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STRUCTURE-FUNCTION ANALYSIS OF A PROTEIN ENCODED BY THE BHV-1 LATENCY RELATED GENE

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STRUCTURE-FUNCTION ANALYSIS OF A PROTEIN ENCODED BY THE BHV-1 LATENCY RELATED GENE

by

Devis Sinani

A DISSERTATION

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Bovine herpes virus 1 (BHV-1) is a significant viral pathogen in cattle that induces a myriad of clinical symptoms. These symptoms include: conjunctivitis, upper respiratory tract infections, genital disorders, and abortions. BHV-1 infection can also lead to transient immune-suppression, which predisposes cattle to secondary bacterial infection leading to life-threatening pneumonia referred to as bovine respiratory disease (BRD). Following acute infection, BHV-1 establishes latency in sensory neurons within trigeminal ganglia. Reactivation of the virus can occur periodically, resulting in virus transmission. The latency-related (LR) RNA is the only abundantly expressed transcript in latently infected sensory neurons and it encodes several proteins, including ORF2, as well as two micro-RNAs. My dissertation work has focused on trying to understand how the LR gene is able to promote establishment and maintenance of latency, specifically through elucidating the mechanism of function of the LR encoded protein ORF2. ORF2 inhibits apoptosis and also interacts with Notch signaling receptors, inhibiting their ability to activate certain BHV-1 promoters and enhance productive infection. The studies presented here identified, through mutational analysis, distinct domains in ORF2 that regulate its stability, localization, and functional properties. Furthermore, ORF2 is able to affect different cellular processes and promote a mature neuronal phenotype through inhibition of the Notch pathway. Lastly, a subset of ORF2 was associated with
chromatin and preferentially associated with single stranded DNA. Collectively, these studies suggest that ORF2 by interfering with apoptosis and the Notch pathway and through its ability to bind DNA plays a role in regulating certain aspects of the latency-reactivation cycle. ORF2, in general, enhances survival of infected neurons, promotes a mature neuronal phenotype and consequently increases the pool of latently infected neurons.
<table>
<thead>
<tr>
<th>Table of Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title...</td>
<td>i</td>
</tr>
<tr>
<td>Abstract..................................................................................................................</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents.................................................................................................</td>
<td>iv</td>
</tr>
<tr>
<td>Dedication...............................................................................................................</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgements....................................................................................................</td>
<td>vi</td>
</tr>
<tr>
<td>Chapter 1 – Introduction ...</td>
<td>1</td>
</tr>
<tr>
<td>Literature review....................................................................................................</td>
<td>2</td>
</tr>
<tr>
<td>Figures....................................................................................................................</td>
<td>32</td>
</tr>
<tr>
<td>Goals of this study.................................................................................................</td>
<td>39</td>
</tr>
<tr>
<td>Materials and methods.............................................................................................</td>
<td>42</td>
</tr>
<tr>
<td>Chapter 2 – Localization of sequences in a protein encoded by the latency related gene of bovine herpesvirus 1 (ORF2) that inhibits apoptosis and interferes with Notch1 mediated trans-activation of the bICP0 promoter ...</td>
<td>53</td>
</tr>
<tr>
<td>Abstract...................................................................................................................</td>
<td>54</td>
</tr>
<tr>
<td>Introduction.............................................................................................................</td>
<td>55</td>
</tr>
<tr>
<td>Results......................................................................................................................</td>
<td>59</td>
</tr>
<tr>
<td>Discussion...............................................................................................................</td>
<td>71</td>
</tr>
<tr>
<td>Figures....................................................................................................................</td>
<td>75</td>
</tr>
<tr>
<td>Chapter 3 – A protein encoded by the latency related gene of bovine herpesvirus 1 (ORF2) promotes neurite sprouting in the presence of Notch1 or Notch3 ...</td>
<td>96</td>
</tr>
<tr>
<td>Abstract...................................................................................................................</td>
<td>97</td>
</tr>
<tr>
<td>Introduction.............................................................................................................</td>
<td>98</td>
</tr>
<tr>
<td>Results......................................................................................................................</td>
<td>102</td>
</tr>
<tr>
<td>Discussion...............................................................................................................</td>
<td>109</td>
</tr>
<tr>
<td>Figures....................................................................................................................</td>
<td>114</td>
</tr>
<tr>
<td>Chapter 4 – A protein (ORF2) encoded by the latency related gene of bovine herpesvirus 1 interacts with DNA ...</td>
<td>128</td>
</tr>
<tr>
<td>Abstract...................................................................................................................</td>
<td>129</td>
</tr>
<tr>
<td>Introduction.............................................................................................................</td>
<td>130</td>
</tr>
<tr>
<td>Results......................................................................................................................</td>
<td>133</td>
</tr>
<tr>
<td>Discussion...............................................................................................................</td>
<td>137</td>
</tr>
<tr>
<td>Figures....................................................................................................................</td>
<td>141</td>
</tr>
<tr>
<td>Chapter 5 – Effect of LR gene expression on autophagy ...</td>
<td>147</td>
</tr>
<tr>
<td>Abstract...................................................................................................................</td>
<td>148</td>
</tr>
<tr>
<td>Introduction.............................................................................................................</td>
<td>150</td>
</tr>
<tr>
<td>Results and discussion............................................................................................</td>
<td>153</td>
</tr>
<tr>
<td>Figures....................................................................................................................</td>
<td>156</td>
</tr>
<tr>
<td>General Conclusions...............................................................................................</td>
<td>158</td>
</tr>
<tr>
<td>References...............................................................................................................</td>
<td>165</td>
</tr>
</tbody>
</table>
DEDICATION

Ja dedikoj prindërve të mij Vangji dhe Pajtim. Sakrificea juaj nuk është harruar...

Dedicated to my parents Vangji and Pajtim. Your sacrifice has not been forgotten...
ACKNOWLEDGEMENTS

During this journey I have not been walking alone. There are many people that have borne the weight with me along the way that I would like to acknowledge. First and foremost I owe a debt of gratitude to my adviser Dr. Clinton Jones. Thank you for taking a chance on me and for the continued support. You have shown me that it is possible to be a leading scientist in your field without having to sacrifice your family life. You are a great scientist, mentor, and colleague and above all I am honored to call you friend. Thank you for everything.

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Next I would like to acknowledge my beautiful wife Sara and my baby boy Pirro. I love you both very much. You have brought so much joy and happiness to my life and my life is so much better and more interesting with you in it. Thank you “babe” for being
so supportive and understanding during many late nights of lab work and studying. You are not just my wife, but my partner and my best friend. I consider myself very lucky.

Last but not least, a special thank you to my parents. They have sacrificed and continue to sacrifice so much in order for my sister and I to be able to come to this country to get the best possible education and take advantage of the opportunities afforded to us in order to reach our full potential. Whether I have reached my full potential is highly debatable, however the degree of their sacrifice is not. Thank you for everything. Faleminderit për gjithëçka që keni bërë për mua.
CHAPTER I

INTRODUCTION
LITERATURE REVIEW

I. Family *Herpesviridae*

The *Herpesviridae* family members have been detected in most animal species, including essentially all mammals (Roizmann *et al.* 1992). All members share common structural traits as well as similar characteristics in their lifecycle.

Herpesviruses do however display differences among each other in the composition and architecture of their genome, host range, growth characteristics, tissue tropism, duration of infection, cytopathology, and latency (Roizman *et al.* 1981). Based on these biological properties, herpesviruses are grouped into three subfamilies; *Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae* (Roizman *et al.* 2001). Prototypes for each subfamily are, Human simplex virus type 1 (HSV-1), Cytomegalovirus (CMV) and Epstein-Barr virus, respectively. *Alphaherpesvirinae* are characterized by a variable host range, a relatively short replication cycle, rapid spread in culture, high cytopathicity, and ability to establish latency in sensory ganglia. In contrast, *Betaherpesvirinae* have a restricted host range, long reproductive cycle and slow progress of infection during which the infected cells become enlarged before lysis. *In vivo*, they establish latency in macrophages of the lymphoreticular tissues, kidneys and secretory glands for example. One of the major health concerns with beta-herpesviruses is CMV-post transplantation infections. *Gammaherpesvirinae*, similar to the beta subfamily exhibit restricted host range. B and T lymphocytes are the primary target of infection although *in vitro* studies have shown that some types of epithelial cells and fibroblasts
can support viral replication. Latent virus is detected in lymphoid tissue and the typical outcome of infection is cell transformation (Roizman et al. 1991; Roizman et al. 2001).

Herpesviruses possess linear double-stranded DNA genomes (~130–250 kbp) and a complex architecture of infectious particles containing around 35 viral proteins, which are involved in nucleic acid metabolism, DNA synthesis, and protein processing. Herpesvirus genomes encode between 70 to 200 genes resulting in approximately 84 different gene products. Although they differ greatly in terms of genomic sequence, Herpesviruses have similar virion structures and genomic organization. Herpesvirus genomes are usually organized as unique long or short regions bounded by inverted repeats. The average size of the virion is 180–200 nm in diameter and consists of a highly ordered icosahedral-shaped nucleocapsid of about 125–130 nm in diameter which encases the genome. The nucleocapsid is surrounded by an amorphous proteinaceous layer called the tegument, which in turn is enclosed within the host-derived envelope containing multiple copies of more than 10 different kinds of viral glycoproteins that are responsible for viral attachment and entry to host cells (Armstrong et al. 1961; Jones 1998; Tikkoo et al. 1995). Both viral replication and assembly occur in the nucleus of the host cell (Roizman et al. 2001).

II. Bovine Herpesvirus type 1 (BHV-1)

Bovine herpesvirus type 1 (BHV-1) is a member of the Alphaherpesvirinae subfamily further classified within the varicellovirus genus (Brown 1989; Roizman et al. 2001). The BHV-1 genome is 135.3 kilobase pairs (kbp) with a high guanine and cytosine (GC) content arranged as a class D herpesvirus genome (Figure 1.1A) (Plummer
et al. 1969). Class D genomes contain a unique long (UL, 104 kbp) and a unique short (US, 10 kbp) region which is flanked on both sides by long and short inverted internal (IRL and IRS) and long and short terminal repeats (TRL and TRS) each consisting of 11 kbp (Muylkens et al. 2007) (Figure 1.1A). A total of 73 open reading frames (ORFs) have been identified so far and the majority of the BHV-1 genes consist of ORFs that are homologous to genes found in other alpha-herpesviruses and have been generally named to the related genes in the prototype HSV-1 (Muylkens et al. 2007).

All BHV-1 strains isolated belong to one single viral species and are classified into three subtypes based on antigenic and genomic analysis, BHV-1.1, 1.2a, 1.2b (Metzler et al. 1985). Subtype 1 virus isolates are the causative agent for infectious bovine rhinotracheitis (IBR) and are typically found in the respiratory tract and in aborted fetuses. Subtype 1 strains are common in Europe, North and South America. Subtype 2a is associated with a broad range of clinical symptoms in the respiratory and genital tract like infectious pustular vulvovaginitis (IPV) and balanopostitis (IPB) and abortions (van Oirschot 1995). Its prevalence is in Brazil and in Europe before 1970 (van Oirschot 1995). Subtype 2b is also associated with respiratory disease, IPV and IPB but not with abortions and has been isolated in Australia and Europe (D’Arce et al. 2002; van Oirschot 1995).

II. A. Pathogenesis

Herpesviruses share many biological traits across family members including a number of homologous proteins, production of viral progeny that leads to host cell lysis and most importantly the ability to persist in a “latent” state inside the host. However,
certain biological aspects of disease vary greatly, for example host range, length of productive infection, and tissues where a latent infection occurs.

Natural infection of BHV-1 occurs by contact of the virus with mucosal membranes of either upper respiratory or genital tracts of cattle although, there has been one report of BHV-1 virus present in insect vectors (Taylor et al. 1982). Virus entry into the respiratory tract can occur via aerosol or by direct contact with virus present in nasal secretions (Mars et al. 2000). In contrast, genital transmission requires direct contact at mating or through semen containing virus (Kupferschmied et al. 1986). Infection can also be transmitted through inoculation of the conjunctival epithelium.

There are several factors that can influence the clinical symptoms and severity of the disease caused by BHV-1: virulence of the strain, type of tissue infected, resistance factors of the host, age of the host, and secondary bacterial infection. There are two major syndromes associated with BHV-1 infections: (i) Infectious bovine rhinotracheitis (IBR), which occurs in the respiratory tract, and (ii) infectious pustular vulvovaginitis (IPV) (in cows) and balanoposthitis (in bulls), which occurs in the genital tract.

IBR is associated with clinical symptoms such as pyrexia, apathy, increased respiratory rate with persistent harsh cough and anorexia, while adult dairy cows exhibit a severe drop in milk production. Mucopurulent discharge from the nostrils and eyes is associated with pustular lesions in the nasal mucosa and conjunctivitis. The nasal and tracheal turbinates are areas primarily involved (Jones et al. 2007; Jones et al. 2010; Muylkens et al. 2007).

BHV-1 is also one of the major etiological agents of bovine respiratory disease complex (BRDC) commonly known to the cattle industry as the “shipping fever” (Jones
et al. 2010). Since BHV-1 is immunosuppressive, infection can lead to secondary bacterial infection by opportunistic pathogens such as *Pasteurella haemolytica*, *Pasteurella multocida*, and *Histophilus somni* which leads to pneumonia (Babiuk et al. 1988; Griffin et al. 2010; Yates 1982). Approximately 75% of morbidity and 50% of mortalities in feedlot cattle are attributed to BRDC amounting to ~$2 billion annual losses making BRDC the most economically significant disease to the cattle industry (Kapil et al. 1997; Powell 2005).

Other diseases observed are abortions (due to fetal death), fatal multisystemic infection of newborn calves, meningoencephalitis, mastitis, enteritis (Kahrs 1977; Lupton et al. 1980). IPV syndrome is mainly associated with pustular lesions of the genital tract and does not lead to as a severe a disease as IBR, but it is still a cause of concern (Wittmann 1989).

Control measures for the eradication of BHV-1 have relied on serological testing of animals and removal of the seropositive individuals. This approach however can be a daunting task in countries like United States due to the large numbers of animals, the constant movement of herds, and feedlot conditions where large numbers of animals reside in fairly tight quarters. Several modified live vaccines have been developed, however they have the ability to establish latency, and can be pathogenic to young calves and cause abortions (Thiry et al. 1985; van Drunen Littel-van den Hurk et al. 1993). Another problem associated with these vaccines is the inability to detect a naturally infected versus a vaccinated cow. The goal remains to develop a vaccine that can induce a robust immune response, be able to be distinguished from wild type (wt), and perhaps protect against wt virus becoming latent.
II.B. Productive infection

II. B. 1. Overview

BHV-1 infection of permissive cells starts with the entry process which is divided into three steps. First a low affinity interaction occurs between gB and/or gC to cell surface structures like heparin sulfate sugar moieties (Li et al. 1995; Liang et al. 1992; Liang et al. 1991; Okazaki et al. 1994). The initial low affinity interaction facilitates the stable binding of gD to cellular specific receptors. There are several potential candidates however, only nectin-1 has been shown to act as an entry receptor for BHV-1 (Geraghty et al. 1998). After the high affinity interaction fusion of the virion envelope and the plasma membrane occurs releasing the de-enveloped nucleocapsid into the cytoplasm (Campadelli-Fiume et al. 2007). Once in the cytosol, virus nucleocapsids most likely piggyback the dynein motor complexes associated with microtubules to the nucleus to release the DNA (Dohner et al. 2002). In the nucleus, BHV-1 gene expression is regulated in a temporal fashion giving rise to successively immediate early (IE), early (E), and late (L) RNA. These transcripts encode proteins involved in the regulation of viral transcription, viral DNA replication, and morphology of progeny virions respectively. The mature virions are assembled in the nucleus. It is not clear how the virions escape the nucleus to enter secretory vesicles and how this virus particle acquires its envelope is controversial. The current accepted model proposes budding of capsids through the nuclear inner membrane which provides the primary envelope. To be released in the cytoplasm, virions fuse their primary envelope with the outer nuclear membrane. Once released in the cytoplasm, naked capsids acquire their tegument and
their secondary envelope by travelling through the Golgi apparatus and secretory pathway. As with all alpha-herpesviruses, production of new progeny virus apparently results in cell death (Muylkens et al. 2007).

II. B. 2. Viral gene expression

Once BHV-1 enters the nucleus of a permissive cell, the lytic replication cycle begins. Viral gene expression can be divided into three distinct phases, which leads to production of progeny virus and ultimately cell death. In bovine primary cell cultures, BHV-1 DNA synthesis starts within the first two hours post infection (h pi) and peaks at 4-6 h pi (Ghram et al. 1986). Viral proteins can be detected as early as 4 h pi and peak at 6-8 h pi (Ghram et al. 1986). IE genes (bICP0, bICP4, bICP22, and bcirc) are expressed in the first 2 h pi and do not require de novo protein expression. E gene transcription occurs 2-4 h pi and requires IE proteins, but not viral DNA synthesis (Schwyzer et al. 1996; Tikkoo et al. 1995). L gene transcription occurs 6-8 h pi and is dependent on viral protein and DNA synthesis (Seal et al. 1992). Gamma 1 genes are transcribed at low levels even when viral replication is blocked. Gamma 2 genes are dependent on viral replication. IE gene expression is stimulated by the tegument protein BHV-1 alpha-transactivating factor (bα-TIF), which is a homologue of the HSV-1 VP16. Bα-TIF associates with the cellular transcription factor Oct-1 and activates IE gene expression by interacting with the TAATGAGCT sequence present in all BHV-1 IE gene promoters (Dixon et al. 1980; Misra et al. 1994). There are two IE transcription units: IE transcription unit 1 (IEtu1) and IEtu2 (Figure 1.1B) (Misra et al. 1994; Misra et al. 1995). IEtu1 encodes functional homologues of two HSV-1 IE proteins, infected cell protein 0
(bICP0) and ICP4 (bICP4). Circ, is also encoded by IEtu1 and it is a less abundant transcript that encodes a tegument protein and is expressed throughout the viral cycle (Fraefel et al. 1994; Fraefel et al. 1993). IEtu2 encodes a protein homologous to HSV-1 ICP22 (bICP22). The IE proteins have transcriptional regulatory functions. bICP4 and bICP22 inhibit IEtu1 and IEtu2 expression while activating E and L gene promoters (Schwyzer et al. 1993). bICP0, although not essential, plays an important role during productive infection because it can activate all three classes of viral promoters (Wirth et al. 1992) and it is expressed at high levels throughout infection (Everett 2000; Fraefel et al. 1994). The E genes encode proteins involved in DNA synthesis while L genes encode structural proteins and their synthesis culminates in virion assembly and release.

II. C. Latency-Reactivation Cycle

II. C. 1. Overview

The lytic phase of BHV-1 lifecycle produces many virions at the mucosal surface of the initial infection. BHV-1 mature virions can gain access to sensory nerve terminals that innervate the mucosal tissue through cell to cell spread (Enquist et al. 1999). The virus is then transported along the microtubules of axons in a retrograde direction to the neuron body in the nervous ganglion (Tomishima et al. 2001). If the infection is initiated via the oral, nasal cavities or ocular surface, the primary site for latency is sensory neurons within the trigeminal ganglia (TG) (Jones et al. 2010). Although the main site of latency for BHV-1 is TG sensory neurons, evidence suggests that latent infection can also occur in non-neuronal sites like lymphoid follicles and pharyngeal tonsils (Winkler et al.
2000), peripheral blood cells (Fuchs et al. 1999), and lymph nodes and spleen (Mweene et al. 1996).

In contrast to productive infection (lytic phase) where all viral genes are expressed, the hallmarks of a latent infection are minimal viral gene expression, absence of lytic cycle viral proteins, and the absence of virus production. The viral genome is maintained as an episome while promoting survival of the latently infected cell. Latently infected cells represent a pool of virus that can escape the surveillance of the host immune system while periodically reactivating, facilitating transmission and making herpesviruses very successful in nature.

The latency-reactivation cycle has been divided into three operational steps: establishment, maintenance, and reactivation. These steps are discussed in detail below.

II. C. 2. Establishment of latency

Extensive viral gene expression and replication occur within TG for approximately one week following infection of calves with BHV-1 (Schang et al. 1997; Winkler et al. 2000). Around 7 days post infection (dpi) lytic viral gene expression is extinguished. Inhibition of IE gene expression leads to reduced cellular toxicity and productive infection, tipping the balance toward the establishment of latency. Neurons that survive retain viral genomes as episomes associated with nuclear histones (Rock et al. 1983; Rock et al. 1985). Productive replication within the TG increases the number of latently infected neurons; however, replication is not required for establishment of latency because mutant viruses that cannot replicate establish latency but at reduced

The host immune system contributes to the establishment of latency by suppressing infection and limiting the spread of the virus at the synaptic junctions. During acute infection in TG there is an infiltration of immune cells by 7-10 dpi and an increase of interferon-gamma (IFN-γ) and interleukin 4 (IL-4) expression (Erturk et al. 1992; Liu et al. 1996; Shimeld et al. 1995; Stumpf et al. 2001; Winkler et al. 2002). The presence of immune cells in close proximity of infected neurons strongly suggests that the immune system promotes the establishment of latency by inducing a non-permissive environment. This role is also supported by evidence that IFN-γ, tumor necrosis factor alpha (TNF-α), and CD8+ T-cells repress productive infection (Cantin et al. 1999; Cantin et al. 1999; Cantin et al. 1995; Halford et al. 1996). The cytokine environment may also promote latency by influencing neuronal health.

Another important factor in the establishment of latency is LR gene expression. LR gene products promote latency by repressing lytic gene expression (Bratanich et al. 1992; Geiser et al. 2002; Jaber et al. 2010) and inhibiting apoptosis in latently infected neurons (Ciacci-Zanella et al. 1999; Henderson et al. 2004; Lovato et al. 2003; Shen et al. 2008; Sinani et al. 2011) (Discussed in more detail later).

II. C. 3. Maintenance of latency

Maintaining a successful latent infection requires that the viral genome be preserved within the infected cell and that the cell remain alive. Since neurons are non-dividing cells, genome replication and division to daughter cells is not a concern for
alpha-herpesviruses. The genome is maintained as a nucleosomal, circular episome associated with histones. To avoid induction of apoptosis and/or immune detection expression of viral genes, normally required for productive infection, is extinguished. The LR gene is the major player in the maintenance of latency.

II. C. 4. Reactivation from latency

Reactivation from latency is usually triggered by external stimuli like stress or immunosuppression. Transporting cattle from one location to another or weaning of young calves are well known stressful events, which may lead to reactivation events. A single injection of the synthetic corticosteroid dexamethasone (DEX) induces reactivation of BHV-1 in latently infected rabbits and cows 100% of the time (Davies et al. 1973; Homan et al. 1983; Inman et al. 2002; Rock et al. 1992; Sheffy et al. 1972). Virus is present in ocular secretions or TG homogenates from rabbits between 48 and 72 hours after DEX treatment. Within 18 hours after DEX treatment expression of all classes of viral genes can be detected in approximately 20% of neurons in TG (Rock et al. 1992). BHV-1 specific neutralizing antibodies in the serum increase significantly after treatment with DEX, which correlates with reactivation (Homan et al. 1983).

DEX-induced reactivation is biologically relevant because naturally occurring stress increases corticosteroid levels enhancing viral replication and virus shedding (Sheffy et al. 1972; Wyler et al. 1989). In a BHV-1 latently infected rabbit model, DEX-mediated reactivation lead to a decrease of LR gene expression in sensory neurons (Rock et al. 1992). DEX treatment also affects splicing patterns in the absence of protein synthesis and induces expression of cyclin A which is required for cell cycle progression
(Collett et al. 1993; Schang et al. 1996). Cell cycle progression is beneficial to the lytic phase of herpesviruses. Although repeated injections with high levels of DEX can lead to immunosuppression, (Migliorati et al. 1994; Schmidt et al. 1999; Winkler et al. 2002), it is more likely that DEX triggers reactivation in TG by activating viral and host gene expression.

II. C. 5. Latency related gene

In contrast to the 70-80 genes that are expressed during productive infection, LR-RNA is the only abundantly expressed transcript during latency (Kutish et al. 1990; Rock et al. 1992; Rock et al. 1987). In situ hybridization localizes LR-RNA to the nucleus of the latently infected neurons (Kutish et al. 1990; Rock et al. 1987). The full LR gene is approximately 2 kbp long (980 bp of promoter and 1180 bp of transcribed region) and it is transcribed antisense to the bICP0 mRNA (Jones et al. 1990; Kutish et al. 1990). In situ hybridization studies mapped the start site of the LR transcription between nucleotides (nts) 700 and 981 (Liang et al. 1992; Rock et al. 1992). The start site for lytic expression was identified to be at nt 724 which is 24 nts downstream of the AT-rich sequences resembling TATA boxes (Figure 1.2B) (Bratanich et al. 1992). A different start site utilized during latency was determined to reside 200-300 bps upstream of the lytic site (Hossain et al. 1995).

The promoter of the LR gene is contained within the 980 bp PstI fragment and it is down-regulated by DEX (Figure 1.2B) (Jones et al. 1990). The LR promoter has strict orientation, and it is more active in bovine and neuronal cell lines versus non-bovine, non-neuronal ones (Bratanich et al. 1992; Jones et al. 1990). Studies have also suggested
that tissue and species-specific transcription factors may play a role in the regulation of
the LR-gene expression (Delhon et al. 1997).

A small fraction of the LR transcripts in TG are alternatively spliced and
polyadenylated, suggesting these transcripts may be translated into more than one LR
protein (Devireddy et al. 1998; Hossain et al. 1995). LR gene products inhibit S phase
entry by interacting with cyclin dependent kinase 2 (cdk2)/cyclin complexes and also
promotes cell survival following induction of apoptosis (Ciacci-Zanella et al. 1999; Jiang
et al. 1998; Schang et al. 1996). The LR gene sequence includes two well defined open
reading frames (ORFs) (ORF1 and 2) and two reading frames lacking an initiating ATG
(RF-B and C) (Figure 1.2A). Splicing at 1 dpi in TG leads to expression of intact ORF2
(Devireddy et al. 2003; Devireddy et al. 1998). ORF2 coding sequences due to
alternative splicing can be fused at the C-terminus with ORF1 or RF-B coding sequences
at 7 and 15 dpi respectively (Devireddy et al. 2003; Devireddy et al. 1998).

When expressed in the absence of other viral genes, ORF2 inhibits cold shock-
induces apoptosis in certain neuroblastoma cells (Shen et al. 2008). A protein resulting
from the fusion of ORF2 with ORF1 interacts with two pro-apoptotic proteins (Bid and
Cdc42) suggesting a possible mechanism for inhibition of apoptosis (Meyer et al. 2007).
Moreover, the ORF2/ORF1 fusion protein (7dpi) interacts with the cellular transcription
factor CAAT/enhancer binding protein (C/EBP) alpha (Meyer et al. 2007). C/EBP alpha
activates the expression of bICP0, the main BHV-1 viral trans-activator (Workman et al.
2009). This is biologically significant because ORF2, whether in its intact form or fused
with ORF1, could potentially promote establishment and maintenance of latency by
inhibiting apoptosis and interfering with cellular transcription factors that enhance productive infection.

A mutant BHV-1 virus designed with three stop codons at the N-terminus of ORF2 (LR mutant virus) is unable to express ORF2 or RF-C but can express low levels of ORF1 (Inman et al. 2001; Jiang et al. 2004; Meyer et al. 2007). Infection of calves with the LR mutant virus results in reduced virus shedding in ocular tissue, TG, and tonsils plus the LR mutant virus does not reactivate from latency following treatment with DEX (Inman et al. 2001; Inman et al. 2002; Perez et al. 2005). LR mutant virus also induces higher levels of apoptosis in TG of infected calves when compared to wt or rescued BHV-1 suggesting that a protein encoded by the LR gene protects neurons from cell death during the establishment and maintenance of latency (Lovato et al. 2003).

Even though studies from our lab have clearly underlined the importance of proteins encoded by the LR gene, recent evidence provides evidence that non-protein coding LR-RNA regulates certain aspects of the latency-reactivation cycle (Bratanich et al. 1992; Geiser et al. 2002; Jaber et al. 2010). LR gene inhibits bICP0 activation of BHV-1 productive infection in a dose-dependent manner. Insertion of three stop codons at the amino-terminus of the first ORF in the LR gene (ORF2) did not rescue bICP0 inhibition suggesting that LR proteins were not required for this inhibition (Geiser et al. 2002). The expression of the LR transcript is antisense to bICP0 and hybridization of LR-RNA sequences with bICP0 mRNA has been proposed as a possible mechanism for this inhibition. However, there is no experimental evidence that this is the case. LR gene products also inhibit mammalian cell growth (Geiser et al. 2005; Schang et al. 1996). Both of these inhibitory functions of the LR gene have been mapped to a 463-bp XbaI-
Psrl region (XP) (Geiser et al. 2002; Seal et al. 1992; van Drunen Littel-van den Hurk et al. 1993). A recent study in our lab demonstrated that this region has the potential to form stem-loop secondary structures and it gives rise to two families of small non-coding RNAs (sncRNAs) (Jaber et al. 2010). These sncRNAs are precursors that mature into two micro RNAs (miRNAs), which are expressed in the TG of latently infected calves where they promote latency by inhibiting bICP0 expression and productive infection.

LR gene products may also promote latency by inhibiting the host IFN-β response. The LR mutant virus expresses LR-RNA prematurely relative to wt or the LR rescued virus leading to higher IFN-β expression during productive infection of bovine cells in in vitro cultures as well as in the tonsils of calves during lytic infection (Perez et al. 2008). This, along with increased apoptosis in the TG of calves infected with the LR mutant virus may be the reason why this mutant virus exhibits diminished symptoms and fails to reactivate from latency (Inman et al. 2001; Inman et al. 2002; Perez et al. 2005).

III. BHV-1 evasion of the immune system

III. A. Overview

Following primary infection of cattle with BHV-1, cattle mount a robust immune response and for the most part inhibit virus shedding and recovery from disease occurs. However, infected cattle are never able to completely clear BHV-1 and the virus establishes a life-long latent infection.

BHV-1 employs numerous immune evasion strategies. Studies published from our lab have shown that BHV-1 interferes with innate immunity because bICP0 inhibits transcription of type I IFN by inducing proteasome-dependent degradation of interferon
regulatory factor 3 (IRF3) and by a direct interaction with IRF7 (Henderson et al. 2005; Saira et al. 2009; Saira et al. 2007). Both IRF3 and 7 are proteins involved in a regulatory complex responsible for activating transcription of type I IFN. Another strategy employed by BHV-1 to avoid innate immune response is through interaction of glycoprotein C (gC) with the third complement component (C3) which is the key activator of the complement system (Huemer et al. 1993). Innate immunity is also impacted by a conserved alpha-herpesvirus immunomodulatory function through the interaction of gG with several chemokines preventing recruitment and activation of several innate immunity cells (Bryant 2003; Costes et al. 2005). Evading the innate immunity enables an efficient replication of the virus during productive infection.

Several strategies are geared towards avoiding detection by cell mediated immunity. During acute infection, BHV-1 down-regulates antigen presentation by class I and class II major histocompatibility complexes (MHC class I and II) (Hinkley et al. 2000; Hinkley et al. 1998; Koppers-Lalic et al. 2005; Koppers-Lalic et al. 2001; Lipinska et al. 2006; Nataraj et al. 1997). This is achieved through UL49.5 by blocking the transporter associated with antigen processing (TAP) and through virion host shutoff protein (vhs, encoded by UL41) by reducing MHC class I and II mRNA.

BHV-1 also impairs CD4+ T-cell responses because it infects them and induces apoptosis during productive infection (Winkler et al. 1999). Apoptosis is presumably induced through activation of caspases and p53 (Devireddy et al. 1999).

As mentioned earlier, these strategies enable efficient replication of BHV-1 during the acute phase. However, the host immune system clears productive infection and virus shedding at the initial site of infection. The immune system is unable to prevent
establishment and maintenance of latency. During latency, evidence suggests that BHV-1 subverts the immune system possibly by stimulating certain immune factors in order to create a milieu that favors latency. Infection of mice with HSV-1 or cattle with BHV-1 shows infiltration and persistence of immune cells in the TG during latency (Allen et al. 2011; Cantin et al. 1995; Halford et al. 1996; Perez et al. 2006; Shimeld et al. 1995; Winkler et al. 2002). This evidence suggests that the presence of the immune system in close proximity with infected neurons promotes establishment and maintenance of latency by inducing a non-permissive environment.

III. B. Apoptosis

Apoptosis is one form of programmed cell death (PCD), and a natural and essential process in the development and maintenance of multicellular organisms. Apoptosis exhibits morphological characteristics like chromatin condensation (pyknosis), fragmentation of the nucleus (karyorhexis), nucleosomal laddering of the DNA, membrane blebbing and the formation of apoptotic bodies (Danial et al. 2004; Fesik et al. 2001; Kerr et al. 1972; Werlen et al. 2003). Apoptosis is indispensable for maintaining healthy organs and tissues because it eliminates old or unhealthy cells. On the other hand if left unchecked, apoptosis can lead to tissue damage, which has been linked to neurodegenerative disorders like Alzheimer’s, Huntington’s and Parkinson’s diseases.

PCD is triggered by a variety of controlled external and internal stimuli which differentiates it from necrotic death (Elward et al. 2003). Apoptosis differs from necrosis also by the manner with which the cellular debris is disposed of. In necrosis, cleanup by
the immune cells leads to an inflammatory response, while during apoptosis cleanup is mediated by immune cells through phagocytosis without triggering inflammation.

There are two fundamentally distinct stimuli that lead to apoptosis; extrinsic (extracellular) and intrinsic (intracellular). Extrinsic stimuli include hormones, growth factors, toxins, cytokines, and nitric oxide. The intrinsic pathway can be stimulated by signals from abnormal physiological stresses as a result of nutrient deprivation, oxidative stress, radiation, and viral or bacterial infection.

Two ligands that trigger the extrinsic pathway are tumor necrosis factor (TNF) and the Fas-Fas ligand (FasL). Both are type II transmembrane proteins and members of the TNF family. TNF binds to the TNF receptor (TNFR) while FasL binds to Fas receptor (FasR), also known as apoptosis antigen 1 (APO-1) or cluster of differentiation 95 (CD95), which results in apoptosis (Chen et al. 2002; Hsu et al. 2005). Binding of TNF to TNFR activates apoptosis by recruiting TNF receptor-associated death domain (TRADD) and caspase 8 to form caspase 8 activating complexes (Wang et al. 2008). Binding of FasL to CD95 recruits Fas-associated death domain (FADD) which further recruits caspases 8 and 10 and c-FLIP(L), to form a death-inducing signaling complex (DISC). DISC formation leads to activation of caspase 8 and caspase 10 which are released to the cytoplasm where they activate caspase 3. Activation of caspase 3 is considered the point of no return. TNF-related apoptosis-inducing ligand (TRAIL) which shows homology to other members of the TNF superfamily can also induce apoptosis (Thorburn 2004). TRAIL binds to death receptors (DRs) 4 and 5 triggering caspase 8-mediated apoptosis.
The intrinsic pathway involves mitochondrial membrane permeabilization (MMP), which leads to release of apoptotic effectors (Dejean et al. 2006). The main effector is cytochrome c, which is released into the cytoplasm through the mitochondrial apoptosis-induced channel (MAC) (Dejean et al. 2006). Cytochrome c binds to apoptotic protease activating factor 1 (Apaf 1) and dATP, which recruits caspase 9 forming the apoptosome complex. This leads to activation of caspase 9 which in turn activates caspase 3 leading to apoptosis (Fesik et al. 2001).

Members of the Bcl-2 family of proteins are involved in the regulation of the intrinsic apoptotic pathway by controlling formation of MAC (Chao et al. 1998; Dejean et al. 2006; Dejean et al. 2006). The family includes members that are both pro and anti-apoptotic. For example, Bax and/or Bak expression leads to induction of apoptosis while Bcl-2 and Bcl-xL inhibit apoptosis possibly by inhibiting Bax/Bak.

### III. C. Viral modulation of apoptosis

Many viruses are known to induce apoptosis when infecting cells in vitro and in vivo (Hardwick 1998; Razvi et al. 1995). Killing of infected cells by apoptosis can be beneficial to viral infection in vivo, because it reduces inflammation, inhibits immune recognition, and facilitates cell to cell spread (Teodoro et al. 1997). On the other hand, premature apoptosis induction by the infected host cell is a strategy to eliminate infected cells and inhibit viral replication. Therefore it is imperative for viruses to modulate apoptosis in order to utilize this cellular process for their benefit. Viral specific proteins modulate the apoptotic response typically through the mitochondria (intrinsic pathway) (Boya et al. 2004; Galluzzi et al. 2008). Viral apoptotic modulators can be pro and anti-
apoptotic. Pro-apoptotic viral proteins induce apoptosis by increasing MMP, either by inserting into the mitochondrial membranes to facilitate MMP or by activating certain host factors that induce MMP. On the other hand, viral anti-apoptotic modulators inhibit apoptosis: many by mimicking Bcl-2 homologues.

Members of the alphaherpesvirinae subfamily induce apoptosis during productive infection (Delhon 2002; Devireddy et al. 1999; Galvan et al. 1999; Galvan et al. 1998; Sadzot-Delvaux et al. 1995). HSV-1 can induce or inhibit apoptosis in a cell-type dependent manner after infection of cultured cells (Asano et al. 1999; Aubert et al. 1999; Galvan et al. 1999; Galvan et al. 1998; Leopardi et al. 1996). HSV infection leads to induction of apoptosis by possibly several mechanisms. For example, HSV induces DNA damage even in the absence of productive infection (Chenet-Monte et al. 1986; Heilbronn et al. 1989; Schlehofer et al. 1982). DNA damage is a potent apoptosis inducer (Soengas et al. 1999). Two viral proteins U₅₁.₅ and U₅₁.₃ when expressed in the absence of other viral proteins have the potential to activate caspase 3 although the mechanism of activation is not clear (Hagglund et al. 2002). Several anti-apoptotic genes within the HSV-1 genome have been identified (Ahmed et al. 2002; Asano et al. 1999; Aubert et al. 1999; Blaho 2001; Galvan et al. 1999; Inman et al. 2001; Jerome et al. 2001; Jerome et al. 1999; Jerome et al. 1998; Munger et al. 2001; Munger et al. 2001; Perng et al. 2002; Perng et al. 2000). The anti-apoptotic genes include ICP27, U₅₃, U₅₅, gJ, gD, and LAT. ICP27 delays caspase 3 processing of the DNA fragmentation factor (DFF) and poly ADP-ribose polymerase (PARP). U₅₃ is a viral protein kinase that blocks the cleavage and activation of the pro-apoptotic protein BAD. gJ inhibits cytotoxic T-lymphocyte (CTL) Granzyme B and Fas-induced apoptosis by protecting infected cells from DNA
fragmentation. gD can also prevent Fas-induced apoptosis by activating NF-κB. Lastly LAT seems to inhibit the splicing of Bcl-x into the short pro-apoptotic form while stabilizing the long anti-apoptotic version (Peng et al. 2003).

BHV-1 induces apoptosis in bovine peripheral blood mononuclear cells (PBMCs) and CD4+ T-cells *in vitro* (Devireddy et al. 1999; Eskra et al. 1997; Hanon et al. 1999; Hanon et al. 1997; Hanon et al. 1998; Hanon et al. 1996; Winkler et al. 1999). BHV-1 can inhibit apoptosis through the expression of the LR gene. LR gene products block apoptosis in cultured cells and in neurons of infected calves (Ciacci-Zanella et al. 1999; Lovato et al. 2003). LR does this in part by preventing activation of caspase 3 and 9 during productive infection (Henderson et al. 2004). Of particular interest to BHV-1 infection is how apoptosis unfolds in neurons. In sympathetic neurons and differentiated PC12 cells, withdrawal of growth factors leads to induction of apoptosis. The initiators of apoptosis are small GTP-binding proteins Cdc42 and Rac1 (Bazenet et al. 1998; Estus 1994), which signal through c-Jun N-terminal kinase and c-Jun, resulting in apoptosis (Xu et al. 2001). Inflammatory cytokines and environmental stresses such as UV radiation, heat shock, oxidative stress, hyperosmolarity, and axonal injury all trigger Cdc42 and JNK activation in neurons. Many of these stimuli also trigger reactivation of herpesviruses (Jones 2003). A recent study from our lab demonstrated that LR ORF2 and ORF2/ORF1 fusion proteins inhibited cold shock and/or FasL-induced apoptosis in mouse neuroblastoma cells and this inhibition was protein not mRNA dependent (Shen et al. 2008). Because ORF2/ORF1 fusion protein interacts with Bid and Cdc42 this suggests a possible mechanism of inhibition of apoptosis by the LR gene (Meyer et al. 2007).
IV. Notch signaling

IV. A. Overview

The Notch pathway is a highly conserved signaling pathway that occurs between neighboring cells, and is one of the primary pathways by which organisms differentiate precursor cells into specialized tissues and organs. It is indispensable during embryonic development and is also essential for cell fate decisions in the adult organism (Artavanis-Tsakonas et al. 1999). Additionally, Notch regulates cell proliferation, apoptosis, hypoxia, establishment of stem cell niche and stem cell renewal (Kimble et al. 2007; Morrison et al. 2006; Ruas et al. 2007). The Notch pathway is regulated at multiple levels; outside the cell, in the cytoplasm, and within the nucleus. Aberrant Notch signaling is linked to developmental disorders, cardiovascular defects and cancer (Gridley 2003; High et al. 2008; Miele et al. 2006). The core components of the pathway consist of a ligand DSL (Delta, Serrate, Lag-2), a receptor Notch and a transcription factor CSL (CBF1/RBP-Jk, Su(H), Lag-1) (Bray 2006) (Figure 1.3). Both DSL (Delta, Serrate, Lag-2) and CSL (CBF1/RBP-Jk, Su(H), Lag-1) are named for the mammalian, D. melanogaster, and C. elegans orthologous proteins, respectively. Organisms typically encode multiple Notch receptors and ligands, but only one CSL. Mammalians have four Notch receptors (Notch1-4) and five DSL ligands (Delta 1, 3, and 4, Jagged 1, 2) (Table 1.1). Both the ligand and Notch receptor are multidomain type I transmembrane proteins while CSL is a DNA-binding transcription factor that acts both as a repressor and activator of Notch target genes. Upon binding of the ligand from a signal-sending cell to the receptor of a signal-receiving cell, the Notch receptor undergoes two sequential proteolytic cleavages. The first cleavage occurs extracellularly by the membrane-bound
metallo-protease TNFα-converting enzyme (TACE). The second cleavage is carried out inside the membrane by the γ-secretase complex. The result of the second cleavage releases the Notch intracellular domain (NICD) from the membrane into the cytoplasm. NICD contains nuclear localization signals that direct it to the nucleus where it forms a ternary complex with the DNA binding protein CSL and the transcriptional co-activator Mastermind (Petcherski et al. 2000; Petcherski et al. 2000; Tamura et al. 1995; Wu et al. 2000). Formation of the CSL-Notch-Mastermind ternary complex is obligatory for the transcription of Notch target genes.

CSL is the conduit that ultimately converts the extracellular signal into a transcriptional one. In the absence of Notch signaling, CSL forms a repressor complex by interacting with the transcriptional machinery and by recruiting silencing mediator of retinoid and thyroid receptors (SMART)/nuclear receptor co-repressor (N-CoR), CBF1-interacting co-repressor (CIR), and SMRT/HDAC-1-associated protein (SPEN or SHARP) (Hsieh et al. 1999; Kao et al. 1998; Olave et al. 1998; Oswald et al. 2002). The co-repressor complex recruits histone deacetylase complexes (HDACs) to the local chromatin and converts it to the transcriptionally silent configuration. NICD binding to CSL results in the release of the co-repressor complex and recruitment of Mastermind. The ternary complex NICD-CSL-Mastermind activates transcription through interaction with Histone acetyltransferase (HAT) complexes PCAF/GCN5 and CBP/p300 (Fryer et al. 2002; Kurooka et al. 2000; Wallberg et al. 2002). Concomitantly Mastermind also recruits specific kinases that hyperphosphorylate NICD resulting in ubiquitin-mediated proteasomal degradation, release of the co-activator complex, and once again repression of target genes (Fryer et al. 2004).
IV. B. Notch signaling in neuronal tissue

The Notch pathway as mentioned above is critical for organogenesis by regulating an array of cellular processes including stem cell self-renewal, cell fate determination, cellular differentiation and apoptosis. In the central nervous system (CNS), components of the signaling pathway are present not only during the embryonic stages, but also continuously present in the adult nervous system (Presente et al. 2001). During the entire lifetime, Notch signaling is involved with changes in the cellular architecture and function of the nervous system by controlling neurogenesis, growth of axons and dendrites, synaptic plasticity, and neuronal death.

In the developing nervous system the proliferation, differentiation, and survival of neural stem cells (NSCs) are essential processes designed to generate sufficient neurons to form neuronal circuits. The regulation of these processes directly controls architecture, function, and plasticity of the CNS. Activated Notch signaling in NSCs maintains these cells in a proliferating state (Zhong et al. 1997), while Numb, a protein that antagonizes Notch and inhibits the pathway, promotes cell cycle arrest and neuronal differentiation (Li et al. 2003). In this way, Notch signaling controls NSC self-renewal and cell fate specification.

Notch receptors and ligands are also expressed in cells of the adult nervous system suggesting this signaling pathway controls CNS plasticity (Presente et al. 2001). As neurons differentiate from progenitor cells, they rapidly grow neurites to form synapses with other neurons. Mature neurons are capable of neurite outgrowth and retraction, synapse formation or disassembly, and strengthening of existing synapses
(neurite remodeling) (Antonini et al. 1993; Carlisle et al. 2005; Trachtenberg et al. 2002; Yuste et al. 2001). Notch signaling plays a central role in this process. Activated Notch leads to inhibition of neurite growth or promotes their retraction, while inhibition of Notch signaling promotes neurite extension (Berezovska et al. 1999; Redmond et al. 2000; Sestan et al. 1999). Modification of neurite morphology could also provide a mechanism by which activated Notch signaling enhances synaptic plasticity (Engert et al. 1999; Maletic-Savatic et al. 1999). Long-term potentiation (synaptic plasticity) is widely assumed to be the main mechanism of memory formation.

IV. C. Viral regulation of Notch signaling

Notch signaling is an important target of numerous viruses because it controls cellular processes that are crucial for the viral life cycle (Hayward 2004). In general, viruses affect the Notch signaling pathway by three separate mechanisms. One, viral proteins mimic components of the Notch pathway. Two, viral proteins interact with components of the Notch pathway to modulate it. Three, viral proteins activate the Notch pathway at the transcriptional level. Modulation of Notch pathway leads to activation of viral and cellular promoters and induces a state that favors viral replication.

Gamma-herpesviruses Epstein-Barr virus (EBV) and Kaposi Sarcoma’s Herpes Virus (KSHV), fall for the most part in the first two categories. EBV nuclear antigen 2 (EBNA2) is a universal transcriptional activator and is one of the first genes expressed after EBV infection. Functionally EBNA2 seems to mimic NICD very closely by interacting with Notch transcription factor CBF1/RBP-Jκ (Grossman et al. 1994; Henkel et al. 1994; Waltzer et al. 1994). The effect of EBNA2 is counter-regulated by EBNA 3B
and EBNA 3C, which prevent EBNA2 from binding to RBP-Jκ and underscores the importance of a delicate balance of gene activation through RBP-Jκ (Johannsen et al. 1996; Robertson et al. 1995; Waltzer et al. 1996). EBNA3C can also form complexes with HDAC1 to inhibit transcription (Radkov et al. 1999). Another EBV protein, latency BamHI-A rightward transcript (BART) RPMS also interacts with Notch components to antagonize EBNA2. RPMS binds to the co-repressor complex associated with CBF1/RBP-Jκ to inhibit transcription (Zhang et al. 2001).

KSHV is able to modulate Notch signaling through two viral proteins. Replication and transcription activator (RTA) which is the key master switch for viral lytic replication and latency associated nuclear antigen (LANA) which is a critical factor in the establishment and maintenance of latency. Both proteins interact with CBF1/RBP-Jκ to modulate Notch downstream gene expression (Lan et al. 2005; Lan et al. 2005; Liang et al. 2002). The promoters upstream of both RTA and LANA, have RBP-Jκ binding sites, which allows each protein to control expression of the other making the Notch pathway a major protagonist in the latency-reactivation switch. KSHV interacts with the Notch pathway in another novel way. Stability of NICD is tightly regulated by the ubiquitin-proteasome pathway and Sel10, a ubiquitin E3 ligase enzyme, which ubiquitinates NICD and targets it for degradation (Gupta-Rossi et al. 2001; Oberg et al. 2001; Wu et al. 2001). KSHV LANA directly interacts with Sel10 preventing ubiquitination and degradation of NICD, which constitutively activates the Notch pathway (Lan et al. 2007).

CBF1/RBP-Jκ binding sites have also been identified in various adenoviral promoters (Ansieau et al. 2001). This led to the discovery that two viral gene products, DNA-binding protein (DBP) and E1A, interact with cellular proteins involved in the
Notch pathway. E1A is an oncoprotein that activates CBF1/RBP-Jκ-dependent gene expression by binding to CBF1/RBP-Jκ and displacing the co-repressor complex (Ansieau et al. 2001). E1A also interacts with two HAT complexes, which regulate Notch signaling (CBP/p300 and TRRAP/GCN5), by modulating their chromatin remodeling functions (Barbeau et al. 1994; Eckner et al. 1994; Eckner et al. 1994; Lang et al. 2003). SRCAP and p400 are cellular proteins that associate with CBP/p300 complexes and function in chromatin remodeling (Cai et al. 2003; Doyon et al. 2004; Monroy et al. 2001). E1A binds to both proteins and antagonizes SRCAP, while it enhances p400 function (Fuchs et al. 2001; Johnston et al. 1999). A second adenoviral protein DBP also interacts with SRCAP and it inhibits its activity (Xu et al. 2001; Xu et al. 2003).

Another DNA virus that manipulates Notch signaling is SV40. Notch induction is regulated at the transcriptional level and requires expression of two viral proteins, large T-antigen and small t-antigen (Bocchetta et al. 2003).

High-risk HPV16 oncoproteins E6 and E7 stimulates Notch1 expression in murine and human primary cell lines (Weijzen et al. 2003), which consequently has an effect on regulating HPV transcription (Lathion et al. 2003).

HCV is the first RNA virus shown to modulate the Notch signaling pathway (Ghosh et al. 2000; Iwai et al.), however it is not clear whether there is an effect on the Notch pathway under physiological conditions during HCV infection and replication. It is also unclear whether the activation of Notch signaling has an effect on the life cycle of HCV.

**IV. D. Viral regulation of Notch signaling has oncogenic potential**
Notch activation is oncogenic in many contexts, because it typically results in the inhibition of differentiation, enhancement of cell cycle progression and prevention of apoptosis. Intracellular forms of all four Notch homologues function as oncogenes and have transformation ability \textit{in vitro} and in animal models (Bellavia et al. 2000; Callahan et al. 2001; Capobianco et al. 1997). To date, all of the viruses that modulate the Notch pathway have oncogenic properties.

\textit{In vitro} infection of B cells with EBV leads to the establishment of immortal lymphoblastoid cell lines. EBV immortalizing protein EBNA-2, which is necessary for EBV-induced transformation, activates CBF1/RBP-Jκ, thus mimicking NICD (Grossman et al. 1994; Henkel et al. 1994; Hsieh et al. 1997; Miller et al. 1974; Waltzer et al. 1994). The ability of EBNA2 to contribute to B cell immortalization is dependent upon its interaction with CBF1/RBP-Jκ (Yalamanchili et al. 1994).

KSHV infection is essential for the development of Kaposi sarcoma (KS). Notch signaling plays a pivotal role in the KSHV life cycle. RTA activates main lytic phase promoters through binding to RBP-Jκ which is a major end point in the Notch signal transduction pathway (Liang et al. 2002). The latency protein LANA plays a central role in the deregulation of various cellular functions including accumulation of the intracellular domain of Notch in KSHV mediated tumorigenesis (Lan et al. 2005). KSHV also modulates Notch signaling by directly increasing the expression of two Notch ligands (JAG1 and DLL4) through vFLIP and vGPCR respectively in lymphatic endothelia (Emuss et al. 2009). This indicates that KSHV infection manipulates the Notch signaling pathway to induce unregulated cell proliferation and differentiation.
Adenovirus E1A is an oncoprotein because it promotes cell cycle progression, inhibition of differentiation, immortalization and transformation (Bayley et al. 1994). The oncogenic properties of E1A are multifactorial, however the Notch pathway activation through direct binding of CBF1/RBP-Jκ, and its interaction with SRCAP and p400 is important for oncogenesis (Ansieau et al. 2001; Fuchs et al. 2001; Johnston et al. 1999). E1A interactions with p400 are necessary for its transformation ability (Fuchs et al. 2001).

Notch activation has also been implicated as part of the SV40-mediated cell transformation. Large T-antigen and small t-antigen activation of Notch is required for growth of transformed mesothelial cells along with inhibition of the tumor suppressor proteins, Rb and p53 (Bocchetta et al. 2003).

High risk HPV16 encodes two viral oncogenes, E6 and E7, which are necessary for the transformation of cervical epithelial cells (zur Hausen 1996). These viral proteins disrupt the cell cycle by degrading p53 and Rb (Dyson et al. 1989; Scheffner et al. 1990). However, the regulation of p53 and Rb is not sufficient for transformation. E6 and E7 independently upregulate transcription of the Notch receptor, which is important for inducing transformation of human and mouse primary cells (Weijzen et al. 2003).

HCV is thought to play an important role in the development of HCC (Banerjee et al.). Two viral proteins, NS3 and NS5A, which play an important role in viral replication, can independently transform cells in vitro (Gale et al. 1999; Park et al. 2000; Sakamuro et al. 1995; Tsuchihara et al. 1999). Interaction of these proteins with the Notch pathway suggests transformation requires regulation of Notch signaling (Ghosh et al. 2000; Iwai et al.).
FIGURES

Figure 1.1: Schematic of the BHV-1 genome

Panel A: Schematic representation of a class D herpesvirus genome. Class D genomes contain two unique regions (U_L and U_S), each flanked by inverted repeats (TR_L/IR_L and TR_S/IR_S). BHV-1 genome is 135 kb with unique long (U_L, 104 kb) and unique short (U_S, 11 kb) segments, which are flanked by inverted internal repeats (IR) and terminal repeats (TR).

Panel B: Schematic representation of the BHV-1 genome. L and S indicate the unique long and short regions, respectively. The boxes represent the inverted and terminal repeats. The positions of the maps units are below the schematic of the genome. Positions of IE transcripts and the LR transcript are presented. IE/4.2 is the IE transcript that encodes bICP4. IE/2.9 is the IE transcript that encodes bICP0. One IE promoter activates expression of IE/4.2 and IE/2.9 and this IE transcription unit is designated IEtu1. E/2.6 is the early transcript that activates expression of E2.6, which encodes bICP0. Exon 2 (e2) of bICP0 contains all of the protein coding sequences of bICP0. The origin of replication (ORI) separates IEtu1 from IEtu2. IEtu2 encodes a protein bICP22. Solid lines in the transcript position map represent exons (e1, e2, or e3).
Figure 1.1:

A.

B.
**Figure 1.2: Schematic representation of LR gene products**

**Panel A:** Partial restriction map, location of LR-RNA, organization of LR ORF, ORF E, and of bICP0. Reading Frame C contains an open reading frame, but lacks an initiating Met. The (*) denote the position of stop codons that are in frame with the respective ORF.

**Panel B:** Expanded map of the *Pst*I fragment that contains the LR promoter, 5’ terminus of the LR transcript, and the putative ORF-E transcript. The AT-rich sequences located near the start site of the LR-RNA are postulated to promote ORF-E transcription, and the arrow denotes the start sites for LR-RNA transcription (nucleotide 724). The numbers indicate the mapped start sites for LR transcription during latent and lytic infections. The white box represents the neuronal specific binding (NSB) site within the LR promoter. Also shown for reference is the termination site for bICP0 translation (bp 960).
Figure 1.2:

A.

B.
Figure 1.3: The core Notch pathway

Binding of the Delta ligand (green) on one cell to the Notch receptor (purple) on another cell results in two proteolytic cleavages of the receptor. The ADAM10 or TACE (TNFα-converting enzyme) metallo-protease (yellow) catalyses the S2 cleavage, generating a substrate for S3 cleavage by the γ-secretase complex (brown). This proteolytic processing mediates release of the Notch intracellular domain (NICD), which enters the nucleus and interacts with the DNA-binding CSL protein (orange). The co-activator Mastermind (Mam; green) and other transcription factors are recruited to the CSL complex, whereas co-repressors (Co-R; blue and grey) are released (Bray 2006).
Figure 1.3:
Table 1.1: **Names of core components of Notch signaling (ligand, receptor and transcription factor) in different species** (Lai 2004)

<table>
<thead>
<tr>
<th>Core component</th>
<th><em>C. elegans</em></th>
<th><em>D. melanogaster</em></th>
<th>Mammals</th>
</tr>
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<tr>
<td>Ligand</td>
<td>LAG-2</td>
<td>Delta</td>
<td>Delta-like1 (DLL1)</td>
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<td></td>
<td>APX-1</td>
<td>Serrate</td>
<td>Delta-like2 (DLL2)</td>
</tr>
<tr>
<td></td>
<td>ARG-2</td>
<td></td>
<td>Delta-like3 (DLL3)</td>
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<td></td>
<td>F16B12.2</td>
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<td></td>
<td>Jagged 2 (JAG2)</td>
</tr>
<tr>
<td>Receptor (Notch)</td>
<td>LIN-12</td>
<td>Notch</td>
<td>Notch1</td>
</tr>
<tr>
<td></td>
<td>GLP-1</td>
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<td>Notch2</td>
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<td></td>
<td></td>
<td></td>
<td>Notch4</td>
</tr>
<tr>
<td>Transcription factor (CSL)</td>
<td>LAG-1</td>
<td>Suppressor of Hairless [Su(H)]</td>
<td>CBF1/RBPJκ</td>
</tr>
<tr>
<td></td>
<td></td>
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GOALS OF THIS STUDY

The survival strategy of *alphaherpesvirinae* subfamily members relies on their ability to establish latency in sensory neurons innervating the site of primary infection followed by periodic reactivation to facilitate transmission. This aspect of the herpesvirus’s lifecycle is simultaneously one of the most clinically relevant and the least understood. Consequently, the overall goal of this study is to develop an understanding by which a viral protein expressed in latently infected neurons, ORF2, promotes the bovine herpesvirus 1 (BHV-1) latency-reactivation process.

During the lytic phase of BHV-1 infection, there are at least 73 viral genes that are expressed leading to an efficient productive infection through interactions with both viral and host factors. In contrast, during the latent phase, abundant viral gene expression is limited to only the latency related (LR) gene. The LR gene encodes proteins and noncoding small RNAs, which promote efficient establishment and/or maintenance of latency. These LR gene products promote latency by suppressing viral gene expression, promoting cell survival, and interfering with immune recognition.

The LR gene contains 2 major open reading frames (ORFs) ORF2 and ORF1 and two reading frames that lack an initiating methionine, RF-C and RF-B (Kutish *et al.* 1990). Previous genetic studies provided evidence that ORF2 is necessary for the BHV-1 latency-reactivation cycle in cattle. ORF2 inhibits apoptosis in the absence of other viral genes (Shen *et al.* 2008) and interacts with two receptors of the Notch signaling pathway (Workman *et al.* 2011). Since these are two distinct functions of ORF2, the first
hypothesis of these studies is that ORF2 contains multiple domains that mediate the respective functions of ORF2. The goal of the studies in Chapter II was to identify important functional domains, which would help us understand the functions of ORF2. By inhibiting apoptosis and interfering with Notch signaling through ORF2, BHV-1 could efficiently influence (Bray 2006; Ehebauer et al. 2006) neuronal maintenance, development, and differentiation (Berezovska et al. 1999; Cornell et al. 2005; Justice et al. 2002) as well as protect neurons from virus-induced apoptosis, promoting the establishment of latency in sensory neurons.

Our previous studies provided evidence that activated Notch signaling trans-activates certain viral promoters and productive infection, and ORF2 interferes with these functions. Questions still remain with respect to whether ORF2 can influence all cellular functions of activated Notch signaling. My second hypothesis is that ORF2 serves as a universal Notch pathway inhibitor because activated Notch may interfere with a latent infection. Therefore the goal of the studies in Chapter III was to test whether ORF2 inhibits Notch cellular functions. This is important because in neurons, untimely activation of Notch signaling can inhibit neurite sprouting (Berezovska et al. 1999; Franklin et al. 1999; Levy et al. 2002; Sestan et al. 1999) and axon repair (El Bejjani et al. 2012), which can result in neuronal cell death (Coleman et al. 2010; Raff et al. 2002). With respect to BHV-1 infection of sensory neurons, maintaining the health of neurons and promoting their survival is crucial for the establishment and maintenance of life-long latency.
Our examination of the ORF2 amino acid sequence led to the identification of a 29 amino acid domain that resembles cellular transcription factors of the Sp family that specifically bind GC rich DNA sequences. The third hypothesis of these studies is that ORF2 also binds to DNA and that this function plays a role in the latency-reactivation cycle. Therefore the goal of the studies in Chapter IV was to provide evidence of ORF2-DNA interactions.
MATERIALS AND METHODS

Cells and viruses

Murine neuroblastoma cells (Neuro-2A), rabbit skin cells (RS) and human embryonic kidney (Hek293) cells were grown in Earle’s modified Eagle’s medium (EMEM) supplemented with 5% FCS, penicillin (10 U/ml), and streptomycin (100 μg/ml).

A BHV-1 mutant containing the LacZ gene in place of the gC gene was obtained from S. Chowdury (Baton Rouge, LA) (gCblue virus) and has been previously described (Geiser et al. 2002; Geiser et al. 2003; Workman et al. 2010). Stock cultures of gCblue were prepared in established bovine kidney cells (CRIB). The virus grows to similar titers as the wild type (wt) virus and expresses the LacZ gene as a late gene.

Plasmids and transient transfections

The mammalian ORF2 construct was described previously (Shen et al. 2008). Briefly, sequences derived from cDNA at 1 day post infection (dpi) were cloned into pCMV-Tag-2B (Stratagene, La Jolla, CA) downstream of a Flag epitope using BamHI-HindIII restriction enzymes. In pCMV-TAG-2A ORF2 is cloned as a single nucleotide frame shift which expresses the mRNA but not the protein (Shen et al. 2008). Sequences derived from ORF2 cDNA at 1day post infection (dpi) with a nuclear localization signal (NLS) deletion (aa 64-70) (ORF2-NLS), with PKA/PKC (ORF2-P) or all (ORF2-AP) putative phosphorylation sites mutated were synthesized by Integrated DNA Technology (IDT; Coralville, IA) and cloned into the pCMV-Tag-2B plasmid using BamHI-HindIII restriction enzymes. ORF2 was also synthesized as a codon-optimized protein for
bacterial expression. Briefly, codon-optimized ORF2 was synthesized with XhoI and HindIII sites introduced at the 5’ and 3’ ends of the gene, respectively. ORF2 sequences were then cloned downstream of six histidine amino acid residues and an Xpress epitope in the vector, pRSET-A (Invitrogen, USA) at the unique XhoI and HindIII sites. A construct containing the Notch1 intracellular domain (ICD) was cloned into a human cytomegalovirus expression construct and has been previously described (Workman et al. 2011). These constructs were gifts from U. Lendahl, Karolinska Institute, Sweden (Lardelli 1996). The construction and characteristics of the bICP0 early promoter-CAT (chloramphenicol acetyltransferase) construct (EP-172) used in this study were described previously (Workman et al. 2011; Workman et al. 2010). The number in the plasmid name refers to the length of the bICP0 E promoter fragment cloned into the promoterless vector, pCAT-basic. A plasmid containing the firefly-luciferase gene downstream of the HES5 promoter was a kind gift from (R. Kopan, Washington University, St Louis, MO). Transient transfections were performed into Neuro-2A cells using TransIT Neural (MIR2145; Mirus), or into Rabbit Skin cells (RS) using Lipofectamine 2000 (11668-019; Invitrogen) according to the manufacturer’s instructions.

**Generation of ORF2 mutants by EZ::TN in-frame linker insertion**

In-frame linker insertion mutants were prepared according to the manufacturer’s instructions (Epicentre Cat # EZ104KN). ORF2 cDNA was released from pCMV2B-ORF2 using BamHI-SalI endonucleases and cloned into pUC57. This plasmid was used as a target DNA in the reaction. The transposon reaction was set up as follows: 1μl EZ-Tn5 10x reaction buffer, 200 ng target DNA, 1μl EZ-Tn5 <NotI/KAN-3> transposon, 6
μl dH₂O, 1 μl transposase. The reaction mixture was incubated at 37° C for 2 hours. To stop the reaction, 1 μl of EZ-Tn5 10x stop solution was added and the reaction mixture was heated at 70°C for 10 minutes. Top 10 DH5α electrocompetent cells (Invitrogen) were transformed with 2 μl of the reaction mixture and a 1:10 dilution was plated unto plates containing 50 μg/ml kanamycin. DNA was extracted from random colonies and initial mapping was performed by digestion with the restriction endonucleases BamHI and HindIII. Certain clones were then sequenced to determine the precise transposon insertion. A panel of these mutants was digested with NotI to remove the kanamycin gene, re-ligated, then digested with BamHI and HindIII, and re-cloned into the pCMV2B Flag-tagged vector. The Flag-tagged mutant constructs were confirmed by sequencing.

**Yeast 2-hybrid analysis**

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

**Immunofluorescence**

Neuro-2A cells cultured for 48 hours after transfection were washed twice with warm EMEM without serum and fixed in 4% paraformaldehyde for 10 minutes at 37° C. Cells were permeabilized with cold 100% ethanol at -20°C for 5 minutes. After washing three times with 1x PBS, slides were blocked with 3% BSA in PBS for 2 hours, then incubated with the mouse anti-Flag antibody (Sigma F1804) (1:250) (Flag-ORF2) or rabbit anti-cleaved caspase 3 (Cell Signaling 6238S) (1:250) for 2 hours at room temperature. The secondary antibody, goat anti-mouse/Alexa Fluor 633
(Invitrogen/Molecular Probes A21050) (1:100) or goat anti-rabbit/Alexa Fluor 488 (Invitrogen/Molecular Probes A11008) (1:100) was added and cells were incubated for 1 hour at room temperature in the dark. DAPI (Thermo Scientific 46190) (1:1000) was used to stain nuclear DNA. An Olympus IX 81 Inverted confocal laser-scanning microscope was used to collect images (excitation/emission at 488/520 nm and 633/660 nm). For apoptosis assays, cells were incubated in EMEM media with 2% FCS for 12 hours. These cells were then incubated on ice (cold-shock) for 4 hours, and subsequently processed for immunofluorescence.

**Laddering Assay**

Cold-shock induced apoptosis of Neuro-2A cells was performed as previously described (Shen et al. 2008; Shen et al. 2009) with few modifications. Briefly, 24 hours after transfection Neuro-2A cells in T25 flasks were incubated in fresh EMEM media with 2% serum for 12 hours. Cells were then cold-shocked on ice for 1 hour and allowed to recover at 37° C for 3 hours. Cells were subsequently collected and analysis of fragmented DNA was performed as described previously (Shen et al. 2008; Shen et al. 2009).

**β-gal assay**

For apoptosis protection assays, Neuro-2A cells were grown in 60 mm plates and were co-transfected with 2 μg of pCMV-β-Gal plasmid and 2 μg of pCMV-Tag plasmid expressing Flag-tagged ORF2, or ORF2 mutants using TransIT Neural. Twenty-four hours after transfection, cells were cold-shocked on ice for 2 hours. Cells were then fixed,
stained and the number of β-gal+ cells counted as described previously (Workman et al. 2010). The number of β-gal+ cells in cultures expressing the blank vector was set to 100%. The number of blue cells in cultures transfected with ORF2 or ORF2 mutants was divided by the number of β-gal+ cells in cultures transfected with the blank vector to calculate the fold difference. The results are an average of at least three independent experiments.

For BHV-1 productive infection assays, the BHV-1 gCblue virus was used. gCblue and procedures for preparing BHV-1 genomic DNA have been previously described (Workman et al. 2010). RS cells grown in 6 well plates were cotransfected with 0.83 μg of the gCblue viral genome, 1 μg Notch1 ICD and 1 μg ORF2 or ORF2 mutants using Lipofectamine 2000. Twenty-four hours after transfection, cells were fixed, stained, and the number of β-gal+ cells counted. The number of blue cells in cultures transfected with a blank vector was used to calculate the fold difference in cultures transfected with Notch1 and ORF2 or ORF2 mutants. The results are an average of three independent experiments.

**Chloramphenicol acetyl transferase (CAT) reporter assay**

Neuro-2A cells grown in 60 mm dishes were co-transfected with the designated plasmids. At 48 hours after transfection, cell lysate was prepared by three freeze/thaw cycles in 0.25 M Tris-HCl, (pH 7.4). Cell debris was pelleted by centrifugation, and protein concentration was determined by the Bradford assay. CAT activity was measured in the presence of 0.1 μCi (14C-chloramphenicol) (CFA754; Amersham Biosciences) and
0.5 mM Acetyl-CoA (A2181; Sigma). The reaction was incubated at 37° C for 1 hour. All forms of chloramphenicol were separated by thin-layer chromatography. CAT activity in 50 μg cell lysate was quantified using a Bio-Rad Molecular Imager FX (Molecular Dynamics, CA). Levels of CAT activity are expressed as fold induction relative to the vector control.

**Immunohistochemistry**

Immunohistochemistry was performed using an ABC kit (Vector Laboratories) according to the manufacturer’s specifications. Trigeminal ganglia (TG) were collected from calves that were mock infected, infected but in the latency phase (at least 60 days post initial infection) or infected and treated with dexamethasone at different time points to reactivate the virus. TG were fixed with formalin and then embedded in paraffin. Thin sections (4-5 μm) of the ganglia were cut and mounted unto glass slides. The slides were first incubated at 65° C for 20 mins, then washed twice in xylene for 10 mins each, twice in 100% EtOH for 5 mins, once in 90% EtOH for 5 mins, once in 70% EtOH for 5 mins, twice in distilled H₂O for 5mins and three times in 1xTBS for 5mins each. To block any endogenous peroxidase activity, sections were incubated in hydrogen peroxide (0.03% in PBS, pH 7.4) for 20 mins at room temperature. Tissue sections were then washed three times in 1xTBS for 5 mins at room temperature followed by digestion with proteinase K (Dako, #S3020) for 20mins at 37° C. Tissue sections were then blocked with 5% normal serum diluted in 1xTBS containing 0.25% bovine serum albumin for 45 mins at room temperature in a humidified chamber. Slides were incubated in either Notch1 (Cell Signaling, #3268S), Notch3 (Santa Cruz Biotechnology, SC-5593) or ORF2 (Open
Biosystems, F7858) rabbit polyclonal antibodies at a 1:500 dilution, overnight in a humidified chamber at 4°C. The next day slides were washed in 1xTBS and incubated in biotinylated goat anti-rabbit IgG (Vector Labs, PK-6101) for 30 mins at room temperature in a humidified chamber. Avidin-biotinylated enzyme complex was added to the slides for 30 mins at room temperature. After three washes in 1xTBS slides were incubated with freshly prepared substrate (Vector Labs, SK-4800), rinsed with distilled water, and counterstained with hematoxylin for 1 min. The thin sections from mock-infected calves were used as a negative control.

Neurite formation assay

Neuro-2A cells grown in 60mm plates were co-transfected with a CMV-promoter plasmid expressing the Notch1 or Notch3 intracellular domain, a pCMV-Tag plasmid expressing Flag-tagged ORF2, or ORF2 mutants and a pCMV-β-Gal plasmid. To induce neurite sprouting, 24 hours after transfection, cells were seeded into new plates at a low density of 2000 cells/cm² and were starved in media with 0.5% serum for three days. Cells were then fixed, stained and a β-gal assay was performed as described above. The percent of cells with neurites was calculated by dividing the number of β-gal+ cells with neurite length at least twice the diameter of the cell by the total number of β-gal+ cells. The results are an average of three independent experiments.

Dual luciferase reporter assay

Neuro-2A cells were seeded (8 x 10⁵ cells) in 60 mm dishes containing EMEM with 10% FCS 24 hours prior to transfection. Two hours before transfection, the media
was replaced with fresh EMEM containing 0.5 % FCS, to lower the basal levels of HES5 promoter activity. Cells were co-transfected with a plasmid containing the firefly-luciferase gene downstream of the HES5 promoter (1μg), a plasmid encoding Renilla luciferase under control of the herpesvirus TK promoter (50 ng) plus the indicated plasmids. Forty hours after transfection, cells were harvested and protein extracts were subjected to a dual luciferase-assay using a commercially available kit (Promega, E1910), according to the manufacturer’s instructions. Luminescence was measured using a Glomax 20/20 Luminometer (Promega E5331).

**DNA binding assay by affinity chromatography**

The mammalian ORF2 expression construct was transfected into Neuro-2A cells using TransIT-Neural. Forty eight hours after transfection, monolayers were washed twice with cold phosphate-buffered saline, scraped from plates, and then lysed in cell lysis buffer (20 mM Tris-HCl (pH7.4), 10 mM MgCl₂, 500 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol and 100 µg/ml BSA). The lysate was sonicated for 15 seconds prior to rotating at 4°C for 30 min. Cell debris was centrifuged at 20,000 rpm for 15 minutes. The supernatant was subsequently desalted against dialysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 10% glycerol and 100 µg/ml BSA) that was used for DNA chromatography.

DNA-cellulose chromatography was performed by incubating 100 µg of ds- or ss-DNA cellulose (Sigma, USA) with 1 mg (as indicated in the respective figure legend) of mammalian cell lysate containing ORF2 overnight at 4°C in binding buffer (20mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM β-mecaptoethanol, 1 mM EDTA, 100 µg/ml BSA,
10% glycerol, 1 mM Phenylmethanesulfonyl fluoride (PMSF) and 1x protease inhibitor (Roche, USA)). After the protein was incubated with the designated DNA cellulose beads, the beads were washed 5 times in binding buffer containing 100 mM NaCl and bound proteins were eluted with Laemmli SDS-PAGE buffer. Samples were subjected to electrophoresis by SDS-PAGE and analyzed by Western blot. To determine the preference of ORF2 for nucleic acid binding, yeast total RNA or Herring ds-DNA at different concentrations were added to DNA cellulose column with extract in competition experiment prior to washing step. DNA-free cellulose (Sigma, USA) was used as a negative control.

**Western Blot**

**To separate soluble and nuclear fractions:**

Neuro-2A cells in 60 mm dishes were transfected with the designated plasmids and 48 hours after transfection cells were collected, washed once with 1xPBS and suspended in hypotonic buffer (10 mM Tris pH 7.5, 10 mM KCl, 0.5 mM EGTA, 1.5 mM MgCl₂, and 0.5% Triton X-100). Cells were vortexed, then centrifuged at 5000 x G for 2 minutes, and the supernatant collected. The pellet was suspended in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100), and incubated at 4° C for 30 minutes with rotation. Lysate was centrifuged at 14,000 rpm for 10 minutes and the supernatant was collected. Proteins were boiled in Laemmli sample buffer for 5 minutes and separated on a 12% SDS-polyacrylamide gel. Immune-detection of ORF2 and its mutants were performed using a mouse anti-Flag antibody (Sigma F1804) (1:500). A polyclonal Goat anti-Actin
(Santa Cruz SC-1616) antibody was used to confirm that equal protein amounts were loaded. A Goat anti-Histone 3 (Santa Cruz SC-8654) was used as a control for detecting nuclear proteins in the respective cellular fractions.

**For Notch degradation experiments:**

Neuro-2A cells were transfected with the designated plasmids then collected, washed once with 1xPBS and suspended in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% Sodium Dodecyl Sulfate, 1% TritonX-100, protease inhibitor) and incubated at 4° C for 30 mins with rotation. Lysates were cleared by centrifugation at 14k rpm for 10 mins. Protein concentration was measured using the standard Bradford assay. Equal amounts of protein were boiled in Laemmli sample buffer for 5 minutes and separated on a 10% SDS-polyacrylamide gel. Mouse anti-Flag antibody (1:500) was used to detect wt and mutant ORF2 while Notch3 was detected using a rabbit anti-Notch3 antibody (Santa Cruz Biotechnology, SC-5593). Goat anti-Actin was used to confirm equal protein loading. To block protein synthesis, 40 hrs after transfection cells where treated with cycloheximide (CHX) (100μM) at the indicated time points. To block proteasome activity cells were treated with Lactacystin (15 μM) (Cayman Chemical, 70980) 10 hrs prior to CHX treatment. Cells were subsequently processed for Western Blot.

**For cellular fractionation experiments:**

Neuro-2A cells in 60 mm dishes were transfected with the designated plasmids and 48 hours after transfection cells were collected, washed once with PBS and
suspended in Lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% TritonX-100) with protease inhibitors (Roche #11836153001). Cell lysate was subsequently incubated at 4°C for 30 minutes, centrifuged at 13k rpm (Beckman Avanti 30 centrifuge, F3602 rotor) for 10 minutes at 4°C, and the supernatant collected. The pellet was suspended in Lysis buffer with benzonase (Sigma #E1014), incubated at 4°C for 30 minutes, and then centrifuged for 10 minutes at 14k rpm and the supernatant was collected. The pellet was suspended in RIPA buffer (50 mM Tris-HCl, pH 8, 500 mM NaCl, 0.5% sodium deoxycholate, 0.1% Sodium Dodecyl Sulfate, 1% TritonX-100) and incubated at 4°C for 30 minutes with rotation. Lysate was centrifuged at 14k rpm for 10 minutes and the supernatant collected. The pellet or the respective samples from each fraction were boiled in Laemmli sample buffer for 5 minutes and all samples were separated on a 12% SDS-polyacrylamide gel. Immunodetection of ORF2 and its mutants were performed using mouse anti-Flag antibody (1:500). Endogenous cdk2 was used as a nuclear control and was detected using a mouse anti-cdk2 (Santa Cruz SC-6248).

**Statistical analysis of data**

The standard Student t-test was used to identify statistical difference. A $p$-value $< 0.05$ was considered to be significant.
CHAPTER II

Localization of sequences in a protein encoded by the latency related gene of bovine herpesvirus 1 (ORF2) that inhibits apoptosis and interferes with Notch1 mediated trans-activation of the bICP0 promoter

The studies in this chapter are included in two separate manuscripts published in the Journal of Virology


ABSTRACT

Bovine herpes virus 1 (BHV-1) infection induces clinical symptoms in the upper respiratory tract, inhibits immune responses, and can result in life-threatening secondary bacterial infections. Following acute infection, BHV-1 establishes latency in sensory neurons within trigeminal ganglia. Periodically, reactivation from latency occurs resulting in virus transmission. The latency-related (LR) RNA is abundantly expressed in latently infected sensory neurons suggesting that LR gene products regulate the latency-reactivation cycle. A LR mutant virus with stop codons at the amino-terminus of the first ORF in the LR gene (ORF2) does not reactivate from latency, in part, because it induces higher levels of apoptosis in infected neurons. ORF2 inhibits apoptosis in transiently transfected cells suggesting that it plays an important role in the latency-reactivation cycle. ORF2 also interacts with Notch1 or Notch3, and consequently inhibits their ability to trans-activate the bICP0 early and glycoprotein C promoters. In this study, we identified ORF2 sequences that were necessary for inhibiting cold-shock induced apoptosis or Notch1 mediated trans-activation of the bICP0 early promoter and stimulation of productive infection. Relative to ORF2 sequences necessary for inhibiting apoptosis, distinct domains in ORF2 were important for interfering with Notch1 mediated trans-activation. Five consensus protein kinase A and/or protein kinase C phosphorylation sites within ORF2 regulate the steady state levels of ORF2 in transfected cells. A nuclear localization signal in ORF2 was necessary for inhibiting Notch1 mediated trans-activation, but not apoptosis. In summary, ORF2 has more than one functional domain that regulates its stability and functional properties.
INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is a significant viral pathogen of cattle (Turin et al. 1999) that suppresses immune responses. Infection can result in life-threatening pneumonia due to secondary bacterial infections (reviewed in (Jones 2009; Jones et al. 2007; Jones et al. 2010)). Increased susceptibility to secondary infection correlates with depressed cell-mediated immunity after BHV-1 infection (Carter et al. 1989; Griebel et al. 1990; Griebel et al. 1987; Griebel et al. 1987). CD8⁺ T cell recognition of infected cells is impaired by repressing expression of major histocompatibility complex class I and the transporter associated with antigen presentation (Hariharan et al. 1993; Hinkley et al. 1998; Nataraj et al. 1997). CD4⁺ T cell function is impaired during acute infection of calves because BHV-1 infects CD4⁺ T cells and induces apoptosis (Winkler et al. 1999). Three viral genes (UL49.5, bICP0, and gG) can inhibit specific immune responses in the absence of other viral genes, reviewed in (Jones et al. 2007; Jones et al. 2001). The ability of bICP0 to inhibit IFN dependent transcription is crucial for pathogenesis because BHV-1 does not grow in mice unless they lack IFN receptors (Abril 2004). BHV-1, due to its immune-suppressive properties, is a significant risk factor for a multi-factorial disease commonly referred to as bovine respiratory disease complex.

Like other alpha-herpesvirinae subfamily members, the primary site for BHV-1 latency is sensory neurons within trigeminal ganglia (TG). Lytic cycle viral gene expression (Schang et al. 1997) and infectious virus (Inman et al. 2002) are detected in
TG, but latency is subsequently established. Increased corticosteroid levels, as a result of stress, can initiate BHV-1 reactivation from latency (Jones et al. 2010). Administration of the synthetic corticosteroid dexamethasone to calves or rabbits latently infected with BHV-1 reproducibly leads to reactivation from latency, virus shedding, and a secondary antibody response (Inman et al. 2002; Jones 1998; Jones 2003; Jones et al. 2000; Jones et al. 2006; Rock et al. 1992). Induction of lytic cycle viral gene expression is consistently detected in TG neurons of calves latently infected with BHV-1 following dexamethasone treatment.

The BHV-1 latency related (LR) gene expresses an abundant transcript (LR-RNA) in latently infected sensory neurons (Rock et al. 1987; Rock et al. 1987). The LR gene contains 2 major open reading frames (ORF) ORF2 and ORF1 and two reading frames that lack an initiating methionine, RF-C and RF-B (Kutish et al. 1990). The LR gene is also antisense with respect to an important viral transcriptional regulator, bICP0, suggesting LR-RNA reduces bICP0 levels. Small non-coding RNAs, including two micro-RNAs, encoded within the LR gene inhibit bICP0 protein expression in transient transfection assays (Jaber et al. 2010). A mutant BHV-1 strain with 3 stop codons at the N-terminus of ORF-2 (LR mutant virus) does not express detectable levels of ORF-2 or RF-C (Jiang et al. 2004) but expresses reduced levels of ORF1 in cultured cells during productive infection (Meyer et al. 2007). The LR mutant virus grows less efficiently in the ocular cavity or trigeminal ganglia, and does not reactivate from latency following dexamethasone treatment (Inman et al. 2001; Inman et al. 2002) suggesting that expression of LR proteins is required for the latency- reactivation cycle in cattle. During
establishment of latency, the LR mutant virus induces higher levels of apoptosis in TG neurons of infected calves (Lovato et al. 2003), and plasmids with the same stop codon mutations exhibit little or no anti-apoptosis activity (Ciacci-Zanella et al. 1999; Henderson et al. 2004). ORF2 coding sequences, in the absence of other viral genes, inhibit apoptosis in transiently transfected cells (Shen et al. 2008) suggesting ORF2 is a dominant function encoded by the LR gene.

Notch receptor family members (Notch1-4) are membrane tethered transcription factors that regulate numerous developmental and physiological processes (Bray 2006; Ehebauer et al. 2006). For example, Notch promotes neuronal maintenance, development, and differentiation (Berezovska et al. 1999; Cornell et al. 2005; Justice et al. 2002). Notch3 (Wang et al. 2007) and Notch1 (Naidr et al. 2003; Sade et al. 2004) promote cell survival by activating a protein kinase, AKT, that inhibits apoptosis. Other studies have concluded that Notch family members induce apoptosis (Bray 2006; Ehebauer et al. 2006) suggesting Notch influences cell survival in a cell-type dependent fashion. When the Notch receptor is engaged by its ligands, the Notch intracellular domain (NICD) is cleaved by specific proteases, and is subsequently translocated to the nucleus where it interacts with members of the CSL family of transcriptional repressors. In general, the NICD-CSL complex binds to specific DNA sequences in certain promoters and subsequently activates genes that regulate growth, cell survival, and differentiation (Bray 2006; Ehebauer et al. 2006).
In the current study, we provide evidence that ORF2 interacts with Notch1 and Notch3 proteins. Additional studies suggest that Notch family members may play a role in reactivation from latency. For example, the Notch1 ICD stimulates productive infection as well as the bICP0 immediate early and early promoters. Secondly, Notch1 and Notch3 trans-activated a late BHV-1 promoter, glycoprotein C (gC). Thirdly, ORF2 interfered with the ability of the Notch1 or Notch3 ICD to trans-activate the bICP0 E and gC promoters. A panel of ORF2 mutants was designed and analyzed for their ability to inhibit apoptosis and interfere with the trans-activation potential of Notch1. Compared to domains in ORF2 that were important for anti-apoptosis, distinct domains were necessary for interfering with Notch1 mediated trans-activation of the bICP0 early promoter and productive infection. Localization of ORF2 to the nucleus was necessary for inhibiting Notch1 mediated trans-activation of the bICP0 E promoter, but not inhibiting apoptosis. Mutating putative protein kinase A (PKA) and/or PKC phosphorylation sites stabilized ORF2 steady state levels, but had no obvious effect on inhibiting apoptosis or interfering with Notch1 mediated trans-activation of the bICP0 early promoter. In summary, distinct domains within ORF2 were necessary for inhibiting apoptosis and interfering with Notch1 mediated trans-activation of the bICP0 early promoter.
RESULTS

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

We previously found that a protein encoded by an alternatively spliced LR transcript (7 dpi cDNA) interacts with a cellular transcription factor, c/EBP-alpha (Meyer et al. 2007). The protein encoded by the 7 dpi cDNA is a fusion between ORF2 and ORF1 (Devireddy et al. 2003; Devireddy et al. 1998) (Figure 2.1). Approximately 2/3 of this fusion protein is derived from ORF1. Recent studies have demonstrated that just the expression of ORF2 aa sequences (Figure 2.1) reduces cold-shock induced apoptosis in transfected mouse neuroblastoma cells (neuro-2A) (Shen et al. 2008). The LR mutant virus that does not express LR proteins, including ORF2, (Jiang et al. 2004) induces higher levels of apoptosis in TG neurons during late stages of acute infection (Lovato et al. 2003) indicating that the anti-apoptosis functions of ORF2 are important for the latency-reactivation cycle. Consequently, we were interested in identifying cellular proteins that interact with just ORF2. To this end, a yeast two-hybrid assay was performed using only ORF2 sequences. Multiple clones of Notch1 and Notch3 were identified in the yeast two-hybrid screen suggesting that ORF2/Notch interactions were stable. Since Notch family members regulate many developmental processes (Berezovska et al. 1999; Cornell et al. 2005; Justice et al. 2002), we focused our studies on the interactions between ORF2 and Notch1 or Notch3, and whether Notch family members have the potential to regulate productive infection.

**ORF2 colocalizes with Notch1 and Notch3 and interferes with their function**
Additional studies were performed to verify that ORF2 interacted with Notch1 and Notch3. Initially, studies were performed to localize ORF2, Notch1, and Notch3. A plasmid that expresses a Flag-tagged ORF2 (Shen et al. 2008) was transfected into mouse neuroblastoma cells (neuro-2A) to determine its sub cellular localization by confocal microscopy. We have consistently detected ORF2 near the periphery of the nucleus (Figure 2.2.A). As expected, the Flag-specific monoclonal antibody did not stain mock-transfected cells (Sinani et al. 2011). Plasmids that express the ICD of Notch1 and Notch3 were used for these studies because they are constitutively activated and the respective proteins localize to the nucleus (Bray 2006; Ehebauer et al. 2006). As expected, Notch1 ICD (Figure 2.2.B) and Notch3 ICD (Figure 2.2.C) were diffusely localized throughout the nucleus of neuro-2A cells.

When the ORF2 expression plasmid was cotransfected with the Notch1 ICD, we consistently found that in over 97% (38 out of 39) of cells expressing both Notch1 and ORF2, ORF2 was diffusely localized throughout the nucleus (Figure 2.3.B), and appeared to co-localize with Notch1. Conversely, the Notch3 ICD localized to the peripheral area within the nucleus in cells that expressed both proteins (Figure 2.3.D) in 18/25 cells, which suggested Notch3 co-localized with ORF2. Consistent with the results shown in Figure 2.2.A, ORF2 localized to peripheral areas of the nucleus in the absence of Notch1 ICD or Notch3 ICD (Figure 2.3.A and 2.3.B). In summary, these results supported the yeast two-hybrid findings demonstrating that Notch1 and Notch3 interacted with ORF2.

Workman et al. also found that Notch1, but not Notch3 enhances BHV-1
productive infection. Only Notch1 activates the BHV-1 immediate-early transcription unit 1 (IEtu1) and bICP0 early promoters: whereas Notch1 and Notch3 both trans-activated the late BHV-1 glycoprotein C (gC) promoter. ORF2 interferes with the ability of Notch1 to trans-activate the bICP0 early promoter and Notch1 or Notch3 mediated activation of the gC promoter (Workman et al. 2011) suggesting this function is important for establishing and/or maintaining latency.

**Construction of plasmids that express ORF2 mutant proteins**

ORF2 is a 181 amino acid protein (Figure 2.4.A and B) that is expressed in a subset of trigeminal ganglionic sensory neurons of latently infected calves (Hossain et al. 1995; Jiang et al. 1998). ORF2 has anti-apoptosis activity (Shen et al. 2008) and ORF2 interacts with Notch1 and Notch3, which results in interfering with trans-activation of certain viral promoters (Workman et al. 2011). Furthermore, an alternatively spliced LR-transcript encodes a protein containing part of ORF2 fused with ORF1, and this protein interacts with C/EBP-alpha (Meyer et al. 2007; Workman et al. 2011). Since ORF2 appears to have at least two distinct functions, we hypothesized that ORF2 has more than one functional domain. A BLAST analysis of ORF2 did not reveal extensive similarity to known proteins, and thus did not provide insight into the mechanism by which ORF2 inhibits apoptosis or regulates transcription. To identify functional domains within ORF2, a panel of transposon insertion mutants was constructed in which a 57-nucleotide sequence was randomly inserted into the template (see Figure 2.4.B for the insertion site of the respective mutants). The EZ::TN linker insertion method was previously used to successfully generate a panel of bICP0 mutants, which is also a high GC template (Zhang
et al. 2005). This approach was used because ORF2 deletion mutants that were constructed did not encode a stable protein, whereas the transposon mutagenesis yielded a panel of mutants that spanned the protein.

Each transposon mutant was cloned into a Flag-tagged CMV expression plasmid and transfected into Neuro-2A cells. At 48 hours after transfection, proteins were extracted using hypotonic buffer. Nuclei as well as other debris were separated from the supernatant by centrifugation, and the pellet was then solubilized with RIPA buffer. Western blots were performed using an anti-Flag antibody to assess expression levels of the respective transposon mutants. ORF2 is predicted to encode a 19 kDa protein and a prominent band migrating near the predicted molecular weight was detected (Figure 2.4.C; closed circle). The ORF2 transposon mutants migrated at an approximate molecular weight of 23 kDa (closed triangle) because of the additional 19 amino acids in the transposon insertion (Figure 2.4.C). The higher molecular weight bands detected by the Flag antibody (denoted by the bracket) were specific for cells transfected with wt ORF2 or the respective transposon mutants of ORF2, and were not detected in cells transfected with the empty vector. We suggest these higher molecular weight bands were post-translationally modified ORF2. β-actin levels in the respective lanes were similar indicating equivalent amounts of protein were loaded. Except for ORF2-95, the other transposon mutants expressed similar levels of protein as wt ORF2 (Figure 2.4.C). In the pellet of cells transfected with ORF2, ORF2-240, ORF2-271, ORF2-333, ORF2-469, or ORF2-529, an ORF2 specific band was readily detected suggesting that ORF2 and certain mutants were tightly associated with cellular structures. This results was
consistent with a previous study demonstrating that ORF2 is localized to distinct regions of the nucleus (Workman et al. 2011). Low levels of histone 3 were detected in the supernatant indicating this fraction contained low levels of nuclear proteins as well as cytoplasmic proteins. High levels of histone 3 were present in the pellet, as expected, confirming that tightly bound nuclear proteins were associated with the pellet. Other extraction buffers yielded variable results (data not shown) further suggesting that ORF2 was associated with cellular structures and extraction of ORF2 was dependent on the buffers used.

Within ORF2, there are 15 potential phosphorylation sites (grey shaded amino acids; Figure 2.4.A), which may explain why ORF2-specific bands migrating between 26-30 kDa were detected in transfected cells. Among the 15 putative phosphorylation sites, five consensus protein kinase A (PKA) and/or PKC phosphorylation sites were present (grey shaded amino acids with white lettering; Figure 2.4.A). A mutant (ORF2-AP) containing all 15 putative phosphorylation sites and a mutant with the 5 PKA/PKC phosphorylation sites (ORF2-P) was synthesized in which the serine, threonine, or tyrosine residues in the wt sequence were replaced with alanine. Mutating the putative phosphorylation sites to alanine increased the stability of ORF2-AP (Figure 2.4.C, lane AP) or ORF2-P (lane P) relative to wt ORF2. Furthermore, higher levels of the ORF2 phosphorylation mutants were detected in the pellet. The phosphorylation mutants also had little or no higher molecular weight bands adding support to the prediction that ORF2 may be phosphorylated by a cellular protein kinase.
A consensus nuclear localization signal (NLS) (aRRcRRc) is located between amino acids 64-70 of ORF2 (Devireddy et al. 2003) (Figure 2.4.A; underlined amino acids and 2.4.B). To test whether this NLS was required for nuclear localization, a mutant (ORF2-ΔNLS) containing a deletion of aa 64-70 was synthesized, cloned into the pCMV-FLAG vector, and transfected in Neuro-2A cells. The ORF2-ΔNLS protein levels were similar to wt ORF2, but ORF2-ΔNLS migrated primarily as a 29 kDa protein (Figure 2.4.D). Unlike wt ORF2, ORF2-ΔNLS was not detected in the nuclear pellet suggesting this protein was not present in the nucleus.

Subcellular localization of ORF2 mutants

ORF2 localized to the nuclear periphery in transfected Neuro-2A cells (Figure 2.5), which was consistent with a previous study (Workman et al. 2011). The ORF2-95 mutant (Figure 2.5), which was expressed at lower levels in transfected cells (Figure 2.4.C), was more diffusely localized throughout the nucleus relative to cells expressing the wt ORF2. The other transposon mutants had a similar localization as wt ORF-2 (data not shown), which was somewhat surprising because certain transposon mutants appeared to be tightly associated with structures in the nucleus (Figure 2.4.C).

The ORF2-ΔNLS mutant was localized to the peripheral area of the cytoplasm confirming the results in Figure 2.4.D, which indicated that deletion of ORF2 amino acids 64-70 disrupted nuclear localization. The phosphorylation mutants, ORF2-AP or ORF2-P, appeared to have similar nuclear localization as wt ORF2 even though they were more difficult to extract from transfected cells. During productive infection, ORF2
is diffusely localized to the nucleus and cytoplasm {Meyer et al. 2007 and data not shown} suggesting that a viral encoded or induced factor regulates ORF2 localization. In summary, we have constructed and characterized a panel of ORF2 mutants that can be used to analyze the known functions of ORF2.

**Analysis of anti-apoptosis activity of ORF2 mutants**

Transient transfection assays were performed to compare the anti-apoptosis functions of ORF2 to the respective mutants described in Figure 2.4. For these studies, plasmids expressing the respective ORF2 mutants were transfected into Neuro-2A cells and cold-shock induced apoptosis was performed as previously described (Carpenter et al. 2007; Shen et al. 2008; Shen et al. 2009). At 24 hours after transfection, cells were starved in 2% fetal calf serum for 12 hours, cultures were incubated on ice for 1 hour, and then returned to 37°C for 3 hours. DNA was then extracted and analyzed by agarose electrophoresis. Neuro-2A cells or Neuro-2A cells placed on ice for 1 hour contain no detectable DNA laddering (Shen et al. 2008; Shen et al. 2009). However, extensive DNA laddering, indicative of apoptosis, occurs when Neuro-2A cells are returned to 37°C for 3 or 6 hours (Shen et al. 2008; Shen et al. 2009). ORF2 and Bcl-2, an inhibitor of the intrinsic apoptotic pathway (Yang et al. 1997), inhibited cold shock-induced apoptosis with similar efficiency (Figures 2.6.A and B). ORF2-310 and ORF2-469 mutants were unable to inhibit cold-shock induced apoptosis when compared to wt ORF2 (Figure 2.4.A and B). The anti-apoptosis activity of wt ORF2 was significantly different than ORF2-310 or ORF2-469 (p<0.008 or 0.01 respectively). The ability of the other transposon insertion mutants to inhibit cold-shock induced apoptosis in Neuro-2A cells
was not significantly different than wt ORF2 (p>0.05). Although the ORF2-95 mutant was expressed at lower levels relative to wt ORF2, it still retained anti-apoptosis activity suggesting that low levels of ORF2 were sufficient for inhibiting apoptosis.

Neuro-2A cells expressing ORF2-ΔNLS (Figure 2.6.C) were protected against cold-shock induced apoptosis with similar efficiency as wt ORF2, which was surprising because ORF2-ΔNLS was localized to the cytoplasm. Both phosphorylation mutants (ORF2-P or ORF2-AP) inhibited cold shock-induced apoptosis relative to the control (Figure 2.6.C, p<0.05).

To confirm the results in Figure 2.6, we used a β-Gal co-transfection assay (Hsu et al. 1995; Kumar et al. 1994) that has been modified to measure the effects of various genes on apoptosis (Ciacci-Zanella et al. 1999; Henderson et al. 2002; Inman et al. 2001; Jin et al. 2003; Perng et al. 2000). When a known apoptosis stimulator is used, the number of surviving cells can be accurately measured using the β-Gal co-transfection assay. For these studies, apoptosis was induced using cold shock treatment of Neuro-2A cell. Neuro-2A cells transfected with a plasmid that expresses wt ORF2 exhibited at least a 3 fold increase in cell survival after cold shock induced apoptosis, as judged by an increase in the number of β-Gal+ cells (Figure 2.7). As expected, plasmids expressing ORF2-310 and ORF2-469 did not enhance survival. Consistent with the studies performed in Figure 2.6, the other ORF2 transposon insertion mutants, ORF2-ΔNLS, and the two phosphorylation mutants (ORF2-AP and ORF2-P) inhibited apoptosis with similar efficiency as wt ORF2. Cell survival was consistently higher in cells expressing
ORF2-ΔNLS, or the phosphorylation mutants (ORF2-AP and ORF2-P) compared to wt ORF2: however the difference was not statistically different (Figure 2.7).

Confocal microscopy was performed to confirm that cells expressing ORF2-310 or ORF2-469 do not protect cells against apoptosis. For these studies, we tested whether cleaved caspase 3 was present in cells expressing either wt ORF2 or the ORF2-310 and ORF2-469 mutants. Cleavage of caspase 3 is considered to be the “point of no return” during intrinsic and extrinsic induced pathways of apoptosis, and thus is an established marker for apoptosis induction (Granville *et al.* 1998; Nicholson *et al.* 1997; Schmitz *et al.* 2000). Neuro-2A cells transfected with the respective plasmids were cold-shocked, incubated at 37° C, processed for immunofluorescence, and then visualized with a confocal microscope. Cleaved caspase 3 was not detected in ORF2 positive cells. However, cleaved caspase 3 was readily detected in cells not expressing ORF2, which was expected because cells that were not transfected would undergo cold shock induced apoptosis. In contrast, cells expressing ORF2-310 or ORF2-469 were consistently positive for cleaved caspase 3 confirming these mutants do not efficiently inhibit apoptosis (Figure 2.8).

**Identification of ORF2 mutants that interfere with trans-activation of the bICP0 E-promoter by Notch1**

As discussed above, ORF2 interacts with the cellular transcription factor Notch1, a component of the Notch signaling pathway that controls differentiation and development of all tissues (Workman *et al.* 2011). Notch1 trans-activates the bICP0
early promoter and ORF2 reduces the trans-activation potential of Notch1.

To identify domains in ORF2 that interfered with Notch1 mediated trans-activation of the bICP0 early promoter, transient transfection assays were performed in Neuro-2A cells. Notch1 over-expression activated the bICP0 early promoter approximately 12 fold, and ORF2 reduced Notch1 mediated trans-activation to 3 fold (Figure 2.9) and (Workman et al. 2011). Four transposon mutants, ORF2-95, ORF2-134, ORF2-240 and ORF2-271, did not significantly reduce the ability of Notch1 to stimulate bICP0 early promoter activity when compared to wt ORF2 (p<0.05). With respect to ORF2-95, we cannot distinguish whether this was due to lower protein expression levels or if an essential inhibitory domain was disrupted. The remainder of the transposon mutants inhibited trans-activation of the bICP0 early promoter by Notch1 with similar efficiency as wt ORF2. The phosphorylation mutants, ORF2-P and ORF2-AP, but not ORF2-ΔNLS, also inhibited Notch1 mediated trans-activation of the bICP0 early promoter with similar efficiency as wt ORF2 (p>0.05).

Identification of ORF2 mutants that interfere with productive infection induced by Notch1

Additional studies were performed to identify ORF2 mutants that interfere with the ability of Notch1 to stimulate productive infection. We hypothesized there may be differences in these results compared to the ability of ORF2 to interfere with the ability of Notch1 to trans-activate the bICP0 early promoter. The rationale for this prediction is summarized below. Eleven known consensus binding sites for CSL (RBP-Jκ) exist
(Persson 2010). The Notch ICD interacts with members of the CSL family of transcriptional repressors (Bray 2006; Ehebauer et al. 2006). The BHV-1 genome contains 82 potential binding sites for CSL, of which 23 are located in non-coding regions. Thus, certain ORF2 mutants may interfere with the ability of Notch1 to trans-activate the bICP0 promoter, but may not interfere with productive infection.

To determine whether ORF2 interfered with the ability of Notch1 to stimulate BHV-1 productive infection, rabbit skin (RS) cells were co-transfected with a plasmid expressing the Notch1 ICD or the Notch1 ICD and ORF2 plus the BHV-1 gCBlue virus genome, and the efficiency of productive infection measured. The gCblue virus contains the LacZ gene downstream of the gC promoter, which allows for measuring the efficiency of productive infection by counting β-Gal+ (beta-galactosidase positive) cells. The number of β-Gal+ cells directly correlates with plaque formation (Geiser et al. 2002; Geiser et al. 2003; Meyer et al. 2007; Workman et al. 2010; Workman et al. 2009). RS cells were used for these studies because they are permissive for BHV-1 and can be readily transfected. The Notch1 ICD expression plasmid consistently stimulated productive infection approximately four fold (Figure 2.10): however ORF2 reduced the number of β-Gal+ cells to background levels. The effects of the respective ORF2 mutants on Notch1 mediated activation of productive infection were then analyzed. Five transposon mutants (ORF2-95, ORF2-134, ORF2-240, ORF2-271, ORF2-310) and the ORF2-ΔNLS mutant were unable to significantly interfere with the ability of Notch1 to stimulate productive infection (p<0.05). Interestingly, ORF2-310 did not interfere with Notch1 mediated trans-activation of the bICP0 early promoter suggesting that the ability
of Notch1 to stimulate productive infection was not entirely dependent on interfering with bICP0 early promoter activity. ORF2-P and ORF-2AP, but not ORF2-ΔNLS, reduced productive infection as efficiently as wt ORF2. ORF2-333, ORF2-469, ORF2-529, in general, had an intermediate effect on the ability of Notch1 to stimulate productive infection, which was not significantly different than wt ORF2 (p>0.05). In general, the results in Figures 2.9 and 2.10 suggested that the amino-terminal sequences in ORF2 were important for inhibiting Notch1 mediated trans-activation of the bICP0 early promoter activity and productive infection.
DISCUSSION

LR protein expression is necessary for dexamethasone induced reactivation from latency in cattle (Inman et al. 2002), in part, because a LR mutant virus containing stop codons at the N-terminus of ORF2 induces higher levels of apoptosis in sensory neurons during the establishment of latency (Lovato et al. 2003). In contrast to BHV-1, other alpha-herpesvirinae subfamily members (HSV-1 and HSV-2 for example) abundantly express several small non-coding RNAs that are proposed to mediate the latency-reactivation cycle in small animal models (Jones 1998; Jones 2003; Perng et al. 2010). BHV-1 encoded ORF2 (Ciacci-Zanella et al. 1999; Shen et al. 2008) and HSV-1 LAT (Ahmed et al. 2002; Branco et al. 2005; Hamza et al. 2007; Inman et al. 2001; Perng et al. 2000) inhibit apoptosis, which promotes the survival of infected sensory neurons. The reduced reactivation phenotype of a HSV-1 LAT null mutant is restored to wild type levels when an anti-apoptosis gene is inserted in the LAT locus and is expressed during latency (Jin et al. 2008; Jin et al. 2005; Mott et al. 2003; Perng et al. 2002) underscoring the importance of enhancing survival of infected neurons. In addition to inhibiting apoptosis, ORF2 interacts with at least three cellular transcription factors, C/EBP-alpha, Notch1, and Notch3 (Meyer et al. 2007; Workman et al. 2011), suggesting ORF2 regulates viral and/or cellular transcription. Thus, identifying domains in ORF2 that are important for inhibiting apoptosis relative to its other known functions may provide insight into how ORF2 regulates the latency-reactivation cycle.

A previous study identified a NLS in ORF2 that matches the NLS in the cellular
transcription factor Sp1 (Devireddy et al. 2003). Deletion of the NLS (ORF2-ΔNLS) prevented localization of this mutant to the nucleus. However, the ORF2-ΔNLS mutant protein was localized at or near the plasma membrane of transfected cells suggesting ORF2 can interact with membrane components. Surprisingly, ORF2-ΔNLS inhibited cold shock induced apoptosis with similar efficiency as ORF2 suggesting nuclear localization of ORF2 was not required for its anti-apoptosis functions or low levels of the ORF2-ΔNLS protein were present in the nucleus. Mutating all of the putative phosphorylation sites within ORF2 or just the consensus PKA/PKC phosphorylation sites led to increased levels of ORF2 in neuro-2A cells but had no effect on inhibiting apoptosis or Notch1 functions. At this point, it is not clear whether phosphorylation regulates an unknown function of ORF2 or if it simply destabilizes ORF2. Phosphorylation renders many proteins susceptible to degradation, reviewed in (Hochstrasser 1996); including the p53 tumor suppressor protein (Zhang et al. 2001) and proteins that regulate cell cycle checkpoints (Hartwell et al. 1989). The presence of PKA phosphorylation sites in ORF2 may have biological significance because cAMP, which activates PKA (Das et al. 2007), stimulates HSV-1 reactivation from latency (Leib et al. 1991; Smith et al. 1992).

ORF2-310 and ORF2-469 were unable to inhibit cold shock induced apoptosis, but these mutants inhibited Notch1 mediated trans-activation of productive infection. These results suggested that ORF2 contains two non-overlapping functional domains and the amino terminus of ORF2 was important for inhibiting Notch1 mediated trans-activation of productive infection. For Notch family members to activate transcription in
the canonical Notch signaling pathway, the Notch intracellular domain must interact with a CSL family member that specifically binds DNA, reviewed in (Bray 2006; Ehebauer et al. 2006). At least two non-canonical Notch signaling pathways exist (Sanalkumar et al. 2010), suggesting ORF2 interferes with one or more of these pathways. ORF2-Notch interactions may also maintain “neuronal health” during life-long latency because activation of Notch signaling interferes with neurite formation (Franklin et al. 1999; Levy et al. 2002), which is crucial for normal neuronal functions. Conversely, ORF2 may capture Notch1 and Notch3 to regulate steps that are necessary for establishing or maintaining life-long latency in cattle. Since Notch1 and Notch3 RNA levels are increased in trigeminal ganglia after dexamethasone treatment, a known stimulus for reactivation from latency (Workman et al. 2011), there may be more than one reason to overcome the deleterious effects of Notch on latently infected neurons. Further studies designed to understand the effects of ORF2 on Notch signaling pathways are currently being pursued.

In summary, we predict that ORF2 promotes survival of infected neurons by at least two distinct mechanisms: 1) inhibits apoptosis (Meyer et al. 2007; Shen et al. 2008; Workman et al. 2011) and 2) interferes with viral transcription by sequestering cellular transcription factors such as Notch1, Notch3 (Workman et al. 2011), or C/EBP-alpha (Meyer et al. 2007). C/EBP-alpha is induced during dexamethasone induced reactivation from latency (Meyer et al. 2007) and cooperates with bTIF, the HSV-1 VP16 homologue, to trans-activate the immediate early transcription unit 1 promoter (Meyer et al. 2008). The ability of ORF2 or an ORF2 fusion protein to interact with cellular transcription
factors that stimulate productive infection is believed to promote the establishment and/or maintenance of latency. It is unlikely that ORF2 plays a direct role in reactivation from latency because LR promoter activity (Jones et al. 1990) and LR-RNA levels (Rock et al. 1992) are reduced dramatically during dexamethasone induced reactivation from latency. ORF2 may not be the only important product encoded by the LR gene because other proteins are encoded by the LR gene (Inman et al. 2004; Meyer et al. 2007) and two micro-RNAs encoded by the LR gene reduce bICP0 protein levels (Jaber et al. 2010). The mechanism by which the additional LR gene factors support ORF2 during life-long latency is being examined.
FIGURES

Figure 2.1: Schematic of protein coding regions within the LR genes and ORF2 isoforms encoded by alternatively spliced LR transcripts

ORF-1 and ORF-2 are the open reading frames present in the LR gene (Kutish et al. 1990). Reading Frame B and C (RF-B and RF-C) each contain an open reading frame that lacks an initiating methionine. The numbers in parenthesis are the approximate size of ORFs (Kd) that are located in LR gene sequences. The ORF2 isoforms encoded by alternatively spliced LR transcripts detected in TG at 1 dpi and latency, 7 dpi, and 15 dpi, (Devireddy et al. 2003) are shown as a comparison to ORF2 present in the LR gene. Although the LR-RNA is differentially spliced at 1 day after infection in TG of cattle, both transcripts encode an intact ORF2. The (*) denotes the position of stop codons that are in frame with the respective ORF.
Figure 2.1:
**Figure 2.2: Localization of ORF2 and Notch family members in neuro-2A cells**

Neuro-2A cells were transfected with 4μg of plasmids expressing Flag-tagged ORF2 (Panel A), Notch1 ICD (Panel B), or Notch3 ICD (Panel C). Cultures were prepared for confocal microscopy at 48 hours after transfection as described in the materials and methods. Cells were stained with anti-Flag antibody (red), anti-Notch1 or Notch3 antibody (green) or DAPI to visualize ORF2, Notch1, Notch3 and the nucleus respectively. DIC (differential interference contrast) was used to show the unstained cells. The images are representative of more than 5 experiments.
Figure 2.2:
Figure 2.3: Co-localization of ORF2 with Notch1 and Notch3

Panels A and C. Neuro-2A cells were transfected with 4μg of the plasmid expressing N-terminally Flag-tagged ORF2, and then prepared for confocal microscopy as described in Materials and Methods. Notch1 and Notch3 antibodies (green) and DAPI (blue) were used to stain cells.

Panels B and D. Neuro-2A cells were cotransfected with 4μg of plasmids expressing N-terminally Flag-tagged ORF2 and Notch1 or Notch3 ICD. Empty pCMV-Tag-2B was used to equalize DNA amounts. At 48 hours after transfection, cells were prepared for confocal microscopy. Cells were stained with anti-Flag antibody (red), anti-Notch1 or Notch3 antibody (green) and DAPI (blue) to visualize ORF2, Notch1, Notch3 and the nucleus respectively. The images are representative of at least three independent experiments.
Figure 2.3:
Figure 2.4: Generation of BHV-1 ORF2 mutants

Panel A: Amino acid sequence of ORF2. NLS (underlined), 15 putative phosphorylation sites (grey shaded amino acids) and 5 consensus protein kinase A (PKA) and/or PKC phosphorylation sites (grey shaded amino acids that have white lettering) are shown. The plus signs denote every 10th amino acid in ORF2.

Panel B: ORF2 coding sequences (BamHI-SalI) were cloned into the pUC57 vector and transposon linker insertion reactions performed as described in the materials and methods. Initial mapping was performed by restriction digestion and the precise location of the transposon insertion was confirmed by sequencing. Vertical lines with the respective numbers indicate the nucleotide position of the respective transposon insertion. The relative position of the consensus nuclear localization signal (NLS) is denoted by the white rectangle.

Panel C: The transposon mutants and the two phosphorylation mutants were cloned into the pCMV-Tag-2B vector and transfected into Neuro-2A cells. At 48 hours after transfection, cells were collected and lysed using hypotonic buffer as described in the materials and methods. After centrifugation, the supernatant was removed. The pellet was suspended in RIPA buffer, the solubilized proteins were collected after centrifuging the residual pellet, and this fraction designated the pellet. Detection of flag-tagged ORF2 mutants was performed using an Anti-Flag antibody. A β-actin antibody was used to confirm that equal amounts of protein were loaded in each lane. A histone 3 antibody was used to identify nuclear proteins in the supernatant or pellet fraction. ORF2 is predicted to migrate as a 19 kDa protein and the black circle denotes the position of this protein. The arrow denotes the higher molecular weight ORF2 specific bands that
migrated at approximately 30 kDa, which were detected in certain samples. The transposon mutants are predicted to migrate as a 22 kDa protein and are denoted by a closed triangle. The * denotes the position of the ORF2-specific bands that migrated slower than expected. For each lane, 100 µg protein was loaded.

Panel D: Neuro-2A cells were transfected with the designated plasmids and the respective samples collected at 48 hours after transfection as described above. These results are representative of more than 3 independent experiments.
Figure 2.4:
Figure 2.5: Localization of ORF2 mutants in neuro-2A cells

Neuro-2A cells were transfected with 4μg of the designated plasmids that express a Flag-tagged ORF2 or ORF2 mutants. Cultures were prepared for confocal microscopy at 48 hours after transfection as described in the materials and methods. ORF2+ cells were stained with the anti-Flag antibody (red), or DAPI was used to visualize ORF2 the nucleus (blue). DIC (differential interference contrast) was used to show the unstained cells. The images are representative of more than 3 experiments.
Figure 2.5:

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- **Flag**: Expression of ORF2 constructs with different C-terminal modifications.
- **Merge**: Overlay of fluorescence and DIC images.
- **DIC**: Differential Interference Contrast images for cell morphology.
Figure 2.6: Inhibition of cold shock-induced DNA laddering by ORF2 mutants

Panel A: Neuro-2A cells were transfected with 4μg of the plasmid expressing N-terminally Flag-tagged ORF2 or the designated ORF2 transposon mutants. Cells were cold-shocked at 4°C for 1 hour and allowed to recover at 37°C for 3 hours. Neuro-2A cells transfected with the empty vector were used as a negative control, while Bcl-2 or ORF2 expressing cells were used as positive controls. The agarose gel images are representative of 5 independent experiments.

Panel B: The relative amounts of fragmented DNA in each lane in Panel A were quantified using a Bio-Rad molecular imager FX. The average of 5 independent experiments is shown with the respective standard deviation.

Panel C: Neuro-2A cells transfected with the empty vector, ORF2, ORF2-ΔNLS, ORF2-P or ORF2-AP were cold shocked as in Panel A and fragmented DNA quantified as described in Panel B. An asterisk denotes significant differences (p<0.05) from the control samples (empty vector) as determined by the Student T-test.
Figure 2.6:

A

B

C

Degraded DNA

ORF2

ORF2-95

ORF2-240

ORF2-271

Control

Degraded DNA

ORF2

ORF2-∆NLS

ORF2

ORF2-P

ORF2-AP

Control

Degraded DNA

* * *
Figure 2.7: Survival of Neuro-2A cells after transfection with ORF2 mutants

Neuro-2A cells were co-transfected with 2 μg of the pCMV-β-Gal plasmid and 2 μg of the designated ORF2 expression plasmids. Cells were cold-shocked for 2 hours and the β-Gal assay was performed as described in the materials and methods. The number of β-gal+ cells in cultures expressing the blank vector was set to 100%. The number of blue cells in cultures transfected with the blank vector to calculate the fold difference divided the number of blue cells in cultures transfected with ORF2 or ORF2 mutants. The results are an average of three independent experiments. An asterisk denotes significant differences (p<0.05) from the control samples (empty vector) as determined by the Student T-test.
Figure 2.7:

Cell Survival (fold induction)

ORF2
ORF2-95
ORF2-134
ORF2-240
ORF2-271
ORF2-310
ORF2-333
ORF2-469
ORF2-529
ORF2-∆NLS
ORF2-P
ORF2-AP
Figure 2.8: Induction of caspase 3 cleavage by ORF2 transposon mutants

Neuro-2A cells were transfected for 24 hours, incubated in EMEM media with 2% FCS for 12 hours, and then cold-shock induced apoptosis performed as described in the materials and methods. Cells were then processed for immunofluorescence as described in the materials and methods. Mouse anti-Flag antibody (Red) was used to detect Flag-tagged ORF2 and the designated transposon mutants. Rabbit anti-cleaved caspase 3 antibody (Green) was used to detect activated cleaved caspase 3. DAPI (Blue) was used to stain the nucleus.
Figure 2.8:
Figure 2.9: Identification of ORF2 sequences that interfere with Notch1 mediated trans-activation of the bICP0 early promoter

Neuro-2A cells were co-transfected with the bICP0 E promoter construct (EP-172), a CMV-promoter plasmid expressing the Notch1 intracellular domain and the designated ORF2 expressing constructs. At 48 hours post transfection, cells were collected and processed for CAT activity as described in the materials and methods. CAT activity of cells transfected with the control CAT vector was set to one. All other values are expressed as fold activation with respect to the control. These studies are the average of at least three independent experiments. An asterisk denotes significant differences (p<0.05) from the control samples (empty vector) as determined by the Student T-test.
Figure 2.9:
Figure 2.10: Identification of ORF2 sequences that interfere with Notch1 mediated trans-activation of productive infection

Neuro-2A cells were co-transfected with the gCblue virus, a CMV-promoter plasmid expressing the intracellular domain of Notch1 and the designated ORF2 constructs. At 48 hours after transfection, cells were fixed and a β-Gal assay performed as described in the materials and methods. The number of β-gal+ cells in cultures expressing the empty vector was set to 1. The number of blue cells in cultures transfected with the empty vector to calculate the fold difference divided the number of blue cells in cultures transfected with Notch1 or co-transfected with Notch1 and ORF2 or ORF2 mutants. The results are an average of at least three independent experiments. An asterisk denotes significant differences (p<0.05) from the control samples (empty vector) as determined by the Student T-test.
Figure 2.10:
CHAPTER III

A protein encoded by the latency related gene of bovine herpesvirus 1 (ORF2) promotes neurite sprouting in the presence of Notch1 or Notch3

The studies in this chapter are included in a manuscript in press in the Journal of Virology

ABSTRACT

Bovine herpes virus 1 (BHV-1) infection induces clinical symptoms in the upper respiratory tract, inhibits immune responses, and can lead to life-threatening secondary bacterial infections. Following acute infection, BHV-1 establishes latency in sensory neurons within trigeminal ganglia, but stress can induce reactivation from latency. The latency-related (LR) RNA is the only viral transcript abundantly expressed in latently infected sensory neurons. A LR mutant virus with stop codons at the amino-terminus of the first ORF in the LR gene (ORF2) does not reactivate from latency, in part, because it induces higher levels of apoptosis in infected neurons. ORF2 inhibits apoptosis in transiently transfected cells suggesting it plays a crucial role in the latency-reactivation cycle. ORF2 also interacts with Notch1 or Notch3, and inhibits their ability to trans-activate certain viral promoters. Notch3 RNA and protein levels are increased during reactivation from latency suggesting Notch promotes reactivation. Activated Notch signaling interferes with neuronal differentiation, in part, because neurite and axon generation is blocked. In this study, we demonstrated that ORF2 is able to restore neurite formation in mouse neuroblastoma cells over-expressing Notch1 or Notch3. ORF2 also interfered with Notch mediated trans-activation of a promoter that regulates expression of Hairy Enhancer of Split 5, which is a Notch target and required for inhibiting neurite formation. Additional studies provided evidence that ORF2 promotes degradation of Notch3 in a proteasome dependent manner. In summary, these studies suggest that ORF2 promotes a mature neuronal phenotype, which enhances survival of infected neurons and consequently increases the pool of latently infected neurons.
INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is a significant viral pathogen of cattle (Turin et al. 1999) that suppresses immune responses. BHV-1, due to its immune-suppressive properties, is a significant risk factor for a multi-factorial disease commonly referred to as bovine respiratory disease complex (reviewed in (Jones 2009; Jones et al. 2007; Jones et al. 2010)). Like other alpha-herpesvirinae subfamily members, the primary site for BHV-1 latency is sensory neurons within trigeminal ganglia (TG). Lytic cycle viral gene expression (Schang et al. 1997) and infectious virus (Inman et al. 2002) are detected in TG during acute infection, but latency is subsequently established. Increased corticosteroid levels, as a result of stress, can initiate BHV-1 reactivation from latency (Jones et al. 2010). Administration of the synthetic corticosteroid dexamethasone (DEX) to calves or rabbits latently infected with BHV-1 reproducibly leads to reactivation from latency and virus shedding (Inman et al. 2002; Jones 1998; Jones 2003; Jones et al. 2000; Jones et al. 2006; Rock et al. 1992). Induction of lytic cycle viral gene expression is consistently detected in TG neurons of calves latently infected with BHV-1 following DEX treatment.

The BHV-1 latency related (LR) RNA is the only abundant viral transcript expressed in latently infected sensory neurons (Rock et al. 1987; Rock et al. 1987). The LR gene contains 2 major open reading frames (ORF2 and ORF1) and two reading frames that lack an initiating methionine (RF-C and RF-B) (Kutish et al. 1990). Two micro-RNAs encoded within the LR gene inhibit bICP0 protein expression (Jaber et al. 1992).
and promote cell survival (da Silva et al. 2012). A mutant BHV-1 strain with 3 stop codons at the N-terminus of ORF-2 (LR mutant virus) does not express detectable levels of ORF-2 or RF-C (Jiang et al. 2004) but expresses reduced levels of ORF1 during productive infection of cultured cells (Meyer et al. 2007). The LR mutant virus grows less efficiently in the ocular cavity or trigeminal ganglia, but does not reactivate from latency following DEX treatment (Inman et al. 2001; Inman et al. 2002) suggesting expression of LR proteins is required for the latency-reactivation cycle in cattle. During establishment of latency, the LR mutant virus induces higher levels of apoptosis in TG neurons of infected calves (Lovato et al. 2003), and plasmids with the same stop codon mutations exhibit little or no anti-apoptosis activity (Ciacci-Zanella et al. 1999; Henderson et al. 2004).

ORF2 protein expression, in the absence of other viral genes, inhibits apoptosis in transiently transfected cells (Shen et al. 2008) suggesting ORF2 is a dominant function encoded by the LR gene. ORF2 also interacts with the intracellular domain of Notch1 and Notch3, components of the Notch signaling pathway (Workman et al. 2011). Notch1, but not Notch3 enhances BHV-1 productive infection and activates the BHV-1 immediate-early transcription unit 1 (IEtu1) and bICP0 early promoters: whereas both Notch1 and Notch3 trans-activate the late BHV-1 glycoprotein C (gC) promoter. ORF2 interferes with the ability of Notch1 to trans-activate the bICP0 early promoter and Notch1 or Notch3 mediated activation of the gC promoter (Workman et al. 2011) suggesting this function promotes the establishment and/or maintenance of latency.
Notch receptors (Notch1-4) are membrane tethered transcription factors that regulate differentiation and development of nearly all cell types [reviewed in (Bray 2006; Cornell et al. 2005; Ehebauer et al. 2006; Justice et al. 2002)]. When the Notch receptor is engaged by one of its five trans-membrane ligands (Jagged1, Jagged2, Delta-like1, Delta-like3, and Delta-like4), the Notch intracellular domain (ICD) is cleaved by specific proteases, the Notch ICD translocates to the nucleus, and is considered to be “active”. Notch ICD interacts with members of the CSL family of transcriptional repressors, CBF1, Su(H), or Lag1 (also referred to as RBP-jκ for mammalians) and mastermind (MAML). The Notch-CSL-MAML complex binds to specific DNA sequences, RNA pol II co-activators are recruited to the Notch-CSL-MAML complex, and consequently transcription occurs (Bray 2006; Ehebauer et al. 2006). In the absence of an activated Notch family member, a CSL complex, which includes repressor proteins, inhibits transcription. Activation of Notch signaling in post-mitotic-neurons or neuroblastoma cells inhibits neurite sprouting (Berezovska et al. 1999; Franklin et al. 1999; Levy et al. 2002; Sestan et al. 1999) and axon repair (El Bejjani et al. 2012), which can lead to neuronal degeneration and apoptosis (Coleman et al. 2010; Kwok et al. 1994; Raff et al. 2002). Conversely, neurite sprouting correlates with regeneration of damaged axons and dendrites (El Bejjani et al. 2012). During neural development, activation of Notch is crucial for maintaining neuro-progenitors and suppressing neuronal differentiation (Justice et al. 2002; Montminy 1997). Consequently, disruption of Notch1 in mice leads to depletion of neural progenitor cells (Daniel et al. 1998; Goldman et al. 1997).
In this study, we found that Notch1 or Notch3 inhibit neurite formation in serum arrested mouse neuroblastoma cells (Neuro-2A). When ORF2 is co-transfected with Notch1 or Notch3, neurite formation is restored suggesting ORF2 impairs Notch dependent signaling. ORF2 also interfered with Notch mediated trans-activation of the promoter that encodes Hairy Enhancer of Split 5, which interferes with neurite formation when over-expressed. The N-terminal domain of ORF2 and the nuclear localization signal in ORF2 were important for inhibiting Notch dependent signaling. Conversely, mutation of five consensus protein kinase A and/or protein kinase C phosphorylation sites within ORF2 did not negatively affect the ability of ORF2 to impair Notch signaling. We suggest that the ability of ORF2 to promote neurite formation in the presence of activated Notch promotes normal neural functions following infection.
RESULTS

Expression of Notch and ORF2 in trigeminal ganglia during the latency-reactivation transition

A previous study demonstrated that Notch3 RNA levels were increased in TG during dexamethasone induced reactivation from latency (Workman et al. 2011). Immunohistochemistry (IHC) was used to determine if Notch protein expression occurred in TG neurons during DEX induced reactivation from latency. By 90 minutes after DEX treatment, Notch3 positive neurons were detected in a subset of TG neurons, and by six hours after DEX treatment Notch3 positive neurons were readily detected (Figure 3.1.A). Conversely, Notch3 positive neurons were not readily detected in mock infected TG sections, latently infected TG, or 24 hours after DEX treatment. Although Notch1 was also detected in TG after DEX treatment (Figure 3.1.B), in general fewer neurons were positive and it did not appear that the signal was as intense as Notch3.

As a comparison, we examined ORF2 protein expression in TG samples prior to and during the early stages of DEX induced reactivation from latency. Relative to mock infected TG sections, ORF2 was readily detected in TG sections of latently infected calves (Figure 3.1.C). Since LR RNA expression is reduced during DEX-induced reactivation from latency (Rock et al. 1992), it was not surprising to find that the number of ORF2 positive neurons was reduced at 1.5 or 3 hours after DEX treatment (Figure 3.1.C). In summary, these results provide evidence that Notch3 protein expression was
induced by DEX in TG neurons; in contrast, the number of neurons expressing ORF2 was reduced.

**ORF2 antagonizes Notch inhibition of neurite formation in mouse neuroblastoma cells**

ORF2 stably interacts with Notch3 or Notch1 and these interactions impair their ability to stimulate viral gene expression and productive infection (Sinani et al. 2011; Workman et al. 2011). Although these previous studies provided evidence that ORF2 interferes with the ability of Notch members to stimulate viral promoters and productive infection, they did not address whether ORF2 influences cellular functions of activated Notch signaling pathways. Activation of Notch signaling in neurons also inhibits neurite sprouting (Berezovska et al. 1999; Franklin et al. 1999; Levy et al. 2002; Sestan et al. 1999) and axon repair (El Bejjani et al. 2012), which can result in neuronal cell death (Coleman et al. 2010; Raff et al. 2002). Conversely, neurite sprouting is synonymous to regeneration of damaged axons and dendrites (El Bejjani et al. 2012). With respect to BHV-1 infection of sensory neurons, maintaining neuronal health is important for lifelong latency.

To test whether ORF2 affects Notch functions in mammalian cells, mouse neuroblastoma cells (Neuro-2A) were transfected with a plasmid that expresses activated Notch3 and examined the effect ORF2 has on neurite formation. When Neuro-2A cells were transfected with a Lac Z expression vector and growth factors removed, β-Gal+ neurons with extended neurites were readily detected when cells were seeded at low
density and then serum starved for three days (Figure 3.2.A, Control panel). As expected (Franklin et al. 1999; Levy et al. 2002), Notch1 or Notch3 over-expression inhibited neurite formation after growth factor withdrawal (Notch1 or 3 panels). When ORF2 was co-transfected with the Notch1 or the Notch3 expression plasmid, β-Gal+ Neuro-2A cells with long neurites were frequently detected. Approximately ½ of the β-Gal+ cells consistently sprouted neurites when Neuro-2A cells were co-transfected with Notch1 or Notch3 and ORF2, which was significantly higher relative to Neuro-2A cells transfected with Notch1 or Notch3 and an empty expression vector (Figure 3.2.B).

To localize domains in ORF2 that interfere with Notch inhibition of neurite formation, the neurite formation assay was performed in Neuro-2A cells using a panel of mutants previously described (Sinani et al. 2011) (a schematic of the respective ORF2 mutants is shown in Figure 2.4.A and B). Four N-terminal transposon mutants, ORF2-95, ORF2-134, ORF2-240 and ORF2-271, failed to restore neurite formation when compared to wt ORF2 (Figure 3.3.A and B). Since ORF2-95, unlike the other transposon mutants, expresses lower protein levels in Neuro-2A cells compared to wt (Sinani et al. 2011), it is difficult to distinguish whether its impaired function was due to protein expression levels or if an essential inhibitory domain was disrupted. The remainder of the transposon mutants restored neurite formation similar to wt ORF2 regardless of whether they were co-transfected with Notch1 or Notch3 (Figure 3.3.A and B). The phosphorylation mutants, ORF2-P and ORF2-AP, but not ORF2-ΔNLS, promoted neurite formation in the presence of Notch1 or Notch3 with similar efficiency as wt ORF2 suggesting nuclear localization, but not the phosphorylation status of ORF2, was
important. In conclusion, these studies provided evidence that ORF2 interfered with activated Notch1 or Notch3 signaling and promoted neurite formation in Neuro-2A cells. The N-terminus of ORF2 and nuclear localization of ORF2 were important for this function.

**ORF2 inhibits activation of the HES5 promoter, a downstream target of Notch**

To further examine the ability of ORF2 to interfere with Notch signaling, we tested whether ORF2 influences Notch mediated trans-activation of the Hairy Enhancer of Split 5 (HES5) promoter. The HES family of transcription factors contains basic helix-loop-helix (bHLH) domains, they generally repress transcription, and Notch trans-activates promoters of HES family members, reviewed in (Bray 2006; Cornell *et al.* 2005; Ehebauer *et al.* 2006; Monroy *et al.* 2003). Since Hes1 and Hes5 proteins are transcription factors that inhibit neuronal differentiation (Ohtsuka *et al.* 1999), examining the effect of ORF2 on HES5 promoter activity is relevant to the finding that ORF2 promoted neurite formation in the presence of Notch. A dual luciferase assay was performed with a reporter plasmid that contains the HES5 promoter upstream of the firefly luciferase gene. Increasing amounts of Notch1 (Figure 3.4.A) and Notch3 (Figure 3.4.B) increased HES5 promoter activity in a dose dependent fashion. In general, Notch1 is a more efficient trans-activator than Notch3 (Monroy *et al.* 2001), which is consistent with our results.

Neuro-2A cells were co-transfected with plasmids expressing ORF2, Notch1 or Notch3, the HES5 promoter and luciferase activity measured at 48 hours after
transfection to test whether ORF2 influenced trans-activation of the HES5 promoter by Notch1 or Notch3. ORF2 consistently inhibited Notch1 dependent trans-activation of the HES5 promoter by 4 fold, which was significantly different when compared to the empty vector control (Figure 3.4.C). ORF2 also reduced the ability of Notch3 to trans-activate HES5 promoter activity in a dose-dependent fashion (Figure 3.4.D).

Additional studies were performed to identify ORF2 mutants that interfere with the ability of Notch to trans-activate its target genes. The effects of the respective ORF2 mutants on Notch1 or Notch3 mediated activation of HES5 promoter was analyzed. Surprisingly, all of the transposon mutants inhibited Notch1 and Notch3 activation of HES5 promoter similar to wt ORF2. In contrast, three ORF2 mutants, ORF2-ΔNLS and ORF2-P and ORF2-AP phosphorylation mutants, were unable to significantly interfere with Notch mediated transactivation of the HES5 promoter when compared to the empty vector (Figure 3.5). In summary, these results confirmed that ORF2 has the potential to interfere with the functional properties of activated Notch1 or Notch3.

**ORF2 promotes Notch3 degradation**

Studies were subsequently performed to test whether ORF2 influenced the steady state levels of Notch3. The rationale for this study is the assembly of the Notch/CSL complex at a promoter results in recruitment of the cyclin-dependent kinase-8 (CDK8)/cyclin C complex (Johannsen et al. 1996). CDK8/cyclin C phosphorylates Notch and phosphorylated Notch is a substrate for the nuclear E3 ubiquitin ligase Sel10. Sel10 interacts with the C-terminal PEST region of Notch, and this is required for
proteasomal-dependent degradation. When Notch is degraded, the co-activator complex dissociates from CSL leading to the recruitment of a co-repressor complex and repression of gene expression.

To examine whether ORF2 had an effect on Notch3 steady state levels, co-transfection studies were performed and Notch3 protein levels examined by Western Blot analysis. Cells were also treated with cycloheximide (CHX) to block de novo protein synthesis. In the absence of ORF2, Notch3 levels were reduced within 2 hours after CHX treatment unless the proteasome inhibitor lactacystin was added to cultures (Figure 7A). In the presence of ORF2, Notch3 protein levels were consistently less than the empty vector control, even before CHX treatment (Figure 3.6.A). One hour after CHX treatment, the levels of Notch3 were also reduced in cells co-transfected with ORF2 when compared to the empty vector. This effect was dependent on the ORF2 protein because a frameshift mutant that expresses mRNA but not protein (2A/ORF2) had no effect on Notch3 stability (Figure 3.6.B)

To identify ORF2 sequences that influence Notch3 stability, Neuro-2A cells were co-transfected with the Notch3 expression plasmid plus the designated ORF2 mutants and western blot was performed to examine Notch protein levels. Three transposon mutants that did not stimulate neurite formation (ORF2-134, ORF2-240, and ORF2-271) and one that behaved like wt (ORF2-529) were used for these studies. ORF2-240, but not the other transposon mutants, had slightly higher levels of Notch3 when compared to wt ORF2 (Figure 3.6.C). The ORF2-NLS mutant, but not the two phosphorylation
mutants, had little or no effect on Notch3 protein stability (Figure 3.6.D). These studies indicated that ORF2 reduced the half-life of Notch3 and that nuclear localization of ORF2 was necessary for this function.
DISCUSSION

In this study, we demonstrated that expression of ORF2 in Neuro-2A cells restored neurite formation in the presence of Notch1 or Notch3. As expected, Notch1 or Notch3 interferes with neurite formation in serum-arrested Neuro-2A cells (Berezovska et al. 1999; Franklin et al. 1999; Levy et al. 2002; Sestan et al. 1999). Previous studies demonstrated that ORF2 interfered with the ability of Notch1 and Notch3 to activate productive infection and trans-activate certain viral promoters (Workman et al. 2011). Since these studies did not test whether ORF2 influenced the ability of Notch to regulate known cellular functions, it was important to test whether ORF2 influenced cellular functions of Notch signaling. Since activated Notch is required for the growth of certain human tumors (Armstrong et al. 1961; Bosch et al. 1999; El-Serag 2004), ORF2 may also have therapeutic value with respect to inhibiting the growth of tumors that are dependent on Notch for proliferation.

Many other genes, in addition to Notch, negatively regulate neurite sprouting. These include, A1 adenosine receptor, merlin, adenomatous polyposis coli protein, β-catenin, Cdc42-interacting protein 4, harmine, HES-1, HES-5, proline-serine-rich coiled-coil protein 1 (also known as DDA3), and Van Gogh 1 (Cai et al. 2003; Doyon et al. 2004; Kalamvoki et al. 2004; Moradpour et al. 2004; Ogawa et al. 2003; Ohtsuka et al. 1999; Polesskaya et al. 2000; Polesskaya et al. 2001; Satoh et al. 2000). Conversely, a number of genes promote neurite sprouting. For example, a complex containing diacylglycerol kinase ζ, Rac1, and syntrophin promotes neurite outgrowth (Nakajima et
In addition, the Brn-3a transcription factor, CD47 (also referred to as integrin-associated protein), degenerin/epithelial Na\(^+\) channel protein, Dickkopf-1, insulin–like growth factors I and II, NF-κB, plasticity-related gene 5, Prickle 1 or 2 genes, protruding, retina derived growth factor, and Wnt-3a promote neurite outgrowth (Boyes et al. 1998; Chen et al. 2007; Foehr et al. 2000; Fuchs et al. 2001; Hung et al. 1999; Ogryzko et al. 1996; Oswald et al. 2001; Sakaguchi et al. 1998; Sano et al. 2001; Wallberg et al. 2002). Several proteins that positively or negatively regulate neurite outgrowth belong to the Wnt signaling pathway (adenomatous polyposis coli protein, β-catenin, Wnt-3a, and Dickkopf-1). The Wnt and Notch signaling pathway have considerable cross-talk and thus activating or repressing the Notch signaling pathway impacts Wnt signaling (Kopan et al. 2009; Song et al. 2000). It is not clear whether ORF2 influences the Wnt signaling pathway, and/or other genes that regulate neurite outgrowth.

There was a nice correlation between the ability of the respective ORF2 mutants to inhibit Notch activation of certain BHV-1 promoters and productive infection and Notch inhibition of neurite formation, however the effect of ORF2 mutants on Notch activation of the HES5 promoter differed. Two phosphorylation mutants (ORF2-P and ORF2-AP) did not effectively inhibit Notch mediated trans-activation of the HES5 promoter whereas the same mutants promoted neurite formation and inhibited Notch activation of BHV-1 promoters and productive infection with wild-type efficiency. In contrast, the ORF2 N-terminal mutants that fail to restore neurite growth blocked by Notch expression and are unable to inhibit Notch1 mediated trans-activation of the bICP0
early promoter were able to inhibit Notch activation of HES5 promoter similar to wt ORF2 (Sinani et al. 2011). This suggests ORF2 has differential effects on Notch mediated trans-activation. For activated Notch to stimulate transcription, Notch, CSL, and MAML form a complex at a consensus CSL binding site, reviewed in (Bray 2006; Ehebauer et al. 2006). Consequently, ORF2 may: 1) interfere with the formation of the Notch/CSL/MAML complex, 2) influence the Notch/CSL/MAML complex from interacting with certain CSL consensus binding sites, or 3) interfere with certain co-activators recruited to the Notch-CSL-MAML complex, (Bray 2006; Ehebauer et al. 2006). The finding that ORF2 reduced the steady state levels of Notch3 would also interfere with the trans-activation potential of Notch3. We speculate that the ORF2 phosphorylation mutants influence trans-activation of the bICP0 early promoter but not the HES5 promoter. Since the ORF2 phosphorylation mutants are more stable than ORF2 (Sinani et al. 2011), the differential effects of inhibiting Notch mediated trans-activation were not merely due to lower levels of mutant ORF2 in transfected cells. Additional studies are necessary to understand the mechanism by which ORF2 and the phosphorylation mutants interfere with Notch mediated trans-activation of promoters.

ORF-2, in the absence of other viral genes, inhibits apoptosis (Shen et al. 2008; Sinani et al. 2011) in transiently transfected cells. We predicted that the anti-apoptosis functions of ORF2 are crucial for the latency-reactivation cycle because a LR mutant virus that contains stop codons at the amino-terminus of ORF2 induces higher levels of apoptosis in TG neurons during establishment of latency (Lovato et al. 2003) does not reactivate from latency after dexamethasone treatment (Inman et al. 2002). The finding
that ORF2 promotes neurite formation in the presence of Notch may maintain normal neuronal functions, including enhancing survival, after infection. BHV-1 productive infection induces Notch1 protein levels (Workman et al. 2011) suggesting that during the establishment of latency ORF2 ensures that infected neurons maintain axonal projections by promoting neurite sprouting in the presence of activated Notch. In the absence of ORF2 (LR mutant virus for example), we predict that certain infected neurons are more susceptible to loss of axonal projections because activated Notch is present. It is well established that neurons in which their axons have been damaged or cut can undergo Wallerian degeneration, a slow form of neuronal death suggesting that ORF2 may protect infected and damaged neurons from Wallerian cell death (Coleman et al. 2010; Robertson et al. 1995). The fact that Notch stimulates productive infection (Workman et al. 2011) also favors virus induced neuronal cell death. In neuronal progenitor cells, Notch activation induces apoptosis by a p53-dependent pathway (Kwok et al. 1994). Activated Notch also interferes with neuronal differentiation in the ophthalmic branch of trigeminal ganglia (Tamura et al. 1995) adding further support that that ORF2 may promote latency by interfering with Notch dependent signaling in sensory neurons. Collectively, these observations, suggest that the ability of ORF2 to restrain Notch functions promotes the establishment and maintenance of life-long latency.

During dexamethasone induced reactivation from latency, Notch3 RNA levels are induced (Workman et al. 2011) and HES6 is induced within six hours after dexamethasone treatment (Workman et al. 2012). Examination of dexamethasone induced transcription in trigeminal ganglia of calves latently infected with neurons
revealed that many genes activated by Notch signaling are induced (data not shown) suggesting that the Notch signaling pathway enhances reactivation from latency or is merely activated by dexamethasone. Since ORF2 protein expression is reduced during dexamethasone induced reactivation, Notch family members would not be restrained by ORF2 suggesting Notch destabilizes normal neuronal functions and may even induce neuronal apoptosis.
FIGURES

Figure 3.1: Expression of Notch and ORF2 in the TG

TG were collected from calves that were mock infected, latently infected (at least 60 days post infection), or latently infected and treated with dexamethasone at the indicated time points to induce reactivation from latency. Thin sections were cut and subjected to immunohistochemistry with either Notch3 (Panel A), Notch1 (Panel B) or ORF2 (Panel C) as described in the materials and methods. Biotinylated goat anti-rabbit IgG was used as a secondary antibody. Thin sections from mock-infected calves were used as a negative control. Black arrows indicate positive neurons.
Figure 3.1:

A

B

C
Figure 3.2: ORF2 antagonizes Notch inhibition of neurite formation

Panel A: Neuro-2A cells were co-transfected with a plasmid expressing the Notch1 or Notch3 intracellular domain, a plasmid expressing ORF2, and a plasmid expressing the Lac Z gene (transfection control). To induce neurite sprouting, 24 hours after transfection, cells were seeded into new plates at a low density of 2000 cells/cm² and were starved in media with 0.5% serum for three days. Cells were then fixed, stained and the β-gal assay was performed as described in the materials and methods.

Panel B: The percent of β-Gal+ cells containing neurites was calculated by dividing the number of β-gal+ cells with a neurite length at least twice the diameter of the cell by the total number of β-gal+ cells. The average of at least 3 independent experiments is shown with the respective standard deviation.
Figure 3.2:
**Figure 3.3: Localization of ORF2 sequences necessary to inhibit neurite formation in the presence of Notch1 or Notch3**

Neuro-2A cells were co-transfected with a plasmid expressing the intracellular domain of Notch1 (Panel A), or Notch3 (Panel B), a plasmid expressing WT or the designated mutant ORF2 construct, and a plasmid expressing Lac Z. Neurite formation was calculated as described in Figure 3.2. The results represent the average of at least 3 independent experiments with the respective standard deviation.
Figure 3.3:

A.

B.
Figure 3.4: ORF2 interferes with Notch mediated trans-activation of the HES5 promoter

Neuro-2A cells were co-transfected with a plasmid containing the Firefly luciferase gene downstream of the HES5 promoter, a plasmid expressing Notch1 (Panels A, C) or Notch3 (Panels B, D) intracellular domain and increasing amounts of a plasmid expressing ORF2 (Panels C, D). Promoter activity was measured using a dual luciferase assay. A plasmid expressing Renilla luciferase under control of the herpesvirus TK promoter was used as an internal control. The results are an average of at least three independent experiments with the respective standard deviation.
Figure 3.4:
Figure 3.5: Localization of ORF2 sequences important for Notch1 and Notch3 mediated trans-activation of the HES5 promoter

Neuro-2A cells were co-transfected with a plasmid expressing the intracellular domain of Notch1 (Panel A) or Notch3 (Panel B), a plasmid expressing WT or mutant ORF2, and a plasmid containing the Firefly luciferase gene downstream of the HES5 promoter. Luciferase assay was performed as described in Figure 5. The results represent the average of at least three independent experiments with the respective standard deviation.
Figure 3.5:

A

B
**Figure 3.6: ORF2 reduces the steady state levels of Notch3**

**Panel A:** Neuro-2A cells were transfected with the designated plasmids then collected and processed for western blot. Cultures were treated with 100 μM CHX for one or two hours forty hours after transfection. To inhibit proteasome activity, cells were treated with Lactacystin (15 μM) 10 hours prior to CHX treatment.

**Panel B:** The plasmid 2A/ORF2 contains ORF2 cloned such that there is a one-nucleotide frame shift with respect to the N-terminal Flag epitope. The plasmid 2B/ORF2 contains ORF2 in frame with the N-terminal Flag epitope.

**Panel C** and **D:** The effects of the respective ORF2 mutants on Notch3 steady state protein levels were examined. Neuro-2A cells were co-transfected with ORF2 or the designated ORF2 mutants and the plasmid expressing Notch3. Cultures were treated with CHX as described above. A mouse anti-Flag antibody was used to detect wt and mutant ORF2 while Notch3 was detected using a rabbit anti-Notch3 antibody. Goat anti-Actin was used to confirm equal protein loading.
Figure 3.6:

A. Notch3 + + + + + + + +
   ORF2 - - - + + + +
   Lactacystin - - + - - - +
   CHX (hrs) - 1 2 - 1 2 2

B. 2A/ORF2
   2B/ORF2
   CHX (2hr) - + - +

C. Control ORF2 ORF2-134 ORF2-240 ORF2-271 ORF2-529
   CHX (2hr) - + - + - + + +

D. Control ORF2 ORF2-NLS ORF2-P ORF2-AP
   CHX (2hr) - + - + - + + +
CHAPTER IV

A protein (ORF2) encoded by the latency related gene of bovine herpesvirus 1 interacts with DNA

The studies in this chapter are included in a manuscript submitted to the Journal of Virology

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ABSTRACT

Bovine herpes virus 1 (BHV-1), as other alpha-herpesvirinae subfamily members, establishes latency in sensory neurons. The viral encoded latency-related RNA (LR-RNA) is abundantly expressed in latently infected sensory neurons, and encodes several proteins, including ORF2, as well as two micro-RNAs. A LR mutant virus with stop codons at the amino-terminus of ORF2 does not reactivate from latency after dexamethasone treatment, in part, due to increased apoptosis during the establishment of latency. ORF2 inhibits apoptosis, interacts with three cellular transcription factors (Notch1, Notch3, and C/EBP-alpha), and interferes with Notch mediated trans-activation of certain viral promoters. These observations suggest that ORF2 expression is crucial for the latency-reactivation cycle in cattle. In this study, we identified a 29 amino acid domain within ORF2 that resembles three cellular transcription factors (SP5, SP8, and general transcription factor II F, polypeptide 2). ORF2 is comprised of over 20% basic amino acids suggesting ORF2 interacts with nucleic acids. A subset of ORF2 was associated with chromatin and preferentially associated with single stranded DNA in transfected mouse neuroblastoma cells (Neuro-2A). Alanine substitution of serine, threonine, and tyrosine residues in ORF2 led to a protein that had increased steady state levels in Neuro-2A cells, and a protein that preferentially interacted with double stranded DNA. ORF2 partially purified from bacteria under denaturing conditions preferentially interacted with double stranded DNA. In contrast, ORF2 purified under native conditions preferentially interacted with single stranded DNA. We suggest that interactions between ORF2 and DNA play a role in regulating certain aspects of the latency-reactivation cycle.
INTRODUCTION

Bovine herpesvirus 1 (BHV-1), an alpha-herpesvirinae subfamily member, causes significant economical losses to the cattle industry (Turin et al. 1999). For example, the ability of BHV-1 to suppress the immune system can result in life-threatening bacterial pneumonia. This multi-factorial disorder is referred to as the bovine respiratory disease complex [reviewed in (Jones 2009; Jones et al. 2007)]. When acute infection occurs on mucosal linings within the ocular, nasal, or oral cavity, sensory neurons within trigeminal ganglia (TG) become the primary site for BHV-1 latency. Abundant viral gene expression (Schang et al. 1997) and infectious virus (Inman et al. 2002) are detected during acute infection, but viral gene expression is extinguished and latency is established (Jones et al. 2007; Jones et al. 2006). Stress (due to confinement, transporting cattle, restricting food and water, or weaning) increases corticosteroid levels, and can initiate reactivation from latency (Jones et al. 2010). Administration of the synthetic corticosteroid dexamethasone to calves latently infected with BHV-1 reproducibly induces reactivation from latency (Inman et al. 2002; Jones 1998; Jones 2003; Jones et al. 2000; Jones et al. 2006; Rock et al. 1992). Induction of lytic cycle viral gene transcription is also consistently detected in TG neurons of calves latently infected with BHV-1 following dexamethasone treatment.

Abundant expression of the BHV-1 encoded latency related (LR) RNA occurs in latently infected neurons, however infectious virus is not detected by standard virological assays (maintenance of latency) (Jones 1998; Jones 2003; Jones et al. 2006; Kutish et al.)
LR-RNA is anti-sense relative to the bICP0 gene and has a unique start site in TG (Bratanich et al. 1992; Hossain et al. 1995). The LR gene has two open reading frames (ORF1 and ORF2) and two reading frames that lack an initiating ATG (RF-B and RF-C). A LR mutant virus strain with 3 stop codons at the N-terminus of ORF2 exhibits diminished clinical symptoms, and reduced virus shedding from the eye, TG, or tonsils of infected calves (Inman et al. 2001; Inman et al. 2002; Perez et al. 2005). ORF1, ORF2, and RF-C are expressed when bovine cells are infected with wt or the LR rescued virus, but have reduced or no expression following infection with the LR mutant virus (Jiang et al. 2004; Meyer et al. 2007). Wt LR gene expression is necessary for Dexamethasone (DEX) induced reactivation from latency (Inman et al. 2002), in part, because the anti-apoptosis activity of ORF2 appears to be crucial for the latency-reactivation cycle (Ciacci-Zanella et al. 1999; Lovato et al. 2003; Shen et al. 2008; Sinani et al. 2011). Additional studies demonstrated that ORF2 interacts with cellular transcription factors, Notch1, Notch3, or C/EBP-alpha (Meyer et al. 2008; Meyer et al. 2007; Workman et al. 2011). ORF2 reduces Notch mediated trans-activation of the bICP0 early promoter and glycoprotein C promoter in transient transfection assays providing evidence that ORF2, in part, maintains latency by interfering with viral transcription. Distinct non-overlapping sequences within ORF2 are important for inhibiting apoptosis versus Notch mediated trans-activation of the bICP0 early promoter (Sinani et al. 2011). Additionally, the ability of LR-specific micro-RNAs to inhibit bICP0 expression and regulate the RNA sensor (RIG-I) may enhance life-long latency in cattle by interfering with productive infection and promoting neuronal survival (da Silva et al. 2012; Jaber et al. 2010).
In this study, we identified a 29 amino acid domain in ORF2 resembling cellular transcription factors that specifically bind GC rich DNA sequences (Sp5 and Sp8). ORF2 extracted from mouse neuroblastoma cells preferentially interacted with single stranded-(ss) DNA cellulose compared to double stranded-(ds) DNA cellulose. In contrast, a mutant ORF2 protein with alanine substitutions of all amino acids that can be phosphorylated interacts preferentially with ds-DNA. A similar result was observed when only five consensus protein kinase A (PKA) and/or PKC phosphorylation sites were mutated to alanine. ORF2 partially purified under denaturing conditions from E. coli preferentially interacted with double stranded ds-DNA cellulose while ORF2 partially purified under non-denaturing conditions preferentially interacted with ss-DNA cellulose. When ORF2 was purified from bacteria under denaturing conditions, ds-DNA, but not total RNA, inhibited binding to ds-DNA cellulose. Collectively, these studies provide evidence that ORF2 interacts with DNA suggesting this function could play a potential role in the latency-reactivation cycle.
RESULTS

**ORF2 is a basic protein containing a domain similar to certain cellular transcription factors**

ORF2 is the first open reading frame downstream of the start site of LR-RNA (Figure 4.1.A), the coding sequences of ORF2 completely overlap the bICP0 transcript, and ORF2 is expressed in a subset of latently infected neurons (Jiang et al. 1998; Jones et al. 2011). ORF2 contains over 20% basic amino acids (black shaded amino acids), and 5 consensus protein kinase A (PKA) or PKC phosphorylation sites (grey shaded amino acids) (Figure 4.1.B). BLAST analysis of the entire ORF2 did not reveal significant similarity to known proteins. However, BLAST analysis of a 29 amino acid motif in ORF2 that contain the nuclear localization signal (NLS), a PKA phosphorylation site at amino acid 71 and six basic amino acids (Figure 4.1.B, underlined amino acids) revealed this domain is similar to three transcription factors (Sp5, Sp8, and general transcription factor IIF, polypeptide 2). The nuclear localization signal (NLS) within ORF2 is identical to the NLS in the transcription factor Sp1 (Devireddy et al. 2003) and deletion of amino acids comprising the ORF2 NLS prevents nuclear localization (Sinani et al. 2011). The transcription family that includes Sp1, Sp5, and Sp8 bind GC rich consensus DNA binding sites, reviewed in (Bouwman et al. 2002). Sp5 and Sp8 are known to regulate forebrain development, neuropore closure, and development of olfactory bulb inter-neurons (Bell et al. 2003; Waclaw et al. 2006; Weidinger et al. 2005; Zembrzycki et al. 2007). Collectively, these observations suggested that ORF2 could potentially interact with nucleic acids.
Analysis of ORF2 in transfected cells

Two phosphorylation mutants of ORF2 were synthesized: ORF2-AP contains alanine mutations in all serine, threonine, and tyrosine residues, and ORF2-P contains all of the PKA/PKC consensus sites mutated to alanine (Sinani et al. 2011). ORF2-P also contains mutations in Tyr39 and Ser120, which are adjacent to PKA/PKC sites. In transfected Neuro-2A cells (Figure 4.1.C), wt ORF2 migrated as a 20 and 30 kd band (Figure 4.1.C; closed circles). The ORF2-AP and ORF2-P mutant proteins were expressed at higher steady state levels relative to wt ORF2 (open arrow), which is consistent with a recent study (Sinani et al. 2011). This could be because phosphorylation of ORF2 might play a role in the half-life of ORF2 or the protein changes conformation so that the anti-Flag antibody better recognizes the Flag-tag. However, since the antibody is at the N-terminus of ORF2 the change in ORF2 half-life is the more plausible explanation. The predominant band expressed by ORF2-AP and ORF2-P migrated near 24 kd, and was the only band detected unless the blot was over-exposed to allow detection of wt ORF2. The predicted molecular weight of ORF2 is approximately 19 kd. In cells transfected with the blank expression vector, the Flag specific antibody did not readily detect specific bands.

When expressed in Neuro-2A cells, ORF2 localizes to the nuclear periphery (Figure 4.2.A) (Sinani et al. 2011; Workman et al. 2011). The phosphorylation mutants, ORF2-AP or ORF2-P, appear to have similar nuclear localization as wt ORF2 when visualized by confocal microscopy (Figure 4.2.A). To further examine the localization of
ORF2 in transfected cells, biochemical fractionation of transfected cells was performed and compared to a protein known to localize to the nucleus, cyclin dependent kinase 2 (cdk2). Treatment of cells with .15 M NaCl released most of ORF2 and cdk2 (Figure 4.2.B). Readily detectable levels of wt ORF2 were also released from the nucleus after DNAse I treatment. In contrast to cdk2, ORF2 was readily detected in the nuclear pellet even after RIPA buffer treatment. In general, the alanine substitution mutants (ORF2-P and ORF2-AP) contained less protein in the nuclear pellet but more protein was released from nuclei after treatment with DNAse I and RIPA buffer. The relative levels of the 33 kd ORF2 specific band varied from experiment to experiment, and the fact that this higher molecular weight band was not detected in cells transfected with ORF2-P or ORF2-AP adds support to the concept that ORF2 specific high molecular weight bands were the result of phosphorylation.

The relative amount of ORF2 in the respective samples were estimated by measuring ORF2 protein levels in the gel and comparing these values to the total amount of protein in the respective nuclear fraction (Figure 4.2.C). When the phosphorylation mutants were compared to wt ORF2, the major difference was higher levels of protein was released by DNAse I and RIPA buffer. In summary, these studies confirmed that ORF2 was localized to the nucleus and that phosphorylation of ORF2 has subtle effects on sub-nuclear localization.

**ORF2 expressed in Neuro-2A cells interacts with DNA**
Release of ORF2 from transfected cells by DNAse I treatment added support to the prediction that ORF2 interacts with DNA or structures containing DNA. To test whether ORF2 interacted with DNA, Neuro-2A cells were transfected with a Flag-tagged ORF2 expressing plasmid, ORF2 extracted from transfected cells, extracts incubated with DNA cellulose beads, and retention of ORF2 to DNA cellulose beads detected by Western blot analysis using a Flag specific monoclonal antibody. In several independent studies, ORF2 preferentially interacted with ss-DNA beads relative to ds-DNA beads or blank cellulose beads (Figure 4.3.A). As expected, ORF2 specific bands were not detected by the Flag monoclonal antibodies in mock-transfected cells (Figure 4.3.A, Mock lanes). In contrast to wt ORF2, ORF2-AP (Figure 4.3.B) or ORF2-P (Figure 4.3.C) phosphorylation mutants preferentially interacted with ds-DNA cellulose. In summary, this study provided evidence that wt ORF2 preferentially interacted with ss-DNA whereas both ORF2 phosphorylation mutants preferentially interacted with ds-DNA.

**ORF2 partially purified from bacteria interacts with DNA**

Although the studies in Figure 3 indicated that ORF2 interacted with DNA, the fact that ORF2 interacts with three cellular transcription factors, Notch1, Notch3, and c/EBP-alpha (Meyer et al. 2007; Workman et al. 2011) suggested that the interaction between ORF2 and DNA may occur by an indirect mechanism. To address this concern, Daraporn Pittayakhajonwut over-expressed a His-tagged ORF2 in E. coli, purified this protein, and performed DNA chromatography. She found that when purified under native conditions, ORF2 preferentially interacted with ss-DNA while under denaturing
conditions, it preferentially interacted with ds-DNA cellulose. This interaction could be competed with herring DNA but not with yeast RNA.
DISCUSSION

In this study, we have provided evidence that ORF2, in the absence of other viral proteins, stably associates with DNA. It seems unlikely that the interaction between ORF2 and DNA was due to ORF2 interacting with cellular DNA binding proteins because ORF2 interacted with DNA even after it was partially purified from bacteria under denaturing conditions. Curiously, purifying ORF2 from bacteria under non-denaturing conditions led to a protein that preferentially interacted with ss-DNA, which was similar to wt ORF-2 extracted from transfected Neuro-2A cells. Substituting serine or threonine residues in the consensus PKA/PKC phosphorylation sites in ORF2 to alanine strongly enhanced the ability of ORF2 to interact with ds-DNA, which was similar to ORF2 purified from bacteria under denaturing conditions. These results suggested that conformational and/or the charge of ORF2 influenced its ability to interact with ds- versus ss-DNA. Although these alanine substitution mutants exhibited a higher steady state protein level in transfected cells, they inhibit apoptosis and Notch mediated trans-activation of the bICP0 early promoter with similar efficiency as wt ORF2 (Sinani et al. 2011). It will be informative to identify which ORF2 amino acids, if any, are phosphorylated in sensory neurons of latently infected calves and if differences exist relative to productive infection or transient transfection.

The finding that a 29 amino acid motif in ORF2 was similar to two cellular transcription factors that specifically bind DNA (Sp5 and Sp8) suggested that ORF2 could potentially bind DNA. To date, we have no evidence indicating ORF2 binds DNA.
in a sequence specific fashion. However, we cannot rule out the possibility that ORF2 can bind specific DNA sequences in the presence of a neuronal factor or by specific post-translational modifications. It is also possible that ORF2 preferentially binds a broad-based consensus sequence that is frequently present in the viral genome. Attempts to generate ORF2 deletion mutants to localize the DNA binding domain of ORF2 because proteins encoded by the deletion mutants were not stable in bacteria or in transiently transfected Neuro-2A cells. These negative results suggest that the ½ life of ORF2 is tightly regulated and may have functional significance.

Previous studies suggested ORF2 is a crucial function encoded by the LR gene that promotes the latency-reactivation cycle, reviewed in (Jones et al. 2011). For example, a LR mutant virus containing stop codons at the N-terminus of ORF2 induces higher levels of apoptosis in sensory neurons during the establishment of latency (Lovato et al. 2003), but the LR mutant virus does not reactivate from latency following dexamethasone treatment (Inman et al. 2002). Consequently, the ability of ORF2 (Ciacci-Zanella et al. 1999; Shen et al. 2008; Sinani et al. 2011) to inhibit apoptosis is proposed to promote the survival of infected sensory neurons, in particular during the establishment of latency (Lovato et al. 2003). The ability of ORF2 to interact with cellular transcription factors, Notch1, Notch3, and C/EBP-alpha for example (Meyer et al. 2007; Workman et al. 2011) also appears to be important during the latency-reactivation cycle. These interactions reduced the ability of Notch1 to stimulate productive infection and the ability of Notch1 or Notch3 to trans-activate certain viral promoters (Workman et al. 2011). Furthermore, C/EBP-alpha cooperates with the BHV-
1 VP16 homologue to trans-activate viral immediate early promoters (Meyer et al. 2008) suggesting that an interaction between ORF2 and C/EBP-alpha reduces immediate early gene expression. Finally, support for ORF2 playing a role in the latency-reactivation cycle comes from studies demonstrating that the wt LR gene, but not the ORF2 mutated LR gene, restores wt reactivation levels to a HSV-1 LAT null mutant (Mott et al. 2003; Perng et al. 2002). Although ORF2 is an important regulator of the latency-reactivation cycle, the LR gene encodes for several functions suggesting that all of these products play a role in the life-long latency-reactivation cycle. For example, the LR gene encodes two micro-RNAs that interfere with bICP0 expression and promote cell survival (da Silva et al. 2012; Jaber et al. 2010) as well as other proteins with unknown functions (Jiang et al. 2004; Meyer et al. 2007).

The finding that ORF2 is tightly associated to the nuclear periphery in transiently transfected cells (Sinani et al. 2011; Workman et al. 2011) and that ORF2 interacts with ss- and ds-DNA implies that ORF2 recruits the viral genome to certain sub-nuclear structures in latently infected neurons. Several studies have concluded that if genes are localized to the nuclear periphery or nuclear envelope gene expression is reduced or silenced (Finlan et al. 2008; Green et al. 2012; Shaklai et al. 2007; Vande Vosse et al. 2011). The organization of genes at the nuclear periphery or nuclear envelope can also lead to activation depending on factors located at these respective specific sites, reviewed in (Akhtar et al. 2007). In general, it appears that genes close to the nuclear pore are more active than compared to when they are located further from the nuclear pore. A yeast protein, enhancer of silent chromatin 1, localizes to the nuclear peripheral areas
much like ORF2, and the enhancer of silent chromatin 1 protein recruits heterochromatin to the nuclear periphery (Andrulis 2001; Gatenberg 2000; Taddei et al. 2004). If ORF2 recruits viral genomes to the nuclear periphery, this would interfere with viral genomes entering replication compartments in infected sensory neurons and consequently promote the establishment and/or the maintenance of latency. ORF2 does not regulate basal levels of the bICP0 early promoter, the immediate early promoter that activates bICP4 and bICP0 expression, or the glycoprotein C promoter in transient transfection assays (Sinani et al. 2011; Workman et al. 2011) suggesting that ORF2 is not a sequence-specific repressor of transcription. Conversely, our studies suggest that ORF2 regulates gene expression by sequestering specific cellular transcription factors, and this function does not appear to require sequence specific binding of ORF2 to DNA. Studies designed to understand the precise role that the DNA binding properties of ORF2 play in the latency-reactivation cycle of BHV-1 are underway.
FIGURES

Figure 4.1: Schematic of LR gene and expression of ORF2 in transfected neuro-2A cells

Panel A: Schematic of the LR gene. Partial restriction map of the LR gene is shown. The numbering system of the LR gene and position of ORF2 was derived from a previous study (Kutish et al. 1990). The location of LR-RNA start sites was previously published (Bratanich et al. 1992; Hossain et al. 1995). The position of the 3’ terminus of bICP0 and the bICP0 stop codon was previously described (Wirth et al. 1992; Wirth et al. 1989; Wirth et al. 1991).

Panel B: Amino acid sequence of ORF2. The black shaded amino acids are basic amino acids, the underlined amino acids contain the NLS, and the grey amino acids are the threonine or serine residues that are within consensus PKA or PKC phosphorylation sites.

Panel C: Neuro-2A cells were transfected with the wt ORF2 construct, the ORF2-AP construct, or the ORF-P construct. At 48 hours after transfection, cell lysate was prepared and wt or mutant ORF2 detected by Western blotting using a Flag-specific monoclonal antibody.
Figure 4.1:
Figure 4.2: Localization of WT ORF2 in Neuro-2A cells

Panel A: Neuro-2A cells were transfected with the designated plasmids that express Flag-tagged wt or mutant ORF2. Cultures were prepared for confocal microscopy at 48 hours after transfection as described in the materials and methods. Anti-Flag antibody (red) and DAPI (blue) were used to visualize wt or mutant ORF2 and the nucleus respectively. DIC (differential interference contrast) was used to show the unstained cells. The images are representative of more than 3 independent experiments.

Panel B: Neuro-2A cells were transfected with the wt or ORF2 constructs. At 48 hours after transfection, cells were collected and processed for Western blot analysis as described in the materials and methods. Closed circles denote wt ORF2 specific high molecular weight bands. The position of ORF2, ORF2-P, and ORF2-AP specific proteins is also denoted. 100 µg of protein from the indicated fraction was loaded unto each lane. Cdk2 was used as a control for a protein residing in the nucleus.

Panel C: Precise amounts of protein from the different fractions were loaded into two different gels as described in panel A. One gel was processed for western blotting, the other for silver staining. Band density in each lane was quantified using a Bio-Rad molecular imager FX. The density of the respective bands in the western blot was divided by the density of bands in the appropriate lanes on the silver stained gel. The graph is a representative of three independent experiments.
Figure 4.2:

A

Flag | Merge | DIC
--- | --- | ---
Empty | | |
ORF2 | | |
ORF2-P | | |
ORF2-AP | | |

B

kDa

15 M NaCl | DNase I | RIPA | Pellet
--- | --- | --- | ---
34 | 17 | 26 | 
34 | 17 | 26 | 
26 | 17 | 26 | 
24 | 17 | 26 |

C

Relative ORF2

| .15 M NaCl | DNase I | RIPA | Pellet
--- | --- | --- | ---
1.5 | 1.0 | 0.5 | 0.0
1.0 | 0.5 | 0.0 | 0.0
0.5 | 0.0 | 0.0 | 0.0
0.0 | 0.0 | 0.0 | 0.0

Flag Merge DIC

Empty

ORF2

ORF2-P

ORF2-AP

cdk2
Figure 4.3: ORF2 interacts with DNA following extraction from transfected Neuro-2A cells

Neuro-2A cells (5 x 10^6) were transfected with plasmids expressing wt ORF2 (Panel A), ORF2-AP (Panel B), or ORF2-P (Panel C). DNA chromatography was performed as described in the materials and methods with ss-DNA, ds-DNA, or blank cellulose beads. As a negative control, Neuro-2A cells were mock transfected. One mg of ORF2 expressing cell extract, or mocked transfected cell extract (Panel A), 300 μg of extract prepared from ORF2-AP (Panel B) or ORF2-P (Panel C) was used. Lower concentrations of cell extract were used after transfection with ORF2-AP or ORF2-P because these plasmids express higher levels of ORF2. Input (I) equivalent to 10% cell extract used in the experiments was loaded in the respective panels to verify that ORF2 was expressed. Expression of wt or mutant ORF2 in Neuro-2A cells was analyzed by Western blots using an anti-Flag antibody. The open circle denotes ORF2 or ORF2 mutant specific bands. Molecular weight standards are shown on the left of each panel.
Figure 4.3:
CHAPTER V

Effect of LR gene expression on autophagy
ABSTRACT

Autophagy is a catabolic process involving the degradation of a cell’s own components through the lysosomal machinery. It is a tightly regulated process that plays an essential part in cell growth, development, and homeostasis. Autophagy helps to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular long-lived proteins and organelles in the cytosol. Because of its essential role in maintaining cellular homeostasis, autophagy has been implicated in several diseases including cancer, neurodegeneration, and myopathies. Recent studies have described autophagy as a defense mechanism against intracellular viruses and bacteria because it can deliver whole microorganisms to lysosomes or it can deliver nucleic acids and antigens from the pathogen to endo/lysosomal compartments for activation of innate and adaptive immunity. On the other hand, autophagy is also exploited by certain viruses to enhance viral replication. The alpha-herpesvirinae subfamily member, HSV-1, inhibits autophagy as a function of its virulence. A recent study also showed that infection of bovine kidney cells with a BHV-1 bICP0 null mutant induces the accumulation of autophagosomes in the cytoplasm of infected cells. This suggests BHV-1 must employ a mechanism of evasion of autophagy in order to establish a successful infection. In this study, we examined the effect that expression of the LR gene has on autophagy. The rationale for testing the LR gene is that previous studies demonstrated that the LR gene promotes cell survival, which enhances latency, which implied the LR gene influences autophagy. Starvation of Hek293 cells led to efficient induction of autophagy as
determined by autophagy markers. However, the LR gene had no obvious effect on autophagy.
INTRODUCTION

Autophagy is a cellular process that leads to the lysosomal degradation and recycling of aberrant proteins or damaged organelles (Levine 2005; Levine et al. 2004; Reggiori 2006). This process is quite distinct from endocytosis-mediated lysosomal degradation of extracellular and plasma membrane proteins. Autophagy is mediated by a unique organelle called the autophagosome, which has been observed in all eukaryotes, plants, yeasts and animals. Since autophagosomes function by engulfing portions of the cytosol, it is defined as a non-selective degradation system. Originally identified as a process induced by cellular starvation, autophagy is now believed to be a cellular reaction to a variety of stimuli including environmental stresses and pathogen infection (Orvedahl et al. 2008).

Increasing evidence suggests that autophagy plays a role in activation of innate and adaptive immunity (Levine et al. 2007). First, numerous studies (Dengjel et al. 2005; Nimmerjahn et al. 2003; Paludan et al. 2005; Schmid et al. 2007) have brought forward evidence that autophagy can deliver cytosolic antigen for MHC class II presentation. Second, by sequestering large portions of the cytoplasm in its double membrane structures, which ultimately delivers the contents to the lysosome, autophagy has the ability to target for degradation whole cellular pathogens (xenophagy) (Levine 2005). The best characterized examples of xenophagy are engulfment of *Mycobacterium tuberculosis* in phagosomes (Gutierrez et al. 2004), trapping of group A *Streptococci* in autophagosomes (Nakagawa et al. 2004), and immune escape by *Shigella* (Ogawa et al. 2004).
2005). Pathogens targeted by autophagy can also include DNA and RNA viruses like herpes simplex virus (Talloczy et al. 2006) and Sindbis virus (Liang et al. 1998). On the other hand, viruses employ their own mechanisms to evade or subvert autophagy (Kirkegaard et al. 2004; Orvedahl et al. 2007). RNA viruses like members of the *Picornaviridae* family have acquired mechanisms of subversion in order to utilize autophagy for their own benefit. Studies conducted with Poliovirus (Jackson et al. 2005), Enterovirus 71 (Huang et al. 2009) and Hepatitis C virus (Ait-Goughoulte et al. 2008; Dreux et al. 2009; Mizui et al. 2009) point to induction of autophagy in order to generate membranous vesicles or scaffolds crucial for viral RNA replication.

Perhaps the most compelling evidence of viral evasion of autophagy comes from studies with herpes simplex virus type 1 (HSV-1). HSV-1 infection induces autophagy and infected cells are able to degrade HSV-1 through xenophagy (Talloczy et al. 2006). HSV-1 counteracts the induction of xenophagy through a neurovirulence protein, ICP34.5. ICP34.5 binds protein phosphatase 1 alpha to counter PKR-mediated phosphorylation of eIF2alpha, and also binds the autophagy-promoting protein Beclin 1 (Orvedahl et al. 2007; Talloczy et al. 2002). Through these interactions, ICP34.5 prevents translational arrest and down-regulates the formation of autophagosomes.

A recent study in our lab provided evidence that infection of bovine kidney cells with bICP0 null mutant BHV-1 also induces autophagy (Geiser et al. 2008). Autophagosomes were not induced during infection of cells with a wt BHV-1 strain
suggesting that bICP0 may play a role in BHV-1 autophagy evasion. Conversely, activation of other viral proteins may influence autophagy.
RESULTS AND DISCUSSION

Effect of LR protein on autophagy

HSV-1 inhibits autophagy in infected cells mainly through its neurovirulence protein ICP34.5 (Orvedahl et al. 2007; Talloczy et al. 2002). BHV-1 which is also an alpha-herpesvirinae subfamily member does not encode a known ICP34.5 homologue. The locus of ICP34.5 in the HSV-1 genome lies between ICP0 and ICP4 genes and it is transcribed anti-sense with respect to LAT. In the BHV-1 genome bICP0 and bICP4 are not separated like HSV-1. They are however transcribed anti-sense to the LR gene similar to LAT for HSV-1. To test whether this region of the BHV-1 genome expressed products that could have an effect on autophagy we expressed the full LR gene including its noncoding promoter region in Hek293 cells under starvation conditions that induce autophagy.

One of the hallmarks of autophagy is the change in subcellular localization of the microtubule-associated protein light chain 3 (LC3) (Klionsky et al. 2008). Normally LC3 is localized in a diffused manner throughout the cytoplasm. During induction of autophagy, through a series of proteolytic and covalent modifications involving an ubiquitin-like conjugation system the C-terminus tail of LC3 is cleaved and a phosphatidylethanolamine group is subsequently added to LC3 (Ohsumi 2001). This allows LC3 to become embedded into the membrane of autophagic vesicles and its localization changes from diffused to punctated.

To test autophagy induction, Hek293 cells expressing LC3 N-terminally fused with a green fluorescent protein (GFP) or GFP alone as a negative control, were starved
for 10 hours in HBSS buffer. When cells expressing only GFP were observed using fluorescence microscopy, the localization of GFP as expected was diffuse throughout cells under normal or starvation conditions (Figure 5.1.A). LR expression also had no effect on the localization of wt GFP (Figure 5.1.A). In cells expressing LC3 N-terminally tagged with GFP under normal growth conditions, the LC3-GFP localization was diffused throughout the cell (Figure 5.1.B). The localization became punctuated however, when cells were starved in HBSS indicating induction of autophagy (Figure 5.1.B). Expression of LR gene products did not however seem to inhibit the punctated localization of LC3-GFP when autophagy was induced through HBSS starvation (Figure 5.1.B).

Since the transformation of LC3 during autophagy involves cleavage of its C-terminal tail, two species of LC3 with different molecular weights are produced. Normal LC3 (LC3 I) migrates as an 18 kDa protein while the cleaved species (LC3 II) migrates with an apparent molecular weight of 16 kDa. The difference can be distinguished by Western blotting. To test the effect of LR on autophagy, Hek293 cells co-expressing LC3-GFP with a vector control or LR gene expressing plasmid, were starved for 10 hours in HBSS buffer and total proteins extracted and subjected to western blot analysis. LC3 proteins were detected with an anti-GFP antibody. The antibody recognized a band around 45 kDa which represents GFP-LC3 I (GFP is ~27 kDa). When cells were treated with HBSS, another species at around 43 kDa was detected representing GFP-LC3 II. There was no effect on the amount of LC3-GFP cleavage when the LR gene was
expressed. In summary, these results indicated that LR gene products have no obvious effects on autophagy.
FIGURES

Figure 5.1: Effect of LR gene expression of autophagy

Hek293 cells were co-transfected with either a control plasmid or a plasmid expressing the full LR gene under its natural promoter and a CMV-promoter plasmid expressing GFP (Panel A) or LC3-GFP (Panel B). Twenty four hours after transfection cells were either incubated in normal 10% FCS MEM media (Media) or were starved in HBSS buffer (HBSS) for 10 hours. Cells were then visualized with a fluorescent microscope (Panels A and B) or processed for western blot analysis (Panel C). Mouse Anti-GFP antibody was used to detect LC3-GFP in the western blot. LC3 I indicates the position of GFP-LC3 I and LC3 II indicates the position of the cleaved species GFP-LC3 II. An asterisk denotes degradation products.
Figure 5.1:

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55 43 34

LC3 I

LC3 II

* Actin

55 43

Actin
GENERAL CONCLUSIONS

The overall goal of the work presented in this dissertation was aimed at further elucidating, at the molecular level, the BHV-1 latency-reactivation process. Although immense effort has been focused toward understanding how alpha-herpesviruses establish, maintain and escape from latency, significant gaps in our knowledge still remain. The molecular mechanisms involved in the establishment of latency are of considerable importance in the fight to prevent recurrent disease and transmission.

The lytic and latent phases of BHV-1 lifecycle contrast from one another in overall gene expression and virus production. During the lytic phase, all viral genes are abundantly expressed and virus progeny is produced at high yields. During the latent phase, viral gene expression is not readily detectable with the exception of the LR gene and no virus can be detected. Genetic studies indicated that a protein (s) encoded by the LR gene is/are required for dexamethasone induced reactivation from latency in calves. Additional studies demonstrated that LR gene products promote latency by at least two ways; repressing lytic cycle viral gene expression (Bratanich et al. 1992; Geiser et al. 2002; Jaber et al. 2010) and inhibiting apoptosis in latently infected neurons through the expression of ORF2 (Ciacci-Zanella et al. 1999; Henderson et al. 2004; Lovato et al. 2003; Shen et al. 2008; Sinani et al. 2011). In addition to inhibiting apoptosis, ORF2 interacts with at least three cellular transcription factors: C/EBP-alpha, Notch1, and Notch3 (Meyer et al. 2007; Workman et al. 2011). Furthermore, Notch signaling enhances expression of certain viral promoters and productive infection (Workman et al. 2011).
ORF2 inhibition of Notch suggests that this protein affects viral and/or cellular transcription and perhaps other functions necessary for the latency-reactivation cycle. Thus, identifying functional domains in ORF2 is important in trying to understand how ORF2 regulates the BHV-1 latency-reactivation cycle. For this study, we used a mutational analysis approach. An examination of the ORF2 sequence identified a NLS that matches the NLS in the cellular transcription factor Sp1 (Devireddy et al. 2003) and several putative phosphorylation sites. Deletion of the NLS (ORF2-ΔNLS) prevented localization of this mutant to the nucleus but had no effect on inhibiting apoptosis suggesting that nuclear localization of ORF2 was not required for its anti-apoptosis functions. However, ORF2-ΔNLS was unable to inhibit Notch activation of BHV-1 bICP0 early promoter and productive infection. Mutating all of the putative phosphorylation sites within ORF2 or just the consensus PKA/PKC phosphorylation sites led to increased levels of ORF2 in neuro-2A cells but had no effect on inhibiting apoptosis or Notch1 activation of BHV-1 promoters and productive infection. The presence of PKA phosphorylation sites in ORF2 has a potential biological significance because cAMP, which activates PKA (Das et al. 2007), stimulates HSV-1 reactivation from latency suggesting phosphorylation of ORF2 could play a similar role (Leib et al. 1991; Smith et al. 1992). The lack of known functional domains in ORF2 prompted us to use a random-transposon insertion system where a transposon linker is randomly inserted throughout the gene with the intent of disrupting any putative functional domains. Analysis of the function of the transposon mutants revealed that ORF2 contains two non-overlapping functional domains with the N-terminus being important for inhibiting Notch1 mediated trans-activation of productive infection and the C-terminus playing a
role in the inhibition of apoptosis.

Additional studies were performed to test whether ORF2 influenced the ability of Notch to regulate known cellular functions. To test this hypothesis, we examined whether Notch signaling influences neurite formation, which is crucial for normal neuronal functions (Franklin et al. 1999; Levy et al. 2002). Previous studies have shown that Notch1 and Notch3 inhibit neurite formation in serum-arrested Neuro-2A cells (Berezovska et al. 1999; Franklin et al. 1999; Levy et al. 2002; Sestan et al. 1999). When we overexpressed ORF2 in the presence of Notch1 or Notch3, neurite growth in Neuro-2A cells was restored. Many other genes, in addition to Notch, negatively regulate neurite sprouting. HES1 and HES5 are two Notch target genes that inhibit neurite sprouting and neuronal differentiation (Ohtsuka et al. 1999). Both Notch1 and Notch3 strongly activate HES-5 promoter activity. Additional studies demonstrated that ORF2 inhibits HES5 activation by both Notch1 and Notch3. The N-terminal domain of ORF2 was important in restoring neurite formation in the presence of Notch. However it seemed that ORF2 phosphorylation was necessary for inhibiting Notch mediated activation of the HES5 promoter. This suggests multiple layers of interaction of ORF2 with Notch signaling. For activated Notch to stimulate transcription, Notch, CSL, and MAML must form a complex at a consensus CSL binding site (Bray 2006; Ehebauer et al. 2006). Consequently, ORF2 may: 1) interfere with the formation of the Notch/CSL/MAML complex, 2) influence the Notch/CSL/MAML complex from interacting with certain CSL consensus binding sites, or 3) interfere with certain co-activators recruited to the Notch-CSL-MAML complex, (Bray 2006; Ehebauer et al. 2006). The finding that ORF2
reduced the steady state levels of Notch3 adds to our understanding of how ORF2 controls the trans-activation potential of Notch. Interestingly, ORF2 phosphorylation state influenced Notch trans-activation of the bICP0 early promoter but not the HES5 promoter.

The published results suggest that ORF2 promotes survival of infected neurons by at least two distinct mechanisms: 1) inhibiting apoptosis (Meyer et al. 2007; Shen et al. 2008; Workman et al. 2011) and 2) interfering with viral transcription by sequestering cellular transcription factors such as Notch1, Notch3 (Workman et al. 2011), and/or C/EBP-alpha (Meyer et al. 2007). Anti-apoptosis functions of ORF2 are crucial for the latency-reactivation cycle because a LR mutant virus that contains stop codons at the amino-terminus of ORF2 induces higher levels of apoptosis in TG neurons during establishment of latency (Lovato et al. 2003) and does not reactivate from latency after dexamethasone treatment (Inman et al. 2002). The ability of ORF2 or an ORF2 fusion protein to interact with cellular transcription factors that stimulate productive infection would promote the establishment and/or maintenance of latency. C/EBP-alpha is induced during dexamethasone-mediated reactivation from latency (Meyer et al. 2007) and cooperates with bTIF, the HSV-1 VP16 homologue, to trans-activate the immediate early transcription unit 1 promoter (Meyer et al. 2008). Concurrently, through interactions with the Notch pathway, ORF2 promotes “neuronal health” during life-long latency. For example, BHV-1 productive infection induces Notch1 protein levels (Workman et al. 2011) suggesting that during the establishment of latency ORF2 ensures that infected neurons maintain axonal projections by promoting neurite sprouting in the presence of
activated Notch. In the absence of ORF2 (LR mutant virus for example), we predict that certain infected neurons are more susceptible to loss of axonal projections because activated Notch is present. It is well established that neurons in which their axons have been damaged or cut can undergo Wallerian degeneration, a slow form of neuronal death suggesting that ORF2 may protect infected and damaged neurons from Wallerian cell death (Coleman et al. 2010; Robertson et al. 1995). ORF2 may also sequester Notch1 and Notch3 to regulate steps that are necessary for establishing or maintaining life-long latency in cattle. Since Notch1 and Notch3 RNA and protein levels are increased in trigeminal ganglia during reactivation after dexamethasone treatment leading to activation of BHV-1 promoters and enhancement of productive infection (Workman et al. 2011), inhibition of Notch by ORF2 may be crucial for maintenance of latency by preventing reactivation. However, ORF2 may not be the only important product encoded by the LR gene because other proteins are encoded by the LR gene (Inman et al. 2004; Meyer et al. 2007) and two micro-RNAs encoded by the LR gene reduce bICP0 protein levels (Jaber et al. 2010).

In addition to inhibiting apoptosis and Notch signaling, we have also provided evidence that ORF2, in the absence of other viral proteins, stably associates with DNA preferentially interacting with ss-DNA. Mutation of phosphorylation sites strongly enhanced the ability of ORF2 to interact with ds-DNA, which was similar to ORF2 purified from bacteria under denaturing conditions. These results suggested that conformation and/or the charge of ORF2 influence its ability to interact with ds- versus ss-DNA.
A 29 amino acid motif in the N-terminus of ORF2 that spans the region around the NLS, is similar to two cellular transcription factors that specifically bind DNA (Sp5 and Sp8) suggesting that ORF2 specifically binds DNA. So far we have no evidence that ORF2 binds to a specific DNA sequence. It is however possible that ORF2 can bind specific DNA sequences in the presence of a neuronal factor or by specific post-translational modifications. It is also possible that ORF2 could preferentially bind a broad-based consensus sequence that is frequently present in the viral genome.

The finding that ORF2 is tightly associated to the nuclear periphery in transiently transfected cells (Sinani et al. 2011; Workman et al. 2011) and that ORF2 interacts with ss- and ds-DNA implies that ORF2 recruits the viral genome to certain sub-nuclear structures in latently infected neurons. Several studies have concluded that if genes are localized to the nuclear periphery or nuclear envelope gene expression is reduced or silenced (Finlan et al. 2008; Green et al. 2012; Shaklai et al. 2007; Vande Vosse et al. 2011). Conversely, the organization of genes at the nuclear periphery or nuclear envelope can also lead to activation depending on factors located at these respective specific sites (Akhtar et al. 2007). If ORF2 recruits viral genomes to the nuclear periphery, this could prevent viral genomes from entering replication compartments in infected sensory neurons and consequently promote the establishment and/or the maintenance of latency. Since ORF2 does not regulate basal levels of several BHV-1 promoters tested (Sinani et al. 2011; Workman et al. 2011), this suggests that ORF2 is not a sequence-specific repressor of transcription. Our studies suggest that most likely
ORF2 regulates gene expression by sequestering specific cellular transcription factors, and this function does not appear to require sequence specific binding of ORF2 to DNA.
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175

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