, without affecting salts, glucose, or amino acids. This allows for improved control over glucocorticoid receptor activation with specific concentrations of DEX.

All cells were incubated at 37°C and 5% CO_2 under sterile conditions for the indicated time periods.

2.2 Viruses

A BoHV-1 mutant containing the β-Gal gene in place of the viral gC gene (gC blue virus) was obtained from S. Chowdury (LSU School of Veterinary Medicine, USA) and stocks of this virus grown in CRIB cells. The gC blue virus grows to similar titers as the wt parental virus and expresses the Lac Z gene. Procedures for preparing genomic DNA were described previously [88].

2.3 Extraction of gC blue viral DNA.

MDBK cultures were infected with an MOI of 10 for 1 h, the cultures subsequently rinsed twice with calcium/magnesium-free PBS (CMF-PBS), and new media added. At 16–36 h post-infection (CPE was approximately 80%), the cells with supernatant were collected and freeze thawed 3 times to enhance cell lysis to get the maximum viral yields. Then the samples were clarified by centrifugation (7000 r.p.m., 4 °C, 20 min in a Beckman J2-21 using a JA-20 rotor) and supernatant was collected. The virus was subsequently pelleted using a 30% sucrose/TE cushion (25 ml virus/5 ml sucrose solution) by centrifugation (25000 rpm for 2-6 h in a Beckman L7-65 ultracentrifuge using an SW28 rotor at 4 °C). The pellet was suspended in 1 ml of DNase I-free TE buffer. The virions were disrupted by adding 100 µl 20% SDS, 15 µl 1 mg/ml RNase, and incubating the solution at 37 °C for 30 min. Proteinase K (100 µl of a 10 mg/ml solution) was added and the solution was incubated at 56 °C for 30 min. Three phenol– $CHCl_3$ –isoamyl alcohol (50 :48 :2) extractions were performed, followed by one extraction with CHCl3 –isoamyl alcohol (48 :2). The resulting aqueous layer was subjected to two extractions with anhydrous ether. DNA was precipitated with 2.5 vols 95 % ethanol and sodium acetate (0.3 M). Viral DNA was electrophoresed on an agarose gel to examine its quality and quantity. Known concentrations of DNA standards were used to estimate the amounts of viral DNA.

2.4 Quantification of β-Gal positive cells

Neuro-2A cells grown in 60 mm plates were co-transfected with 1.5 µg of the gC blue viral genome and the designated amounts of plasmid expressing PR or/and KLF4 using Lipofectamine 3000 (catalog no. L3000075; Invitrogen). At 24hrs after transfection designated cultures were treated with P4 and RU486. At 48 h after transfection, cells were fixed with a solution containing 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) and then stained with a solution containing 1% Bluo-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.5M $MgCl_2$ in PBS and then stored at 4°C. Staining media was replaced with PBS and the number of β-galactosidase (β-Gal)-positive cells (cells stained in blue color) was determined.

In brief, the number of β-Gal positive cells in cultures expressing the blank vector (empty vector) was set at 1 for each experiment. To calculate fold change of β-Gal positive cells, the number of blue cells in cultures transfected with the plasmids of interest were divided by the number of blue cells in cultures transfected with the blank vector. Approximately, ten microscopic fields were counted for each sample and the average was taken as the gC blue positive cell count. The effect that KLF4, Progesterone and over-expression of the PR had on productive infection is expressed as fold induction relative to the control. This representation of the data minimized the differences in cell density, Lipofectamine 3000 lot variation, and transfection efficiency.

2.5 Plasmids

The human progesterone receptor A and B (PR A and PR B) isoforms in the pSG5 expression vector were obtained from Pierre Chambon (University of Strasbourg, Strasbourg, France). The KLF4 expression vector was obtained from Dr. Jonathan Katz (University of Pennsylvania, USA). The KLF9 was purchased from Neogen Bio-system. The KLF6 expression vector was obtained from Dr. Bin Guo (North Dakota State University, USA). The PLZF expression vector

was obtained from Dr. Derek Sant'Angelo (Sloan-Kettering Cancer Center). The Slug expression vector was obtained from Paul Wade (NIEHS, Research Triangle Park, NC).

The construction and characteristics of the BoHV-1 bICP0 E promoter and deletion constructs (EP-943, EP-638, EP-328, EP-172, EP-143 and EP-71) used in the study were described previously. Briefly six DNA reporter constructs were generated by PCR using the wt BoHV-1 genome as a template and a common 3′ primer (5′-ctcgagCCTGCTGGGCGACACAAACAACAGA-3′) with the following 5′ primers: EP-943, 5′-ggtaccGCGACGGCGGCAATAAAGACGAGTC-3′; EP-638, 5′-ggtaccGCCCTCGGTCTCGGTCGGAG-3′; EP-172, 5′-gggtaccGCCTTGCGTGGGGGGTTTCG-3′; EP-143, 5′-gggtaccAGCCGGGGGGTGCGGGCC-3′; EP-133, 5′-gggtaccTGCGGGCCTTTCGCCG-3′; or EP-71, 5′-gggtaccGCTCCCGGCGCGTCA-3′. The promoter fragments were cloned upstream of the pGL3-basic luciferase vector at the unique XhoI and KpnI sites to generate plasmids EP-943, EP-638, EP-172, EP-143, EP-133, and EP-71. Numbers in the plasmid name refer to the length of the bICP0 E promoter fragment cloned into pGL3-Basic Vector (Promega) (Chapter 3 Figure 4A). All plasmids were prepared from bacterial cultures by alkaline lysis and 2 rounds of cesium chloride ultracentrifugation.

A plasmid that expresses Akt1 was a gift from Jie Chen (pCDNA3-HA-Akt1; plasmid 73408; Addgene). Sp1 expression vector was obtained from Addgene (CMV-Sp1; cat 12097).

The mouse GR-α construct used for the studies was obtained from Dr. Cidlowski (NIEHS, Research Triangle Park, NC, USA). The ORF2 expression construct and ORF2-Stop was generated in pCMV-Tag-2B vectors (Stratagene; La Jolla USA) as described previously [89, 90]. These plasmids only contain ORF2 coding sequences from BoHV-1 genomic sequences and no untranslated regions. A Flag epitope is present at the N-terminus of ORF2, and the human IE CMV promoter drives its expression. For ORF2-Stop, the genomic sequences of ORF2 were synthesized. The first in-frame ORF2 AUG was deleted and replaced with three stop codons such that translation

will not occur in all three reading frames; this fragment was synthesized by GenScript (Piscataway, USA) and then cloned into the pCMV-Tag-2B vector.

The full-length ICP0 promoter (−800 to +150) was previously described, and provided by the late Dr. Priscilla Schafer [91]. The respective CRM constructs were synthesized by Genscript and inserted into pGL4.23[luc2/minP] (Promega; Madison, WI, USA) at SacI and XhoI unique restriction enzyme sites. The KLF15 expression plasmid was obtained from Deborah Otteson (University of Houston; Houston, TX, USA).

2.6 Transfection and Dual-Luciferase Reporter Assay

Cells (i.e., Neuro-2A, Vero, CV-1 and NIH-3T3) (8×10^5) were seeded into dishes containing MEM with 10% fetal bovine serum (FBS) at 24 h prior to transfection. At 2 h before transfection, cells were cultured with antibiotic-free medium containing 2% charcoal stripped FBS. All plasmids were transfected using Lipofectamine 3000 transfection reagent (L3000075; Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instructions. Cells were co-transfected with the designated plasmids and a plasmid carrying Renilla luciferase under the control of a minimal herpesvirus thymidine kinase (TK) promoter (50 ng). To maintain equal plasmid amounts in the transfection mixtures, an empty expression vector (pGL3 basic or pGL4.24[luc2/minP]) was added as needed. At 24 hrs after transfection designated cultures were treated with either progesterone (100 nM; Tocris Bioscience; 2835) and/or RU486 (1 μM; Sigma), water soluble DEX (Sigma; St Louis, MO, USA; D2915; 10 uM final concentration). At 48 h post transfection, cells were washed, harvested using passive lysis buffer, and stored at −80 °C. The protein lysate subjected to a dual-luciferase assay by using a commercially available kit (catalog number E1910; Promega; Madison, USA) according to the manufacturer's instructions. Luminescence was measured using a GloMax 20/20 luminometer (catalog number E5331; Promega).

2.7 Mouse strains for GR mutant studies

The GR ^{S229A} mice were developed by Dr. John A. Cidlowski (NIEHS) using targeted embryonic stem (ES) cells in which exon 2 of the mouse Nr3c1 (GR) gene was replaced with a neomycin cassette flanked by lox71 and lox2272 sites and a recombinase mediated cassette exchange approach that have been described previously [92]. In brief, an exchange vector was generated that contained exon 2 of the mouse GR gene in which the AGT codon (serine) was mutated to GCT (alanine) at amino acid position 229. The exchange vector also contained a hygromycin selection marker flanked by Frt sites. Flanking the hygromycin and mutant GR ^{S229A} exon 2 cassettes were lox66 and lox2272 sites that permit Cre recombinase-dependent directional insertion of DNA in conjunction with the lox71 and lox2272 sites on the targeted allele. The targeted ES cells were transfected with the exchange vector and Cre, and ES cells undergoing successful cassette exchange were confirmed by PCR. The GRS^{229A} positive ES cell clone was injected into C57BL/6 blastocysts to create chimeric mice. Chimeric males were bred with albino B6J[B6(Cg)-Tyrc-2J] females (Jackson Labs strain 000058) to confirm germline 14 transmission, and the hygromycin cassette was deleted by crossing mice with Flp-deleter mice (B6.129S4-Gt (ROSA) 26Sortm1(FLP1) Dym /RainJ, Jackson Labs strain 009086). The resulting Nr3c1S$^{229A/S229A}$ mice (GR^{S229A} knockin) were maintained on a C57BL/6 (Jackson Labs strain 000664) background. C57BL/6J male and female breeding pairs were purchased from Jackson Labs; GRS^{229A} breeding pairs were developed and gifted by Dr. John A. Cidlowski (NIEHS).

2.8 Primary cell preparation

Kidneys from uninfected GR^{S229A} and wt control animals were aseptically dissected, briefly dipped in 100% ethanol, and immediately stored in minimal essential media (MEM; Corning, Catalogue #15-010-CV) + 10% FBS on ice. The capsule of the kidney was manually removed, and the remaining tissue minced into < 3 mm pieces. Kidney pieces were washed three times with PBS and incubated in 0.25% trypsin for 4-18 h at 4°C with rocking. At 4, 8 and 18 h, supernatant was removed, and tissue pieces were incubated with residual trypsin at 37°C for 30 mins. Trypsin was

removed and an equal volume MEM with L-glutamine, 10% FBS and antibiotics was added. Remaining tissue and cells were gently dispersed by pipetting, filtered through a 100 µM cell strainer, and viability determined via trypan blue assay. Approximately 48 h post-isolation, cells were plated onto 8-well chamber slides (NUNC Lab Tek II, Catalogue # 154534) and grown for 24-48h at 37°C under 5% CO_2. Media was replaced with MEM containing 2% charcoal-stripped FBS (Sigma; Catalogue # F6765) and 10 µM water-soluble DEX (Sigma; Catalogue # D2915) for 4 h prior to fixation using 100% chilled methanol for 5 mins. Samples without DEX were included as controls. Cells were washed with TBS and incubated with either total GR (Cell Signaling, Cat #: 3660: 1:2000 dilution) or phos-GR antibody (Cell Signaling, Catalogue # 4161, 1:1600 dilution) overnight at 4°C. The next day, cells were washed with TBS and incubated with secondary Alexa fluor 488 (Invitrogen, Catalogue # A11029) for 1h at room temp in the dark. Cells were washed with TBS and stained with 4',6- diamidino-2-phenylindole, dihydrochloride (DAPI, Thermo Fisher Scientific, Catalogue # 62247) for 10 mins at room temp prior to mounting of cover slips. Images were obtained with an Olympus BX microscope and CellSense Entry software with exposure times of ~50 millisecond (DAPI) and ~400 millisecond (Alexa).

2.9 Chromatin immunoprecipitation (ChIP) assay

Neuro-2A and Vero cells for ChIP assay were cultured at 37°C under 5% CO_2 in 100 mm dishes until ~80% confluency. Two hours prior to transfection, cells were washed with PBS and antibiotic free media with 2% charcoal stripped FBS added. Cells were transfected with pGL4.24[luc2P/minP] plasmid containing one of the CRM constructs upstream of the minimal promoter (4 ug DNA), using TransIT-X2 (Mirus; Madison, WI, USA; MIR 6003) according to manufacturer's instructions. Where indicated, cells were co-transfected with GR-α (3 ug DNA) and KLF15 (3 ug DNA). At 24 h post transfection, designated samples were treated with DEX (10uM). At 40 h post transfection, cells were washed 3 times with 2ml ice cold PBS and 16% paraformaldehyde was added to crosslink transcription factors to DNA, incubated for 10 minutes

on a shaker. 500ul from 2.5M Glycine was added to the samples to stop crosslinking and incubated for 5 min on a shaker at room temperature. Samples were washed with 5ml of PBS two times and harvested. The samples were centrifuged at 1000g for 5 min. The supernatant was decanted, and the cell pellet was lysed in FA lysis buffer (50 mM HEPES, pH 34 7.5, 140 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) containing Protease Inhibitor cocktail (Thermo Fisher Scientific). Cell lysate was sonicated to shear DNA in to fragments~500bp and pre-cleared with agarose beads and salmon sperm DNA (Millipore) to reduce non-specific binding. Samples for inputs were separated at this time and stored at -80°C. Cleared samples were divided into equal volumes and immunoprecipitated using 2 ug of GR-α (Cell Signaling, Danvers, MA; catalogue number 3660S) or 2 ug of KLF15 (Abcam, Cambridge, MA; catalogue number ab2647) specific antibodies, with non-specific IgG (2 ug) as an isotype control in RIPA buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA [pH 8.0], 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) overnight at 4°C. This process precipitates transcription factor (protein) bound DNA fragments with the Ab. Immunoprecipitated samples were then incubated with Dynabeads protein A beads (Lifetechnologies) for 2hrs at 4°C and the supernatant was removed. Dynabeads with protein bound DNA were washed using low salt wash buffer (2X)- 0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl, high salt wash buffer (2X)- 0.1% SDS, 1% Triton X-100, 2mM EDTA, pH 8, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl, and LiCl wash buffer (1X)- 0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, pH 8 wash buffers. Washed dynabeads were then eluted in elusion buffer (1% SDS, 0.1 M NaHCO3) for 20 min at 30°C and the supernatant was collected and de-crosslinked by adding proteinase K and RNase A at 65°C overnight. DNA purification was carried out by extracting de-crosslinked samples twice with Phenol: chloroform: isoamyl alcohol. Precipitated DNA was amplified by PCR using specific primers mentioned in primers section. Amplified DNA was separated on 1.5% agarose

gel stained with ethidium bromide for visualization. Bands were quantified with ImageLab software (Biorad; Hercules, CA, USA;), and data were presented as a % of the input sample, which represents approximately 13% of cleared cell lysate.

2.10 Primer sets for ChIP studies

1. Forward primers

HSV-1 ICP0 A CRM construct and AΔSp1- 5'-**GAGAGACGATGGCAGGAG-3'** (position 26-43),

D CRM construct DΔSp1ΔAllKLF-5'-**TCCCCCAAATCGGGGGCCG-3'** (position 51-69)

B CRM construct and BΔAllSp1 - pGL4.24 seq F primer **5′-ACATACGCTCTCCATCAAAAC-3′**

2. Reverse primer for all three CRM constructs and their Sp1 site mutants - pGL4.24[luc2P/minP]; **5'-ACAGTACCGGATTGCCAAG-3'**.

The primers were designed using KOS sequences, as all of the ICP0 promoter constructs are derived from KOS sequences [91].

2.11 Co-immune precipitation

Neuro-2A cells grown on 100 mm dishes at 37°C under 5% CO_2 were co-transfected with plasmids that express KLF4 (1.5 μg DNA) and 1ug of PR-A and PR-B expression plasmids. Designated cultures were treated with P4 (100 nM; Tocris Bioscience; 2835) in MEM containing 2% stripped FBS and/or RU486 for 4 hours before harvesting of transfected cultures. Whole cell extracts were prepared with RIPA lysis buffer that included 1× Protease Inhibitor cocktail (Thermo Fisher Scientific) and protein concentration quantified using Brad-ford assay. Protein lysate (500 μg) was combined with anti-PR and /or anti-KLF4 (5 μg) antibodies and the reactions were incubated for overnight at 4°C on a rotator. Protein A Dynabeads® (cat. No 10001D, Life

Technologies) were added and incubated for 2 h at 4° C with rotation. Immunoprecipitates were collected using a magnet (DynaMag™) (cat. No.12321D, Life Technologies), supernatants removed and the Dynabeads® -Ag-Ab complexes washed 3 times with 1ml of washing buffer (20 mM Tris HCL pH (8.0), 500 mM NaCl, 2 mM EDTA, 1% Triton X100, 0.1% SDS). Proteins were eluted from Dynabeads® by incubating with 30 µl of elution buffer (1%SDS, 100 mM $NaHCO_3$) in a water bath at 42° C for 30 minutes and the eluent was used for western blotting as described below.

2.12 Western blot analysis

Neuro-2A and Vero cells were grown in 100mm dishes until confluent and the transfections were carried out in 2% charcoal stripped serum. The designated cultures were treated with DEX (10uM) or Progesterone (P4-100nM) as denoted in the results section for 4hrs before cell collection. After 40hrs from transfection cells were washed in cold PBS and collected in RIPA buffer (50 mM Tris HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA [pH 8.0], 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Thermo Fisher Scientific, Waltham, MA, USA). Samples were boiled in 2X Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 50 mM dithiothreitol, 0.1% bromophenol blue, 10% glycerol) for 5 min, and all samples were separated on an 10% or 12% SDS–polyacrylamide gel.

Proteins were transferred on to PVDF (Bio-rad) membraned via semi dry transfer and blocked for 1hr at RT in 5% non-fat milk in TBS-T. Immunodetection of the respective proteins was performed using the antibodies Cell Signaling: anti-GR (D8H2) (3660S), and Proteintech: anti- KLF4 antibody (11880-1-AP), Thermo Fisher Scientific; anti-PR (MA1–412).

CHAPTER III

PIONEER TRANSCRIPTION FACTORS, PROGESTERONE RECEPTOR AND KRÜPPEL LIKE TRANSCRIPTION FACTOR 4, COOPERATIVELY STIMULATE THE BOVINE ALPHAHERPESVIRUS 1 ICP0 EARLY PROMOTER AND PRODUCTIVE LATE PROTEIN EXPRESSION.

The studies in this chapter are included in two separate manuscripts that were published in Virus research.

Laximan Sawant, **Nishani Wijesekera**, and Clinton Jones, 2020 Pioneer transcription factors, progesterone receptor and Krüppel like transcription factor 4, cooperatively stimulate the bovine herpesvirus 1 ICP0 early promoter and productive late protein expression, Virus Research, volume 288, article 198115 10.1016/j.virusres.2020.198115

Fouad S. El-mayet, Laximan Sawant, **Nishani Wijesekera** and Clinton Jones, 2019, Progesterone increases the incidence of bovine herpesvirus 1 reactivation from latency and stimulates productive infection, Virus Research, volume 276, article 197803 10.1016/j.virusres.2019.197803

Abstract

Reproductive failure in cows vaccinated with one or more modified live vaccines (MLV) for BoHV-1 is a common sequalae among dairy cattle herds throughout the world. BoHV-1 reactivation from latency during pregnancy may be attributed to host factors, including hormonal fluctuations during gestation. Progesterone (P4) is a prominent hormone secreted during pregnancy: consequently, we hypothesized that P4 could increase the incidence of BoHV-1 reactivation from latency. This premise is supported by my studies demonstrating P4 stimulates productive infection in a ligand dependent manner in the presence of its cognate receptor, the progesterone receptor (PR). Since steroid receptors are known to enhance the expression of transcription factors, including Krüppel like factor family members, the cooperative effects of PR with KLF members were examined. My studies demonstrated PR cooperates with KLF4 to stimulate productive infection to a level higher than PR alone. Evaluation of bICP0 early promoter for transactivation by PR and KLF family members demonstrated KLF4 and PR cooperatively transactivated this promoter 16-fold higher compared to PR alone. However, the P4 effect on the bICP0 early promoter was ligand independent. Since both PR and KLF4 are pioneer transcription factors, I hypothesized conversion of silent chromatin to active chromatin occurs by these transcription factors in the absence of viral proteins. Sequences spanning from 943-328 of the bICP0 early promoter are crucial for PR and KLF4 mediated transactivation. This further demonstrates that sequences at the 3' end of bICP0 early promoter are less important for transactivation by PR and KLF4. These studies provide evidence P4 works better with KLF4. Immunoprecipitation studies revealed a stable interaction between PR and KLF4 in mouse neuronal cells (Neuro-2A).

Introduction

Bovine herpesvirus 1 (BoHV-1) is the most frequently diagnosed cause of viral abortion in North America. Even the existing modified live vaccines (MLVs) that contain an attenuated BoHV-1 strain contributes to reproductive failure in naïve heifers and cause huge economic loss to the cattle industry worldwide. Several European countries (Austria, Denmark, Finland, Norway, Sweden and Switzerland) eradicated BoHV-1 thus MLVs used in the USA are prohibited, making IBR a notifiable disease. Only MLVs that have a specific viral marker, usually deletion of glycoprotein E, are used in Europe [93].

Following acute BoHV-1 infection, cattle establish latency in the peripheral nervous system, trigeminal ganglia (TG) [94, 95]. Periodically virus is reactivated due to stressful stimuli and shed through mucosal surfaces [95]. Immunosuppression by corticosteroid treatment also leads to productive infection [96] and reactivation from latency [97]. This is because stressful stimuli signal adrenal gland to secrete more cortisol and cortisol suppresses immune responses by downregulating NF-kB and AP-1 mediated inflammatory responses [98]. Furthermore, high cortisol levels in animals increases the neutrophil numbers in the circulation but also reduces the tissue extravasation of these cells thus predisposing to infections. However, in the case of BoHV-1 this might be less important during the process of reactivation from latency [99].

Stress can be mimicked by administering the synthetic corticosteroid Dexamethasone, intravenously to animals. Previous studies demonstrated DEX treatment of latently infected calves and female rabbits [100] with BoHV-1, reactivate the latent virus leading to clinical disease [101]. Interestingly, like DEX, P4 is also a steroid which is abundantly produced during pregnancy by the placenta, which raises the possibility that P4 contributes to reactivation from latency in pregnant animals.

The majority of BoHV-1 reactivation in pregnant cows result in reproductive failure or abortions [102]. This is supported by the fact that BoHV-1 replicates efficiently in ovary and the corpus luteum and contributes to pathogenesis [103, 104]. One reason for abortions could be increased secretion of P4 towards late stages of gestation [105].

Progesterone binds to its receptor PR which has a similar affinity to DNA sequences bound by GR [106, 107]. Interestingly, BoHV-1 has over 100 glucocorticoid receptor response elements (GREs) in the genome which is known to be bound by the glucocorticoid receptor that is also bound by PR to regulate gene expression and productive infection [108].

As with other *Alphaherpesvirinae* subfamily members, BoHV-1 gene expression is operationally divided into three distinct phases during productive infection of cultured cells: immediate early (IE), early (E), and late (L) [109, 110]. IE gene expression is stimulated by VP16, a tegument protein [111, 112]. An IE gene immediate early transcription unit 1 (IEtu1) encodes two transcriptional regulatory proteins, bICP0 and bICP4, because a single IE transcript is differentially spliced and then translated into bICP0 or bICP4 (Figure 3A) [113, 114]. The bICP0 protein is also translated from an E mRNA (E2.6) because a separate E promoter drives expression of the bICP0 E transcript [113, 115]. The bICP0 protein has properties similar to those of herpes simplex virus 1 (HSV-1)-encoded ICP0 [116]. For example, a C3HC4 zinc RING (really important new gene) finger is located near the amino terminus of bICP0 that is crucial for stimulating viral promoters and productive infection [88, 117] as well as interfering with activation of the beta-interferon promoter [118]. Furthermore, bICP4 is a functional orthologue of HSV-1-encoded ICP4 [114, 119]

Previous studies have shown GR stimulates BoHV-1 productive infection. Also, several other stress induced transcription factors such as KLF family members, cooperate with GR to further enhance BoHV-1 viral replication and gene expression [79, 81, 84]. In essence, GR and KLF15 transactivate

IEtu1 promoter in a cooperative fashion [120]. However, the early (E) promoter of bICP0, which is crucial for ICP0 expression, has not been studied in detail.

Interestingly, the bICP0 early transcript is detected consistently in bovine TG after dexamethasone treatment. Furthermore, previous studies demonstrated fragments of different lengths of the bICP0 early promoter are significantly activated by DEX in cultured Neuro-2A cells [121]. Moreover, my hypothesis was supported by studies done with PR and KLF15 because it demonstrated PR cooperates with KLF15 to enhance BoHV-1 replication and gene expression [84]. With respect to other transcription factors expressed in BoHV-1 TG during DEX induced reactivation, KLF4 was also found in significant levels thus was assessed in the current study together with PR [54].

Studies in this chapter demonstrated BoHV-1 productive infection is significantly enhanced by PR and KLF4, and progesterone further stimulates viral replication. Furthermore, bICP0 early promoter activity is cooperatively transactivated by PR and KLF4. However, P4 did not stimulate bICP0 E promoter activity suggesting PR and KLF4 transactivated the promoter by a ligand independent mechanism. Finally, these studies revealed PR and KLF4 stably interact with each other in cultured Neuro-2A cells.

Results

1.Progesterone and progesterone receptor stimulate the BoHV-1 productive infection

A BoHV-1 recombinant virus that contains the Lac Z gene inserted downstream of the gC promoter in place of the gC ORF (BoHV-1 gC blue virus) was used for this study. The gCblue virus grows to similar titers as wt BoHV-1. β-Gal expression directly correlates with viral replication because the gC promoter is a late promoter and its expression is low prior to viral DNA replication. Twenty-four hours after transfection of rabbit skin cells was used to count β-

Gal + cells to minimize the number of virus positive cells that resulted from virus spread (data not shown). Rabbit skin cells were transfected with BoHV-1 gC blue DNA instead of infecting cells because VP16 and other regulatory proteins in the virion, bICP4 for example [122] diminish the stimulatory effects of DEX and cellular or viral genes on productive infection [80, 108, 120, 123, 124]. As previously reported, BoHV-1 DNA is not very infectious (Figure 1A and B), in part because IEtu1 promoter activity is dependent on the tegument protein, VP16. However, co-transfection with a plasmid that expresses the human P4 receptor (equal amounts from PR A and PR B isoforms were included in the transfection) stimulated viral replication, as judged by a significant increase in the number of β-Gal + cells (Figure 1). The addition of P4 further stimulated viral replication. Addition of the PR and GR antagonist (RU486) [125] reduced the effects of P4. Similar results were observed at 48 h after transfection; however, it was clear β-Gal + cells fused because of BoHV-1 cell-cell spread making it difficult to quantify the results (data not shown). Collectively, these studies revealed P4 and PR significantly stimulated BoHV-1 productive infection in rabbit skin cells.

Figure 1

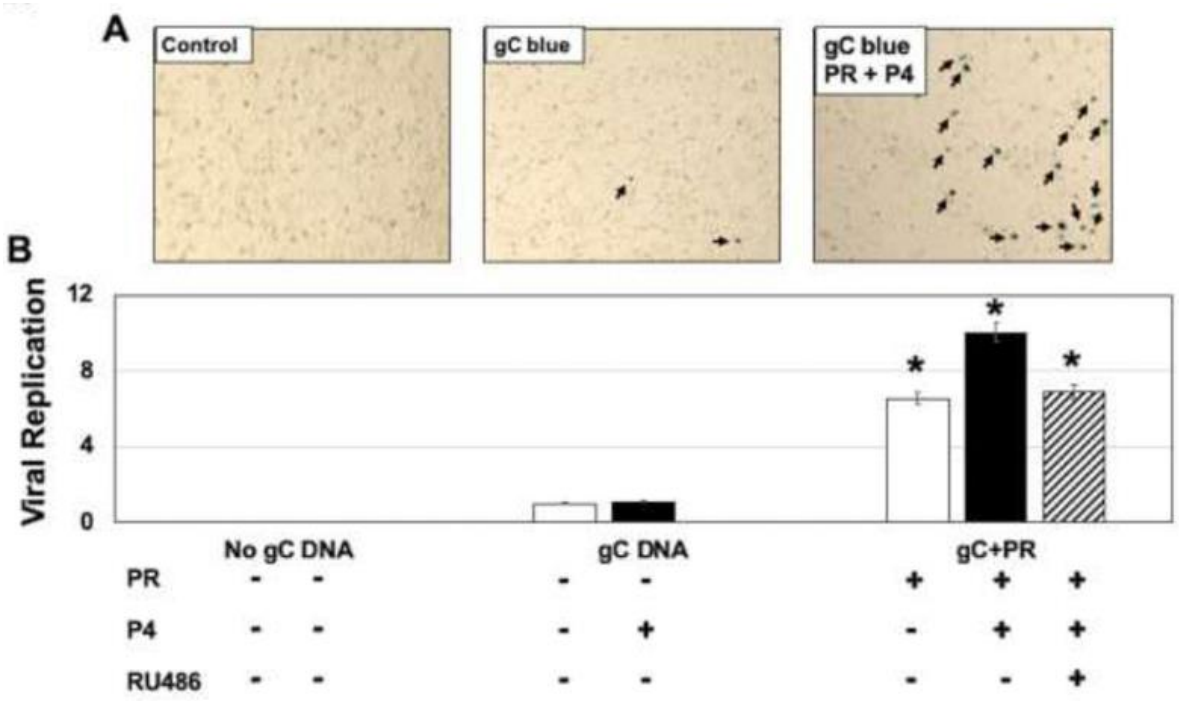


Figure 1. P4 and PR cooperate to stimulate productive infection.

Rabbit skin cells (Panel B) were used for these studies. Twenty-four hours prior to transfection 2% "stripped" FBS was added to media. Stripped FBS was used for these studies because normal serum contains steroid hormones, including P4. Cells incubated with stripped FBS for 24 h contain little or no nuclear GR. Cells were then transfected with 1.5 ug BHV-1 gC-Blue and where indicated a plasmid that expresses human PRA and PRB protein (0.5 ug DNA of each PR isoform). To maintain the same amount of DNA in each sample, empty vector was included. Designated cultures were then treated with water soluble P4 (10 u M; Sigma). At 24 h after transfection, the number of β-Gal + cells were counted. Representative results from 3 consecutive experiments after β-Gal staining are shown (Panel A: arrows denote β-Gal + cells). The value for the control (gC-Blue virus treated with PBS after transfection) was set at 1. The results from P4 treated cultures were compared to the control and are an average of three independent studies

(Panel B). An asterisk denotes a significant difference between rabbit skin cells transfected with BoHV-1 DNA and PR then treated with P4 ($P < 0.05$) when compared to all other combinations tested in these studies, using the students T test.

2. PR cooperates with KLF4 to transactivate the bICP0 early promoter

The organization of the BoHV-1 bICP0 and bICP4-coding regions is unique relative to the HSV-1 genome [126-128]. For example, the IEtu1 promoter drives IE expression of IE/2.9 and IE/4.2 mRNAs, which are derived from a single alternatively spliced transcript (Figure 2A). IE/2.9 mRNA is translated into the bICP0 protein, and IE/4.2 mRNA is translated into the bICP4 protein. Secondly, an E promoter drives expression of E/2.6, an early transcript translated into the bICP0 protein [126-129]. bICP0 RNA is detected more frequently than bICP4 RNA in TG of calves during DEX induced reactivation from latency [130] suggesting the bICP0 E promoter is active during early stages of reactivation in sensory neurons. Of note, previous studies also demonstrated GR and KLF4 cooperatively transactivated the full-length bICP0 E promoter (EP-943) [120]. Based on these observations, we tested whether PR cooperates with stress-induced KLF family members to transactivate EP-943 promoter activity.

PR+KLF4 transactivated EP-943 activity approximately 16-fold in transfected Neuro-2A cells whereas PR alone stimulated promoter activity less than 2-fold (Figure 2B). P4 significantly reduced PR+KLF4 mediated transactivation of EP-943 to approximately 7 fold suggesting PR+KLF4 mediated transactivation occurred via a ligand independent mechanism [131]. Relative to KLF4 and PR, EP-943 was not cooperatively transactivated by PR and KLF6, KLF9, PLZF or SLUG regardless of P4 treatment. These studies demonstrated PR+KLF4 transactivated EP-943 significantly more than other stress-induced transcription factors.

Figure 2

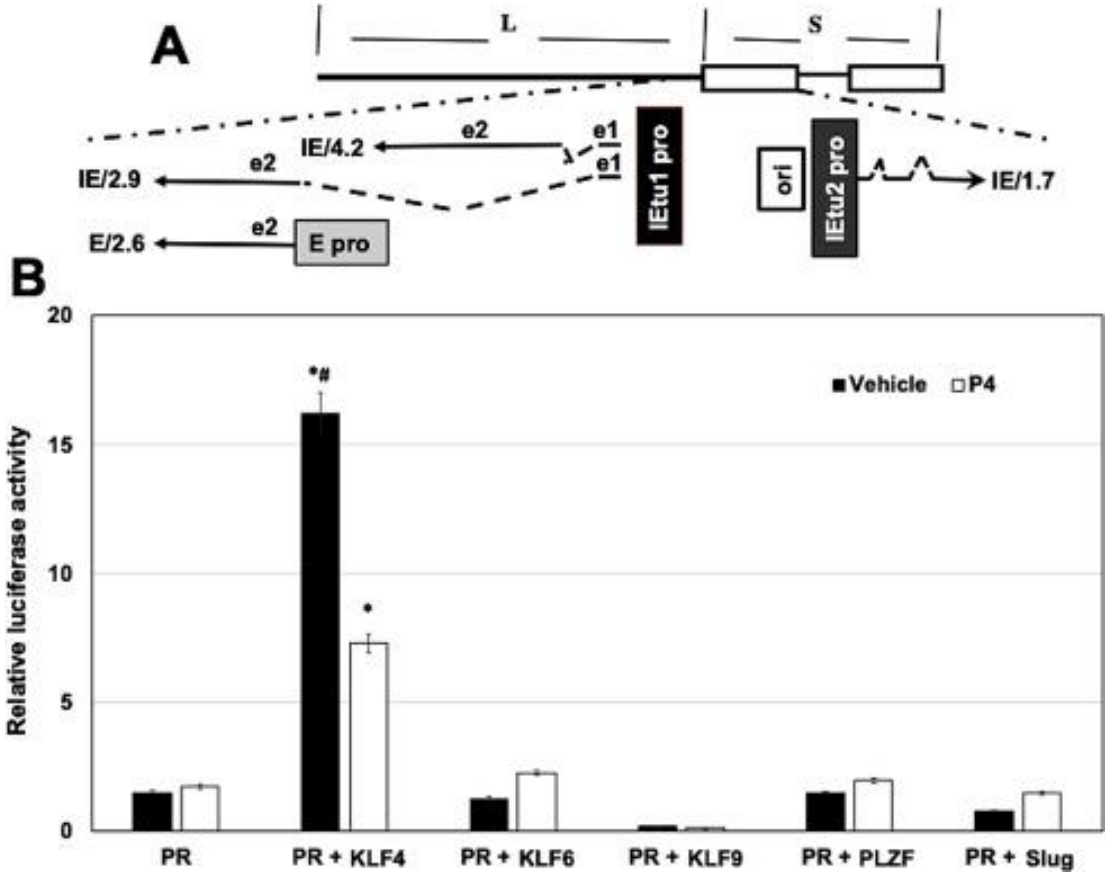


Figure 2. PR+KLF4 cooperate to transactivate the bICP0 E promoter

Panel A: Schematic of BoHV-1 genome and location of unique long (L) region, direct repeats (open rectangles), and unique short region (S). The IE/4.2 mRNA encodes the bICP4 protein and IE/2.9 mRNA encodes the bICP0 protein. A single IE promoter activates expression of IE/4.2 and IE/2.9 and is designated IEtu1 (black rectangle). E/2.6 is the early bICP0 mRNA and is regulated by the bICP0 E promoter (E pro; gray rectangle). bICP0 protein coding sequences are in Exon 2 (e2). Origin of replication (ORI) separates IEtu1 from IEtu2. The IEtu2 promoter (IEtu2 pro) regulates IE1.7 mRNA expression, which is translated into the bICP22 protein. Solid lines in IE/2.9, IE/4.2, and IE/1.7 are exons (e1, e2, or e3) and dashed lines introns.

Panel B: Neuro-2A cells were transfected with the designated bICP0 E promoter construct containing the firefly luciferase reporter gene (0.5 ug DNA) and where indicated a plasmid that expresses the human PR protein (A and B) (1.0 ug of DNA of each) and/or KLF-4, KLF-6, KLF-9, KLF-15, PLZF or SLUG (0.5 ug DNA). To maintain the same amount of DNA in each sample, empty vectors were included in certain samples. At 24 h after transfection, designated cultures were treated with 2% stripped FBS, and then vehicle (DMSO) or P4 (100 nM) added to cultures. At 48 hours after transfection, cells were harvested, and protein lysate subjected to dual-luciferase assay. Levels of promoter activity in the empty luciferase vector EP-943 were normalized to a value of 1 and fold activation for other samples presented. The results are the average of 3 independent experiments and error bars denote the standard error. A pound sign indicates significant differences ($P < 0.05$) in cells transfected with the EP-943 cotransfected with PR plus KLF4 relative to EP-943 sample cotransfected with PR and treated with P4. An asterisk sign indicates a significant difference between EP-943 cotransfected with KLF4 and PR and EP-943 cotransfected with the other stress-induced transcription factors (KLF6, KLF9, PLZF, Slug) with or without P4 treatment. The student *t* test was used for analyzing the results.

3. Localization of bICP0 E promoter sequences important for PR and KLF4 mediated transactivation

bICP0 E promoter deletion mutants (Figure 3A) were used to localize sequences important for PR+KLF4 mediated promoter activation. PR+KLF4 mediated transactivation of EP-638 and EP-398 was significantly reduced relative to EP-943 (Figure 3B). P4 treatment reduced PR+KLF4 mediated transactivation of EP-638 and EP-328: however, RU486 treatment did not significantly

influence promoter activity. The EP-172 construct was not significantly transactivated by PR and/or KLF4 regardless of P4 treatment. Studies in Figure 3 suggested separate enhancer domains

upstream of the TATA box and promoter proximal sequences were transactivated by PR+KLF4 in a cooperative fashion.

In summary, these studies confirmed the bICP0 E promoter was cooperatively transactivated by PR+KLF4 more efficiently and transactivation substantially decreased when sequences between EP 943 to EP 172 were deleted.

Figure 3

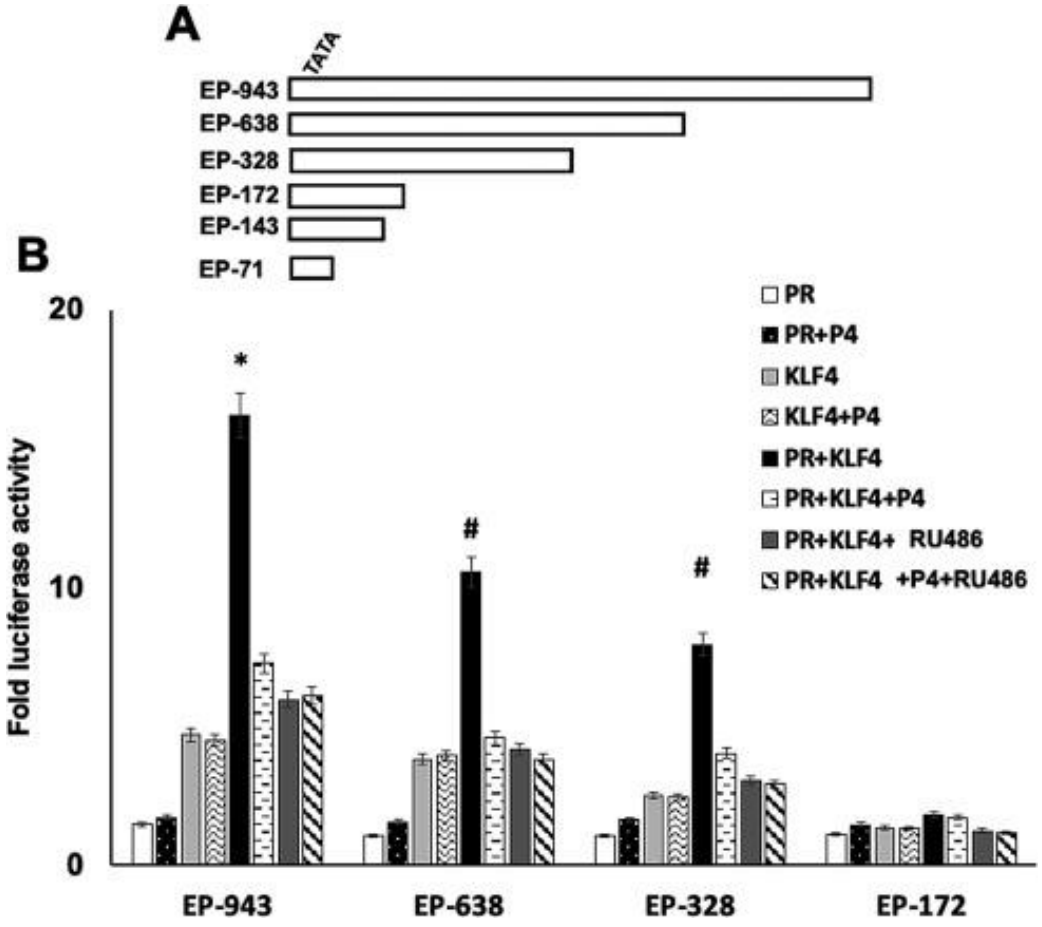


Figure 3- Localization of bICP0 E promoter sequences important for PR+KLF4 mediated transactivation

Panel A: The full-length bICP0 E promoter (EP-943) and deletion constructs were prepared as previously described (Workman et al., 2011). These fragments were cloned upstream of the luciferase vector (pGL3-Basic Vector, Promega) as SacI-HindIII fragments. The position of the TATA box is shown, and fragments extend to the nucleotide prior to the ATG.

Panel B: Neuro-2A cells were transfected with the designated bICP0 E promoter constructs containing the firefly luciferase reporter gene (0.5 ug DNA; see Figure 3A for schematic of constructs) and where indicated a plasmid that expresses human PR protein (A and B) (1.0 ug of DNA of each) and/or KLF4 (0.5 ug DNA). To maintain the same amount of DNA in each sample, empty vector was included in certain samples. At 24 h after transfection, cultures were incubated with MEM containing 2% stripped FBS, and then DMSO (vehicle) or P4 (100 nM; Tocris Bioscience; 2835) was added to cultures. At 48 hours after transfection, cells were harvested, and protein lysate subjected to a dual-luciferase assay. Levels of promoter activity in the empty luciferase vector (pGL3-Promoter Vector) were normalized to a value of 1 and fold activation for other samples are presented. The results are the average of 3 independent experiments and error bars denote the standard error. An asterisk indicates significant differences ($P< 0.05$) in cells transfected with the EP-943 cotransfected with PR plus KLF4 relative to other EP-943 samples. The EP-638 sample cotransfected with KLF4 plus PR was significantly different relative to all other samples transfected with EP-638; the results of EP-638 or EP-943 cotransfected with KLF4 plus PR were significantly different (denoted by pound sign). With respect to EP-328, the pound sign indicates a significant difference from the other EP-328 samples. The results of EP-638 or EP-328 cotransfected with KLF4 plus PR were not significantly different. The results for EP-943 cotransfected with KLF4 and PR were significantly different than EP-328 cotransfected with the same constructs. The student *t* test was used for analyzing the results.

4. KLF4 cooperates with PR to stimulate productive infection

To examine the possibility that PR and KLF4 influence late viral protein expression, mouse neuroblastoma cells (Neuro-2A) were co-transfected with gC-Blue genomic DNA. I used Neuro-2A cells for these studies because they have neuronal like properties [132], approximately 50% of the cells are transfected, and BoHV-1 replicates at low levels in these cells [133]. Neuro-2A cells were transfected with BoHV-1 gC blue DNA versus infecting these cells because VP16 and other regulatory proteins are present in the virion, bICP4 for example [122]. Consequently, the stimulatory effects of cellular or viral proteins on productive infection are reduced or eliminated. β-Gal expression directly correlates with virus replication in highly permissive bovine or rabbit cells because the gC promoter is a late promoter and its expression is low prior to viral DNA replication. Time points between 24–48 h after transfection were used to count cells expressing β-Gal+ cells to minimize the number of virus-infected cells that resulted from virus spread, in essence secondary infection of surrounding cells. However, it is possible that the number of β-Gal+ cells in Neuro-2A cells may not directly reflect productive infection because of the inefficient replication of BoHV-1 in these cells [133]. Hence, for these studies we refer to an increase in the number of β-Gal+ Neuro-2A cells as increasing late viral protein expression. After transfection, cultures were incubated in MEM + 2% stripped FBS in the presence or absence of P4. FBS passed through a column containing “activated” charcoal removes hormones, lipid-based molecules, certain growth factors, and cytokines yielding stripped FBS: however, this process does not remove salts, glucose, and most amino acids. Co-transfection of gC Blue and PR+KLF4 stimulated the number of β-Gal+ Neuro-2A cells approximately 29-fold even when P4 was not added to cultures, which was significantly different compared to the effects seen by PR+ P4 treatment or when transfected with KLF4 alone (Figure 4). P4 treatment did not significantly increase the effects of PR+KLF4, consistent with studies described in Figure 4. Cultures were treated with RU486 because it is a PR specific antagonist and is commonly used to address

whether transcriptional activation by PR is ligand dependent: in essence P4 must bind PR to activate transcription [134, 135]. RU486 decreased the number of β-Gal+ Neuro-2A cells suggesting productive late viral protein expression was stimulated by KLF4+PR in a ligand dependent manner. While I believe over-expressing KLF4 and PR directly transactivates the bICP0 E promoter, it is also possible that KLF4 and PR activate cellular genes, which then stimulate productive late viral gene expression.

Figure 4

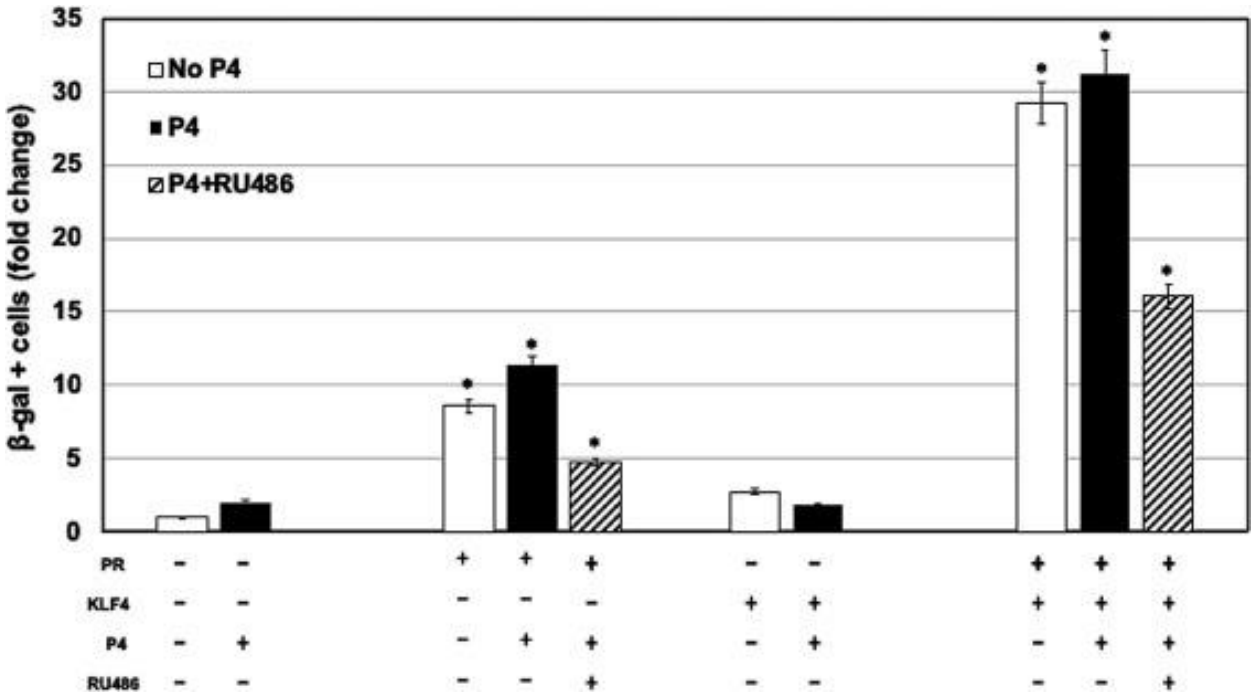


Figure 4. PR+KLF4 cooperate to stimulate late productive infection.

Neuro-2A cells were transfected with 1.5 ug BHV-1 gC-Blue and where indicated a plasmid that expresses the human PR protein (PR-A and PR-B) (1.0 ug DNA of each plasmid) and KLF4 (0.5

ug DNA). To maintain the same amount of DNA in each sample, empty vector was included in samples. Cells were incubated with MEM containing 2% stripped FBS 24 h after transfection. Designated cultures were treated with vehicle (DMSO), P4 (100 nM), DMSO (vehicle), and, where indicated, RU486 (1 uM). At 48 h after transfection, cells were fixed and stained for counting β-Gal+ cells. The value for the control (gC-Blue virus cotransfected with empty vector, treated with the DMSO vehicle after transfection) was set at 1. The results for P4 or RU486-treated cultures were compared to those for the control. The number of β-Gal+ Neuro-2A cells was counted from 4 independent quadrants/plate. The results are the average of 3 independent experiments. An asterisk indicates a significant difference between the control and samples transfected with the PR and/or KLF4 and treated with or without P4 or RU486 ($P < 0.05$) using the student *t* test.

5. PR is associated with KLF4 in transfected Neuro-2A cells

To test whether PR is stably associated with KLF4, Neuro-2A cells were co-transfected with plasmids that express KLF4 and PR, and co-immunoprecipitation (co-IP) studies were performed. Following IP with the PR antibody, we consistently detected KLF4 in the immunoprecipitate when cultures were not treated with P4 (Figure 5A) and when cultures were treated with P4 (Figure 5B). When IP was performed with KLF4 and the Western Blot probed with the PR specific antibody, the immunoprecipitate contained PR in the absence of P4 (Figure 5C) or presence of P4 (Figure 5D). In summary, these studies revealed KLF4, and PR were stably associated with each other in Neuro-2A cells. Furthermore, the association between KLF4 and PR was independent of P4 treatment.

Figure 5

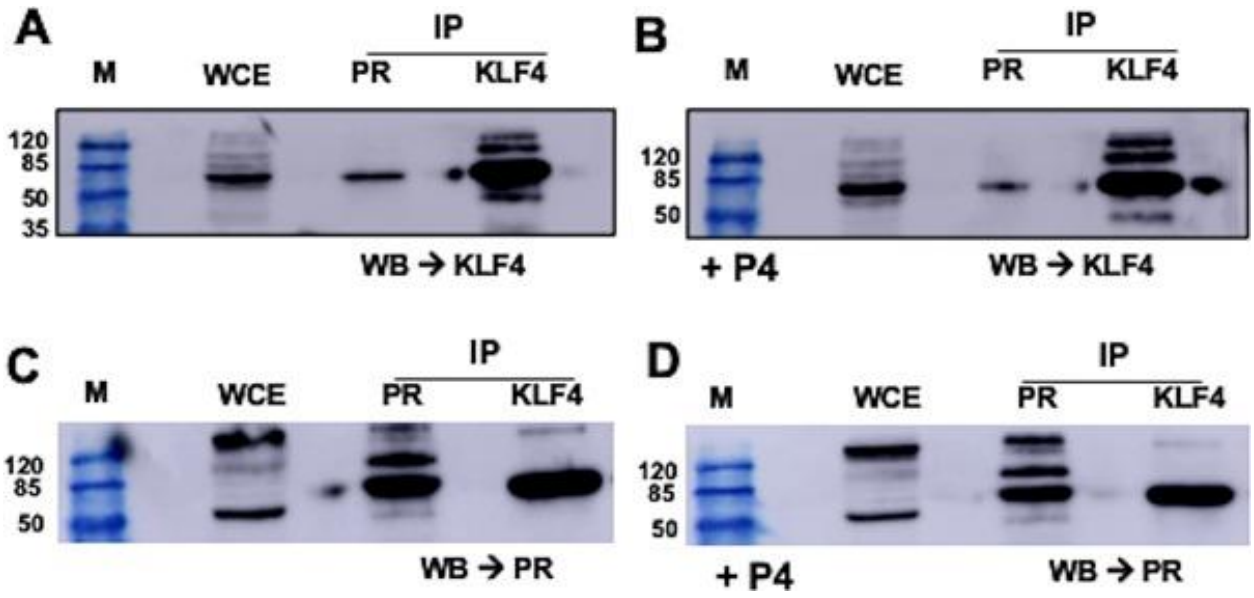


Figure 5. PR and KLF4 are associated with each other in transfected Neuro-2A cells.

Neuro-2A cells were grown to 80% confluence on 100 mm dishes in stripped FBS. Cells were cotransfected with plasmids that express the human PR protein (PR-A and PR-B) (1μg of DNA of each PR construct) and/or KLF4 (1.5 ug DNA). After transfection cultures were incubated with MEM containing 2% stripped FBS **(panel A and C)**. Cultures were treated with P4 at 24 hours after transfection (100 nM; Tocris Bioscience; 2835) in MEM containing 2% stripped FBS for 4 hours **(panel B and D)**. Whole cell extract (WCE) was prepared and co-IP studies performed using the PR or KLF4 antibody as described in material and methods. Following IP with the designated antibody, KLF4 or PR was detected in immunoprecipitates by Western blotting. WCE (50 ug protein) was used as a positive control. This result is representative of 2 experiments conducted consecutively.

Discussion

The KLF/specificity proteins (Sp) superfamily of transcription factors transactivate the same genes as PR in human reproductive tract [136]. This has been investigated because the majority of PR binding gene promoters do not contain PREs. However, these PR responsive promoters do contain KLF/Sp binding sites suggesting KLF members interact with PR and tether PR to these promoters in order to transactivate them [136]. This supports the fact that KLF family members might cooperate with PR to transactivate promoters that do not contain consensus PREs via a KLF mediated mechanism.

In this study, we examined the effects PR and stress-induced transcription factors have on bICP0 E promoter activity. These studies revealed PR and KLF4 cooperatively transactivated the bICP0 E promoter in transient transfection studies. Strikingly, KLF4 cooperated with PR to stimulate bICP0 E promoter activity significantly higher than PR, KLF4 and P4 treatment alone (Figures 2 and 3). Furthermore, P4 did not dramatically increase late viral gene expression when PR and KLF4 were over-expressed (Figure 4). These observations suggest PR and KLF4 stimulated bICP0 E promoter activity and productive late viral protein infection without P4 activating PR, which is commonly referred to as an "unliganded" mechanism. Interestingly, unliganded PR activation requires phosphorylation [137] by cyclin dependent kinases i.e., CDK2. However, CDK2 is important during ligand dependent and ligand independent activation of PR [138].

P4 stimulates KLF4 expression [139], in part by repressing expression of a miRNA that specifically binds KLF4 mRNA and reduces KLF4 protein expression [140]. Based on these published observations, I suggest that interactions between PR and KLF4 (Figure 5) in cells where there is limiting P4 levels or in the absence of P4 triggers bICP0 E promoter activity.

The IEtu1 promoter is activated by PR via a P4 dependent mechanism. In summary, P4-dependent and independent mechanisms are predicted to promote virus replication and spread in

the context of reproductive tissues of cattle. It should also be pointed out that PR and KLF4 may activate expression of cellular genes that activate bICP0 E promoter activity.

The current study demonstrated that sequences located between bICP0 nucleotides 943-328 were important for PR+KLF4 mediated transactivation (Figure 3). These sequences contain numerous Sp1 binding sites, three nucleosome enriched KLF4 binding sites [141], four KLF-like binding sites, and two potential CACC rich KLF binding sites. Additional GC and CA-rich motifs in the bICP0 E promoter may also be crucial because KLF family members were reported to bind GC and/or CA rich sequences [142-145]. Studies performed later demonstrated that mutating KLF4 and Sp1 binding sites or both at once significantly reduce the transactivation by PR+KLF4 and GR+KLF4 on bICP0 early promoter [146]. Furthermore, chromatin immune precipitation (ChIP) studies demonstrated that wt bICP0 enhancer fragments occupy KLF4 and PR strongly compared to KLF4/Sp1 sites mutated enhancer fragments [146]. Furthermore, published studies demonstrated that a Sp family member (Sp1) cooperates with the PR-B isoform to transactivate promoters that lack PREs; for example, the $p21^{WAFI}$ promoter [136]. It is possible that Sp1 and PR-B stably interact with each other, and further studies are necessary to confirm this hypothesis. Furthermore, PR-B isoform interacts with Basic transcription element binding (BTEB)/KLF9 protein and transactivate the promoters which contains PREs [136] even though PR did not cooperate with KLF9 to mediate bICP0 E promoter transactivation. This could be due to the absence of consensus PREs in bICP0 E promoter.

The BoHV-1 genome contains more than 100 putative GREs [96] suggesting DEX and GR differentially induce expression of certain viral and/or cellular genes. Furthermore, the bICP0 E promoter lacks “whole” GREs and independent studies concluded the ½ GREs were not essential for PR+KLF4 mediated transactivation [147].

DEX stimulates reactivation from latency in all rabbits and calves latently infected with BoHV-1 [100, 148]: conversely, P4 sporadically induces reactivation [100]. Since PR and GR cooperate with KLF15 to activate the IEtu1 promoter [108, 120] and PR and GR cooperate with KLF4 to transactivate the bICP0 E promoter [100] merely activating these two key viral regulatory promoters are not the only events necessary for triggering reactivation from latency.

Productive infection studies demonstrate that P4 increases viral DNA replication, which indicates the ligand dependent activity of PR. P4 induces phosphorylation of PR at Serine 294 and 345 and Ser400. Furthermore, phosphorylation of PR is important for ligand dependent and ligand independent receptor activation, receptor turn over and promoter specificity of PR [138]. Thus, differential phosphorylation of serine residues may drive transactivation of several promoters, which results in IE, E or late gene expression and productive infection. Treatment with RU486 reduces P4 induced viral replication, thus demonstrating RU486 impairs PR activation. Furthermore, these studies support the fact that P4 mediated activation of PR during productive infection is ligand dependent in rabbit skin cells and Neuro-2A cells.

Current results show that presence of KLF4 alone does not efficiently activate late viral gene replication: hence, suggesting KLF/Sp binding sites might not play a crucial role during productive infection. However, PR alone or PR together with KLF4 have synergistically stimulated bICP0 promoter activity and productive infection. Since there are many GREs in the BoHV-1 genome, I predict PR interacts with GREs and recruits KLF4 to promoter regions that enhance productive infection.

During DEX induced reactivation from latency, many cellular transcription factors are expressed in bovine TGs. However, these studies imply KLF4, and PR exclusively activate bICP0 EP activity. Studies performed by others identified two Sp1 binding sites are present in the promoter of PR-A suggesting this could be a mechanism by which KLF4 activates PR expression.

Increased expression of PR mediated by KLF4 may also enhance the interaction of PR with the bICP0 EP hence significantly transactivating the promoter [149]. The effect of KLF4 on PR promoter has not yet been examined and could be an important aspect to pursue.

PR and KLF/Sp family members interact with co-transcriptional activators, including steroid receptor co-activator (SRC-1), histone acetylases (HAT); CREB binding protein (CBP). Furthermore, PR, Sp1 and KLF4 physically interact with CBP. Current study demonstrated that in transfected Neuro-2A cells PR isoforms stably interact with KLF4. However, it is not clear whether which PR isoform is interacting with KLF4. Since Sp1 is known to interact with the PR-B isoform and KLF family members, it is highly possible that KLF4 is interacting with PR-B [136]. Further experiments are needed to confirm this hypothesis.

While the mechanisms by which nuclear hormones regulate BoHV-1 pathogenesis and reactivation from latency are unclear, fluctuations in DEX and P4 in cattle have the potential to facilitate virus transmission and survival in nature. Understanding how PR and GR mediate viral replication and reactivation from latency may lead to developing new modified live vaccines that do not reactivate from latency or replicate in certain tissues as well as wild-type BoHV-1. These vaccines would likely reduce economic losses caused by BoHV-1 acute infections and reactivation from latency.

CHAPTER IV

SPECIFIC AKT FAMILY MEMBERS AND A BOVINE ALPHAHERPESVIRUS 1 (BoHV-1) NON-CODING RNA IMPAIR STRESS INDUCED TRANSCRIPTION AND PROMOTE MAINTENANCE OF LATENCY

The studies in this chapter are included in two separate manuscripts that were published in the international Journal of Molecular Sciences and Journal of Virology.

Jing Zhao, Liqian Zhu, **Nishani Wijesekera**, and Clinton Jones 2020 Specific Akt family members impair stress mediated transactivation of viral promoters and enhance neuronal differentiation: important functions for maintaining latency, Journal of Virology, Vol 94, No 21 10.1128/JVI.00901-20

Jing Zhao, **Nishani Wijesekera** and Clinton Jones 2021, Inhibition of Stress-Induced Viral Promoters by a Bovine Herpesvirus 1 Non-Coding RNA and the Cellular Transcription Factor, β-Catenin, International Journal of Molecular Sciences, Vol 22, Issue 2 10.3390/ijms22020519

Abstract

BoHV-1 is the number one viral cause of abortions in dairy cattle in the United States. Current vaccines, including modified live vaccines (MLV) may cause abortions in cows. This is primarily due to reactivation of the virus from latency. Latency is established in trigeminal ganglia after acute infection and the only abundant transcript expressed in TG during latency is the latency related (LR) transcript. Stress can induce reactivation from latency, in part by activating the glucocorticoid receptor (GR), which enhances productive infection and transactivate key viral regulatory promoters in both Bovine herpesvirus 1 (BoHV-1) and Herpes simplex virus 1 (HSV-1). The number of GR expressing neurons are significantly higher during reactivation than during latency adding support to the premise that GR is important for reactivation. In this study I hypothesized that host and viral factors contribute to maintain latency. An open reading frame in the LR gene encodes a protein (ORF2), which supports maintenance of latency by inhibiting apoptosis in latently infected neurons. The current study shows that a mutation in ORF2 sequence (ORF2-stop), which prevents protein coding, but allows transcription of ORF-2 mRNA also supports latency by inhibiting GR induced productive infection in Neuro-2A (N2A) and rabbit skin (RS) cells. Further studies demonstrated ORF2 Stop reduced GR expression in a dose dependent manner in Neuro-2A cells. This observation provides a mechanism by which ORF2 stop encodes a RNA that interferes with GR induced productive infection. Another signaling pathway upregulated during BoHV-1 latency in TG is the Akt serine/threonine protein kinase pathway. Akt family members, Akt-1 and Akt-2, inhibit GR or/and KLF15 mediated transactivation of the BoHV-1 immediate early transcription unit 1 (IEtu1) promoter and the HSV-1 infected cell protein 0 (ICP0) promoter. Collectively these studies identified a viral factor, a non-coding RNA within ORF-2 coding sequences, and the Akt signaling pathway promotes maintenance of latency by inhibiting GR expression in a neuronal cell culture model.

Introduction

Bovine alphaherpesvirus-1 (BoHV-1) is a significant pathogen in the cattle industry, which causes severe pneumonia in animals. BoHV-1 infection predisposes the lower respiratory tract of cattle to opportunistic bacterial infections such as *Mannheimia hemolytica* (MH) which leads to life threatening pneumonia. This poly-microbial disease is known as shipping fever [29].

BoHV-1 establishes latency in trigeminal ganglia (TG) when infection occurs in the nasal, oral, or ocular cavities. If genital infection occurs, the site of latency is the sacral spinal ganglia. Recent evidence revealed pharyngeal tonsils are also a site of latency in cattle. During latency, the infectious virus is not isolated from animals, but viral DNA is detected in the peripheral nervous system, for example TG. The current modified live vaccines (MLV) are unable to prevent BoHV-1 infection and establishment of latency [33].

During acute infection, BoHV-1 IE (Immediate early), E (early) and late (L) genes are expressed in a temporal fashion, which culminates in production of infectious virus. In contrast, during latency only the latency related transcript (LRT) is abundantly expressed, and lytic viral gene expression is extinguished. LRT promotes establishing and maintaining latency, in part by inhibiting apoptosis. The LR gene encodes several proteins and two micro RNAs that are abundantly expressed in TG neurons and are essential for latency-reactivation cycle. The micro RNAs reduce bICP0 protein expression in transient transfection studies. The LR gene contains two open reading frames (ORF), ORF-1 and ORF-2. ORF-2 expression in trigeminal ganglionic neurons inhibits apoptosis. In Neuro-2A cells, ORF-2 localizes to the periphery of the nucleus. But a mutant virus, which contains STOP codons in the open reading frame, does not produce a protein and infection of calves with this mutant virus leads to diminished clinical symptoms during acute infection compared to wt-BoHV-1. Furthermore, LR mutant virus elicits a more robust interferon response in cell culture and tonsils of calves during acute infection. However,

LR mutant virus grows to lower levels in the ocular cavity and TG compared to wt-BoHV-1 and induces more apoptosis in TG neurons. This may explain why the LR-mutant virus causes exhibited reduced levels of reactivation from latency compared to wt-BoHV-1 [150].

There are many factors which contribute to maintenance of latency, including host and viral factors. Cellular signaling pathways which support latency are beta-catenin (Wnt) and Akt signaling pathways. Wnt signaling pathway is important for cell differentiation, fate determination and polarity during embryonic development [151]. Canonical Wnt pathway regulates the cellular beta catenin levels. In the absence of Wnt agonists, the cytoplasmic beta-catenin is degraded by specific destruction complex. However, binding of Wnt agonists stabilizes cytoplasmic beta-catenin. Consequently, beta-catenin enters the nucleus and binds to T cell factor to activate beta-catenin dependent genes [152]. Furthermore, the Wnt pathway supports axonal growth and neuronal survival during BoHV-1 latency. Inhibiting the Wnt pathway reduces viral replication during productive infection [153].

Another cellular signaling pathway, which supports latency, is phosphatidyleinositol-3-kinase (PI3K)/ Akt pathway. The Akt pathway inhibits apoptosis and is hijacked by various viruses to benefit their life cycle inside the host. Akt3 expression is higher in TG neurons of latently infected calves compared to DEX treated or uninfected TG [154]. In the context of HSV-1, suppressing Akt pathway promotes maintenance of latency in a rat neuronal model [63, 155] and human neuronal cell culture model (Lund human mesencephalic -LUHMES) [156].

Previous studies demonstrated that, LR-ORF2 sequences and a mutated ORF-2 sequence which lacks the initial methionine and contains stop codons (ORF2 stop) can inhibit the GR mediated transactivation of BoHV-1 IEtu1 promoter in Neuro 2A and Vero cells but not in a control virus (mouse mammary tumor virus-MMTV) which has many GREs and known to be transactivated by GR and DEX. In the current study, I demonstrated GR, KLF15 and DEX induced productive

infection is inhibited by ORF-2 stop sequences in a dose dependent manner in Neuro-2A and RS cells. I also demonstrated this is due to inhibiting GR steady state protein levels by ORF2 stop sequences. Akt-1 reduces GR mediated transactivation of the BoHV-1 IEtu1 promoter [50] by inhibiting GR expression in transfected Neuro-2A cells in a dose dependent manner.

Results

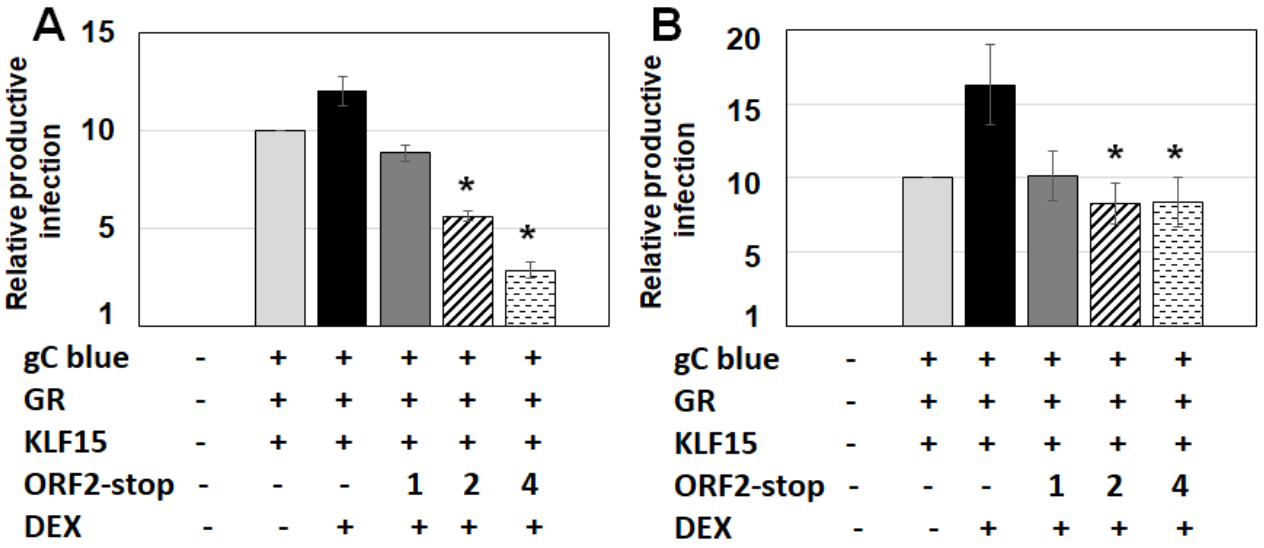
1. ORF2 Impairs Productive Infection

To test whether over-expression of ORF2 RNA influenced productive infection, Neuro-2A cells were transfected with BoHV-1 genomic DNA. A BoHV-1 recombinant virus that contains the Lac Z gene inserted downstream of the gC promoter (BoHV-1 gC blue virus) was used for this study. The gC blue virus grows to similar titers, as wt BoHV-1 and β-Gal expression directly correlates with viral replication because the gC promoter is a late promoter, and its expression is low prior to viral DNA replication. gC blue DNA was used instead of infecting cells because VP16 and other regulatory proteins in the virion, bICP4 for example [157], diminish the stimulatory effects of DEX and expression of regulatory genes on productive infection. Transfecting Neuro-2A cells with BoHV-1 DNA generally leads to a quiescent infection because BoHV-1 yields approximately 10,000 fold less infectious virus relative to bovine cells [158]. As previously reported [159], transfection of Neuro-2A cells with gC-blue, Krüppel-like transcription factor 15 (KLF15), and GR increased the number of β-gal+ cells approximately 10-fold relative to transfecting cells with just gC-Blue DNA (Figure 1A). Addition of DEX further stimulated the number of β-gal+ cells. This result was expected, because GR and KLF15 form a feed-forward transcription loop [160] to stimulate the IEtu1 promoter, a promoter that drives expression of bICP0 and bICP4 [159]. Strikingly, the ORF2-Stop expression construct significantly reduced productive infection when 2 and 4 ug of the expression plasmid were included in the transfection mix. In fact,

the number of β-gal+ cells were less than the control, implying ORF-2 RNA sequences interfered with additional aspects of infection in Neuro-2A cells. I also examined the effects of increasing concentrations of ORF2-Stop on productive infection in Rabbit Skin (RS) cells because BoHV-1 grows efficiently in these cells. As expected, GR and KLF15 strongly stimulated productive infection, and DEX further enhanced productive infection (Figure 1B). Consistent with the results in Neuo-2A cells, ORF2-Stop significantly reduced productive infection. GR, KLF15, and DEX have no effect on gC promoter activity. Furthermore, ORF2 has no effect on gC basal promoter activity nor when GR, KLF15, and DEX are transfected with the gC promoter, suggesting ORF2 and ORF2-Stop do not directly influence gC promoter activity (data not shown).

GR-mediated transactivation is complicated, and many factors regulate (positively or negatively) the efficiency of activating stress-induced promoters [161, 162]. Additional studies tested whether ORF2 RNA influenced GR protein levels in transfected Neuro-2A cells. ORF2-Stop consistently reduced mouse GR protein levels from a co-transfected plasmid (Figure 1C). In summary, these studies suggest ORF2 RNA sequences impaired productive infection and GR-mediated transactivation, in part, because it reduced steady-state GR protein levels.

Figure 1


A
Relative productive infection
15
10
5
1
*
*
gC blue - + + + + +
GR - + + + + +
KLF15 - + + + + +
ORF2-stop - - - 1 2 4
DEX - - + + + +
B
Relative productive infection
20
15
10
5
1
*
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gC blue - + + + + +
GR - + + + + +
KLF15 - + + + + +
ORF2-stop - - - 1 2 4
DEX - - + + + +

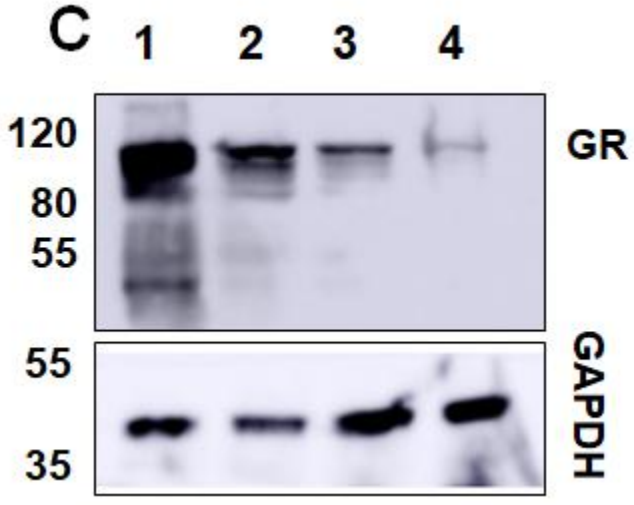

C
1 2 3 4
120
80
55
GR
55
35
GAPDH

Figure 1- ORF2 Impairs Productive Infection and GR expression.

Latency-related (LR) ORF2 RNA sequences interfere with productive infection and reduce GR steady-state protein levels. Panel (A): Neuro-2A cells were transfected with 1.5 ug BoHV-1 gC-Blue, and where indicated, a plasmid-expressing mouse GR protein (1.0 ug DNA), Krüppel-like transcription factor 15 (KLF15) (0.5 ug DNA), and ORF2-Stop (1, 2, or 4 ug plasmid). To maintain the same amount of DNA in each sample, an empty vector was included in samples. After transfection, 2% stripped FBS was added to media. Then, designated cultures were treated with water-soluble DEX (10 uM; Sigma; St. Louis, MO, USA). At 48 h after transfection, the number of β-Gal+ Neuro-2A cells was counted from four independent quadrants/plate. Panel (B): Rabbit skin (RS) cells were transfected with the designated plasmids as described in Panel A. At 24 h after transfection, the number of β-Gal+ RS cells was counted from four independent quadrants/plate. Twenty-four hours was used for RS cells because at later times after transfection, virus shedding occurs in the initial transfected cell and spread to surrounding cells, which culminates in disruption of the monolayer due to productive infection. For Panels A and B, the results are the average of three independent experiments. The value for the control (gC-Blue virus co-transfected with empty vector and then treated with PBS after transfection) was set at 1. An asterisk indicates a significant difference between control and samples transfected with the GR and/or KLF15 and treated with DEX ($p < 0.05$) using the student t-test. Panel (C): Neuro-2A cells were co-transfected with the GR expression construct (1.0 ug DNA; lane 1) and 1 (lane 2), 2 (lane 3), or 4 (lane 4) ug of the ORF2 Stop plasmid (ug DNA). To maintain the same amount of DNA in each sample, an empty vector was included in certain samples. Cells were incubated with 2% stripped FBS 24 h after transfection, and cultures were treated with DEX (10 μM) until 48 h after transfection. Then, WCL was prepared, and Western blots were performed to measure GR: 10 ug WCL was used for each lane. The position of molecular weight markers is shown. The result is representative of 3 independent experiments.

2. **Akt1 impairs GR expression in transfected N2A cells**.

To address whether Akt1 influenced GR steady state proteins, increasing amounts of Akt1 were cotransfected with the GR expression construct and Western blots performed. The GR construct we used for these studies expresses a 120 kd protein, as previously demonstrated [159]. In the presence of DEX, we consistently observed that 3 ug Akt1 reduced GR protein levels approximately 50% relative to the loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Figure 2).

Figure 2

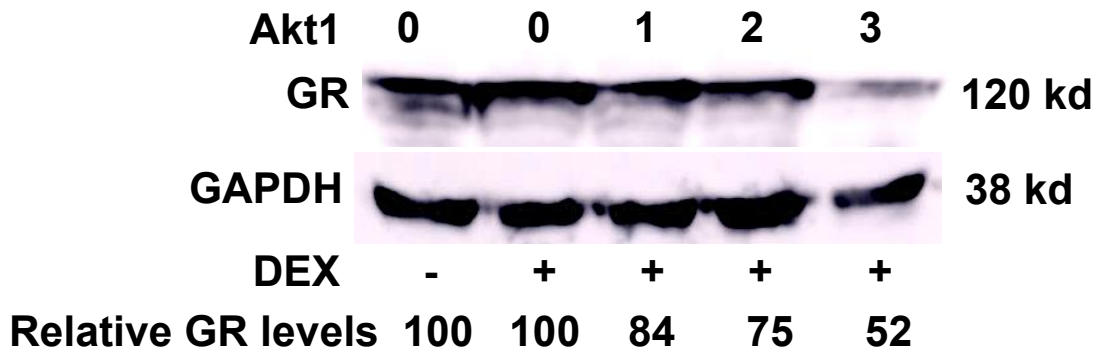


Figure 2-Akt-1 impairs GR steady state protein levels in Neuro-2A cells.

Neuro-2A cells were transfected with a plasmid that expresses Akt1 (1, 2, or 3 μg of the expression vector as denoted), and a plasmid that expresses GR (1.0 μg). Cells were incubated with 2% stripped FBS 24 hours after transfection and then certain cultures were treated with DEX (10 μM). At 48 hours after transfection, cells were harvested, cell lysate prepared, and Western blots performed to detect GR and the loading control GAPDH. 50 ug protein was loaded in each lane. The results are representative of 3 independent experiments

Discussion

BoHV-1 establishes latency in TG because LR gene products are expressed abundantly while leaky expression of lytic cycle viral genes occurs [33]. However, the mechanisms which support latency is not very well understood. The current study investigates whether host cellular transcription factors and viral factors actively support the maintenance of latency.

The LR gene encodes ORF2 but insertion of stop codons (ORF2 stop) and lack of initial methionine abolishes protein synthesis. However, the ORF2 stop construct still expressed ORF2 RNA and current study demonstrated ORF2 RNA inhibited productive infection in Neuro-2A and RS cells in a dose dependent manner. However, results produced by infection of calves with the LR-ORF2 mutant virus, demonstrated reduced reactivation from latency and viral shedding during acute infection [33]. This could be due to the fact that, ORF-2 protein expression supports neuronal cell survival by inhibiting apoptosis, which enhances survival of neurons latently infected by BoHV-1 [163]. Furthermore, previous studies demonstrated that mutations in the initial coding sequence of LR gene produced a virus that sheds 3 to 4 log less infectious virus from the ocular cavity during acute infection compared to LR rescued and wt Cooper strain [59].

The LR gene is anti-sense relative to the bICP0 gene and cellular transcription factors such as Notch-1 and C-EBPα interact with bICP0 to activate bICP0 protein expression [121, 164]. Notch-1 also impairs neurite formation and wt-ORF2 antagonizes this function: consequently, ORF2 supports neurite formation. This function has the potential to maintain latency by increasing the number of latently infected neurons [165].

Furthermore, the wt-ORF2 construct inhibited GR and DEX mediated transactivation of IEtu-1 promoter. However, ORF2 stop construct also has a similar action on IEtu-1 promoter, which further shows that expression of ORF-2 encoded protein is not essential for trans-repression of the IEtu-1 collapsed promoter [166].

Finally, this current study demonstrated Akt-1 inhibits the expression of GR in Neuro-2A cells. As mentioned before, Akt pathway is upregulated significantly during latency thus inhibiting GR

during latency by Akt signaling could assist maintenance of latency by suppressing lytic viral gene expression. In support of this, parallel studies demonstrated, Akt family members can suppress GR, KLF15 and DEX mediated transactivation of the BoHV-1 and HSV-1 lytic viral promoters [154]. Studies done by others on GR and Akt pathway demonstrated that, activated GR can activate Akt pathway in COS-7 cells when treated with DEX [167]. However, the current study suggests otherwise because Akt-1 reduces the steady state GR protein expression Neuro-2A cells. A reason for the differences in these studies are different cell lines and unexplained effect from endogenous GR in Neuro-2A cells. COS-7 cells do not express GR, so did they transfect GR in these cells.

CHAPTER V

INDEPENDENT CIS-REGULATORY MODULES WITHIN THE HUMAN ALPHAHERPESVIRUS 1 INFECTED CELL PROTEIN 0 (ICP0) PROMOTER ARE TRANSACTIVATED BY KRÜPPEL-LIKE FACTOR 15 AND GLUCOCORTICOID RECEPTOR

The studies in this chapter are included in the manuscript that was published in journal of "Viruses".

Nishani Wijesekera, Nicholas Hazell and Clinton Jones 2022 Independent Cis-Regulatory Modules within the Herpes Simplex Virus 1 Infected Cell Protein 0 (ICP0) Promoter Are Transactivated by Krüppel-like Factor 15 and Glucocorticoid Receptor, Viruses, 1*4*(6), 1284. https://doi.org/10.3390/v14061284

Abstract

A corticosteroid antagonist impairs Human alphaherpesvirus 1 (HSV-1) productive infection and explant-induced reactivation from latency, suggesting corticosteroids and the glucocorticoid receptor (GR) mediate certain aspects of these complex virus–host interactions. GR-hormone complexes regulate transcription positively and negatively, in part, by binding GR response elements (GREs). Recent studies revealed infected cell protein 0 (ICP0), ICP4, and ICP27 promoter/cis-regulatory modules (CRMs) are cooperatively transactivated by GR and Krüppel-like factor 15 (KLF15), which forms a feed-forward transcription loop. I hypothesized the ICP0 promoter contains independent CRMs that are transactivated by GR, KLF15, and the synthetic corticosteroid dexamethasone (DEX). This hypothesis is based on the finding that the ICP0 promoter contains multiple transcription factor binding sites including specificity protein 1 (Sp1) binding sites, and GR and KLF15 cooperatively transactivate the full-length ICP0 promoter. This hypothesis is also supported by the fact that Sp1 belongs to the same superfamily as KLF15. ICP0 promoter sequences spanning −800 to −635 (CRM A) were efficiently transactivated by GR, KLF15, and DEX in monkey kidney cells (Vero), whereas GR and DEX significantly enhanced promoter activity in mouse neuroblastoma cells (Neuro-2A). Chromatin immunoprecipitation (ChIP) studies revealed that GR tightly binds to the wt-A fragment when Neuro-2A cells are treated with DEX. Furthermore, ICP0 fragment B (−458 to −635) was efficiently transactivated by GR, KLF15, and DEX in Vero cells, but not Neuro-2A cells. GR showed stable interaction with wt-B fragment in Vero cells but not KLF15 when transfected with both GR, KLF15 and treated with DEX. Finally, fragment D (−232 to −24) was transactivated significantly in Vero cells by GR, KLF15, and DEX, whereas KLF15 and DEX were sufficient for transactivation in Neuro-2A cells. ChIP studies revealed GR stably interacts with wt-D fragment compared to DΔSp1ΔAll KLF. Collectively, these studies revealed efficient transactivation of three

independent CRMs within the ICP0 promoter by GR, KLF15, and/or DEX. Finally, GC-rich sequences containing specificity protein 1 (Sp1) binding sites were essential for transactivation.

Introduction

Sensory neurons in trigeminal ganglia (TG) are important sites for Human alphaherpesvirus 1 (HSV-1) latency following acute infection of the oral, ocular, or nasal cavity [43, 168]. Primary ocular infections can lead to follicular conjunctivitis, superficial punctate keratitis, and dendritic ulcers. In fact, HSV-1 is the leading cause of infectious blindness worldwide, and there are approximately 50,000 new cases each year. Approximately 20% of patients develop recurrent stromal keratitis, which is the result of reactivation from latency. Furthermore, HSV-1-induced recurrent encephalitis is generally due to reactivation from latency [169]. Hence, the ability of HSV-1 to reactivate from latency is crucial for virus transmission and recurrent eye disease. Identifying cellular factors that trigger productive infection and/or reactivation from latency may yield novel strategies to impair productive infection and reduce the incidence of reactivation from latency.

HSV-1 encodes five immediate early (IE) viral mRNAs that are expressed in the absence of de novo protein synthesis during productive infection: ICP0, ICP4, ICP22, ICP27, and ICP47 [170]. A viral tegument protein (VP16) interacts with two host transcription factors, Oct1 and host cellular factor 1 (HCF1), to transactivate IE gene expression, reviewed in [171, 172]. ICP0, a product of the RL2 gene, is a promiscuous activator of promoters, has E3 ubiquitin ligase activity (reviewed in [173, 174]), and enhances histone removal and acetylation on viral DNA [175]. ICP0 expression promotes reactivation from latency [176, 177] , in part, because it impairs innate immune responses during early stages of reactivation [178]. Collectively, ICP0 functions stimulate productive infection.

The incidence of HSV-1 reactivation from latency in humans correlates with increased stress: for example, exposure to UV light, heat stress (fever), and trauma [168, 179-184]. Stressful stimuli generally increase corticosteroid levels, which enter a cell, and bind to the glucocorticoid receptor (GR) or mineralocorticoid receptor (MR), reviewed in [56]. The MR or GR corticosteroid complex enters the nucleus, specifically binds a glucocorticoid response element (GRE), alters chromatin confirmation, and activates transcription. In response to stress, GR and Krüppel-like transcription factor 15 (KLF15) regulate gene expression by a positive feed-forward loop [185-187]. For example, GR activates KLF15 expression, and then, GR directly binds KLF15; consequently, a novel set of genes are synergistically activated by GR and KLF15 when compared to GR or KLF15.

Growing evidence has demonstrated that corticosteroids and KLF15 mediate HSV-1 replication and reactivation from latency, as summarized below. For example, primary human gingival fibroblasts treated with the synthetic corticosteroid dexamethasone (DEX) prior to HSV infection yielded significantly higher levels of the virus relative to controls only infected with HSV-1 [188]. This study further revealed that infection increased nuclear GR levels after infection when compared to controls. Our recent studies indicated HSV-1 infection of a Neuro-2A that was treated with DEX also yield higher levels of the infectious virus [85]. Furthermore, the corticosteroid-specific antagonist, CORT-108297, significantly reduces HSV-1 replication in Neuro-2A cells, but has no effect on cell viability [85]. We and others demonstrated that DEX accelerates explant-induced reactivation from latency, as judged by increased virus production [189-191]. In accordance with the findings that DEX promotes explant-induced reactivation from latency, CORT-108297 significantly reduces virus shedding during explant-induced reactivation [191]. Finally, HSV-1 induces (KLF15) steady state protein levels during productive infection, and silencing KLF15 significantly reduces viral replication [83]. Collectively, these compelling studies indicate GR and DEX promote viral replication and reactivation from latency.

To probe the mechanism by which GR and stress stimulate viral replication and reactivation

from latency, we tested whether GR and stress-induced cellular transcription factors transactivate key viral promoters. These studies demonstrated that HSV-1 IE promoter/cis-regulatory modules (CRMs) that drive expression of key viral transcriptional regulators (ICP0, ICP4, and ICP27) are cooperatively transactivated by GR and KLF15 [85, 192, 193]. Surprisingly, the ICP0, ICP4, and ICP27 promoters do not contain consensus GREs. KLF family members and specificity protein 1 (Sp1) belong to the same super family of transcription factors, and these family members bind GC-rich sequences [76, 194]. Mutagenesis studies suggest Sp1 and/or GC rich motifs are important for cooperative transactivation of HSV-1 IE promoters [85, 192, 193]. These studies further imply interactions between GR and KLF15 overcome the requirement that GR must bind a consensus GRE. Understanding how stress-induced transcription factors, including GR and KLF15, trigger HSV-1 gene expression is important because the viral genome exists as silent chromatin during latency [32, 195]. Since viral transcriptional regulatory proteins are not abundantly expressed in latently infected cells, it is reasonable to suggest that cellular transcription factors trigger viral gene expression following stressful stimuli.

These studies were focused on identifying CRMs upstream of the ICP0 TATA box that increase transcription of a heterologous minimal promoter; test whether CRMs are cooperatively transactivated by GR, KLF15, and/or DEX; and identify CRM sequences important for transactivation. These studies provide new insight into how GR and KLF15 increase ICP0 expression and productive infection.

Results

5.1 GR and KLF15 transactivate specific ICP0 promoter CRM fragments.

The ICP0 gene is located within the long repeats (TR_L and IR_L) of the HSV-1 genome (Figure 1A) and the ICP0 promoter contains numerous putative transcription factor binding sites **[91]** (Figure 1B and C). Previous studies identified a GR/KLF15 responsive region (RR) at the 5'-

terminus of the ICP0 promoter (-800 to -458; Figure 1B). To identify ICP0 non-coding sequences that can stimulate transcription, 4 ICP0 fragments upstream of the TATA box were synthesized (Figure 1B) and cloned at the 5'-terminus of a minimal promoter in the luciferase reporter construct (pGL4.24[luc2P/minP]).

Transcriptional activity was measured in a mouse neuroblastoma (Neuro-2A) and monkey kidney cell line (Vero). The rational for using these two cell lines is they can be readily transfected, and Vero cells are highly permissive for HSV-1 infection. Furthermore, Neuro-2A cells are neuronal-like cells that can be differentiated into dopaminergic-like neurons [196]. Neuro-2A cells express low levels of GR and KLF15 [159]. Silencing KLF15 significantly impairs HSV-1 replication [197] and the corticosteroid-specific antagonist (CORT-108297) significantly reduces HSV-1 replication [198] in Neuro-2A cells. GR gene is comprised of 9 exons and the first and last are alternatively spliced. The most abundant GR isoforms in cells are GR-α and GR-β [199]. The endogenous GR in Neuro-2A cells is smaller than the protein produced by the GR-α, the GR expression plasmid used for transient transfection studies, and the endogenous GR does not transactivate HSV-1 as efficiently as when GR-α is expressed [198, 200, 201] and data not shown. Other GR isoforms, generated by alternative splicing, do not have the transcriptional activation potential as high as GR-α [202] suggesting the GR expressed in Neuro-2A cells is an isoform that does not activate gene expression as efficiently as GR-α or the GR gene has a deletion in Neuro-2A cells. A GR specific band migrating with similar mobility as GR-α, and a smaller band are expressed at low levels in Vero cells (Figure 2) and GR-α transactivates HSV-1 promoters more efficiently than the endogenous GR protein [198, 200, 201]. In general, there is no selective pressure to express GR at high levels in established cell lines because GR expression can induce apoptosis and cell cycle arrest in certain cells, reviewed in [161, 203]. The A fragment consistently activated the minimal promoter approximately 60-fold in Neuro-2A (Figure 1D) and Vero cells (Figure 1E). The B fragment luciferase construct exhibited significantly

higher than pGL4.24[luc2P/minP in Neuro-2A but not in Vero cells. While CRM activity of fragment C was less than 10-fold higher in Vero cells compared to the empty vector, it was significantly higher than the empty vector. Since fragment C exhibited similar activity as fragment B, I did not pursue additional studies with C because sequences in fragment B were primarily responsible for CRM activity. The fragment D construct was not significantly different than the empty vector in Vero cells because it yielded variable results. In summary, these studies revealed the ICP0 promoter contained independent CRMs and the GR/KLF15 RR contained two fragments with CRM activity.

Figure 1

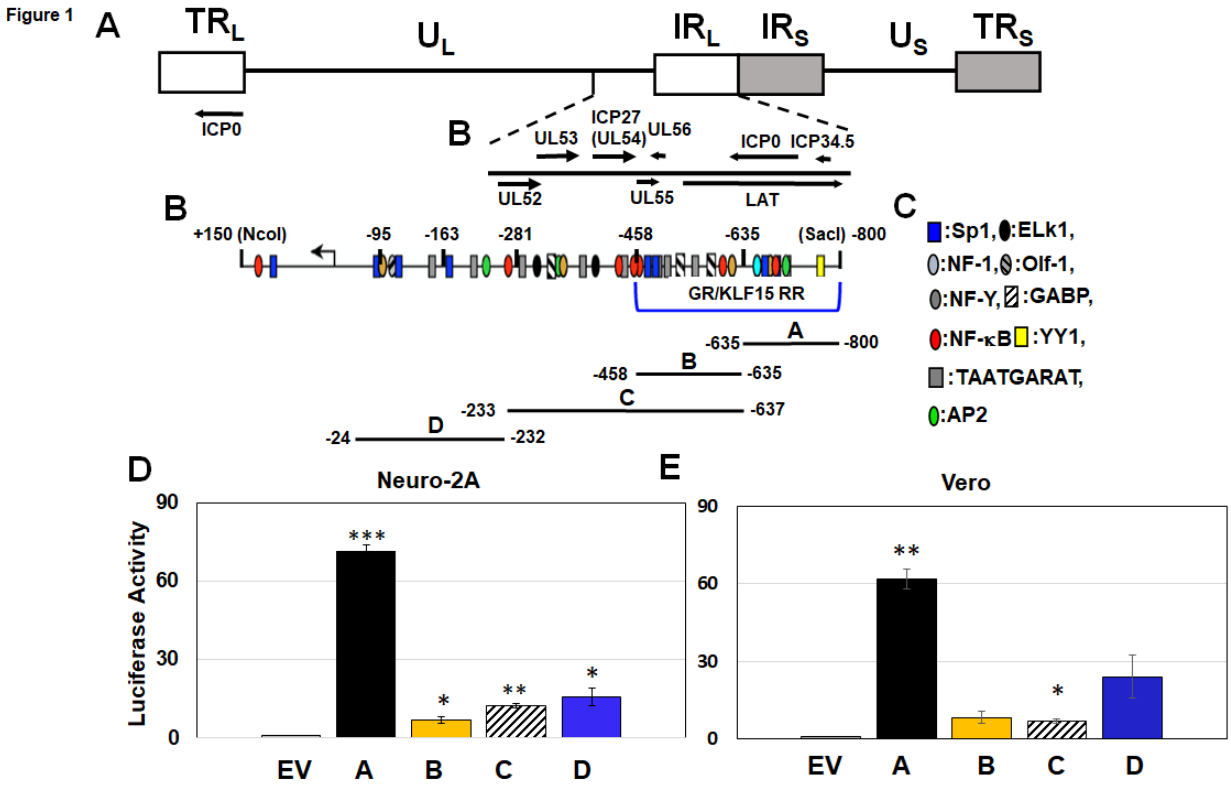


Figure 1: Location of ICP0 gene and promoter/enhancer sequences within the IR_L and TR_L repeats of the HSV-1 genome. Panel A: Schematic of HSV-1 genome. Unique long (U_L) and unique short (U_S) segments are flanked by the long internal or terminal repeats (IR_L and TR_L: white rectangles) and short internal or terminal (IR_S and TR_S: gray rectangles). Location of known genes in IR_L, including ICP0 are shown. A copy of ICP0 is also present in the TR_L. **Panel B:** Schematic of ICP0 promoter, and location of potential transcription factor binding sites (**Panel C**) relative to the start site of ICP0 mRNA (arrow). Four fragments (A-D) used in this study are shown and were cloned upstream of pGL4.24[luc2/minP] firefly luciferase reporter plasmid. Neuro-2A (**Panel D**) or Vero (**Panel E**) cells were transfected with 0.5 ug of the denoted CRM constructs and a Renilla luciferase plasmid (0.05ug). At 48 hours after transfection, dual luciferase activity was performed. Fold activation was calculated relative to pGL4.24[luc2P/minP] for each construct. Results are the mean of 3 experiments and error bars indicate standard error. Statistical analysis was performed as described in the materials and methods.

Figure 2-Endogenous GR expression in Vero cells

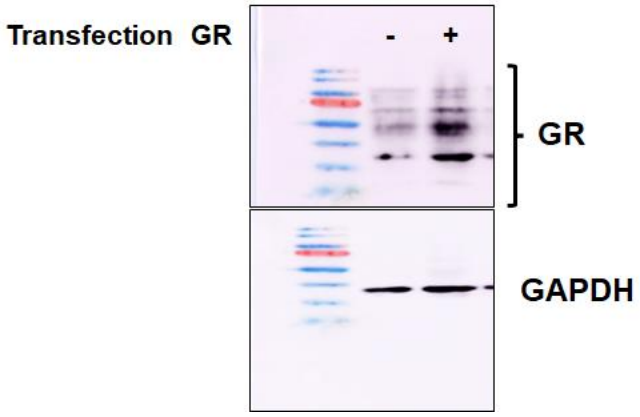


Figure 2: Vero cells were transfected with a plasmid that expresses GR (2 µg) as designated in the figure. Whole cell lysate was prepared using RIPA buffer, proteins separated by SDS-PAGE (50 µg in each lane) and GR expression detected by western blotting. Cell lysate from non-transfected Vero cells were used to check endogenous GR expression.

5.2. Localization of fragment A CRM activity

Since fragment A exhibited the highest cis-activation of the luciferase construct, additional sub-fragments were synthesized, and transcriptional activity measured. The fragment A contains a Sp1 binding site (GGCGGG), two complements of a consensus Sp1 binding site (denoted cSp1: CCGCCC), and a 15 base alternating purine/pyrimidine motif, which has the potential to form Z-DNA [204] (Figure 3A). The Sp1 and cSp1 binding sites have the potential to form a stem-loop structure suggesting secondary structures exist in fragment A. Our previous studies demonstrated that mutating certain Sp1 sites within the ICP4 and ICP27 enhancers reduced the ability of GR and KLF15 to transactivate these promoters [200, 201] suggesting Sp1 or cSp1 sites were important for CRM activity of fragment A. Surprisingly, fragment A2 exhibited significantly higher levels of CRM activity relative to intact fragment A in Neuro-2A and Vero cells (Figure 3B and C). However, fragment A1 had significantly lower CRM activity relative to wt-A fragment. Fragments A3 and Z were not significantly different than wt-A fragment in Neuro-2A or Vero cells suggesting the Z DNA motif had little effect on transcriptional activity. Interestingly, transcriptional activity of fragment AΔSp1 was significantly reduced in Vero, but not in Neuro-2A cells. In summary, these studies revealed sequences encompassing -720 to -635 (fragment A2) exhibited stronger transcriptional activity than wt-A and Sp1 plus cSp1 binding sites were crucial for stimulating transcriptional activity in Vero cells.

Figure 3

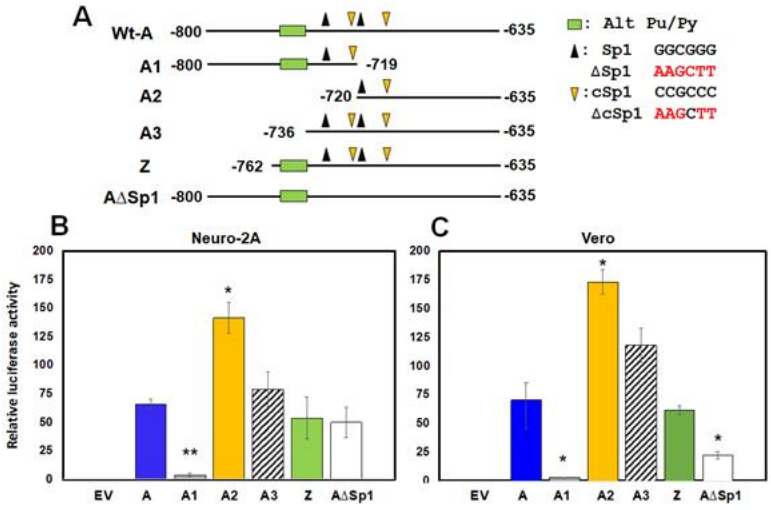


Figure 3. Localization of ICP0 fragment A that exhibits CRM activity. Panel A: Schematic of ICP0 wt-A fragment with transcription factor binding sites and mutants used in the study. A1 and A2 contain the 5' half and 3' half of the wt-A fragment. Fragment A3 contains all Sp1 and cSp1 binding sites but lack the 15 base alternating purine/pyrimidine motif. Fragment Z contains the alternating purine/pyrimidine motif (CGCGCATATATACGCTTG) and all Sp1/cSp1 binding sites. The AΔSp1 construct has mutations in all Sp1/cSp1 binding sites. All constructs were cloned into the pGL4.24[luc2P/minP] vector such that the fragment is upstream of the minimal promoter. Nucleotide position numbers shown are relative to the ICP0 mRNA initiation site. Nucleotide sequence of Sp1 and cSp1 sites and mutants are shown to the right of panel A. Neuro-2A (**Panel B**) or Vero (**Panel C**) cells were transfected with 0.5 µg with the wt A construct or designated A mutant and Renilla luciferase plasmid (0.05 µg). 48 hours after transfection cells were harvested and dual luciferase assay performed. Luciferase activity was calculated relative to pGL4.24[luc2P/minP] for each construct. Results are the mean of 3 experiments and the error bars indicate standard error. Statistical analysis was performed as described in the materials and methods.

5.3. Localization of fragment A sequences that mediate transactivation by GR, KLF15, and/or DEX.

The ability of GR, KLF15, and/or DEX to transactivate the wt-A fragment and the respective mutants (Figure 3A) was examined in Neuro-2A and Vero cell lines (Figure 4A and 4B). The wt-A fragment was significantly transactivated by GR and DEX in Neuro-2A cells; however, DEX and KLF15 addition did not further increase promoter activity. Interestingly, the wt-A fragment was significantly transactivated by GR, KLF15 and DEX treatment in Vero cells. Fragment A1was not transactivated by GR and/or KLF15 in both cell lines regardless of DEX treatment confirming this fragment inhibits promoter activity. In Neuro-2A cells, fragments A2 and A3 were not significantly transactivated by GR, KLF15, and/or DEX treatment when compared to the wt-A fragment. Conversely, fragment A2 was significantly transactivated by GR or KLF15 in Vero cells when compared to the wt-A fragment; but adding GR reduced KLF15 and DEX mediated transactivation. Fragment A3 exhibited reduced transactivation by KLF15 and DEX when compared to the A2 fragment in Vero cells. In both cell lines, the ability of GR and KLF15 to transactivate fragment Z was not higher than the wt-A fragment.

The AΔSp1 fragment, which contains mutations in the Sp1 and cSp1 binding sites of fragment A, was compared to the wt-A fragment for transactivation by GR, KLF15, and/or DEX in Vero and Neuro-2A cell. The A-ΔSp1 construct was not significantly transactivated by GR and KLF15 regardless of DEX treatment in Neuro-2A cells. Transactivation of the A-ΔSp1 mutant by GR, KLF15, and DEX treatment in Vero cells was significantly reduced when compared to wt fragment A. In contrast to the results in Vero cells, the A-ΔSp1 construct did not dramatically reduce luciferase activity relative to the wt-A or A2 fragment in Neuro-2A cells. Collectively, these studies revealed cell type dependent effects were observed with respect to transactivation of the wt-A and A2 fragments in Vero versus Neuro-2A cells.

Figure 4

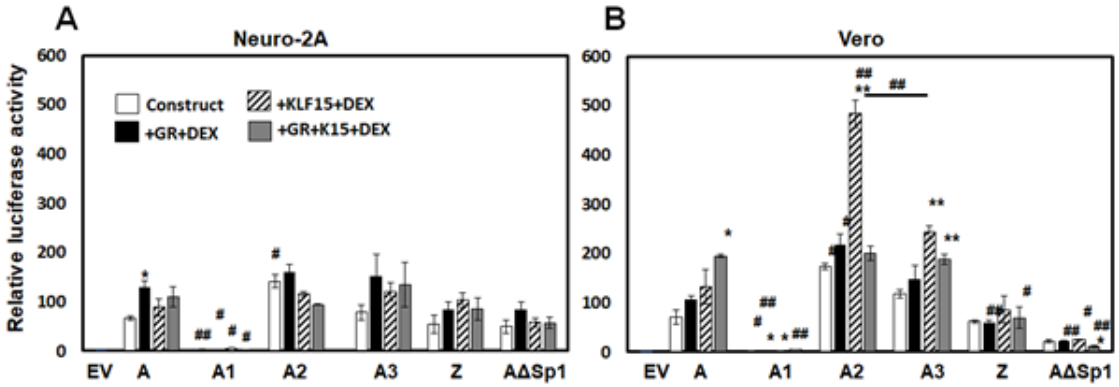


Figure 4. Identification of sequences in ICP0 fragment A that mediate transactivation by GR, KLF15, and/or DEX. Neuro-2A (**Panel A**) or Vero (**Panel B**) cells were transfected with 0.5 µg of the wt A luciferase construct, or the designated A mutant fragments and Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. Where indicated, the denoted cultures were co-transfected with GR (1ug) and/or KLF15 (0.5ug) expression plasmids. Empty vector (pGL4.24[luc2P/minP]) was added to cultures to maintain the same DNA amount. 24 hours after transfection certain cultures were treated with DEX (10uM). Cells were harvested at 48 hours and dual luciferase assay performed. Luciferase activity was calculated relative to empty vector (EV) luciferase value. Results are the mean of 3 experiments and error bars denote the standard error. An * denotes a significant difference compared to luciferase construct alone. A # denotes a significant difference in the denoted mutant construct relative to the respective wt A transfected samples. A */# denotes a significant difference of $P<0.05$ whereas a **/## denotes a signficant difference of $P<0.005$, as determined by student's t-test.

5.4. Occupancy of wt-A enhancer fragment by GR requires Sp1 binding sites

To test whether GR or KLF15 occupies the wt-A enhancer fragment, Neuro-2A cells were transfected with the wt-A enhancer construct and GR expression plasmid. Cultures were subsequently treated with DEX and chromatin immune-precipitation assays (ChIP) performed as described in Figure 5. The reason for transfecting with only GR is GR and DEX transactivated the A fragment more efficiently than GR+KLF15 in Neuro-2A cells. GR occupancy of the wt-A enhancer fragment was significantly higher than isotype and KLF15 in Neuro-2A cells (Figure 5) and GR occupancy of AΔSp1 mutant. However, the variation between experiments was higher in GR occupancy of wt-A CRM construct. In summary, GR occupancy of the wt-A enhancer fragment in Neuro-2A cells correlated with efficient transactivation by GR and DEX treatment in luciferase studies.

Figure 5

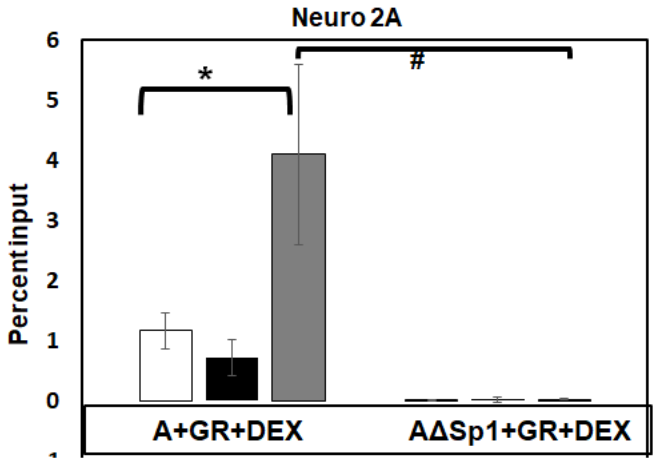


Figure 5

GR occupancy of ICP0 A enhancer fragment requires Sp1 binding sites. Neuro-2A cells were incubated in 2% stripped FBS and then transfected with the wt-A enhancer fragment (4μg) (**Panel A**) or AΔSp1 mutant (**Panel B**). Cultures were cotransfected with a GR-α (3μg) expression plasmid. 24 hours after transfection cells were treated with DEX (10μM). 40 hours after transfection cells were subjected to ChIP. ChIP was performed using GR (5μg) and KLF15 (5μg) antibodies. Nonspecific Isotype IgG (rabbit) was used as the control antibody. After immunoprecipitation, samples were subjected to phenol-chloroform extraction, ethanol precipitation, and the enhancer fragment amplified by PCR using forward and reverse primers as described in the materials and methods. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. Relative binding of DNA by GR or KLF15 via immunoprecipitation was quantified by image lab software (Biorad; Hercules, CA, USA) and are shown as a percentage of input. All results are the mean of 3 experiments and error bars denote standard error of the mean. Significant difference between GR occupancy with wt-A versus AΔSp1 is denoted by # and was determined by student's t-test ($P<0.005$).

5.4. Sp1 and cSp1 binding sites in the B fragment mediate CRM activity

Although CRM activity of the wt-B fragment was modest, it was examined further because it contains 2 consensus Sp1 binding sites and 2 cSp1 binding sites (Figure 6A) and is contained within the GR/KLF15 RR of the ICP0 promoter (Figure 6B) and [198]. Since Sp1 and certain GC rich motifs that resemble Sp1 binding sites are crucial for GR, KLF15, and/or DEX mediated transactivation [200, 201] mutants of both Sp1 (B-ΔSp1), cSp1 (B-ΔcSp1), or Sp1 and cSp1 binding sites (BΔAll) were prepared. In Vero (Figure 6C) but not Neuro-2A cells (Figure 6B), all three fragment B mutants exhibited significantly reduced transcriptional activity when compared to the-wt B fragment. Notably, the BΔAll construct exhibited the least CRM activity suggesting Sp1 and cSp1 binding sites were important for stimulating transcription in Vero cells. Although

wt-B and the three mutants did not have significantly different CRM activity in Neuro-2A cells due to variability in the individual results, the three mutant constructs exhibited reduced transcriptional activity, with BΔAll having the lowest activity.

Figure 6

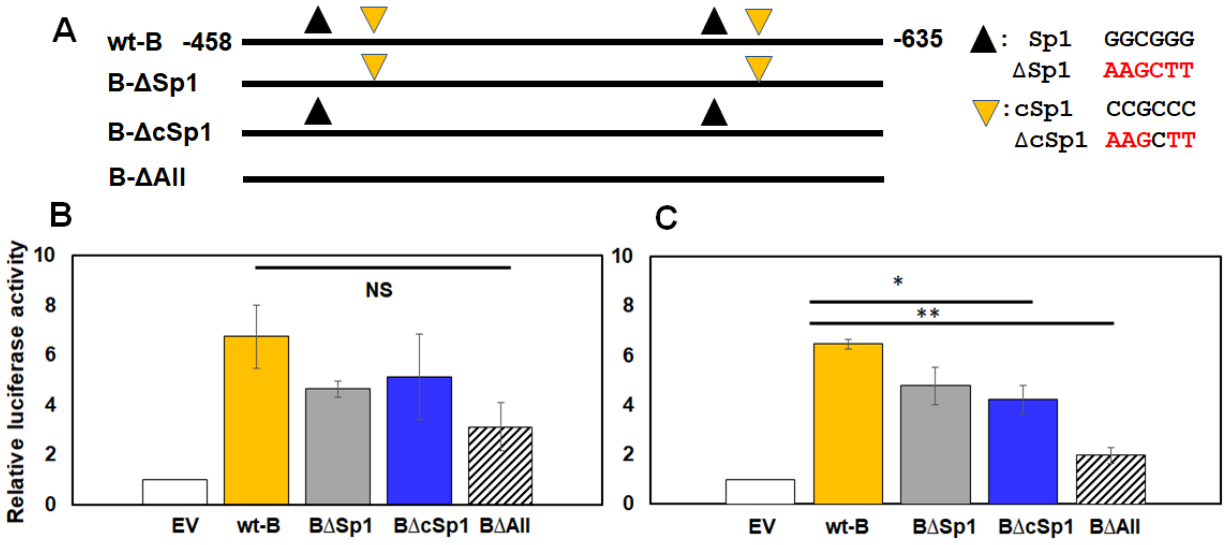


Figure 6: Localization of ICP0 B fragment CRM activity. Panel A: ICP0 wt-B fragment, location of Sp1 and cSp1 binding sites, and fragment B mutants are shown. Nucleotide position numbers are relative to the transcription initiation site of ICP0 mRNA. All constructs were cloned into pGL4.24[luc2P/minP]. The nucleotide sequence of Sp1, cSp1 sites and their mutated form is shown to the right of the figure. Neuro-2A (**Panel B**) or Vero (**Panel C**) cells were incubated in 2% stripped FBS and transfected with 0.5 ug of wt-B construct or denoted B mutant construct and a Renilla luciferase plasmid (0.05ug). 48 hrs after transfection cells were harvested and dual luciferase assay performed. Fold activation was calculated relative to pGL4.24[luc2P/minP] or empty vector (EV) for each construct. Results are the mean of 3 experiments and error bars denote standard error. Statistical analysis was performed as described in the materials and methods.

5.5. Sp1 binding sites mediate transactivation of B fragment by GR, KLF15, and DEX in Vero cells

In Neuro-2A cells, the wt-B fragment was not readily transactivated by GR, KLF15, and/or DEX (Figure 7A). Surprisingly, there was a significant reduction of promoter activity when transfected with KLF15+DEX or GR+KLF15+DEX. Furthermore, fragment B mutants were not affected by GR, KLF15, and/or DEX transactivation relative to the wt-B fragment. These results were consistent with the finding that Sp1 and cSp1 binding sites in wt-B fragment did not significantly reduce transcriptional activity in Neuro-2A cells.

In contrast to Neuro-2A cells, the wt-B fragment was significantly transactivated by GR and DEX or KLF15 and DEX or GR, KLF15, and DEX in Vero cells (Figure 7B). These studies also revealed that transactivation of fragment B was significantly reduced when Sp1 binding sites (BΔSp1) or cSp1 binding sites (BΔcSp1) were mutated and then transfected with GR, KLF15, and/or DEX. When all Sp1 and cSp1 binding sites were mutated, promoter activity was at basal levels following transfection with GR, KLF15, and/or DEX. Collectively, these studies indicated that Sp1 and cSp1 binding sites were crucial for transactivation of the fragment B by GR, KLF15, and DEX in Vero cells.

Figure 7

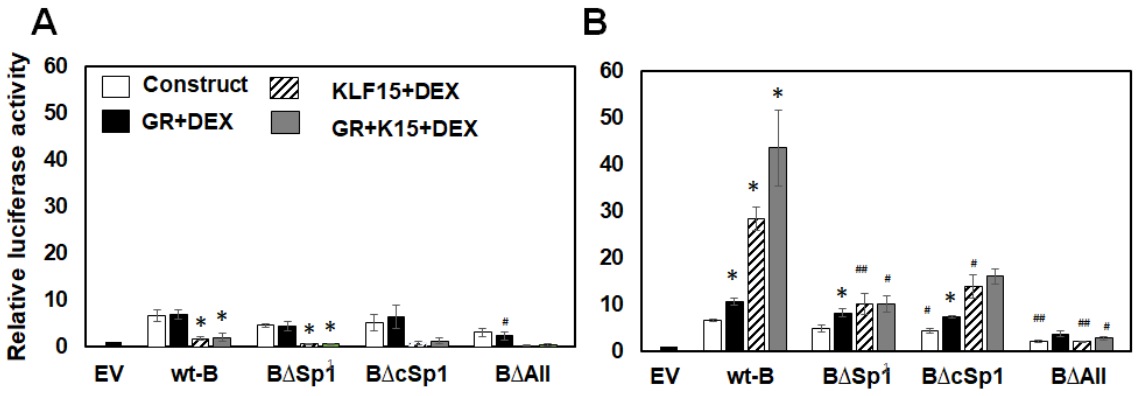


Figure 7. Transactivation of fragment B and mutants by GR, KLF15, and/or DEX. Neuro-2A (**Panel A**) or Vero (**Panel B**) cells were incubated in 2% stripped FBS and transfected with 0.5 ug of the denoted B constructs and a Renilla luciferase plasmid (0.05ug). Denoted samples were co-transfected with GR (1 ug) and/or KLF15 (0.5 ug) expression plasmids. Empty vector (pGL4.24[luc2P/minP]) was added to cultures to maintain the same DNA concentration. 24 hours after transfection certain cultures were incubated with water soluble DEX (10uM). Cells were harvested at 48 hours and dual luciferase assay performed. Luciferase activity is calculated relative to empty vector luciferase activity. Results are the mean of 3 experiments and error bars indicate standard error. Statistical analysis was performed as described in the materials and methods.

5.6. Occupancy of wt-B enhancer fragment by GR requires Sp1 and cSp1 binding sites.

To test whether GR or KLF15 occupies the wt-B enhancer fragment, Vero cells were transfected with the wt-B enhancer construct, GR, and KLF15 expression plasmids. Cultures were subsequently treated with DEX and chromatin immune-precipitation assays (ChIP) performed as previously described. The rationale for examining occupancy of B enhancer fragment in Vero

cells, and not Neuro-2A cells, was transactivation of the wt-B enhancer fragment was higher in Vero cells (Figure 7). GR occupancy of the wt-B enhancer fragment was significantly higher than KLF15 in Vero cells (Figure 8). The Isotype control antibody yielded high variability, which was unexpected. Occupancy of the wt-B enhancer fragment was also significantly higher than GR occupancy of the BΔAll mutant. In summary, GR occupancy of the wt-B enhancer fragment in Vero cells correlated with efficient transactivation by GR, KLF15 and DEX treatment and intact Sp1 and cSp1 binding sites in the ICP0 enhancer fragment B.

Figure 8

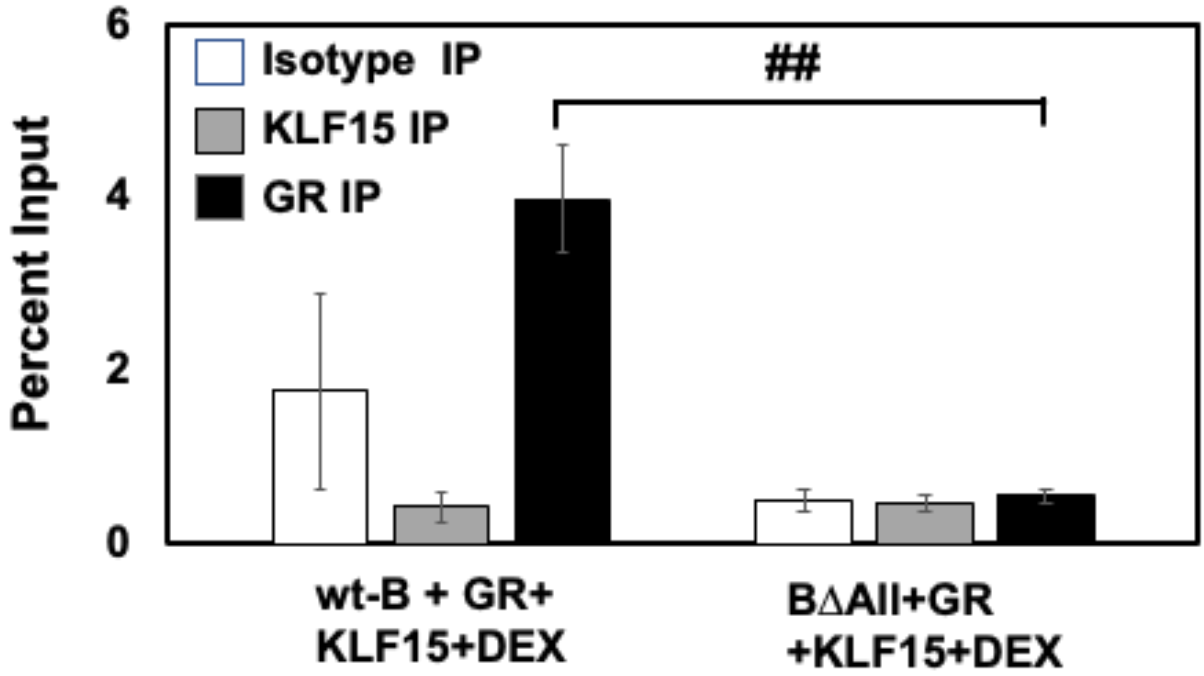


Figure 8

GR occupancy of ICP0 B enhancer fragment requires Sp1/cSp1 binding sites. Vero cells were incubated in 2% stripped FBS and then transfected with the wt-B enhancer fragment (4µg) (**Panel A**) or BΔAll mutant (**Panel B**). Cultures were cotransfected with a GR-α (3µg) and KLF15 (3 µg) expression plasmid. 24 hours after transfection cells were treated with DEX (10µM). 40 hours after transfection cells were subjected to ChIP. ChIP was performed using GR (5µg) and KLF15 (5µg) antibodies. Nonspecific Isotype IgG (rabbit) was used as the control antibody. After immunoprecipitation, samples were subjected to phenol-chloroform extraction, ethanol precipitation, and the enhancer fragment amplified by PCR using forward and reverse primers as described in the materials and methods. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. Relative binding of DNA by GR or KLF15 via immunoprecipitation was quantified by image lab software (Biorad; Hercules, CA, USA) and are shown as a percentage of input. All results are the mean of 3 experiments and error bars denote standard error of the mean. Significant difference between GR occupancy with wt-B versus BΔAll is denoted by ## and was determined by student's t-test ($P<0.005$).

5.7. Sp1 binding sites in fragment D are necessary for stimulating transcription in Vero cells.

To identify sequences within fragment D that possess CRM activity, the wt-D fragment and the respective D mutants (Figure 9A and B) were transfected into Neuro-2A or Vero cells and transcriptional activity measured. The wt-D fragment consistently stimulated promoter activity 5-10-fold higher than the empty luciferase reporter construct in Neuro-2A (Figure 9C) and Vero cells (Figure 9D). Within fragment D, I identified a motif similar to a consensus Egr-1 binding site (GCGGGGGCG) [205]. Egr-1 binding sites downstream of the HSV-1 and HSV-2 start sites of VP16 transcription were reported to enhance virulence and reactivation from latency [206]. Interestingly, binding of Egr-1 to a consensus binding site in its own promoter or the type II

collagen promoter reduces gene expression [205, 207]. Mutating the Egr-1 like site in fragment D (D-ΔEgr-1) significantly increased CRM activity in Neuro-2A and Vero cells. Mutating the two Sp1 binding sites also significantly reduced transcriptional activity in Vero, but not Neuro-2A, cells. The KLF or KLF-like sites in fragment D did not significantly reduce transcriptional activity in Neuro-2 or Vero cells.

Figure 9

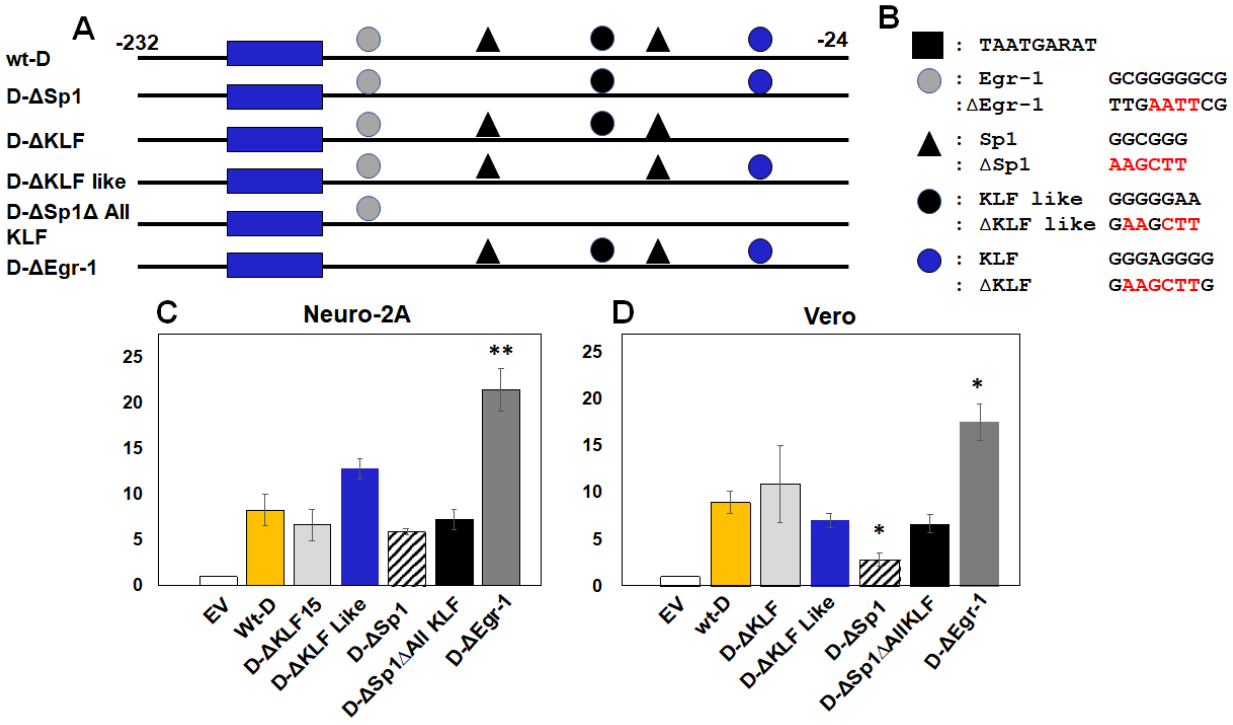


Figure 9: Examination of transcriptional activation of a minimal promoter by fragment D. **Panel A:** ICP0 wt-D fragment, location of transcription factor binding sites, and schematic of the respective mutants are shown. All constructs were cloned upstream of the minimal promoter of pGL4.24[luc2P/minP]. Nucleotide position numbers are shown relative to the transcription initiation site of ICP0 mRNA. **Panel B**: nucleotide sequences of Sp1, KLF, KLF like and Egr-1 sites and the respective mutants are shown. Neuro-2A (**Panel C**) or Vero (**Panel D**) cells were incubated in 2% stripped FBS and transfected with 0.5 ug of the wt-D construct or denoted

mutants and Renilla luciferase expression plasmid (0.05 ug). 48 hrs after transfection cells were harvested and dual luciferase assays performed. Luciferase activity is calculated relative to pGL4.24[luc2P/minP] for each construct. Results are the mean of 3 experiments and error bars indicate standard error. Statistical analysis was performed as described in the materials and methods.

5.8. Cell-type dependent transactivation of fragment D by GR, KLF15, and DEX

KLF15 and DEX significantly transactivated wt-D fragment in Neuro-2A cells (Figure 10A); however, GR transactivation did not significantly increase promoter activity. Conversely, significant transactivation of wt-D fragment only occurred when Vero cells were transfected with GR, KLF15, and cultures treated with DEX (Figure 10B). Mutating Sp1 binding sites significantly reduced KLF15 and DEX mediated transactivation in Neuro-2A cells. GR, KLF15, and DEX mediated transactivation of fragment D was also significantly reduced in Vero cells when Sp1 binding sites were mutated. Mutating putative KLF or KLF-like binding sites significantly reduced GR, KLF15, and DEX mediated transactivation in Vero, but not Neuro-2A cells. Mutating the Egr-1 like binding site significantly enhanced the ability of KLF15 and DEX to transactivate the wt-D fragment construct in Neuro-2A cells. Although mutating the Egr-1 binding site significantly increased GR, KLF15, and DEX mediated transactivation in Vero cells, the effects were not significant compared to the wt-D fragment.

Figure 10

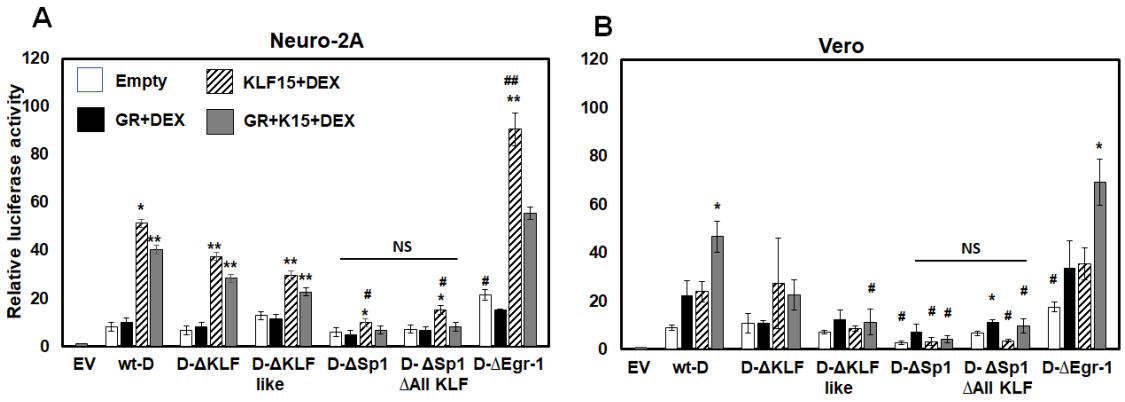


Figure 10: Transactivation of fragment D construct and mutants by GR, KLF15, and/or DEX. Neuro-2A (**Panel A**) or Vero (**Panel B**) cells were incubated in 2% stripped FBS and transfected with 0.5 ug of wt-D construct or denoted mutant constructs and Renilla luciferase plasmid (0.05 ug). Where indicated, samples were co-transfected with GR (1 ug) and/or KLF15 (0.5 ug) expression plasmids. Empty vector (pGL4.24[luc2P/minP]) was added to cultures to maintain the same DNA amount. 24 hours after transfection certain cultures were treated with DEX (10 uM) as denoted. Cells were harvested at 48 hrs, and dual luciferase assays performed. Luciferase activity was calculated relative to the empty vector luciferase activity. Results are the mean of 3 experiments and error bars denotes standard error. An * denotes a significant difference in a luciferase construct when compared to that luciferase construct cotransfected with GR, KLF15 and/or treated with DEX. A # denotes a significant difference in the mutant luciferase construct compared to relevant wt D transfected samples. A */# denotes a significant difference at P<0.05 whereas a **/## denotes a signficant difference as P<0.005, as determined by student's t-test. NS denotes a non-significant difference between the denoted constructs.

5.9. ChIP studies of fragment D

To assess KLF15 or GR occupancy of the D enhancer fragment, Neuro-2A cells were transfected with the wt-D enhancer construct and KLF15 expression vector and then treated with DEX. GR

was not co-transfected with the wt-D or mutant D enhancer fragment because it did not increase transactivation (Figure 10). ChIP was subsequently performed using KLF15- and GR- specific antibodies. Endogenous GR occupancy of the wt-D fragment was significantly higher than the isotype control antibody (Figure 11: denoted by **). In contrast to the wt-D fragment, occupancy of the D-ΔSp1ΔAll KLF mutant by GR was significantly reduced (denoted by ##). Furthermore, significant differences were not detected using a KLF15 or GR specific antibody when compared to the Isotype control antibody. In summary these studies support the finding that Sp1 and cSp1 binding sites mediate GR occupancy and promote KLF15 mediated transactivation.

Figure 11

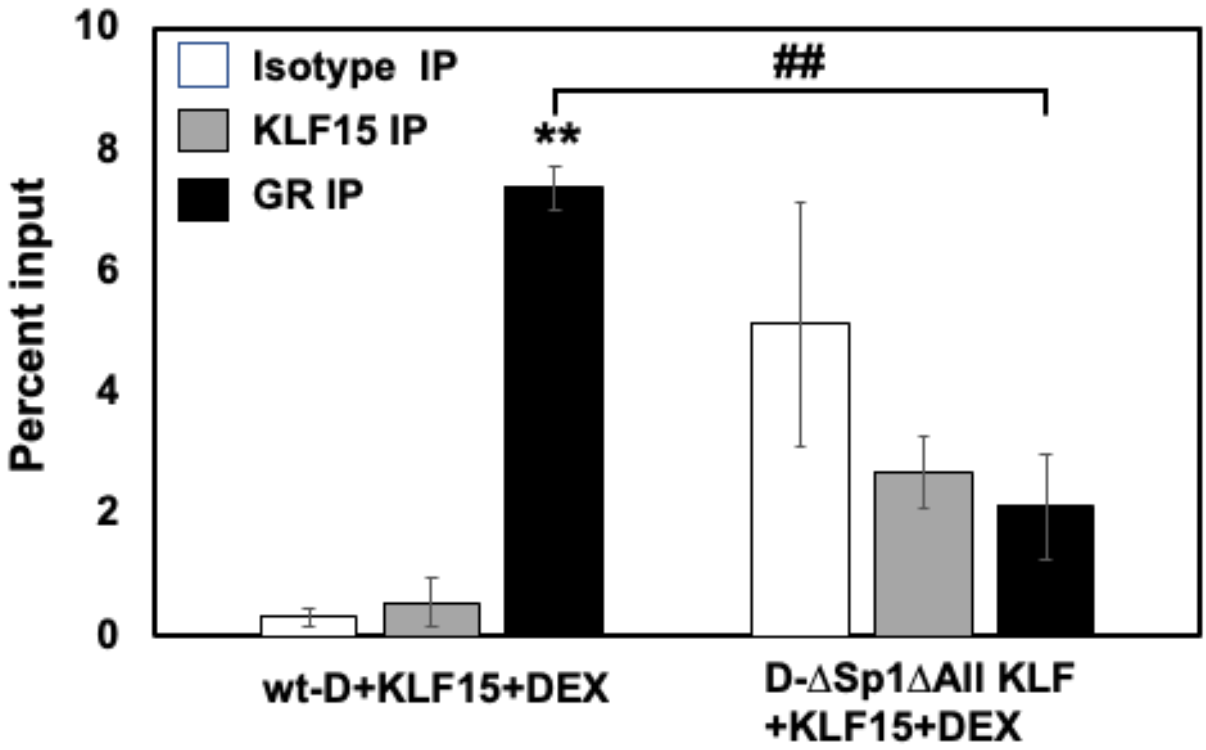


Figure 11

GR occupancy of ICP0 D enhancer fragment requires KLF15, KLF like and Sp1 binding sites. Neuro-2A cells were incubated in 2% stripped FBS and then transfected with the wt-D

enhancer fragment (4µg) (**Panel A**) or DΔSp1ΔAll KLF mutant (**Panel B**). Cultures were cotransfected with KLF15 (3 µg) expression plasmid. 24 hours after transfection cells were treated with DEX (10µM). 40 hours after transfection cells were subjected to ChIP. ChIP was performed using GR (5µg) and KLF15 (5µg) antibodies. Nonspecific Isotype IgG (rabbit) was used as the control antibody. After immunoprecipitation, samples were subjected to phenol-chloroform extraction, ethanol precipitation, and the enhancer fragment amplified by PCR using forward and reverse primers as described in the materials and methods. PCR products were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. Relative binding of DNA by GR or KLF15 via immunoprecipitation was quantified by image lab software (Biorad; Hercules, CA, USA) and are shown as a percentage of input. All results are the mean of 3 experiments and error bars denote standard error of the mean. Significant difference between GR occupancy with wt-D versus DΔSp1ΔAll KLF is denoted by ## and was determined by student's t-test ($P<0.005$).

Discussion

In this study, I present evidence that 3 distinct CRMs in the ICP0 promoter were transactivated by GR, KLF15, and or DEX. Surprisingly, fragment A contains sequences (-800 to -719) that significantly reduced transcriptional activity in Neuro-2A and Vero cells. Furthermore, mutagenesis of an Egr-1 like binding site in fragment D significantly increased CRM activity in Neuro-2A and Vero cells. Transactivation by GR, KLF15, and/or DEX treatment was also increased in these respective ICP0 CRM fragments, when negative regulatory sequences were deleted or mutated. While it was surprising to identify negative regulatory sequences in ICP0 CRM sequences, I suggest they mediate transcriptional activity during productive infection, promote establishment and maintenance of latency, or enhance ICP0 expression in certain cell types.

We [159] and others [160] demonstrated GR and KLF15 stably interact with each other and form a feed-forward transcription loop [160, 208]. Consensus GREs are not present in any of the ICP0 fragments that stimulated transcription suggesting the GR/KLF15 complex interacts with Sp1 or cSp1 binding sites. ICP0 fragments A, B, and D contain Sp1 or cSp1 binding sites crucial for GR, KLF15, and/or DEX mediated transactivation in Vero cells and to a lesser extent in Neuro-2A cells. Interestingly, ICP0 fragments A and B contain adjacent cSp1 and Sp1 binding sites separated by 4-6 nucleotides (Figure 12A). Fragment A2, which contains a cSp1 and Sp1-like binding site (GGCGGG), was strongly transactivated by KLF15 and DEX relative to the intact fragment A in Vero cells.

Fragment A1, which contains a distinct Sp1-like and cSp-1-like motif, was not transactivated by GR, KLF15, and/or DEX suggesting under certain circumstances adjacent sequences can have positive or negative effects on transcription. Recent studies also identified a Sp1 and cSp1 site within HSV-1 ICP27 promoter sequences that is crucial for GR, KLF15, and DEX mediated transactivation [200]. While the ICP4 enhancer is strongly transactivated by GR, KLF4 or KLF15, and DEX, these sequences do not contain a cSp1 [201]. However, 2 KLF4 consensus binding sites that encompass a consensus Sp1 site are located in the ICP4 enhancer (Figure 9A). Mutation of the 3' KLF4 binding site had the same effect as mutating both sites with respect to GR, KLF4 or KLF15, and DEX mediated transactivation in Neuro-2A and Vero cells. ChIP studies generally revealed a correlation between reduced GR occupancy to mutant Sp1 and/or cSp1 sites and transcriptional activation of ICP4 [201] and ICP27 [200] regulatory sequences. Under normal circumstances, Sp1 and other transcriptional coactivators (denoted by X) are predicted to occupy Sp1 and cSp1 binding sites, which results in basal levels of transcription (Figure 12B). Following a stressful stimulus, GR is activated and KLF15 expression is induced. Consequently, the GR/KLF15 complex is predicted to occupy Sp1 and/or cSp1 binding sites instead of Sp1; thus, transcription is significantly increased (Figure 12C). This model also

predicts GR and KLF15 interact with cell-type specific transcriptional coactivators (denoted as X) that play a role in transcriptional activation. I predict that the levels of GR, KLF15, and other transcriptional regulators in these two cell lines are important with respect to the differences I have observed. Studies designed to test whether the GR/KLF15 complex displaces Sp1 and whether there are other Sp1 family members bound to these sites will provide insight into how GR and KLF15 activate ICP0 promoter activity. It is also of interest to compare whether GR and KLF15 mediated transactivation of the ICP0, ICP4, and ICP27 CRMs utilize the strategy outlined Figure 12B and C. Finally, not all GC rich motifs are transactivated by GR and KLF15 because mutating the GC-rich Egr-1 like motif significantly increased GR and KLF15 mediated transactivation of fragment D in Neuro-2A and Vero cells.

Under certain circumstances, DEX does not stimulate GR and/or KLF15 mediated transactivation of the ICP0 fragments or the ICP4 enhancer [201]. While DEX generally enhanced GR and KLF15 mediated transactivation of the ICP0 fragments in Vero cells, transactivation of fragment A2 by GR and DEX was significantly reduced by KLF15 transfection. Although GR is generally activated by increased hormone levels (ligand-dependent activation), GR can also be activated when corticosteroids are not increased (ligand-independent activation). For example, GR phosphorylation is induced by UV light, which correlates with GR-mediated transcriptional activation and induction of certain enzymes, but corticosteroid levels are not increased [209-211]. This observation is relevant because UV light increases the incidence of HSV-1 and HSV-2 reactivation from latency [212-214]. Unliganded GR also increases expression of a tumor suppressor gene BRCA via the b-subunit of the Ets transcription factor GA-binding protein [215]. Thirdly, unliganded GR activation increases involucrin expression but is impaired when the c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinases (ERK) are inhibited in keratinocytes [216]. Finally, ethanol can increase GR-mediated transcription in the absence of increased corticosteroid levels [217]. Regardless of the ligand-independent stimulus that

activates GR, phosphorylation of certain GR serine or threonine residues must occur [209]. Hence, GR-mediated activation of HSV-1 and bovine herpesvirus 1 (BoHV-1) gene expression and productive infection [159, 218, 219] can be stimulated by ligand-dependent and independent mechanisms.

Figure 12

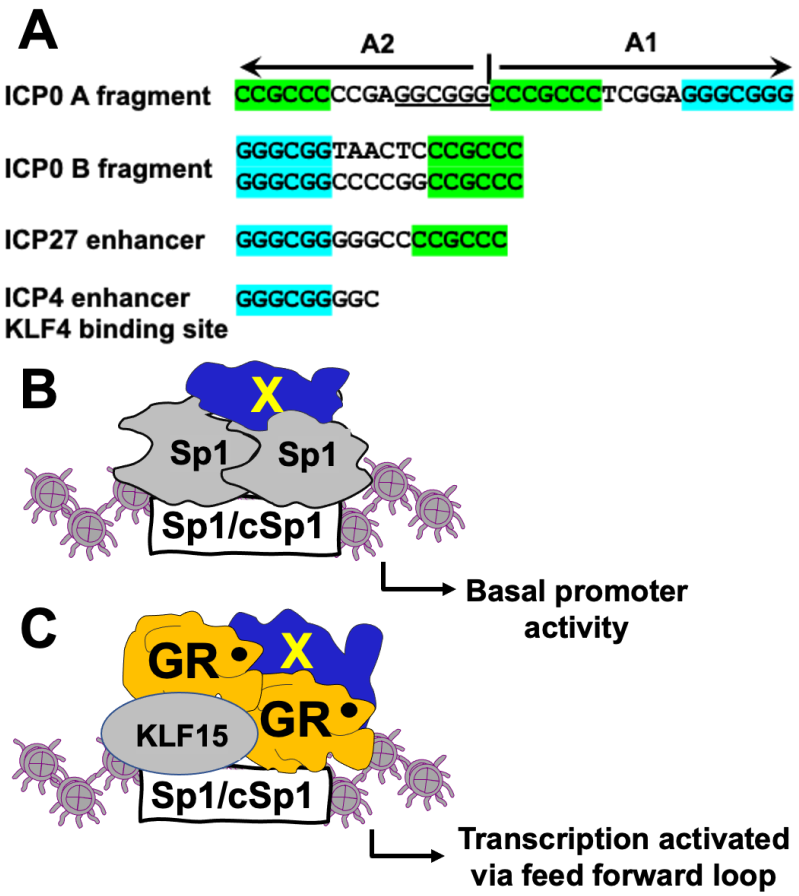


Figure 12

Summary of GR/KLF15 mediated transactivation of Sp1/cSp1 binding sites in HSV-1 IE promoters. Panel A: Location of Sp1 (GGGCGG), cSp1 sites (CCGCCC), or a Sp1-like motif (GGCGGG) in ICP0 A or B fragments, and the ICP27 enhancer **[200]**. ICP4 enhancer sequences contain KLF4 consensus binding sites important for KLF4 or KLF15 and GR mediated

transactivation. The KLF4 binding site also contains a Sp1 binding site **[201]**. Panel B: Model depicting Sp1 occupies Sp1 and cSp1 binding sites. X are unknown transcriptional coactivators. These interactions lead to basal promoter activity. Panel C: Putative model depicting a complex that contains GR, KLF15, DEX (black circle associated with GR), and unknown transcriptional coactivators (denoted by X) are bound to Sp1 and/or cSp1 binding site following a stressful stimulus. These interactions culminate in transcriptional activation. Grey circles denote histones.

5. Conclusions

These studies provide new insight into the complexity of ICP0 promoter activation and how a feed-forward transcription loop (GR and KLF15) transactivates the full-length ICP0 promoter. Sp1 and cSp1 sites located in ICP0 CRM sequences were crucial for GR, KLF15, and/or DEX mediated transactivation. Since ICP0 encodes multiple functions crucial for productive infection and reactivation from latency, understanding how stressful stimuli trigger ICP0 promoter activity is important. Interestingly, these studies revealed that mutating just one important cis-acting motif in the full-length ICP0 promoter may not abolish its ability to be transactivated by stressful stimuli: for example, GR, KLF15, and/or DEX.

CHAPTER VI

CIS REGULATORY MODULES IN THE HUMAN ALPHAHERPESVIRUS 1 INFECTED CELL PROTEIN 0 (ICP0) PROMOTER ARE TRANSACTIVATED BY GLUCOCORTICOID RECEPTOR, SPECIFICITY PROTEIN 1 AND KRÜPPEL LIKE FACTOR 4

Abstract

Krüppel like factor (KLF) family members are transcription factors (TFs) that regulate transcription by specifically binding GC rich sequences in promoters [85, 86]. Previously I demonstrated that Human alphaherpesvirus-1 (HSV-1) ICP0 promoter cis regulatory modules (CRMs) are significantly transactivated by a nuclear hormone receptor (NHR); glucocorticoid receptor (GR) and Krüppel like factor 15 (KLF15) when treated with synthetic corticosteroid dexamethasone (DEX). DEX activates GR which leads to nuclear translocation and initiation of GR dependent transcription. Binding sites for KLF15 are consensus binding sites for the cellular transcription factor, specificity protein 1 (Sp1). These observations lead me to hypothesize that Sp1 can transactivate ICP0 CRMs via Sp1 binding sites. Surprisingly, current studies demonstrated over-expression of Sp1 did not transactivate the ICP0 CRMs in two monkey kidney cell lines, Vero, and CV-1. However, Sp1 cooperated with GR to transactivate ICP0 promoter sequences spanning from −800 to −635 relative to the ICP0 transcription initiation site (Fragment A), −458 to −635 (Fragment B) and −232 to −24 (Fragment D) CRMs similar to GR,

KLF15 and DEX in CV-1 but not Vero cells; CV-1 cells were used for these studies because they do not express endogenous GR unless transfected by the GR- α expression construct. GR and Sp1 mediated transactivation was significantly reduced by mutating Sp1 binding sites in each CRM fragment in CV-1 cells. Additional studies examined the ability of KLF4, a pioneer TF, to transactivate the ICP0 CRMs. KLF4 consistently transactivated the ICP0 B and D CRMs~5-fold in mouse neuroblastoma cells (Neuro-2A cells). Conversely, only A and D CRMs were transactivated 2-fold by KLF4 in mouse embryonic fibroblasts (NIH-3T3). KLF15 and KLF4 did not cooperatively transactivate the ICP0 CRMs in CV-1 or NIH-3T3 cell lines.

Introduction

Human alphaherpesvirus-1 (HSV-1) is a significant viral pathogen worldwide that can cause blindness or fatal encephalitis. Approximately, 2/3 of people under the age of 50 have HSV-1. Following acute infection, lifelong latency is established in neurons; trigeminal ganglia (TG) are a significant site of latency. During latency, infectious virus is undetectable but viral DNA exists as a circular episome in latently infected neurons. Periodically cellular stressors trigger virus shedding, which is referred to a reactivation from latency. Recurrent eye disease and encephalitis are primarily due to reactivation from latency [195].

During acute infection the first genes transcribed are immediate early (IE) genes. There are several IE proteins expressed during HSV-1 lytic cycle such as ICP0, ICP4, ICP22, ICP27, and ICP47. Infected cell protein 0 (ICP0) is a viral protein that can activate expression of all viral genes including IE, early (E) and late (L). ICP0 has E3 ubiquitin ligase activity, which is a crucial property for evading host immunity. ICP0 is an important protein for efficient viral replication because at low multiplicities of infection wt-HSV-1 has a growth advantage over ICP0 null viruses [220].

HSV-1 reactivation from latency does not readily occur in mouse models of infection. Consequently, reactivation from latency in mice is studied by a method called explant induced reactivation where TG are dissected from latently infected mice and incubated with tissue culture medium [195] and/or treated with the synthetic corticosteroid Dexamethasone [191]. During explant induced reactivation, key viral regulatory proteins are expressed in TG. Previous in vivo studies demonstrated that 13% of neurons express ICP0 when TG are treated with DEX at 16hrs post treatment [54]. Thus, ICP0 expression correlates with reactivation from latency.

ICP0 protein expression is regulated by transactivation of cis regulatory modules (CRMs) present in the ICP0 promoter. Krüppel like factor 15 (KLF15), SLUG, Sam pointed domain containing Ets transcription factor (SPDEF) family members and glucocorticoid receptor (GR) are among the cellular transcription factors (TFs) that are significantly upregulated during explant induced reactivation of HSV-1. These TFs are expressed in significantly higher amounts in trigeminal ganglia (TG) of latently infected mice at 8 hrs post-explant when treated with DEX [54].

ICP0 promoter sequences contain multiple specificity protein 1 (Sp1) binding sites. Interestingly, Sp1 is a cellular transcription factor that also belongs to the same super family as KLF members. Previous studies demonstrated KLF15 and GR transactivate the independent CRMs within the ICP0 promoter and Sp1 binding sites are important for transactivation [86]. Sp1 is a host cellular transcription factor important for maintaining cellular homeostasis. Thus, Sp1 binding sites are present in promoters of many housekeeping genes that regulates cell differentiation, metabolism, cell growth and cell death [221, 222]. Sp1 binds to GC (GGGCGG and CCGCCC) boxes and genes that respond for Sp1 contain multiple GC boxes. However, a single Sp1 binding site is sufficient for the transactivation of certain promoters [223].

Based on the observations, I hypothesized Sp1 also transactivates the ICP0 CRMs. In the current study I show that Sp1 cooperates with GR to transactivate different ICP0 CRMs when treated with DEX.

Moreover, several transcription factors are upregulated during BoHV-1 reactivation from latency: for example, KLF4, KLF15, KLF6 and PLZF (promyelocytic leukemia zinc finger). KLF4 belongs to the KLF family and transactivates the BoHV-1 bICP0 [224] and HSV-1 ICP4 IE promoters [193]. KLF4 also cooperates with the progesterone receptor (PR) to stimulate BoHV-1 productive infection [224]. Furthermore, KLF4 can bind “silent” chromatin and activate gene transcription: thus KLF4 is considered to be a pioneer transcription factor [225]. Phosphorylation,

acetylation, ubiquitination, or methylation of KLF4 can lead to either transactivation or trans-repression of genes responsive for KLF4. Furthermore, KLF4 is an essential TF that plays a major role in establishing pluripotent stem cells [226]. In this current study, I also investigated how KLF4 modulate the HSV-1 ICP0 promoter.

Results

6.1 Sp1 cooperates with GR to transactivate ICP0 CRMs in CV1 cells and Sp1 binding sites are important for the transcriptional activity.

Previous studies demonstrated that mutation of specificity protein 1 (Sp1) binding sites, in ICP0 CRM/promoter fragments A, B and D [86] (Figure 1A and B) significantly reduces GR/KLF15 and DEX mediated transactivation. Since Sp1 binding sites are occupied by Sp1 transcription factor during normal cellular processes, and SV40 early and late promoters are known to be transactivated by Sp1[227] I hypothesized that Sp1 transactivates the ICP0 CRM fragments via Sp1 sites. To test this hypothesis, monkey kidney cells (Vero) and CV-1 cells (an African Green Monkey kidney cell line that does not express GR [228]) were used for these studies. The reason for selecting CV-1 cells is to avoid interfering with transfected GR-α because in some cell lines the predominant isoform of endogenous GR is GR-β, which can inhibit GR-α [229]. These cell lines were transfected with ICP0 CRM fragments A, B or D and increasing concentrations of the Sp1 expression vector (0.25, 0.5 and 1ug). Sp1 alone reduced transcriptional activity in both cell lines (Figure 2A and B).

Additional studies tested whether GR and/or KLF15 cooperates with Sp1 to transactivate the respective ICP0 CRMs. In Vero cells, Sp1 did not cooperate with GR to stimulate the A, B or D CRM fragments (Figure 3A). However, Sp1 cooperated with GR to transactivate ICP0 CRMs in CV-1 cells, and the levels of cooperation were like GR+KLF15+DEX mediated transactivation of ICP0 CRM A, B and D fragments (Figure 3B).

Furthermore, Sp1+KLF15+DEX transactivated B and D CRM constructs 2-fold in CV-1 cells but only B CRM construct was transactivated in Vero in a similar pattern as GR+KLF15+DEX (Figure 3C and 3D).

GR+KLF15+Sp1+DEX mediated transactivation in Vero (Figure 3E) or CV-1 cells (Figure 3F), promoter activity was lower compared to GR+KLF15+DEX mediated transactivation. In summary, these studies revealed GR and Sp1 cooperatively transactivated ICP0 CRM A in CV-1 but not Vero cells.

To test whether Sp1 binding sites were important for GR, Sp1 and DEX mediated transactivation, the Sp1 site mutants of the ICP0 CRM fragments were transfected into CV-1 cells and promoter activity measured (Figure 4). As expected, Sp1 mutants of ICP0 A (AΔSp1), B (BΔAll Sp1) and D (DΔSp1ΔAllKLF) fragments were not transactivated by GR and Sp1 when treated with DEX. These results suggest either GR and/or Sp1 binds to Sp1 sites in ICP0 CRM fragments to stimulate transcription.

Figure 1

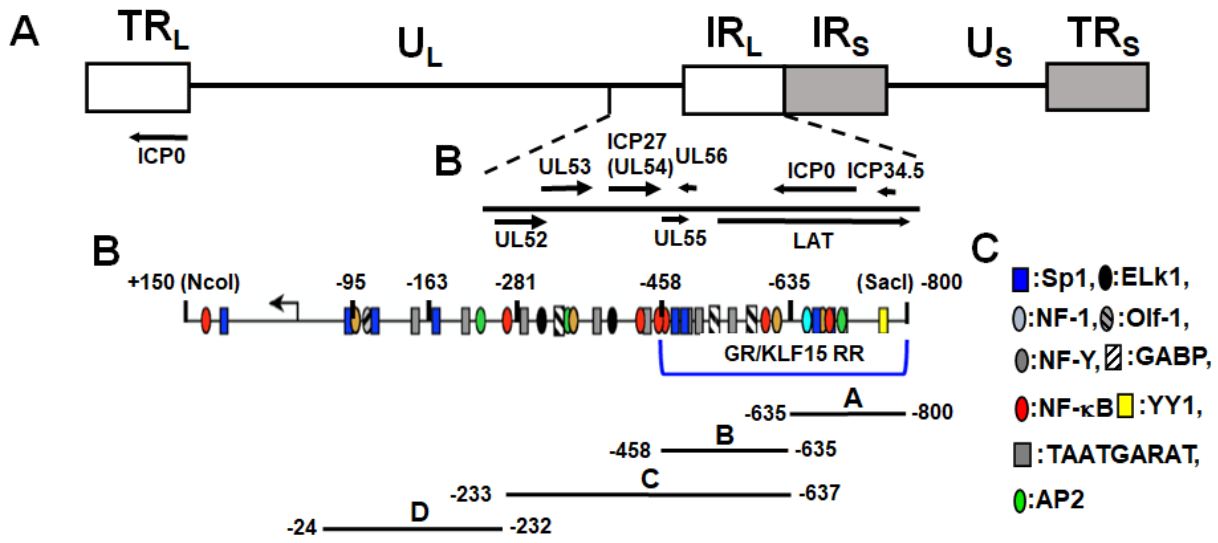


Figure 1. Location of ICP0 gene and promoter/CRM construct sequences within the IR_L and TR_L repeats of the HSV-1 genome. Panel A: Schematic of the HSV-1 genome. Unique long (U_L) and unique short (U_S) segments are flanked by the long internal or terminal repeats (IR_L and TR_L: white rectangles) and short internal or terminal (IR_S and TR_S: gray rectangles). Location of known genes in IR_L, including ICP0 are shown. A copy of ICP0 is also present in the TR_L.

Figure 2

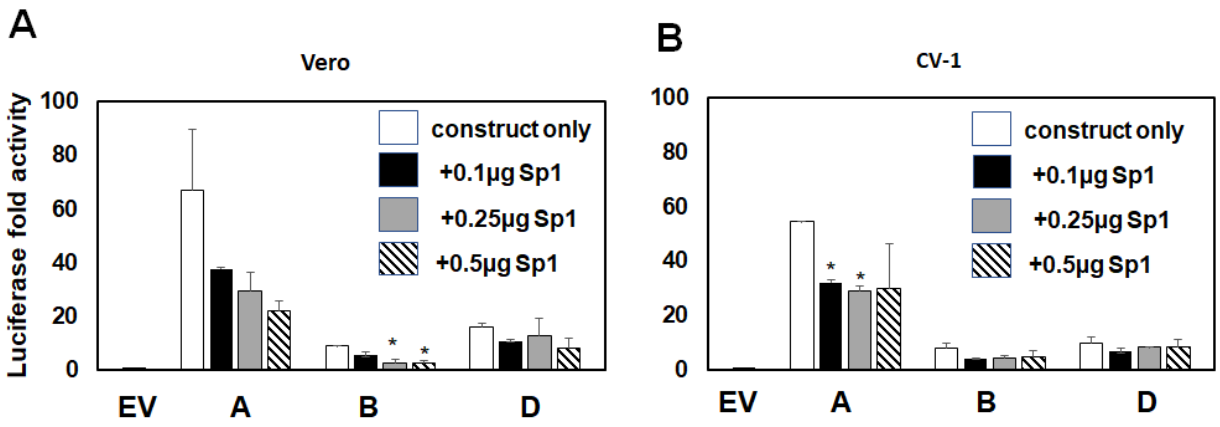


Figure 2. Identification of Sp1 effect on HSV-1 ICP0 CRM constructs. Vero (**Panel A**) or CV-1 (**Panel B**) cells were transfected with 0.5 µg of the wt A or wt- B or wt-D CRM construct, increasing amounts of Sp1 expression vector (0.1/ 0.25/0.5ug) and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. Empty vector (pGL4.24[luc2P/minP]) was added to cultures to maintain the same DNA amount. Cells were harvested at 24 hours and dual luciferase assay performed. Luciferase activity was calculated relative to empty vector (EV) luciferase value. Results are the mean of 2 experiments and error bars denote the standard error. An * denotes a significant difference compared to respective CRM construct alone. A * denotes a significant difference of $P<0.05$ whereas as determined by student's t-test.

Figure 3

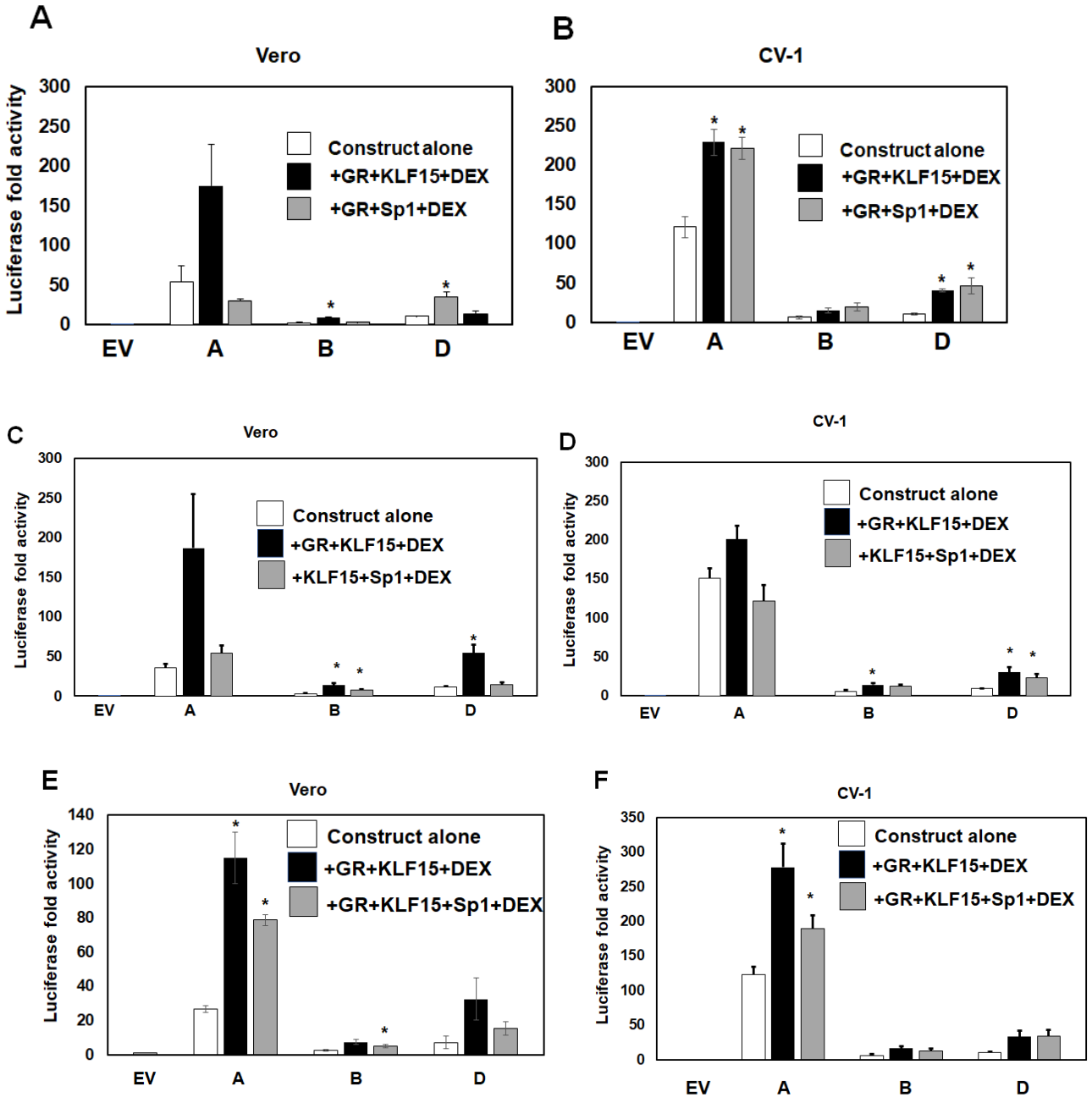

A
Vero
Luciferase fold activity
300
250
200
150
100
50
0
Construct alone
+GR+KLF15+DEX
+GR+Sp1+DEX
EV
A
B
D
B
CV-1
Construct alone
+GR+KLF15+DEX
+GR+Sp1+DEX
EV
A
B
D
C
Vero
Luciferase fold activity
Construct alone
+GR+KLF15+DEX
+KLF15+Sp1+DEX
EV
A
B
D
D
CV-1
Luciferase fold activity
Construct alone
+GR+KLF15+DEX
+KLF15+Sp1+DEX
EV
A
B
D
E
Vero
Luciferase fold activity
140
120
100
80
60
40
20
0
Construct alone
+GR+KLF15+DEX
+GR+KLF15+Sp1+DEX
EV
A
B
D
F
CV-1
Luciferase fold activity
350
300
250
200
150
100
50
0
Construct alone
+GR+KLF15+DEX
+GR+KLF15+Sp1+DEX
EV
A
B
D

Figure 3. Identification of ICP0 CRM constructs that are transactivated by GR, Sp1 and DEX. Vero (**Panel A, C, E**) or CV-1 (**Panel B, D, F**) cells were transfected with 0.5 µg of wt A, wt-B or wt-D CRM construct, and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. Where indicated, denoted cultures were co-transfected with GR (1ug) and/or KLF15 (0.5ug) and/or the Sp1 (0.5ug) expression plasmids. Empty vector (pGL4.24[luc2P/minP]) was added to the transfection mixture to maintain the same DNA amount. 24 hours after transfection certain cultures were treated with DEX (10uM). Cells were harvested at 48 hours and dual luciferase assays performed. Luciferase activity was calculated relative to the empty vector (EV) luciferase value. Results are the mean of 3 experiments and error bars denote the standard error. An * denotes a significant difference compared to the respective CRM construct alone. A */# denotes a significant difference of $P<0.05$ as determined by student's t-test.

Figure 4

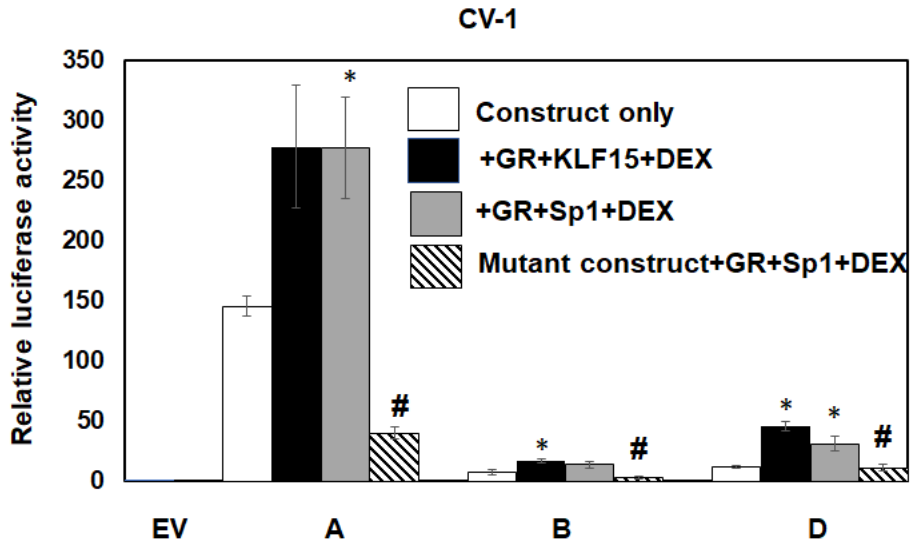


Figure 4. **Transactivation of ICP0 CRM constructs and their mutants by GR, Sp1, and DEX**

CV-1 cells were transfected with 0.5 µg of the wt A, wt-B or wt-D CRM construct or their respective mutants (AΔSp1, BΔAllSp1 and DΔSp1ΔAll KLF) and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. Where indicated, the denoted cultures were co-transfected with GR (1ug) and KLF15 (0.5ug) or Sp1 (0.5ug) expression plasmids. Empty vector (pGL4.24[luc2P/minP]) was added to cultures to maintain the same DNA amount in the transfection mixture. 24 hours after transfection certain cultures were treated with DEX (10uM). Cells were harvested at 48 hours and dual luciferase assay performed. Luciferase activity was calculated relative to empty vector (EV) luciferase value. Results are the mean of 3 experiments and error bars denote the standard error. An * denotes a significant difference compared to the respective wt-CRM construct alone. An # denotes a significant difference compared to the respective wt-CRM construct+GR+Sp1+DEX. A */# denotes a significant difference of $P<0.05$ as determined by student's t-test.

6.2 KLF4 transactivates ICP0 CRMs

KLF4 expression is significantly upregulated in TG neurons following dexamethasone treatment [191]. Previous studies demonstrated that HSV-1 ICP4 CRM sequences are transactivated by KLF4 and GR: consequently, I tested whether KLF4 can transactivate ICP0 CRM fragments. This hypothesis was tested using mouse neuroblastoma cells (Neuro-2A) because they are readily transfected and possess neuron like properties [230]. Neuro-2A cells were transfected with either ICP0 A, B or D CRM fragments and co-transfected with a KLF4 expression vector. ICP0 B and D CRM constructs were transactivated 7-fold and 8-fold respectively when transfected with KLF4; however, the ICP0 CRM A construct was transactivated less than 2-fold (Figure 5A).

Additional studies revealed 0.25-1ug KLF4 had an incremental effect on transactivation and the highest transactivation was achieved at 1ug of KLF4 in Neuro-2A cells (Figure 5B). These results were drawn from a single experiment because KLF4 demonstrated steady incremental pattern of transactivation of ICP0 CRM transactivation.

Figure 5

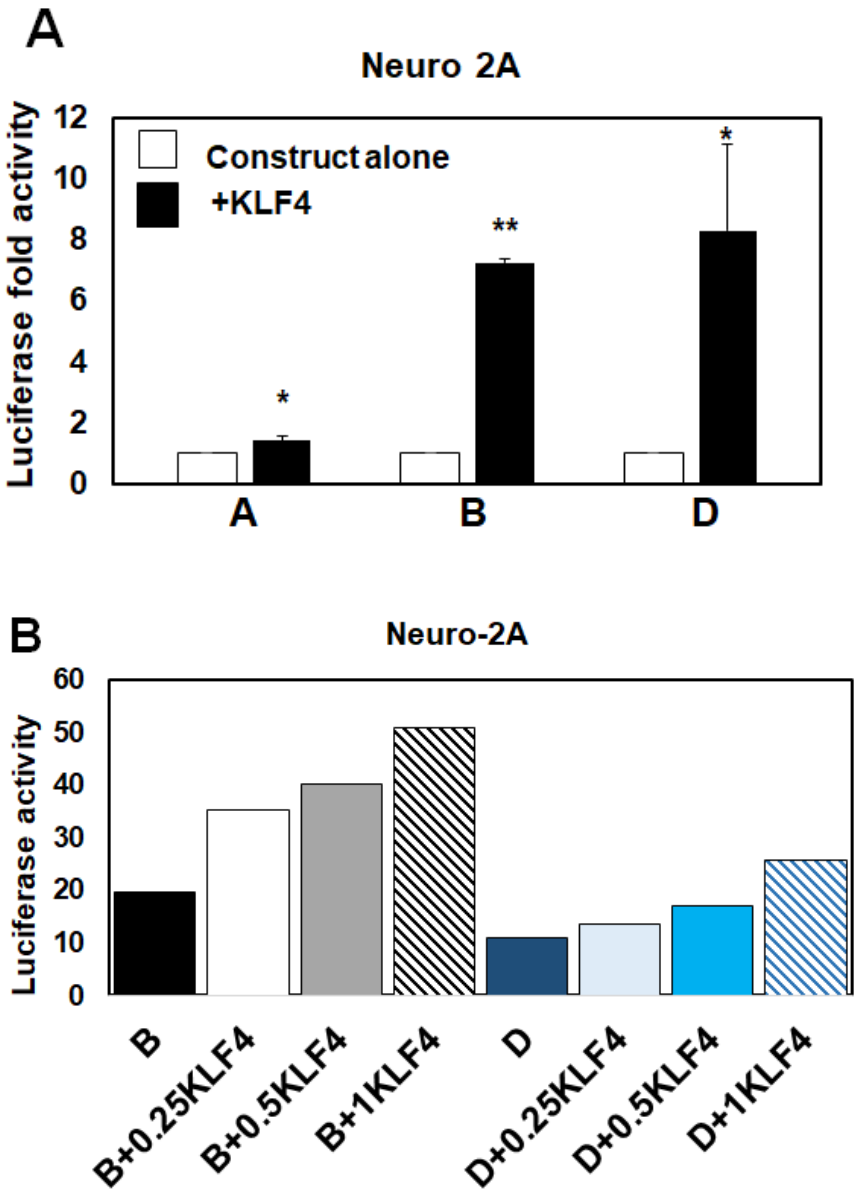


Figure 5. Transactivation of ICP0 CRM constructs by KLF4

Panel A-Neuro-2A cells were transfected with 0.5 µg of the wt A, wt-B or wt-D CRM construct and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. Where indicated, the denoted cultures were co-transfected with KLF4 (0.5ug) expression plasmids. Empty vector (pGL4.24[luc2P/minP]) was added to cultures to maintain the same DNA amount in thr transfection mixture. Cells were harvested at 24 hours and dual luciferase assay performed. Fold activation was calculated relative to each CRM construct. Results are the mean of 3 experiments and error bars denote the standard error. An * denotes a significant difference compared to the respective *wt*-CRM construct alone. A * denotes a significant difference of $P<0.05$ and ** denotes a significant difference of $P<0.005$ as determined by student's t-test.

Panel B. Neuro-2A cells were transfected with 0.5 µg of the wt-B, wt-D CRM construct, and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. Where indicated, the denoted cultures were co-transfected with KLF4 expression plasmids in increasing concentrations (0.25, 0.5 and 1ug). Empty vector (pGL4.24[luc2P/minP]) was added to cultures to maintain the same DNA amount in the transfection mixture. Cells were harvested at 24 hours and dual luciferase assay performed. Results are from a single experiment.

6.3 Sp1 and cSp1 sites are crucial for KLF4 mediated transactivation of ICP0 B CRM construct.

ICP0 B CRM construct contains 2 consensus Sp1 binding sites (GGCGGG) and two complementary Sp1 (cSP1) binding sites (CCGCCC) [86]. The mutants of Sp1 (BΔSp1) or cSp1 (BΔcSp1) or both Sp1 and cSp1 sites (BΔAll) (Figure 6A) were tested for transactivation by

KLF4 (Figure 6B). All three mutants significantly reduced the KLF4 mediated transcriptional activity of the B CRM construct. Notably, transactivation of BΔAll by KLF4 exhibited the lowest transcriptional activity demonstrating Sp1 and cSp1 sites were crucial for KLF4 mediated transactivation of the ICP0 B CRM construct (Figure 6B). This result is consistent with previous findings demonstrating KLF15 and GR mediated transactivation of the B CRM construct was significantly reduced in BΔAll [86].

Figure 6

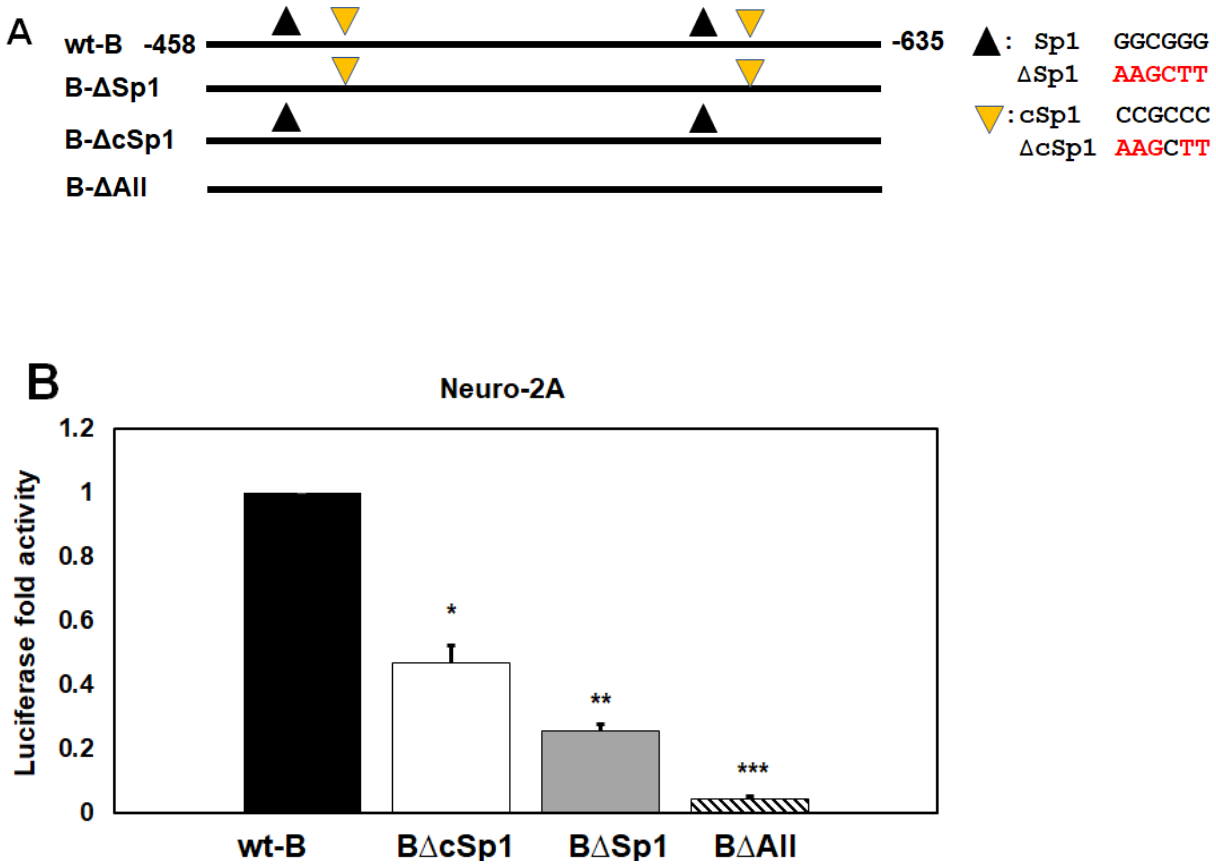


Figure 6. Localizing sequences important for KLF4 mediated transactivation of ICP0 CRM construct B

Panel A- ICP0 wt-B fragment, location of Sp1 and cSp1 binding sites, and fragment B mutants are shown. Nucleotide position numbers are relative to the transcription initiation site of ICP0 mRNA. All constructs were cloned into pGL4.24[luc2P/minP]. The nucleotide sequence of Sp1, cSp1 sites, and their mutated form (red) is shown to the right of the figure

Panel B-Neuro-2A cells were transfected with 0.5 µg of the wt-B CRM construct or its denoted mutants and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. Cultures were also co-transfected with KLF4 (1ug) expression plasmid. Cells were harvested at 24 hours and dual luciferase assay performed. Results are the mean of 2 experiments and error bars denote the standard error. Luciferase fold activity was calculated taking wt-B+KLF4 luciferase activity as 1-fold. An * denotes a significant difference compared to the wt-B+KLF4 luciferase activity. A * denotes a significant difference of $P<0.05$, ** denotes a significant difference of $P<0.005$ and *** denotes a significant difference of $P<0.0005$ as determined by student's t-test.

6.4 Both Sp1 and KLF binding sites mediate transactivation of D CRM construct by KLF4.

Since wt-D CRM construct was transactivated significantly by KLF4, I performed studies to test whether the Sp1 binding sites were important for KLF4 mediated transactivation. The mutants of the D CRM construct (Figure 7 A and B) [86] were co-transfected with KLF4 in Neuro-2A cells and luciferase activity measured compared to wt-D CRM activity transfected with KLF4. Surprisingly mutations in KLF binding sites (D-ΔKLF), KLF like binding sites (D-ΔKLFlike), Sp1 binding sites (D-ΔSp1) and Egr-1 (early growth response protein-1) binding site (D-ΔEgr-1) reduced KLF4 mediated transactivation of D CRM construct below 0.5-fold (Figure 7C). Mutation of all Sp1 binding sites resulted in the lowest level of luciferase activity indicating KLF4 transactivates wt-D CRM construct primarily via Sp1 binding sites.

Figure 7

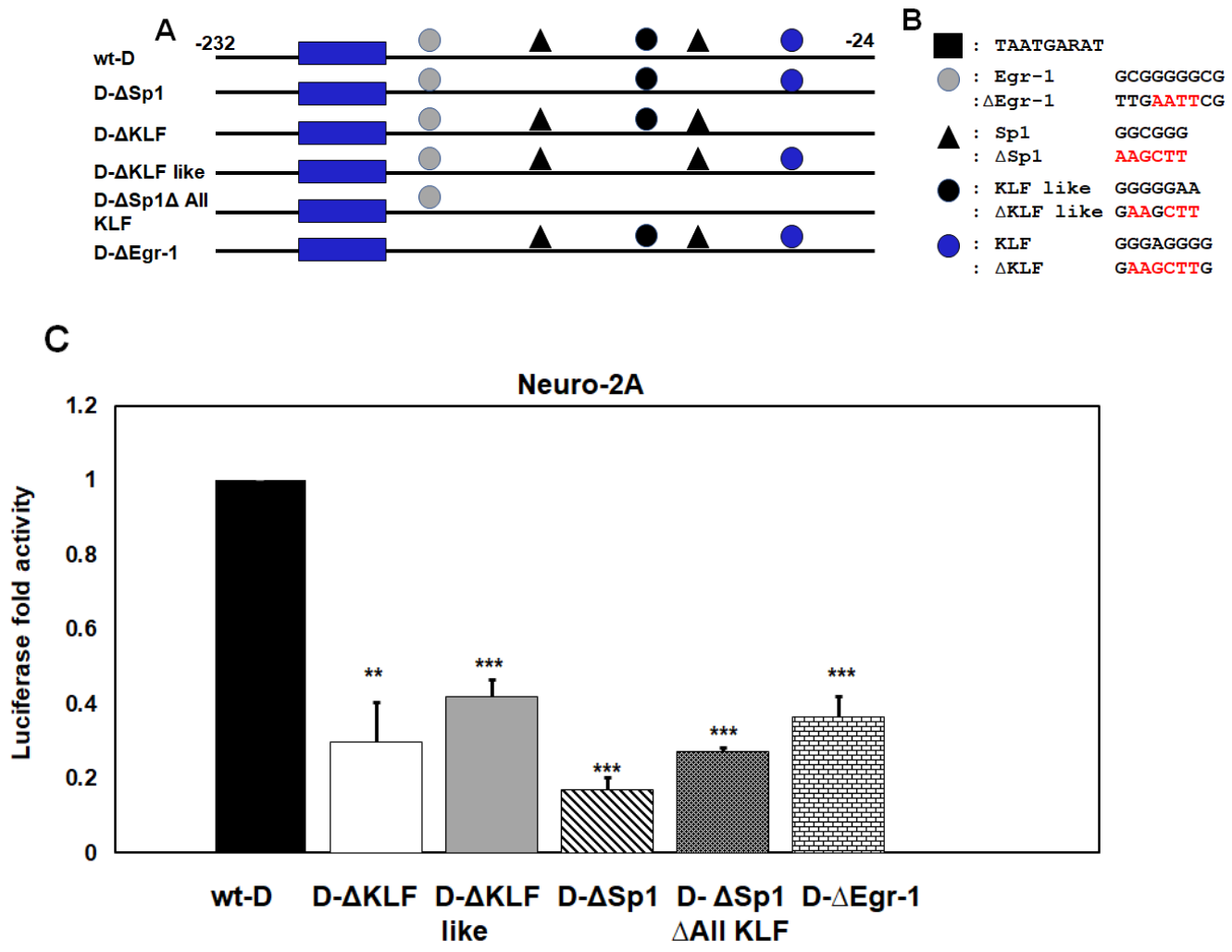

A
-232
-24
wt-D
D-ΔSp1
D-ΔKLF
D-ΔKLF like
D-ΔSp1Δ All KLF
D-ΔEgr-1
B
: TAATGARAT
: Egr-1 GCGGGGGCG
:ΔEgr-1 TTGAATTCG
: Sp1 GGCGGG
: ΔSp1 AAGCTT
: KLF like GGGGGAA
: ΔKLF like GAAGCTT
: KLF GGGAGGGG
: ΔKLF GAAGCTTG
C
Neuro-2A
Luciferase fold activity
1.2
1
0.8
0.6
0.4
0.2
0
**
***
***
***
***
wt-D
D-ΔKLF
D-ΔKLF like
D-ΔSp1
D- ΔSp1 ΔAll KLF
D-ΔEgr-1

Figure 7. Localizing sequences important for KLF4 mediated transactivation of ICP0 CRM construct D

Panel A: ICP0 wt−D CRM construct, location of transcription factor binding sites, and schematic of the respective mutants are shown. All constructs were cloned upstream of the minimal promoter of pGL4.24[luc2P/minP]. Nucleotide position numbers are shown relative to the transcription initiation site of ICP0 mRNA. **Panel B**: nucleotide sequences of Sp1, KLF, KLF-like, and Egr−1 sites, and the respective mutants (red) are shown.

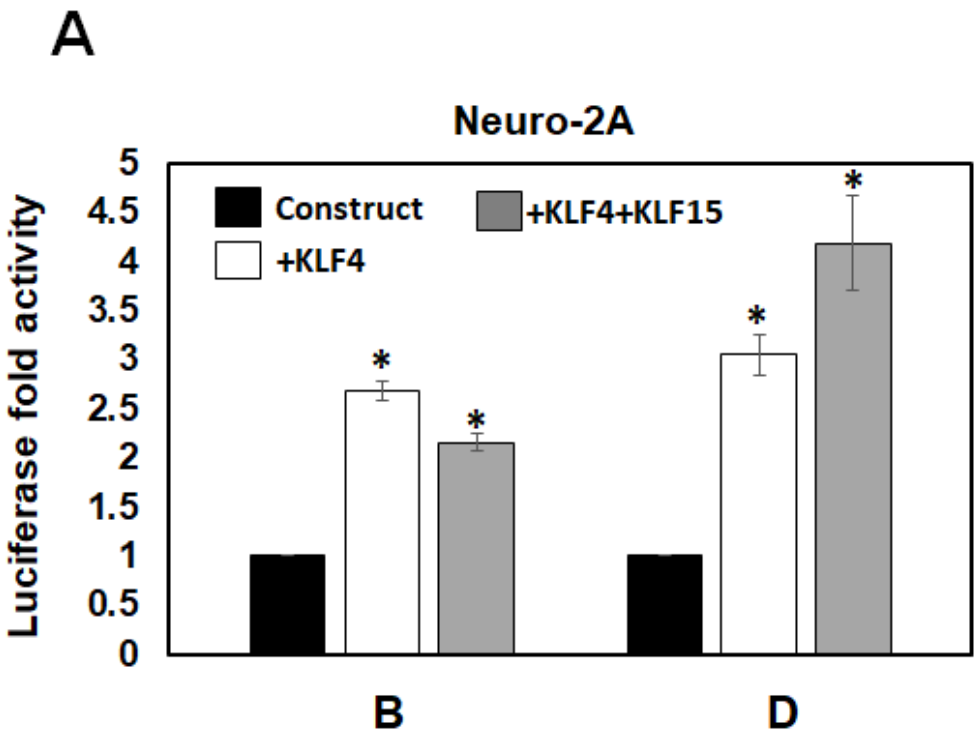
Panel C-Neuro-2A cells were transfected with 0.5 µg of the wt-D CRM construct or its mutants and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. The cultures were also co-transfected with KLF4 (1ug) expression plasmid. Cells were harvested at 24 hours and dual luciferase assay performed. Results are the mean of 3 experiments and error bars denote the standard error. Luciferase fold activity was calculated taking wt-D+KLF4 luciferase activity as 1-fold. An * denotes a significant difference compared to the wt-D+KLF4 luciferase activity. ** denotes a significant difference of $P<0.005$ and *** denotes a significant difference of $P<0.0005$ as determined by student's t-test.

6.5 KLF15 and KLF4 do not synergistically transactivate wt-D fragment in Neuro-2A cells.

Since previous studies demonstrated KLF15 cooperates with GR to transactivate ICP0 CRM constructs, I hypothesized that KLF4 may cooperate with KLF15 to transactivate ICP0 B and D CRM constructs. To test this hypothesis, Neuro-2A cells were transfected with wt-B or wt-D CRM construct and co-transfected with KLF4 and KLF15. KLF15 slightly increased the wt-D fragment's transactivation compared to KLF4 alone which shows there is an additive effect on ICP0 D CRM construct (Figure 8A).

Experiments to localize sequences important for KLF4 and KLF15 mediated transactivation revealed that mutation of all Sp1 binding sites in wt-D CRM construct reduced KLF4 and KLF15 mediated transactivation to basal levels. Mutation of the Egr-1 site had the least effect on KLF4+KLF15 mediated transactivation (Figure 8B). In summary, KLF4 and KLF15 mediate D CRM construct's transactivation through Sp1 binding sites.

Figure 8



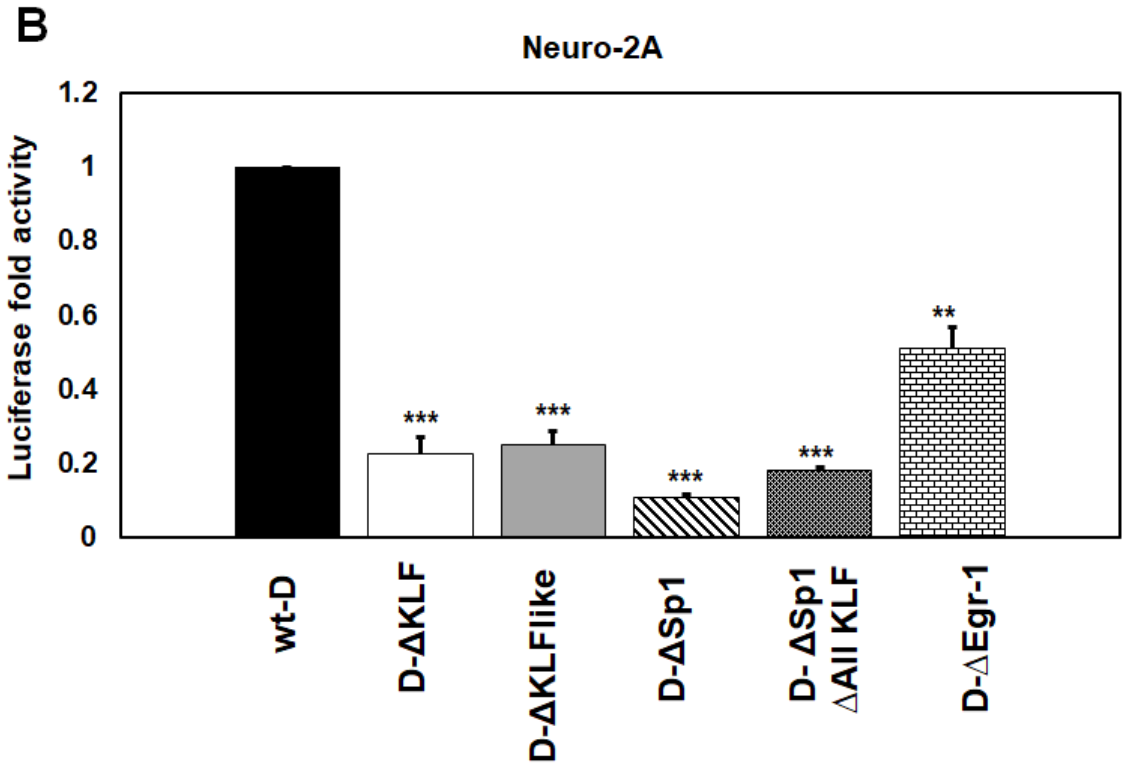


Figure 8. Transactivation of ICP0 CRM constructs B and D by KLF4 and KLF15

Panel A-Neuro-2A cells were transfected with 0.5 µg of the wt-B CRM construct and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. The cultures were also co-transfected with KLF4 (1ug) or/and KLF15 (0.5ug) expression plasmids. Cells were harvested at 24 hours and dual luciferase assay performed. Results are the mean of 3 experiments and error bars denote the standard error. Luciferase fold activity was calculated taking wt-CRM constructs' luciferase activity as 1-fold. An * denotes a significant difference compared to the wt-

CRM construct's luciferase activity. A * denotes a significant difference of P<0.05 as determined by student's t-test.

Panel B-Neuro-2A cells were transfected with 0.5 µg of the wt-D CRM construct or its mutants and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. The cultures were also co-transfected with KLF4 (1ug) and KLF15 (0.5ug) expression plasmids as indicated. Cells were harvested at 24 hours and dual luciferase assay performed. Results are the mean of 3 experiments and error bars denote the standard error. Luciferase fold activity was calculated taking wt-D luciferase activity as 1-fold. An * denotes a significant difference compared to the wt-D luciferase activity. ** Denotes a significant difference of P<0.005 and *** denotes a significant difference of P<0.0005 as determined by student's t-test.

6.6 KLF15 and KLF4 do not synergistically transactivate the wt-D fragment in NIH 3T3 cells

NIH-3T3 cells are mouse embryonic fibroblasts and were used to test how KLF4 and/or KLF15 transactivate ICP0 CRM constructs in non-neuronal cells. Both ICP0 A and D CRM constructs were transactivated almost 2-fold by KLF4 alone in 3T3 cells. However, the ICP0 B CRM construct was not transactivated by KLF4 in this cell line, which is different compared to the results in Neuro-2A cells.

The experiments to test whether KLF15 cooperates with KLF4 revealed that both A and D CRM constructs had slightly higher transactivation with KLF15 addition but only in the D fragment (Figure 9).

Figure 9

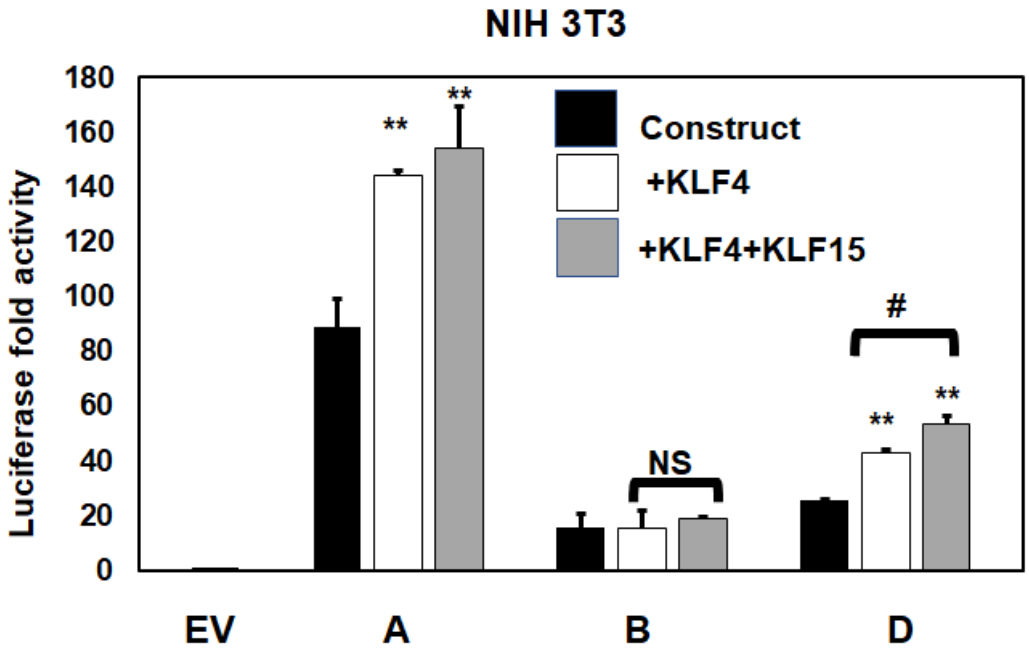


Figure 9. Transactivation of ICP0 CRM constructs by KLF4 and KLF15

NIH-3T3 cells were transfected with 0.5 µg of the wt A or wt-B or wt-D CRM construct and a Renilla luciferase expression plasmid (0.05 µg) as described in the materials and methods. Where indicated, the denoted cultures were co-transfected with KLF4 (0.5ug) and/or KLF15 (0.5ug) expression plasmids. Empty vector (pGL4.24[luc2P/minP]) was added to cultures to maintain the same DNA amount. Cells were harvested at 24 hours and dual luciferase assay performed. Fold activation was calculated relative to empty vector. Results are the average of 3 experiments and error bars denote the standard error. ** denotes a significant difference compared to the respective *wt*-CRM construct alone and # denotes a significant difference compared to the wt CRM construct+KLF4 versus wt CRM construct+KLF4+KLF15. ** denotes a significant difference of $P<0.005$ and # denotes a significant difference of $P<0.0\ 5$ as determined by student's t-test.

Discussion

In the current study I provide evidence that Sp1 cooperates with GR to transactivate HSV-1 ICP0 CRMs using cell line (CV-1) that does not express detectable levels of GR [228]. Interestingly, Vero cells, developed from an African Green Monkey expresses GR but does not support GR and Sp1 mediated transactivation (Figure 2). For these studies, I transfected cells with a GR expression plasmid that only expresses the GR-α protein, which is the GR isoform that has the strongest transactivation potential [231]. Hence, Vero cells may express a GR protein isoform that is unable to transactivate the ICP0 CRMs. Independent studies demonstrated Sp1 transactivates promoters with consensus Sp1 binding sites, including the mineralocorticoid response element (MRE) within the epidermal growth factor receptor (EGFR) [232] and the human progesterone receptor A (PR-A) promoter [149]. Previous studies demonstrated Sp1 binding sites in the HSV-1 ICP4 and ICP0 promoters are required for GR, KLF15 or KLF4 mediated transactivation [86, 193]. Notably, HSV-1 ICP4 and ICP0 promoters do not contain consensus GR response elements (GREs), which raises an important question; How does GR mediate transactivation of promoters that lack the consensus GRE? Interestingly, chromatin immune-precipitation (ChIP) studies revealed GR occupies wt-ICP0 CRMs but does not interact with the same CRM sequences with Sp1 mutations. Furthermore, mutating Sp1 binding sites significantly reduced GR-mediated transactivation. While KLF15 does not appear to bind wt-ICP0 CRMs when cells are transfected with GR+KLF15+DEX, this was surprising because KLF15 and Sp1 belongs to the same superfamily of transcription factors [233] and Chapter 5.

Transfected Sp1 trans-repressed the ICP0 CRMs in Vero and CV-1 cells (Figure 1) indicating Sp1 alone was not essential for basal transcription of ICP0 CRMs. This observation was unexpected because Sp1 is a TF that contributes to basal transcription of many cellular genes [234]. Furthermore, Sp1 is essential for maximal promotor activity of cellular promoters, including a receptor tyrosine kinase (c-kit) promoter [235].

Even though GR cooperated with KLF15 in Vero cells to transactivate ICP0 A, B and D fragments, GR did not cooperate with Sp1. Interestingly, inclusion of Sp1 in the transfection mix reduced the transcriptional activity when cultures were treated with DEX (Figure 2A). Since ChIP studies revealed GR occupies wt-ICP0 CRMs during GR+KLF15+DEX mediated transactivation (Chapter 5), Sp1 may form a complex with GR and inhibits GR from binding to ICP0 CRMs: thus, Sp1 impairs transcriptional activity in Vero cells. Further studies are necessary to prove there is an interaction between GR and Sp1.

Studies by others concluded KLF4 cooperates with Sp1 to transactivate the promoter: that drives expression of the pregnancy specific glycoproteins (PSG) gene [236]. This could be due to presence of Sp1 binding sites in the KLF4 promoter region [237]. While KLF4 and Sp1 did not cooperatively transactivate the HSV-1 ICP0 CRMs (data not shown), KLF4 transactivated the ICP0 B and D CRMs. Further studies are essential to investigate whether GR cooperates with KLF4 to transactivate ICP0 CRMs. Furthermore, previous studies demonstrated KLF family members bind to the HSV-1 ICP4 CRM via Sp1/KLF4 binding sites [86, 193]. Thus, the current study demonstrating mutating Sp1 binding sites in the ICP0 CRM fragments significantly reduced KLF4 mediated transactivation of ICP0 B and D CRMs are consistent with previous findings. Moreover, KLF4 and KLF15 influence adipogenesis via upregulating the promoter that drives expression of the PR-A receptor [238] . However, there is no evidence that KLF4 cooperates with KLF15 to transactivate the promoters examined. To assess whether KLF4 and KLF15 stably interact, additional studies are necessary.

In summary the current study demonstrated that HSV-1 ICP0 CRMs are regulated by KLF family members independently of GR.

CHAPTER VII

A MOUSE STRAIN WHICH CONTAINS A MUTANT GLUCOCORTICOID RECEPTOR DOES NOT EXPRESS PHOSPHORYLATED GLUCOCORTICOID RECEPTOR UPON SYNTHETIC CORTICOSTEROID DEXAMETHASONE TREATMENT.

Abstract

Following acute human alpha herpesvirus 1 (HSV-1) infection, sensory neurons in trigeminal ganglia (TG) are an important site for life-long latency. Reactivation from latency can cause recurrent HSV-1 disease, including herpes labialis, herpes simplex keratitis, and encephalitis. Acute or chronic stress correlates with increased viral gene expression, viral replication, and reactivation from latency. Based on these observations, we hypothesized that the glucocorticoid receptor (GR) enhances reactivation from latency. To test this hypothesis, mice containing a serine 229 to alanine mutation in the GR (GR^{S229A}) were used to compare the HSV-1 latency-reactivation cycle to wild-type mice. Although serine 229 phosphorylation is essential for GR-mediated transcriptional activation, the mutant mice are viable and healthy. The current study demonstrates that primary cells derived from kidneys of GR^{S229A} mice do not contain phosphorylated serine at position 229 when treated with the synthetic corticosteroid dexamethasone (DEX). However, primary cells derived from wild-type (wt) mouse kidneys were phosphorylated at serine 229 and GR was localized in the nucleus after DEX treatment. As

expected, both GR^{S229A} and wt mouse primary cells were positive for total GR when treated with DEX. In summary, this study confirmed GR^{S229A} mice do not phosphorylate GR at position 229.

Introduction

Stress is a trigger for alpha herpes virus reactivation from latency [183, 184, 239]. However, administration of the synthetic corticosteroid dexamethasone (DEX) alone does not consistently induce Human alphaherpesvirus 1 (HSV-1) reactivation from latency in mice. In contrast, DEX consistently induces BoHV-1 reactivation in calves or rabbits latently infected [240]. Consequently, a procedure called explant induced reactivation is used to induce reactivation ex-vivo [191]. During explant induced reactivation of HSV-1 glucocorticoid receptor (GR), KLF15, SPDEF (Sam pointed domain containing Ets factor) and SLUG expression is upregulated in trigeminal ganglionic neurons compared to latency or uninfected mice [54]. Furthermore, GR consistently transactivates Bovine herpes virus-1 (BoHV-1) promoters [79, 81, 241] and HSV-1 [85, 86, 192, 193] regulatory promoters. Finally, incubating TG latently infected with HSV-1 in charcoal stripped fetal bovine serum (FBS) reduces viral shedding during explant induced reactivation because stripped FBS contains significantly lower levels of nuclear hormones and growth factors [191]. However, treatment with DEX consistently led to explant induced reactivation of HSV-1. Furthermore, a GR antagonist (CORT-108297) reduced the efficiency of explant induced reactivation confirming GR accelerates explant induced reactivation [191].

GR phosphorylation and ligand binding enhances transcriptional activation. Human GR (hGR) mutations at Serine 113 (S113), S141, S203, S211, S226 and S404 have reduced the transcriptional activity [242, 243]; in particular, S211 is essential for transcriptional activation [244]. S211 of hGR is S229 of the mouse GR and a serine to alanine mutation is also crucial for efficient transactivation [244]. In addition to reduced transcriptional activity, phosphorylation at

S229 leads to a confirmational change of GR which increases the affinity to bind with GREs [243].

Previous studies demonstrated that mutations in the GR protein can be examined in transient transfection studies [245]. Although GR knockout mice were developed [246], most pups die at birth because of respiratory failure [247]. A conditional GR mutant that does not express GR in the nervous system (Gr^{NesCre}) was also developed [248]; however, Gr^{NesCre} mice are smaller than parental mice and have altered fat deposition because they express higher levels of corticosteroids in serum due to disruption of the hypothalamic/pituitary axis. Thus, this strain did not appear to be suitable for the goals of this study. Therefore, a mouse strain that contains a serine to alanine mutation in position 229 of GR (GR^{S229A}) was used for these studies and was obtained from Dr. John Cidlowski, NIEHS.

My studies demonstrated that GR^{S229A} is not phosphorylated at position 229 when cells were treated with corticosteroids. This further confirms GR^{S229A} mice are a suitable animal model to study effects of GR phosphorylation during the HSV-1 latency reactivation cycle.

Results

Primary kidney cells from GR^{S229A} mice express GR but phosphorylated serine 229 is not detected.

To test whether GR plays a role in HSV-1 gene expression during reactivation from latency, a mutant mouse strain where the murine GR contains a serine 229 to alanine mutation (GR^{S229A}) was used. Unlike GR knockout mice, GR^{S229A} breed efficiently, pups are healthy, and similar numbers of male and female pups are born. [243].

To compare GR phosphorylation at position Ser 229 in GR^{S229A} mice versus parental C57BL/6J mice (referred to as wt mice hereafter), primary kidney cells were prepared from each mouse strain and an antibody that specifically recognizes phosphorylated GR on serine 211/229 used. Upon addition of DEX to cultures primary kidney cells, phosphorylated GR (Phos-GR) localized to the nucleus of wt mice, which was expected [243, 249] (Figure 1A). Conversely, Phos-GR staining in kidney cells derived from GR^{S229A} mice was not detected after DEX treatment (Figure 1A). However, a monoclonal antibody that recognizes total GR revealed strong fluorescence in wt and GR^{S229A} mice (Figure 1B).

Figure 1

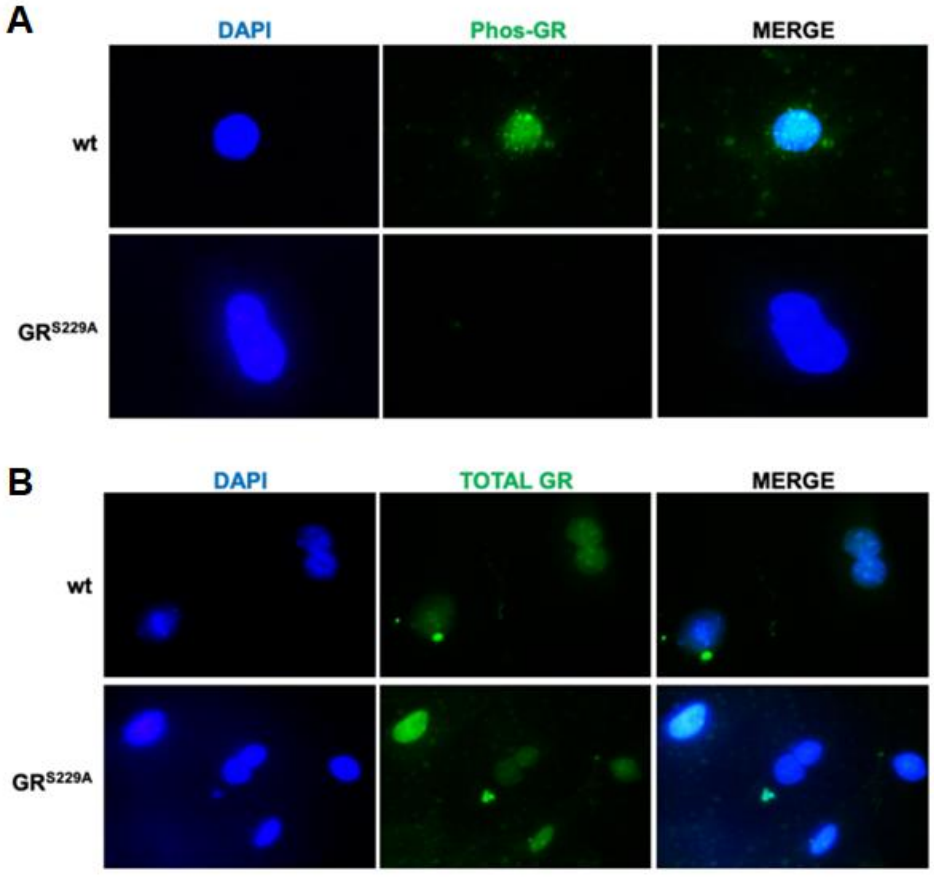


Figure 1

Figure 1: Comparison of serine 229A phosphorylation in GR^{S229A} mice versus wt mice.

Primary kidney cells from wt or GR^{S229A} male or female mice (n=5 mice/group) were prepared. These cells were treated with DEX for 4hrs and stained for serine 229 phosphorylated GR (panel A, green) or total GR (panel B, green). DAPI nuclear staining (blue) was included in all samples to visualize GR nuclear localization. Representative images from female mice are shown. 40X magnification. For this study, a total of 32 mice were used. Four mice/group were used, and two separate experiments performed. The micrographs are representative of three microscopic fields per condition.

Discussion

The glucocorticoid receptor (GR) transactivates many HSV-1 and BoHV-1 promoters by itself and in combination with certain stress induced transcription factors [54, 192, 241, 250-254]. The current study proves GR^{S229A} mice express normal levels of GR in primary kidney cells but GR^{S229A} mice do not contain GR that is phosphorylated at position 229.

In general, only activated GR translocates to the nucleus. Phosphorylation at S229 leads to binding to ligands, nuclear translocation and transcriptional activation [255]. In S2229A mouse derived kidney cells, nuclear GR is detected via total GR antibody when treated with DEX. This nuclear GR found in GR^{S229A} mouse kidney cells may be a result of phosphorylation of GR at a different site other than S229 which does not make GR transcriptionally active. As expected, an antibody detecting S229 phosphorylation was unable to detect GR in kidney cells from GR^{S229A} mice.

Hence, the GR^{S229A} mouse strain will be useful for examining the effects this mutant GR has on viral replication and the latency-reactivation cycle.

CHAPTER VIII

FUTURE DIRECTIONS

Both viral and host factors prevent reactivation of alphaherpesviruses under low levels of stress. LR gene supports latency by many mechanisms and one of which is by coding a protein named ORF2. ORF2 mRNA itself can inhibit immediate early (IE) gene expression mediated by glucocorticoid receptor (GR) via interfering Notch signaling. Furthermore, ORF2-STOP/ORF2 RNA inhibits GR, KLF15 and DEX induced BoHV-1 productive infection in both Neuro-2A and rabbit skin cells. However, it is not clear which ORF-2 sequences are important for this activity. Thus, constructing additional ORF2 mutants and testing the effects of these mutants on productive infection and trans-repression of the IEtu1 promoter and bICP0 early promoter could be performed.

Additionally, there are many host cellular signaling pathways that are upregulated during latency such as beta catenin pathway, Akt pathway and NF-kB pathway. The Akt signaling pathway is known to support neurite formation and inhibit IE gene expression of BoHV-1 and HSV-1. Previous studies demonstrated GR, KLF15 and dexamethasone transactivate the ICP0 full length promoter and cis regulatory modules in a cell type dependent manner. Interestingly, Akt-1 inhibits GR+KLF15+DEX mediated transactivation of HSV-1 ICP0 full length promoter. However, which sequences of ICP0 promoter are important for trans-repression is unknown.

Thus, it would be interesting to assess whether transcriptional activity of ICP0 CRM constructs (A, B, C and D) are impaired by Akt-1.

In addition to, GR, KLF15 and DEX mediated transactivation of ICP0 CRMs, GR also cooperates with specificity protein 1 (Sp1) to transactivate HSV-1 ICP0 CRM A, B and D in CV-1 cells. Furthermore, previous studies demonstrated that Sp1 binding sites are crucial for this transcriptional activity. However, it is not clear whether Sp1 occupies ICP0 CRMs during GR, Sp1 and DEX mediated transactivation. Thus, it is important to assess Sp1 or GR occupancy of the Sp1 binding sites during transactivation by performing ChIP assay.

Since preliminary studies revealed KLF4 transactivates ICP0 CRM B and D constructs in Neuro-2A cells, it is also possible GR cooperates with KLF4 because both are pioneer transcription factors. Therefore, conducting luciferase studies in a cell line deficient of endogenous GR i.e., either COS-7 or CV-1 would be a good cell culture model to pursue this hypothesis. If GR cooperates with KLF4, it is possible GR and KLF4 stably interact with each other in CV-1 cells. Thus, it would be interesting to test whether GR and KLF4 interact. Co-immune precipitation could be used to test if this interaction occurs.

Finally, my studies demonstrated GR^{S229A} mice express a GR mutant that has reduced transactivation potential. Thus, it would be interesting to assess the level of ICP0 protein expression in TG during explant induced reactivation of HSV-1 in this mouse strain compared to the wild type mice. This will demonstrate whether there is downregulation of ICP0 expression levels in GR^{S229A} mice during reactivation from latency due to loss of GR mediated transactivation of ICP0 promoter.

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APPENDICES

aGR	Activated GR
AIDS	Acquired immune deficiency syndrome
bICP0	Bovine infected cell protein 0
BoHV-1	Bovine alphaherpesvirus-1
BRDC	Bovine respiratory disease complex
BRTV	Bovine rhino tracheitis virus
BVDV	Bovine viral diarrhea virus
CBP	Creb binding protein
CDK	Cyclin dependent kinase
ChIP	Chromatin immune precipitation
CNS	Central nervous system
CSF	Cerebro-spinal fluid
CPE	Cytopathic effects
CRM	Cis regulatory module

DBD	DNA binding domain
DEX	Dexamethasone
Akt	Serine/threonine kinase
E	Early
Egr-1	Early growth response protein-1
EP	Early promoter
ERK	Extra cellular signal regulated kinases
ES	Embryonic stem
Ets	Erythroblast transformation specific
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gB	Glycoprotein B
GR	Glucocorticoid receptor
GSK	Glycogen synthase kinase
HAT	Histone acetyltransferase
HCF-1	Host cellular factor-1
HCMV	Human cytomegalovirus
HR	Hinge region

HSE	Herpes simplex encephalitis
HSK	Herpes simplex keratitis
Hsp	Heat shock protein
HSV-1	Human alphaherpesvirus-1
IBR	Infectious bovine rhino tracheitis
IE	Immediate early
IEtu1	Immediate early transcription unit 1
IGF	Insulin like growth factors
IPB	Infectious pustular balanoposthitis
IPV	Infectious pustular-vulvovaginitis
KLF	Krüppel like factor
KSHV	Kaposi's sarcoma associated herpesvirus
L	Late
LAT	Latency associated transcript
LH	Luteinizing hormone
LRT	Latency related transcript
MDBK	Madin-Darby bovine kidney cells
MH	*Manheimia hemolytica*

MLV	Modified live vaccine
MR	Mineralocorticoid receptor
MRE	Mineralocorticoid receptor response elements
MRI	Magnetic resonance imaging
Neuro-2A	Murine neuroblastoma cells
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NGF	Nerve growth factor
nGRE	Negative glucocorticoid receptor response elements
NHR	Nuclear hormone receptor
NIH-3T3	Mouse embryonic fibroblast
Oct-1	Octamer transcription factor-1
ORF	Open reading frame
P4	Progesterone
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLZF	Promyelocytic leukemia zinc finger protein
PR	Progesterone receptor
PRE	Progesterone receptor response element

RING	Really interesting new gene
RR	Responsive region
S	Unique shorter region
Sp	Specificity protein
SPDEF	SAM Pointed Domain Containing ETS Transcription Factor
SRC	Src family kinases
SV40	Simian virus 40
TAD	Transactivation domain
TCF	T cell factor
TF	Transcription factor
TG	Trigeminal ganglia
TK	Thymidine kinase
TRD	Trans-repressor domain
VEGF	Vasculo-endothelial growth factor
VP16	Virion protein 16

VITA

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