B Virus Uses a Different Mechanism to Counteract the PKR Response

Li Zhu
B VIRUS USES A DIFFERENT MECHANISM FROM HSV-1 TO COUNTERACT THE PKR RESPONSE

by

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ABSTRACT

B virus (*Cercopithecine herpesvirus 1*), which causes an often fatal zoonotic infection in humans, shares extensive homology with human herpes simplex virus type 1 (HSV-1). The $\gamma_{134.5}$ gene of HSV-1 plays a major role in counteracting dsRNA-dependent protein kinase (PKR) activity. HSV-1 Us11 protein, if expressed early as a result of mutation, binds to PKR and prevents PKR activation. The results of experiments in this dissertation revealed that although B virus lacks a $\gamma_{134.5}$ gene homolog, it is able to inhibit PKR activation, and subsequently, eIF2$\alpha$ phosphorylation. The initial hypothesis was that B virus Us11 protein substitutes for the function of $\gamma_{134.5}$ gene homolog by blocking cellular PKR activation. Using western blot analysis, Us11 protein (20 kDa) of B virus was observed early following infection (3 h post infection). Expression of B virus Us11 protein was not blocked by phosphonoacetic acid (PAA), an inhibitor of DNA replication, confirming Us11 is not a “true late” gene of B virus as it is in HSV-1. Analysis of these results suggested that B virus Us11 protein compensates for the lack of the $\gamma_{134.5}$ gene homolog and prevents PKR activation. Next, the results demonstrated that B virus Us11 recombinant protein prevented PKR activation by dsRNA *in vitro*. A B virus Us11 protein stable expression cell line (U373-BVUs11) was established to
investigate whether Us11 protein inhibited PKR activation \textit{in vivo}. Experiments revealed that B virus Us11 protein stably expressed in U373 cells prevented PKR activation and subsequent eIF2\(\alpha\) phosphorylation induced by the infection of these cells with \(\Delta\gamma_134.5\) of HSV-1. As the consequence of preventing PKR activation and subsequent eIF2\(\alpha\) phosphorylation, B virus Us11 protein complemented \(\Delta\gamma_134.5\) HSV-1 in U373 cells as evidenced by restoration of virus protein synthesis and replication in U373 cells. Furthermore, pull-down assays showed that B virus Us11 protein binds to PKR. In addition, the results demonstrated that B virus Us11 protein stably expressed in U373 cells counteracted the inhibiting effect of IFN-\(\alpha\) on HSV-1 replication by preventing PKR activation. These data suggested that B virus and HSV-1, two closely related viruses, use different mechanisms to counteract PKR activity.

\textbf{INDEX WORDS:} B virus, Us11 protein, Temporal kinetics, PKR activation, Type I IFN, PACT, herpes B virus, eIF2\(\alpha\), protein synthesis inhibition
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THE PKR RESPONSE

by

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LIST OF ABBREVIATIONS

DsRNA-dependent protein kinase                             PKR
Interferon                                                IFN
Cytomagalovirus                                           HCMV
Herpes simplex virus type 1                               HSV-1
Herpes simplex virus type 2                               HSV-2
\(\gamma_{1,34.5}\) deletion mutant of HSV-1                 \(\Delta\gamma_{1,34.5}\) of HSV-1
Wild type HSV-1                                           WT HSV-1
Viral host shutoff protein                                 vhs
Intradermal                                               ID
Intracranial                                              IC
Interferon receptor                                       IFNR
Interferon-stimulated gene factor 3                       ISGF-3
Interferon-stimulated regulatory element                  ISRE
DsRNA binding motifs                                      DRBMs
Protein activator of PKR                                  PACT
The \(\alpha\) subunit of eukaryotic translation initiation factor 2 eIF2\(\alpha\)
Inhibitor of NF-\(\kappa\)B                                   I-\(\kappa\)B
Interferon regulatory factor 3                            IRF-3
Initiator tRNA                                            tRNA\(\text{Ai}\)
Fas-associated death domain containing protein            FADD
Tumor necrosis factor-α (TNF-α)
Lipopolysaccharide (LPS)
Growth arrest and DNA damage-inducible protein (Gadd34)
Protein phosphatase 1 (PP1)
I-κB kinase (IKK)
Nitric oxide synthase 2 (NOS2)
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Glycoprotein C (gC)
Phosphate-buffered saline (PBS)
Phenylmethylsulfonyl fluoride (PMSF)
Phosphonoacetic acid (PAA)
β-mercaptoethanol (β-ME)
Kinase domain (KD)
Varicella-Zoster virus (VZV)
SECTION I. INTRODUCTION

PART I: B VIRUS, A MEMBER OF FAMILY HERPESVIRIDAE, CAUSES LETHAL INFECTION IN HUMANS

FAMILY HERPESVIRIDAE.

General Introduction

Herpesviruses are widely disseminated in nature. To date, almost 100 herpesviruses have been isolated and partially characterized. Of these, eight herpesviruses have been isolated from humans and more than 30 herpesviruses have been identified in nonhuman primates (Huff and Barry 2003). These viruses belong to the same family, Herpesviridae, because they share the same virion architecture and genome structure. A typical herpes virion consists of a core containing linear double-stranded DNA, an icosadeltahedral capsid approximately 100-nm in diameter containing 162 capsomers, an amorphous material designated as tegument, which is localized between the capsid and envelope, and an envelope containing viral glycoprotein spikes on its surface. Each of the known herpesviruses has four biological properties: (1) each possesses large quantities of enzymes involved in nucleic acid metabolism, DNA synthesis, and processing of proteins, (2) synthesis of DNA and the assembly of capsids of each herpesvirus occur in the nucleus, (3) production of infectious progeny virus is accompanied by destruction of host cells, and (4) each herpesvirus is able to remain latent in its natural hosts.

Classification of the Family Herpesviridae.

The family, Herpesviridae, has been classified into 3 subfamilies based on their biological properties: the alphaherpesvirinae, the betaherpesvirinae, and the
gammaherpesvirinae. The subfamily, betaherpesvirinae, contains genera Cytomegalovirus (HCMV) and Muromegalovirus (murine cytomegalovirus). The viruses of this subfamily have a long reproductive cycle and the capacity to establish latency in cells of secretory glands with infected cells becoming very large. The subfamily, gammaherpesvirinae, contains the genera Lymphocryptovirus (EBV) and Rhadinovirus (HHV8, herpesvirus ateles and herpesvirus saimiri). The viruses of this subfamily specifically infect either T or B cells.

**SUBFAMILY ALPHAHERPESVIRINAE.**

Herpesviruses evolved over a period of 400 million years. About 180-210 million years ago, the alphaherpesvirinae subfamily separated from the other subfamilies. This subfamily contains genera Simplexvirus (HSV-1, HSV-2, Cercopithecine herpesvirus 1, bovine mammallitis virus), Varicellovirus (varicella-zoster virus (VZV), pseudorabies virus, and equine herpesvirus 1), Mardivirus (Gallid herpesvirus 2, Gallid herpesvirus 3, and Meleagrid herpesvirus 1), and Iltovirus (Gallid herpesvirus 1)(Thiry et al., 2005). In this subfamily, viruses have a variable host range, short reproductive cycle, the ability to rapidly destroy infected cells, and the capacity to establish latency primarily in sensory ganglia. The complete genomic sequences of many viruses of this subfamily are available, including HSV-1and HSV-2 in humans, simian agent 8 (SA8) in baboons, VZV in humans, simian varicella virus (SVV) in Old World monkeys, equine herpesvirus 1 and 4 (EHV-1 and EHV-4) in horses, bovine herpesvirus 1 and 5 (BHV-1 and BHV-5) in cattle, and pseudorabies virus (PRV) in pigs (Mori and Nishiyama 2005).
B virus (*Cercopithecine herpesvirus 1*), along with HSV-1 and HSV-2, belongs to the subfamily *alphaherpesvirinae*. HSV-1 was the first human herpesvirus to be discovered and is among the most intensively studied viruses. It serves as the prototype of the subfamily *alphaherpesvirinae*.

**HSV Molecular Biology**

**Genome organization.** The total HSV-1 genome length is 152260 base pairs (bp), with a base composition of 68.3% G+C (McGeoch et al., 1988). The genome is composed of two components, designated long (L) and short (S), which consist of unique sequence (UL and US) flanked by a pair of inverted repeat elements (TRL-IRL and IRS-TRS), respectively. These two components can invert relative to each other, resulting in the generation of four linear isomers of the viral genomes (Wadsworth et al., 1975). In the inverted repeats flanking the long unique sequence of the HSV-1 genome, there is a gene, designated as $\gamma_{134.5}$, which enables HSV-1 to counteract the PKR response and cause lethal infection in the mouse. Investigators have shown that a $\Delta\gamma_{134.5}$ deletion mutant ($\Delta\gamma_{134.5}$) of HSV-1 is more than 100,000 times less virulent following intracerebral inoculation (IC) of mice compared with the wild type (WT) HSV-1 (Chou et al., 1990).

**Three groups of HSV genes.** HSV genes form three groups for which expression has to be coordinately regulated in a cascade fashion. The first group of genes is called $\alpha$ genes, or immediate early genes, and can be expressed in the absence of *de novo* viral protein synthesis. These are expressed first and reach the peak rate of synthesis at approximately 2 to 4 hr post infection (Ackermann et al., 1984). There are six $\alpha$ proteins
in HSV that are ICP0, ICP4, ICP22, ICP27, ICP47, and Us1.5. All α proteins, except for ICP47, have been shown to be required for the synthesis of the subsequent protein groups. The second group of genes is called β, or early genes, for which expression is greatly enhanced in the presence of functional α proteins (Honess and Roizman 1974; Honess and Roizman 1975). Expression reaches the peak rate of synthesis at approximately 5 to 7 hr post infection (Honess and Roizman 1974; Honess and Roizman 1975). Early gene expression is not dependent on viral DNA synthesis. On the contrary, expression is enhanced rather than reduced in the presence of DNA replication inhibitors. Most β proteins are involved in viral nucleic acid metabolism. The synthesis of β proteins signals the onset of DNA replication. The third group of genes is called γ, or late genes, for which expression depends on DNA replication (Conley et al., 1981). These genes have been divided into two groups: γ1, or leaky late genes, whose expression occurs relatively early in infection and is minimally affected by the inhibition of DNA replication, and γ2, or true late genes, whose expression is totally blocked in the presence of the inhibitors of DNA replication (Conley et al., 1981).

**Replicative cycle of HSV.** During the infection, the virion attaches to cell surface receptors, an event which is followed by the fusion of the virion envelope with plasma membrane. The capsid and tegument proteins are released into the cytoplasm. The capsid is then transported to the nuclear pore, where the viral DNA is released into the nucleus. Some tegument proteins, such as viral host shutoff protein (vhs), cause degradation of mRNAs in cytoplasm. Other tegument proteins, such as VP16, are transported into nucleus to promote α gene expression. Once viral DNA is released into the nucleus, it
circularizes and is transcribed by host RNA polymerase II producing $\alpha$ mRNAs (Alwine et al., 1974). The $\alpha$ gene promoters contain the consensus sequence 5’-GyATGnTAATGArATTGnTTGnGGG-3’ in one or more copies that can act as binding sites for the host transcription factor Oct-1 (Gaffney et al., 1985). A tegument protein, VP 16 of HSV-1, was identified as a viral protein responsible for the transactivation of $\alpha$ genes (Campbell et al., 1984). Once released into the cytoplasm, VP 16 is carried into the nucleus by a cellular protein called host cell factor (HCF), and the HCF-VP 16 complex binds Oct-1, which is bound to viral DNA, forming the activator complex that promotes $\alpha$ gene expression (La Boissiere et al., 1999). Five of the six $\alpha$ proteins act to regulate viral $\beta$ gene expression in the nucleus. Once $\beta$ proteins appear, the onset of DNA replication begins. The replication of viral DNA stimulates the expression of $\gamma$ genes that function in assembling the capsid in the nucleus and modifying the membranes for virion formation. The filled capsid buds through the inner nuclear membrane to form an enveloped virion, which fuses with the outer nuclear membrane, resulting in de-envelopment of the nucleocapsid and subsequent entry into the cytoplasm. The nucleocapsid buds into the trans-Golgi network, and the re-enveloped virion is then released through the secretory pathway (Roizman B and Knipe DM, 2001).

B Virus Background and Significance.

**Discovery of B virus.** In 1932, a physician (WB), who had been bitten by an apparently healthy rhesus monkey, developed localized erythema at the site of animal bite. This apparently localized infection was followed by lethal encephalomyelitis three days later. It was reported that an ultrafilterable agent was recovered from the brain and
cord specimens of WB that was lethal to rabbits following intradermal (ID) or intracranial (IC) injection (Gay FP and Holden M, 1933). Investigators considered the virus to be a variant of herpes simplex virus and suggested the name W virus. A second group, working independently, reported in 1934 that this virus could be isolated not only from numerous neurological tissues but also from peripheral organs (spleen) and resulted in lethal diseases when inoculated by either the ID or IC routes into rabbits (Sabin AB and Wright WM, 1934). They named the agent B virus because the agent was originally isolated from the patient whose name was WB. This name was universally adopted and is used today as a common name. According to the present nomenclature, the virus is designated the *Cercopithecine herpesvirus 1*.

**B virus shares extensive homology with HSV genetically and serologically.** The genomic organization of B virus is very similar to that of HSV-1 and HSV-2: the unique long (UL) and unique short (US) segments flanked by inverted long (RL) and short (RS) repeat sequences covalently joined in four possible isomeric configurations (Harrington et al., 1992). The G+C content of B virus DNA is 74.5%, the highest among all the herpesviruses. Fifty-six and twelve genes were identified in the unique long and unique short segments, respectively, and sequence homology to proteins of HSV was observed (Ohsawa et al., 2002; Ohsawa et al., 2003). Further, B virus shares a close serological relationship with HSV. The antibodies to B virus are cross-reactive with HSV antigens (Hilliard et al., 1987).

**B virus infections in natural hosts.** B virus is indigenous to certain Asian Old World monkeys, such as rhesus (*Macaca mulatta*) and cynomolgus (*M. fascicularis*)
(Keeble et al., 1958; Keeble 1960). The percentage of seropositive rhesus monkeys in a given group ranged from 10% to 70% during the period of transport from the trapping site to a U. S. colony (Palmer 1987). Investigators reported that 72% of the sera from cynomolgus caught in the jungles in Indonesia were found to contain antibodies to B virus (Zeitlyonok et al., 1966). Because B virus usually does not cause severe disease in its natural hosts except for some immunosuppressed individuals (McClure 1970), the first description of the natural disease in rhesus monkeys did not appear until 1958 (Keeble et al., 1958). The disease was described in newly imported rhesus monkeys. Lesions were observed usually on the tongue, less frequently on the lips. Oral lesions appeared in the monkeys, first as single or multiple vesicles that ruptured in 3-4 days, producing ulcers. The ulcers were healed by granulation without scarring in 7-14 days. The lesions resembled those encountered in humans experiencing primary or recurrent HSV infection. A latent infection is usually established within sensory ganglia after primary B virus infection as demonstrated by the isolation of B virus from trigeminal ganglia in a clinically normal seropositive rhesus monkey (Boulter 1975).

**B virus infections in humans.** B virus has a unique property among α-herpesviruses: when it jumps to another primate host, such as human, it causes a lethal infection within a relatively short time (Palmer 1987; Weigler 1992). Disease in humans usually results from a monkey bite, a splash, or by injury with a needle contaminated with monkey tissues. The skin is the primary site of replication leading to erythema and chalor. Early symptoms of the human illness include fever and flu-like aches and pains. The symptoms rapidly progress to neurological signs and symptoms, including
hyperesthesias, ataxia, diplopia, agitation, and ascending, flaccid paralysis. Mortality due to B virus infection was as high as 80% in humans if anti-viral treatments were not administered at early stages of infection (Whitley, RJ and Hilliard JK, 2001).

Problem. Macaques are invaluable resources for basic research and medical investigation. Transmission of B virus is a potential risk when dealing with them, because they are natural hosts for B virus. There is a high mortality rate associated with B virus infection in human beings. During the previous 20 years, 23 cases of zoonotic infections have been observed and five of these succumbed to infection while two other cases reactivated resulting in severe morbidity. There is serious consideration of shutting down future importation of macaques, which would reduce by 50,000 a year the number of animals available for research. This would severely impact a huge number of projects, but particularly HIV/AIDS work.

PART II: VIRUS-HOST INTERACTION

HOST INNATE DEFENSES MEDIATED BY PKR AND IFN α/β

General Introduction

PKR is expressed constitutively and exists as a latent form.

According to the new nomenclature, the current name of dsRNA dependent protein kinase (PKR) is eukaryotic translation initiation factor 2-alpha kinase 2 (Eif2aK2). It is a serine/threonine kinase that plays one of the key roles in the function of type I IFN. Both the human 68 kDa and murine 65 kDa PKR proteins contain two dsRNA binding motifs (DRBMGs), that are localized at the N-terminal end of the kinase and one kinase domain
(KD) localized at the C-terminal end (Clemens and Elia 1997). The kinase domain is composed of N-terminal and C-terminal lobes. PKR is expressed constitutively at a basal level in cells, and its expression is induced several fold by treatment with type I IFN (IFN I) or viral infection. In addition, PKR normally exists as a latent form with the DRBM-II interacting with the catalytic domain, inhibiting kinase activity (Wu and Kaufman 1997). Several observations summarized by Cole (2007) support an autoinhibition model for PKR. First, deletion of the DRBM of PKR can constitutively activate this kinase capable of autophosphorylation and phosphorylation of eIF2α in the absence of dsRNA. Second, NMR studies of the DRBM indicate that resonances associated with DRBM are broadened or disappear when the catalytic domain is added, suggesting that DRBM contacts the catalytic domain.

The activation of PKR.

Although normally latent, PKR is activated after binding to its activator (Dar et al., 2005). The best-characterized activator of PKR is dsRNA that usually results from virus infection. In RNA viruses, because RNA serves as templates for both transcription and translation, production of dsRNA is an obligate part of the genomic replicative cycle. In DNA viruses, dsRNA mainly comes from transcription from opposing transcription units. Abundant dsRNA arising from symmetric transcription is present in HSV-1 infected Hep-2 cells. The dsRNA, when denatured, results in about 50 to 55% of viral DNA forming DNA-RNA hybrids (Jacquemont and Roizman 1975). As such, dsRNA acts as a molecular indicator of virus infection. When dsRNA binds to the DRBMs of PKR, binding of two PKR molecules to the same dsRNA leads to the formation of stable dimers
and dissociation of the inhibitory interaction between DRBM-II and the kinase domain. Formation of PKR dimers also facilitates trans-autophosphorylation of multiple sites by the two kinase moieties in a PKR dimer (Huang et al., 2002). The importance of homodimerization in PKR activation has been emphasized by biochemical and genetic data. Replacement of the DRBM of PKR with an unrelated domain that is able to dimerize constitutively activates PKR (Ung et al., 2001).

Besides dsRNA, type I or type II IFN induces tyrosine (Tyr) phosphorylation of PKR (Su et al., 2007). PKR physically interacts with either JAK1 or TYK2 and acts as a substrate of activated JAKs in IFN-treated cells. In addition, the lack of JAK1 or TYK2 in cells results in a lack of induction of PKR phosphorylation and impaired induction of eIF2α phosphorylation and inhibition of protein synthesis by IFN. Therefore, type I or type II IFN induces Tyr phosphorylation of PKR through activated JAK1 or TYK2.

A P53-associated cellular protein-testes derived (PACT, also known as P2P-R, RBBP6) also can activate PKR in a dsRNA independent manner in response to diverse stress signals. Exposure of cells to stress signals, e.g., serum starvation, has been demonstrated to result in rapid phosphorylation of endogenous PACT, followed by enhanced association of PACT with PKR, which, in turn, leads to conformational change and activation of PKR (Patel et al., 2000; Huang et al., 2002). PACT, or its mouse orthologue RAX, acts as a physiological mediator that links a wide range of different cell stresses to PKR. After activation, PKR is involved in antiviral activities, regulation of cell proliferation, and apoptosis by phosphorylation of a variety of substrates, including the α
subunit of eukaryotic translation initiation factor 2 (eIF2α), inhibitor of NF-κB (I-κB), IRF-1 (Samuel 1993; Uetani et al., 2000), and P53 (Cuddihy et al., 1999).

**Antiviral activities of PKR mediated by phosphorylation of eIF2α.**

When PKR is activated, the phosphorylation on Thr446 promotes substrate recognition and phosphorylation (Garcia et al., 2006). The α subunit of eIF2 is the best-studied substrate of PKR. The αG helix of PKR in the surface of the C lobe of KD interacts initially with eIF2α to promote a conformational change in this factor that brings the S51 residue close to the PKR phoso-acceptor site for catalysis. This change in eIF2α involves local unfolding of S51, which results in full accessibility of this residue to the catalytic cleft of PKR and subsequent phosphorylation of eIF2α on Ser-51 (Dar et al., 2005), an event which leads to a general protein synthesis shutoff in infected cells (Colthurst et al., 1987).

Cellular eIF2 is a heterotrimeric G protein, which plays a significant role in the initiation of translation as reviewed recently. The subunits of eIF2 have different functions. The α subunit performs a regulatory function, the β subunit binds the initiator tRNA (tRNAi), and the γ subunit binds guanine nucleotides (GTP). The binding of GTP and tRNAi to eIF2 results in the formation of a ternary complex, which subsequently associates with 40S ribosomal subunits. With the help of other initiation factors, this 40S-eIF2 complex is recruited to the 5’ cap of mRNA. This complex moves forward (5’ to 3’) along mRNA and searches for the first start codon. Translation begins from the first start codon encountered by the small ribosome complex, and translation initiation factors dissociate from the small ribosomal subunit to enable the large ribosomal subunit to
assemble with it, completing the formation of the ribosome. Cellular eIF2 dissociates from the ribosome after hydrolysis of GTP and changes into an inactive GDP-bound form. The inactivate eIF2 is recycled into its active GTP-bound form by the action of guanine nucleotide exchange factor eIF2B. Phosphorylated eIF2α has a greater affinity for the eIF2B guanine nucleotide exchanger than does the nonphosphorylated eIF2α isoform. This increased affinity impedes the eIF2B guanine nucleotide exchanger function that recycles GDP to GTP on eIF2 and results in the accumulation of inactive GDP-bound eIF2. In addition, because eIF2B is present in limiting amounts, small changes in levels of phosphorylated eIF2α will have a large impact on the rates of protein synthesis by sequestering most of the available eIF2B (Mohr 2004). With the accumulation of inactive GDP-bound eIF2, protein synthesis in cells is globally blocked.

Viruses completely depend on host translation machinery and need continual protein synthesis to produce virus progeny. Once global protein synthesis is shut off, virus replication is inhibited. This cell function has been selected and conserved as a way of sacrificing a single infected cell for the benefit of the whole population.

**Antiviral activities of PKR mediated by induction of apoptosis.**

Apoptosis is the process used by cells, when no longer needed, to commit suicide by activating an intracellular death program. One mechanism of apoptosis, the extrinsic pathway, is initiated through ligation of cell surface death receptors such as Fas. This event leads to the recruitment and activation of an adaptor protein, Fas-associated death domain containing protein (FADD), and to the subsequent activation of caspase cascades, including caspase 8 (Chinnaiyan et al., 1995). The other mechanism is called the intrinsic
pathway. Mitochondria play a key role in the activation of this pathway. Cytochrome c released from mitochondria binds adaptor protein, Apaf-1, followed by the activation of caspase cascades, including caspase 9 (Li et al., 1997).

PKR induces apoptosis in response to different stress signals, e.g., dsRNA, tumor necrosis factor-α (TNF-α), or lipopolysaccharide (LPS) (Der et al., 1997). Overexpression of PKR in HeLa cells resulted in rapid cell death characteristic of apoptosis. Rapid cell death was not observed when the mutant PKR (Lys296→arg) was overexpressed in HeLa cells (Lee and Esteban 1994), suggesting observed cell death is the result of PKR expression and activation.

The mechanism by which PKR expression activates apoptosis is still not clear. PKR-mediated apoptosis seems to be associated with the phosphorylation of eIF2α on Ser-51. Expression of a Ser51Ala mutant of eIF2α results in the protection of cells from serum deprivation or TNF-α induced apoptosis (Srivastava et al., 1998). This expression also protected PKR-overexpressing HeLa cells from undergoing apoptosis (Gil et al., 1999). In addition, expression of a Ser51Asp mutant eIF2α, mimicking phosphorylated eIF2α, was sufficient to induce apoptosis in HeLa cells (Srivastava et al., 1998). Because phosphorylated eIF2α leads to global protein synthesis shut-off in host cells, one hypothesis is that translation inhibition depletes the cell of short lived proteins that provide anti-apoptotic functions, e.g., apoptosis inhibitors (Deveraux and Reed 1999). In addition to causing translation inhibition, phosphorylated eIF2α can induce the expression of growth arrest and DNA damage-inducible (Gadd34) genes (Novoa et al., 2001). The carboxyl domain of Gadd34 protein can complex with protein phosphatase 1 (PP1) and
dephosphorylate eIF-2α, enabling protein synthesis to recover (Kojima et al., 2003). The amino terminal domain, in particular, and the overall function of this protein up-regulate ATF4 and CHOP gene expression and are involved in cell growth suppression and apoptosis (Hollander et al., 2003). In addition to induction of apoptosis by phosphorylation of eIF-2α, PKR was also shown to induce Fas expression and activate FADD, critical steps in the extrinsic apoptosis pathway (Balachandran et al., 2000).

PKR plays a pivotal role in antiviral activities by inducing apoptosis in response to viral infection. Activation of apoptosis by host cells in response to virus infection precludes further activity of the virus, whose growth is totally dependent on the host cells. Treating murine fibroblasts or PKR-overexpressing cells with type I IFN greatly enhanced influenza A-induced apoptosis and reduced virus replication. In contrast, cells expressing a dominant-negative variant of PKR were completely resistant to virus-induced apoptosis (Balachandran et al., 2000). Therefore, activated cellular PKR and phosphorylated eIF-2α limit viral replication not only through inhibiting protein synthesis, but also through inducing apoptosis.

**Antiviral activities of PKR mediated by phosphorylation of I-κB subunits and IRF-1 activation.**

Nuclear factor kappa B (NF-κB) is a dimeric transcription factor that plays an essential role in regulating a variety of genes. In unstimulated cells, NF-κB is found localized in the cytoplasm as an inactive, heterodimeric complex bound to the inhibitor of κB (I-κB). I-κB sequesters NF-κB in the cytosol by masking its nuclear localization signal and the DNA binding domain (Whiteside and Israel 1997). A large I-κB kinase (IKK) complex can
stimulate the phosphorylation of I-κB subunits on two critical serine residues and initiate polyubiquitination and subsequent degradation of I-κB by the 26S proteasome, resulting in the activation of NF-κB (Chen et al., 1995) (Zamanian-Daryoush et al., 2000). The IKK complex was shown to be physically associated with PKR by coimmunoprecipitation and its activity was dependent on PKR, as demonstrated using PKR null cell lines, in which double-stranded RNA failed to stimulate IKK activity and subsequent release of NF-κB from I-κB complexes compared to cells from an isogenic background wild type for PKR (Zamanian-Daryoush et al., 2000).

Activated PKR not only stimulates de novo synthesis of IRF-1, in addition to phosphorylation of I-κB, but also binds and phosphorylates IRF-1, events which are followed by IRF-1 translocation into the nucleus (De Logu et al., 1998; Uetani et al., 2000). With activation of these transcription factors, NF-κB and phosphorylated IRF-1, certain antiviral genes, e.g., IFN-β and nitric oxide synthase 2 (NOS2) are transcribed (Kirchhoff et al., 1995). In one such example, it was shown that treatment of human airway epithelial cells with synthetic dsRNA resulted in rapid activation of PKR and subsequent activation of signaling components including NF-κB and IRF-1, followed by NOS2 gene expression. In PKR null cells, NOS2 gene expression was markedly diminished and NF-κB and IRF-1 activation was impaired in response to dsRNA (Uetani et al., 2000). Nitric oxide (NO) produced by NOS2 has potent antiviral activity against a number of viruses. The exact antiviral mechanism of NO is still not clear, although inhibition of ribonucleotide reductase and deamination of viral DNA may be important mechanisms (Karupiah et al., 1993).
Regulation of cell differentiation and proliferation by PKR.

PKR regulates cell differentiation to different lineages. PKR, constitutively expressed in epidermal and epithelial keratinocytes in the skin, is necessary for keratinocyte differentiation. Loss of PKR in these cells induces increased cell proliferation and altered keratinocyte differentiation (Kuyama et al., 2003). In addition, an influence of PKR on the immune system is indicated by controlling the growth of mature T lymphocytes (Kadereit et al., 2000).

The role of PKR in controlling the cell proliferation is indicated by the dramatic effects caused by overexpression of different PKR inhibitors. Almost all PKR inhibitors studied have been shown to cause oncogenic transformation. The NS5A protein of HCV can bind to PKR, inhibit PKR function, and has oncogenic potential (Gale et al., 1999). The E3L protein of VV inhibits PKR and transforms NIH 3T3-E3L cell.

PKR could regulate functions of some tumor suppressors. IRF1 acts as a tumor suppressor in mouse models and is deleted in leukemias (Willman et al., 1993). PKR is suggested to mediate the activities of IRF-1 (Uetani et al., 2000). PKR also was shown to regulate the function of P53 that can sense genotoxic stress. Activation of P53 results in cell cycle arrest or apoptosis. Studies showed that PKR could interact directly with the C-terminal part of P53 and phosphorylate it on the Ser392 residue (Cuddihy et al., 1999). In addition, PKR can influence apoptosis in U937 cells induced by TNF-α, correlating the ability of PKR to induce P53 (Yeung et al., 1996). The function of P53 was impaired in PKR−/− MEF.
General Introduction of IFN α/β.

The interferon (IFN) α/β system, a critical innate antiviral mechanism, involves three major elements. A virus infection can induce IFN-β gene expression by activating the IFN-β gene promoter. This promoter contains positive regulatory domains for the binding of specific transcription factors, e.g., ATF-2/c-Jun, NF-κB, and members of the interferon regulatory factor family, e.g., interferon regulatory factor 3 (IRF-3). When these transcription factors are activated by virus infection, IFN-β gene will be expressed and IFN-β will be secreted. Once secreted, interferon α/β will bind to the receptor, IFNAR, on infected and neighboring uninfected cells. Binding of interferon α/β to IFNAR results in the activation of the kinases JAK1 and TYK2 that are associated with the cytoplasmic tails of the two subunits of IFNAR. Once activated, the JAK1 and TYK2 kinases cause phosphorylation of downstream substrates STAT1 and STAT2. Then STAT1 and STAT2 form heterodimers and associate with IRF-9, forming the STAT1/STAT2/IRF-9 complex, also known as interferon-stimulated gene factor 3, or ISGF-3. This complex translocates into the nucleus and binds to the specific DNA sequence containing the common motif: interferon-stimulated regulatory element, or ISRE. Hundreds of genes with a promoter that contains an ISRE will then be transcribed. The best-characterized IFN-induced antiviral pathways utilize PKR and the 2'-5’OAS system (Stark et al., 1998).

VIRUS EVASION MECHANISMS FROM HOST INNATE DEFENSES MEDIATED BY PKR.
Viruses have evolved diverse mechanisms to prevent PKR activation and maintain low levels of phosphorylated eIF2α, ensuring their replication success. These mechanisms include (i) synthesis of proteins that bind and sequester dsRNA to avoid activating PKR; (ii) synthesis of proteins or RNAs that bind PKR and prevent its activation; (iii) inhibition of PKR expression or induction of PKR degradation; and (iv) activation of phosphatases that dephosphorylate eIF2α and PKR. For example, adenoviruses encode VA RNAI, a small abundant highly-structured RNA, which competitively binds the dsRNA-binding domain of PKR and prevents its activation (Schneider and Mohr 2003). Because only one end of VA RNAI interacts with PKR in the dsRNA-binding site and blocks PKR dimerization and activation by viral dsRNA. Alternatively, some viruses encode proteins that can bind and sequester dsRNA to prevent PKR activation. Proteins, e.g., HSV-1 Us11 (Mulvey et al., 1999), vaccinia virus E3L (Beattie et al., 1995), reovirus σ3 (Lloyd and Shatkin 1992), and influenza virus NS1 (Salvatore et al., 2002) bind dsRNA, prevent PKR activation, and maintain normal protein synthesis rates during virus infection. In addition to binding dsRNA, some viral proteins, e.g., EBV SM (Poppers et al., 2003), HSV-1 Us11 (Cassady and Gross 2002), and vaccinia virus E3L (Romano et al., 1998) can physically interact with PKR to prevent activation. Moreover, some viruses have evolved mechanisms that prevent eIF2α phosphorylation. HSV-1 encodes γ134.5 gene, a gene product which complexes with protein phosphatase 1 α (PP1α) and dephosphorylates eIF2α (He et al., 1997). In addition, vaccinia virus encodes K3L protein, which is structurally similar to eIF2α, but lacks the critical phospho-acceptor, serine residue and provides a pseudo-substrate of PKR preventing eIF2α activation (Carroll et al., 1993).
B VIRUS ENCODES SPECIFIC PROTEIN(S) THAT CAN INHIBIT CELLULAR PKR ACTIVITY.

We hypothesize that B virus encodes protein(s) that can circumvent the antiviral response mediated by PKR based on the fact that B virus replicates efficiently in neuronal cells. If a virus lacks a protein that can counteract PKR activity, it will fail to replicate efficiently in neuronal cells as a result of the activation of the host cellular protein synthesis shutoff. The γ134.5 deletion mutant of HSV-1 is one example. It cannot replicate in human neuronal cells or human neuroglial cells because host cellular protein synthesis shutoff is mediated by PKR activated by virus infection (He et al., 1996; Mulvey et al., 2003). B virus can replicate efficiently in human neuronal cells and human neuroglial cells (Patrusheva, et al., unpublished data), which suggests that B virus contains gene(s) that encode proteins that counteract the antiviral activity of cellular PKR, as does wild-type HSV-1. Discovery of the identity of the B virus gene product(s) that can circumvent the effects of PKR activity was the goal of the investigations described here.

HSV-1 GENE PRODUCTS THAT COUNTERACT CELLULAR PKR ACTIVITY.

The γ134.5 Gene Encodes a Product That Can Inhibit the Action of PKR.

The γ134.5 gene, present in two copies per genome, has been shown to be located in the inverted repeats flanking the unique long component of viral DNA. The terminal 500-bp a sequence serves as its promoter while the adjacent b sequences serve as the coding sequences (Chou and Roizman 1986). This gene encodes a 263-amino acid protein that consists of three domains, an amino-terminal domain of ~160 amino acids,
10 repeats of three amino acids (Ala-Thr-Pro)\textsubscript{10}, and a 73-amino acid carboxyl-terminal domain (Chou and Roizman 1990). It has been shown that a $\gamma_134.5$ deletion mutant ($\Delta\gamma_134.5$) of HSV-1 was more than 100,000 times less virulent upon intracerebral inoculation of mice compared with the wild type (WT) HSV-1 (Chou et al., 1990). In addition, a significant amount of virus can be recovered from the brains of animals inoculated with WT viruses, but not with $\Delta\gamma_134.5$ viruses, suggesting that $\Delta\gamma_134.5$ of HSV-1 replicates inefficiently in central nervous system.

The reason why $\Delta\gamma_134.5$ of HSV-1 replicates inefficiently in the central nervous system remains unknown initially. Later studies showed that there is a premature protein synthesis shutoff in neuroblastoma cells infected with $\Delta\gamma_134.5$ of HSV-1, suggesting one of the functions of the HSV-1 $\gamma_134.5$ gene is to preclude host protein synthesis shutoff in order to enable the protein synthesis necessary for viral replication (Chou and Roizman 1992). The $\gamma_134.5$ gene function of preventing protein synthesis shutoff was mapped to the carboxyl-terminal domain which was found to be homologous to a corresponding stretch of amino acids of the carboxyl terminus of a murine protein known as MyD116 (Lord et al., 1990) and a Chinese hamster protein known as GADD34 (Fornace et al., 1989). The precise functions of MyD116 and GADD34 genes are not completely known, however, it is thought that each can suppress cell division after DNA damage resulting in cell death (Zhan et al., 1994). It has been shown that the carboxyl terminus of the MyD116 gene substitutes for the corresponding domain of the $\gamma_134.5$ gene of HSV-1 to
preclude the premature shutoff of total protein synthesis in infected human cells (He et al., 1996).

Chou et al. (1992) demonstrated that in Δγ134.5 HSV-1 infected neuroblastoma cells, the onset of DNA synthesis triggers late viral mRNA accumulation. Double-stranded RNA generated by opposing transcripts of HSV-1 late genes activates PKR, which subsequently phosphorylates eIF2α, causing premature protein synthesis shutoff. Wild type HSV-1 encoding γ134.5 precludes this premature protein synthesis shutoff.

Chou et al. (1995) found that eIF2α was only phosphorylated in the cells infected with Δγ134.5 HSV-1, suggesting the product of γ134.5 gene prevents phosphorylation of eIF2α. Experiments designed to investigate the mechanism by which this occurs revealed that in vitro the carboxyl terminus of γ134.5 protein binds protein phosphatase 1 α (PP1). Additionally, these studies revealed that the phosphatase activity specific for phosphorylated eIF2α was abundant in WT HSV-1-infected cells and significantly decreased in Δγ134.5 of HSV-1-infected cells. These data suggested that the product of γ134.5 gene associated with cellular PP1α and direct this phosphatase activity specific for phosphorylated eIF2α-P (He et al., 1997).

Us11 Inhibits the Action of PKR When Expressed as an Early Gene

The HSV-1 Us11, a true late gene, encodes a 21-kDa phosphoprotein, one of the abundant tegument proteins expressed at late times post-infection (Johnson et al., 1986; Roller and Roizman 1990; Roller and Roizman 1992). The gene product of Us11 which
performs multiple functions in infected cells can bind dsRNA and highly structured RNA both in sequence-specific and conformation-specific manners, and has been shown to be involved in post-transcriptional regulation of gene expression, nucleo-cytoplasmic and intercellular transport of specific mRNAs, and packaging of specific mRNAs into HSV-1 particles (Roller and Roizman 1990; Roller and Roizman 1991; Diaz et al., 1996; Roller et al., 1996; Schaerer-Uthurralt et al., 1998; Koshizuka et al., 2001; Khoo et al., 2002; Sciortino et al., 2002). Interestingly, Us11 was shown to effectively substitute for the missing ICP34.5 function in HSV-1 γ134.5 null mutants, providing the ability to sustain near wild-type translation rates when expressed at early time points of infection as a result of mutation (He et al., 1997; Cassady et al., 1998). It has been shown that Us11 protein prevents PKR activation by binding and sequestering dsRNA. The RNA-binding activity was mapped to the C-terminal domain consisting of 24 RXP repeats (Rixon and McGeoch 1984; Roller et al., 1996). In addition, Us11 protein was shown to bind to a number of cellular proteins and modulate their activities. Us11 was shown to interact with PKR physically preventing PKR activation. By forming a stable complex with inactive PKR and/or sequestering dsRNA, Us11 can prevent PKR activation by dsRNA and PACT and, consequently, block the accumulation of phosphorylated eIF-2α and translation inhibition in infected cells (Cassady and Gross 2002; Peters et al., 2002). PKR-binding activity was also mapped to the C-terminal half of Us11 (Peters et al., 2002). The function of Us11 in its natural WT context as a true late or γ2 gene remains unknown at present. Cassady et al. (1998) suggested that Us11, produced early in infection binds to PKR and precludes the phosphorylation of PKR and subsequent eIF-2α phosphorylation, whereas Us11 driven by
its natural promoter and expressed late in infection is ineffective in this regard. Mulvey et al. (2004) thought that both the Us11 and \( \gamma_{134.5} \) genes are required for HSV-1 to inhibit the action of PKR. The Us11 expressed as a true late protein in the natural context of HSV-1 was thought to be critical for maintaining proper late cell translation rates, whereas \( \gamma_{134.5} \) was thought to play an essential role in maintaining proper early cell translation rates (Mulvey et al., 2004). In \( \Delta \gamma_{134.5} \) HSV-1 infected cells, there were no late proteins translated although late mRNA accumulates from the onset of DNA synthesis or a closely related event, late mRNA accumulation, triggers protein synthesis shutoff. Therefore, \( \gamma_{134.5} \) mutants are not only deficient in \( \gamma_{134.5} \) functions, but fail to synthesize the gene products specified by viral \( \gamma_{2} \) genes, one of which is Us11, which prevents PKR activation. The phenotype of \( \gamma_{134.5} \) mutants of HSV-1 results from deficiency of both \( \gamma_{134.5} \) and Us11 genes. The function of HSV-1 Us11, a true late protein, remains unknown.

**GOALS OF DISSERTATION**

The observation that B virus replicated efficiently in neuroglial cells suggested that B virus contains genes that can inhibit the action of PKR. In addition, B virus shares extensive homology with HSV-1 genetically, and with the knowledge that the \( \gamma_{134.5} \) gene product of HSV-1 plays a major role in counteracting PKR activity, it was reasonable to hypothesize that B virus encoded a \( \gamma_{134.5} \) gene homolog of HSV-1. In these dissertation studies, the sequence of the termini of B virus genome was completed to identify the \( \gamma_{134.5} \) gene homolog of HSV-1. The sequencing results revealed that the long terminal repeats of B virus genome lacked the \( \gamma_{134.5} \) gene homolog, prompting an
investigation to determine whether B virus counteracted PKR activity preventing subsequent eIF2α phosphorylation in the absence of γ134.5 gene homolog. The investigation revealed that although B virus lacked the γ134.5 gene homolog of HSV-1, it retained the capacity to prevent PKR directed eIF2α phosphorylation, suggesting that B virus utilizes a different mechanism, or sequence of mechanisms, to counteract the action of PKR when compared with HSV-1.

Although the B virus genome lacks a γ134.5 gene homolog, it contains all other homologs of HSV-1, including HSV-1 Us11 (Ohsawa et al., 2002; Perelygina et al., 2003). B virus Us11 polypeptide contains 20 copies of the RXP repeats in its carboxyl-terminal domain, whereas the carboxyl-terminal domain of HSV-1 Us11 protein contains 24 copies of the RXP repeats which are critical for its functionality. The N-terminal domain of B virus Us11 protein shares little homology to the corresponding part of the HSV-1 homolog (Figure 1). Although no information on B virus Us11 protein expression or function was available, sequence conservation of the C-terminal domain suggested that Us11 functions mapping to this domain were also conserved in B virus. We hypothesized that B virus Us11 protein was responsible for the inhibition of PKR. It was reported that PKR activation was triggered by viral DNA replication, usually 4 to 5 h post infection (Chou and Roizman 1992). Therefore, PKR activation occurs early prior to the late gene synthesis. Furthermore, it was demonstrated that HSV-1 Us11 protein, only if present prior to the activation of PKR, can prevent PKR activation efficiently (Cassady et al., 1998). These observations taken together, suggest that B virus Us11 gene should be expressed at early times of infection if it plays a role in blocking PKR. Therefore,
whether Us11 is expressed at early times of infection or whether it is a true late gene of B virus was investigated as part of the study described in this dissertation. This goal was accomplished by temporal analysis of Us11 expression in B virus infected cells in the presence and absence of the DNA replication inhibitor phosphonoacetic acid, PAA.
Figure 1. Comparative analysis of Us11 protein amino acid sequence of B virus and HSV-1. Red boxes highlight the identical amino acids between the two proteins. The blue line marks the C-terminal domain, which contains 24 RXP repeats in the carboxyl domain of the HSV-1 Us11 protein and 20 RXP repeats in that of B virus Us11 protein.
The subsequent goal of this project was to test whether a function of B virus Us11 protein is to prevent PKR activation. Expressing and purifying the Us11 fusion protein in a bacterial system accomplished this goal, and subsequently testing whether the Us11 fusion protein bound to PKR to prevent PKR activation \textit{in vitro}. In addition, neuroglial cells expressing B virus Us11 (U373-BVUs11) were prepared to determine whether Us11 prevented PKR activation and subsequent eIF2\(\alpha\) phosphorylation when these cells were infected with \(\Delta\gamma_{134.5}\) HSV-1, by compensating the missing function of the \(\gamma_{134.5}\) gene product, and restoring the replication abilities of \(\Delta\gamma_{134.5}\) HSV-1.

PKR has been shown as one of the key players in the function of type I interferon (Langland et al., 2006). The final goal of this dissertation was to test whether B virus Us11 protein played a role in counteracting the innate immune responses mediated by type I IFN by preventing PKR phosphorylation. This goal was accomplished by pre-treating U373-pcDNA or U373-BVUs11 cells with IFN-\(\alpha\), infecting these cells with HSV-1, and analyzing the replication abilities of HSV-1 in these cells. In addition, the phosphorylation status of PKR in these infected neuroglial cells was also analyzed.
SECTION II. MATERIALS AND METHODS

Cells, viruses, and media. *Escherichia coli* NovaBlue (Novagen, Madison, WI) cells were used for plasmid propagation. *E. coli* BL21 (DE3) pLysS (Novagen) cells were used for expression of the fusion protein. Vero (ATCC CCL-81), HeLa (ATCC#CCL-2), and LLC-MK2 (ATCC CCL 7.1) cells were cultured in a Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen) and antibiotic solution (Invitrogen). U373 cells (a kind gift from Dr. Ian Mohr, New York University School of Medicine, New York, NY) were propagated in DMEM supplemented with 5% FBS, 5% calf serum, 50 U of penicillin/ml, and 50 μg of streptomycin/ml. HSV-1 strain KOS (ATCC VR-1493), HSV-2 strain 186 (a kind gift from Dr. R. Courtney, The Pennsylvania State University College of Medicine, Hershey, PA), HSV-1 strain Patton, γ134.5 deletion mutant (Δγ134.5) of HSV-1 (kind gifts from Dr. Ian Mohr, New York University School of Medicine, New York, NY), B virus laboratory strain E2490 (a kind gift from late Dr. R. N. Hull) and B virus clinical isolates were propagated and titrated in Vero cells maintained in DMEM supplemented with 2% FBS. B virus clinical strains, including MR7 from a rhesus macaque (*Macaca mulatta*), and two human isolates, A4 and A5, were provided by the National B Virus Resource Laboratory (Atlanta, GA).

Cloning of B virus genomic DNA termini. The termini of B virus E2490 genomic DNA were blunt-ended by using a Single dA™ tailing kit (Novagen) as suggested by the manufacturer. Briefly, the flushing reaction containing 10 μl of B virus genomic DNA (0.5 μg), 2.5 μl of 10X Flush buffer, 2.5 μl of 10X dNTP mix, 1.25 μl of
100 mM DTT, 1 μl of T4 DNA polymerase (4 units), and 7.75 μl of deionized water was incubated at 11°C for 20 min. The reaction was stopped by heating at 75°C for 10 min. The blunt-ended DNA was digested overnight with restriction enzyme SphI, purified on a Qiaquick spin column (Qiagen, Chatsworth, CA), and ligated into Smal- and SphI-digested vector pUC19 with Fast-Link™ DNA Ligation kits (Epicentre Technologies, Madison, WI). *E. coli* NovaBlue (Novagen) competent cells were transformed with the ligation mixture. Plasmid DNA was extracted and subjected to sequencing.

**Southern blots.** Ten μg of B virus genomic DNA was digested overnight at 37°C with 20 units of SphI (New England Biolabs, Beverly, MA). Digested genomic DNA was ethanol precipitated, dissolved in TE, and electrophoresed on a 0.8% agarose gel with 1X TBE running buffer overnight at 30 V. The DNA was transferred onto a nylon membrane (Roche, Indianapolis, IN) by alkali blotting, according to the instructions supplied with the membranes. B virus genomic termini plasmid DNA (1 μg) was labeled with digoxigenin using a Highprime labeling kit (Roche) according to the instruction of the manufacturer. After prehybridization in 30 ml of Dig Easy Hyb (Roche) for 8 h at 42°C, the membrane was hybridized with probe overnight at 42°C in 30 ml of Dig Easy Hyb. Blots were washed once with 2XSSC containing 0.1% SDS for 15 min at RT, two times with 1XSSC containing 0.1% SDS for 15 min at 65°C and once with 0.1XSSC containing 0.1% SDS for 15 min at 65°C. Washed blots were detected with an ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the instructions provided by the manufacturer.
Sequencing of B virus genomic DNA termini and the Us11 gene in clinical isolates. DNA sequencing was performed using BigDye Terminator (PE Applied Biosystems, Foster city, CA) sequencing chemistry on an automatic DNA sequencer ABI 377 (Applied Biosystems). Sequencing reactions were performed in the presence of Sequence Enhancer A (Gibco, Rockville, Maryland) to resolve sequence compressions due to the high G+C content of templates. A 3:1 mixture of regular and dGTP BigDye sequencing kits (Applied Biosystems) was used in order to overcome premature termination of sequencing reactions in G-rich regions, since B virus DNA is >75% G:C rich. To sequence Us11 genes in different clinical isolates, DNA fragments containing the Us11 gene were amplified from genomic DNA of multiple clinical B virus isolates by using the forward primer 5’-AGCCCTCGACCCGTGAGGC and the reverse primer 5’-AGCTTGCCGCCTCTGCGCG. PCR products were purified using QIAquick Gel Extraction Kit (Qiagen). The amplified DNA fragments were sequenced using each PCR primer. The Us11 sequences were deposited into the GenBank. DNAStar SeqMan program (DNAStar Inc.), which was used to assemble the complete genome sequence from the sequences of the overlapping plasmid clones.

DNA sequence analysis. Identification of open reading frames (ORFs), repeats, and DNA regulatory sequences was performed using DNAStar suite of programs. Genbank database searches were carried out with BlastN, BlastP, and BlastX on default settings. Multiple sequence alignments between B virus, HSV-1 and HSV-2 Us11 genes were performed using DNASTAR MegAlign program (version 4.0.3). Protein analyses were performed using DNASTAR Protean program (version 4.0.3), and the NetPhos 2.0
server. To prove that a homolog of the HSV neurovirulence factor, $\gamma_{134.5}$, was absent in the B virus genome, BlastP and PSI-Blast searches, using the ICP34.5 protein as the query were used against the entire B virus genome translated in six frames. In addition, a Hidden Markov Model profile was created from the conservative domain of the ICP34.5 protein and used to scan the translated B virus genome.

**Us11 expression constructs.** To generate a B virus Us11 prokaryotic expression construct, the Us11 ORF was amplified from genomic DNA of B virus E2490 with the forward primer Us11-2 5’-CTAATGGCGTCAACAACCG-3’ and the reverse primer Us11-R1 5’-ACCCCGGGGGTCGTGTGGC-3’. PCR reaction mixtures (50 μl) containing DMSO (5%), a pair of primers (200 pmols of each primer), and B virus DNA (10 ng) were amplified with HotStarTaq™ DNA polymerase (1.25 U) as described by the manufacturer (Qiagen). The thermal cycling parameters were as follows: 15 min at 95°C, 35 two-step cycles of 20s at 95°C and 20s at 65°C, and 4-min incubation at 72°C. A gel-purified 0.44 kb PCR product was cloned into a pBAD-TOPO vector (Invitrogen) in frame with V5 epitope and histidine tags, resulting in Us11-pBAD plasmid. The second prokaryotic expression construct was developed by subcloning a NcoI-Pmel fragment containing the Us11-V5-histidine module from Us11-pBAD into the NcoI and Pmel sites of a pScreenT vector (Novagen) in frame with the T7 gene 10. The resulting plasmid was designated Us11-pScreenT.

To generate eukaryotic expression constructs of B virus Us11, the Us11 ORF was amplified from each genomic DNA of B virus lab strain E2490 and clinical strain MR7 with the forward primer Us11-F-Hind 5’-AAGCTTGCGCATGCTAATGGCGTCA-3’
and the reverse primer Us11R-Xba 5’-TCTAGAACCCGGGGTCTGTTGGC-3’. The PCR products were cloned into a PCR-XL-TOPO vector (Invitrogen) respectively, resulting in E2490Us11-TOPO and MR7Us11-TOPO plasmids. The HindIII-XbaI fragments containing the Us11 ORF from E2490Us11-TOPO and MR7Us11-TOPO plasmids were subsequently cloned into the HindIII and XbaI sites of a pcDNA3.1A vector. The resulting plasmids were designated as E2490Us11-pcDNA and MR7Us11-pcDNA, respectively.

Eukaryotic expression constructs of HSV-1 Us11 were generated similarly. Briefly, the Us11 ORF was amplified from genomic DNA of HSV-1 strain Patton with the forward primer HUs11-F-Hind 5’-AAG CTTATGAGCCAGACCCAACCCC-3’ and the reverse primer HUs11R-Xba 5’-TCTAGATACAGACCCGCGAGCCGTA-3’. The PCR products were cloned into a PCR-XL-TOPO vector (Invitrogen) initially. Then the HindIII-XbaI fragment containing HSV-1 Us11 ORF was subcloned into the HindIII and XbaI sites of a pcDNA3.1A vector. The resulting plasmids were designated as HSVUs11-pcDNA. All constructs were verified by DNA sequencing.

**Expression and purification of the Us11 fusion protein.** Fresh overnight cultures of *E. coli* BL21 (DE3) pLysS cells harboring plasmid Us11-pScreenT, which contained the Us11 structural gene fused to the 3’ of the sequence encoding the T7 gene 10, were diluted 25-fold into fresh LB medium supplemented with 50 μg/ml ampicillin, and the cultures were grown at 37°C with vigorous shaking until an optical density of 0.6 at A600 was reached. Next, IPTG was added to a final concentration of 0.4 mM to induce the synthesis of T7 gene 10-Us11 fusion protein and the cultures were allowed to grow for
another 4 hrs at 37°C with vigorous shaking. Cell pellets were frozen at -80°C. Thawed pellets (from 250 ml culture) were resuspended in 20 ml PBS buffer, and cells were broken by passing through a French Pressure Cell twice (1000psi). Crude extracts were centrifuged at 18000 rpm for 30 min at 4°C. Synthesized T7 gene 10-Us11 fusion protein formed inclusion bodies that were located in the pellets. To purify the inclusion bodies in the pellet, the pellet was resuspended in 15 ml of B-PER reagent (Pierce, Rockford, IL), and lysozyme was added into the suspension mixture to a final concentration of 200 μg/ml. After incubation at room temperature for 5 min, 100 ml of 1:10 diluted B-PER reagent was added and mixed by vortexing. The inclusion bodies were collected by centrifugation at 27000g for 15 min and resuspended in 100 ml of 1:10 diluted B-PER reagent. Centrifugation and resuspension with 100 ml of 1:10 diluted B-PER reagent were repeated 2 more times. Purified inclusion bodies were solubilized in extraction buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol, and 6 M urea and loaded onto a 1-ml HiTrap heparin affinity column (Amersham) equilibrated with 10 ml of binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10% glycerol). The column was washed with 10 ml of binding buffer and then with the 10 ml of binding buffer containing 500 mM NaCl. Fusion protein was eluted with binding buffer supplemented with 800 mM NaCl.

**Antisera and antibodies.** Polyclonal Us11-specific antiserum was induced in rabbits by immunization with B virus Us11 fusion protein using a protocol described earlier (Perelygina et al., 2005). Briefly, approximately 200 μg of B virus Us11 fusion protein in Freund’s complete adjuvant was injected into multiple sites of the skin of White New
Zealand female rabbit to induce polyclonal Us11 antibody. To boost, 200 μg of Us11 fusion protein in Freund’s incomplete adjuvant was injected every 4 weeks. Pre-immune sera were collected at the time of the first immunization. Test bleeds were carried out on the fourteenth day after each injection. Polyclonal gC-specific antiserum was previously generated (Perelygina et al., 2005). Anti-V5 Mab was purchased from Sigma. Rabbit polyclonal anti-PKR, anti-phospho-PKR, anti-eIF2α, and anti-phospho-eIF2α antibodies were supplied by Cell Signaling (Danvers, MA). The anti-phospho-PKR antibody recognizes the phosphorylation site of PKR on threonine (Thr) at position 446. Two anti-PKR antibodies, (#2766) and (#3072), were provided by the company. The anti-PKR antibody (#2766) was designed to recognize the N-terminus of PKR, while the anti-PKR antibody (#3072) was designed to recognize the C-terminus of PKR. Mouse monoclonal anti-HSV ICP5 antibodies were supplied by Virusys Corporation (North Berwick, ME). Rabbit anti-GAPDH antibodies were supplied by Abcam (Cambridge, MA).

Transfection procedures and establishment of B virus Us11 or HSV-1 Us11 stable expression U373 cell lines (U373-BVUs11 or U373-HSVUs11). Vero cells were transiently transfected with a E2490Us11-pcDNA or MR7Us11-pcDNA construct using Lipofectamine™ 2000 (Invitrogen) in accordance with the protocol recommended by the supplier. To establish B virus Us11 or HSV-1 Us11 stable expression U373 cell lines, U373 cells were transfected with E2490Us11-pcDNA or HSVUs11-pcDNA DNA using GenePORTER transfection reagent (Biocompare, San Diego, CA), in accordance with the protocol recommended by the supplier. After 24 h, the transfected cells were subcultured at 1:30 or 1:60 in antibiotic-free medium. Antibiotic-containing medium (1.3
mg/ml Geneticin®, Sigma, St. Louis, MO) was substituted the following day. Single cell cloning was performed with cloning cylinder (Fisher, Suwanee, GA) to select the colonies resistant to the antibiotics. Immunoblotting analysis was used to evaluate the amount of B virus Us11 or HSV-1 Us11 protein expressed in different clones. U373 cells were also transfected with the pcDNA3.1 A vector as described above. At 48 h posttransfection, transfected cells were split and cultured in medium containing Geneticin®(1.3 mg/ml). Surviving cells were passaged for several generations in Geneticin® containing medium to produce the U373-pcDNA cell line, which served as a negative control in experiments with the Us11 stable expression cell lines.

**Infection of Vero or MK2 cells with B virus different strains.** To analyze Us11 protein synthesis in infected cells, confluent Vero or MK2 cell monolayers in 24-well plates were inoculated with B virus isolates at a MOI of 3 in a volume of 100 μl of DMEM medium per well and incubated for 1 h at 37°C to allow virus to attach. The cells were washed with Hank’s solution to remove the unbound virus, and then the first samples were collected (0 hpi) by adding 60 μl of SDS sample buffer (0.05 M Tris-HCl, 4% SDS, 10 % Glycerol, 4% 2-mercaptoethanol, and 0.018% bromophenol blue) per well. Media were then added to the remaining wells, and the plates were further incubated at 37°C for additional 21 h. Every 2 h, starting at 3 hpi, cell monolayers were rinsed with Hank’s solution and resuspended in 60 μl of SDS sample buffer. Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analyses for B virus Us11. To inhibit viral DNA synthesis, Vero cells were maintained 1 h before, during and after infection in medium containing 300 μg/ml
phosphonoacetic acid (PAA) (Sigma). The PAA treated or untreated Vero cells were infected with B virus strain E2490 at MOI of 3. At 17 hpi, the mock infected and infected cell monolayers were harvested with SDS sample buffer as described above and the cell lysates were analyzed by immunoblotting for Us11 and glycoprotein C (gC), respectively.

Infection of U373-pcDNA, U373-BVUs11 clone 4 and clone 12, or U373-HSVUs11 clone 10 cells with WT or Δγ134.5 HSV-1. U373-pcDNA, U373-BVUs11, or U373-HSVUs11 cell monolayers were infected with WT or Δγ134.5 HSV-1 at a MOI of 5, in a volume of 500 μl of growth medium per well on 6 well plates. At 6 h and 9 h post infection, cell monolayers were rinsed with Hank’s solution, resuspended and boiled in 60 μl disruption buffer (50 mM Tris-Hcl, pH7.0, 2%SDS, 700 mM β-mercaptoethanol, 2.75% sucrose). The mock-infected cell lysates were also harvested. The cell lysates harvested at 6 h post infection were analyzed for total PKR, phospho-PKR, total eIF2α, phospho-eIF2α, ICP5, and GAPDH, respectively, by immunoblotting. The cell lysates harvested at 9 h post infection were analyzed for ICP5 and GAPDH, respectively.

Pretreatment of U373-pcDNA and U373-BVUs11 clone 12 cells with IFN-α and infection of these cells with WT HSV-1. U373-pcDNA or U373-BVUs11 clone 12 cell monolayers were treated with various amounts of IFN-α (0, 1.4, 14, 140, 1400 U/ml) for 16 h and then were infected with WT HSV-1 at a MOI of 5, in a volume of 500 μl of growth medium per well on 6 well plates. At 6 h post infection, cell monolayers were rinsed with Hank’s solution, resuspended and boiled in 60 μl disruption buffer (50 mM
Tris-HCl [pH7.0], 2% SDS, 700 mM β-mercaptoethanol, 2.75% sucrose). The cell lysates were analyzed for total PKR, phospho-PKR, ICP5, or GAPDH, respectively, by immunoblotting. Wild type U373 cells or U373-BVUs11 clone 12 cell monolayers were treated or untreated with 1400 U/ml of IFN-α for 16 h and then were infected with WT HSV-1 at a MOI of 0.1, in a volume of 500 µl of growth medium per well on 6 well plates. At 24 h post infection, the supernatants were harvested and titrated in Vero cells.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analyses.** Proteins were separated on a 10% SDS-PAGE gel and either stained with Coomassie blue or transferred to a nitrocellulose membrane for immunoblotting. The immunoblotting procedure was described previously (Perelygina et al., 2002). Briefly, nitrocellulose containing electrophoretically separated proteins was blocked with blotto (5% skim milk in PBS) for at least 1 h, probed with primary antibodies diluted in blotto for 1 h, and washed 3 times in wash buffer (0.05% TWEEN 20 in PBS) for 10 minutes each. The nitrocellulose was then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in blotto for 1 h, washed 3 times as before and the target proteins were detected using an ECL kit (Amersham) according to manufacturer’s specifications.

**Total RNA extraction and northern blot analysis.** Vero cells in a T25 flask were mock infected or infected with B virus strains E2490 or MR7 at a MOI of 0.1. At 20 hpi, the cells were washed once with PBS, then harvested and homogenized by adding 800 µl of TRI reagent (Molecular Research Center, Cincinnati, OH) followed by the addition of 100 µl of BCP (Molecular Research Center). The mixture was incubated for 10 min at
room temperature and then it was centrifuged at 12000 rpm for 15 min at 4°C. The aqueous phase was transferred into a fresh tube. Following isopropanol precipitation, the RNA pellet was washed with 75% ethanol and dissolved in DEPC-treated water. The RNA (20 µg per lane) was fractionated on a 1.4% formaldehyde-agarose gel (Maniatis et al., 1982), transferred with 20XSSC to a positively charged nylon membrane (Boehringer Mannheim, Santa Maria, CA), UV crosslinked, and then hybridized with αP32-labeled probes generated by random priming from Us11-pBAD plasmid DNA with ready-to-go DNA labeling beads (Amersham) according to the manufacturer’s instructions. Prehybridization and hybridization were carried out at 42°C in hybridization buffer containing 50% formamide, 5X SSC, 5X Denhardt’s solution, 0.5% SDS, 0.5 mg/ml heparin, 8% dextran, and 50 μg/ml denatured salmon sperm DNA (Sigma). The membranes were washed and exposed to a Fuji X-ray film with an intensifier screen at -70°C for 2 to 72 h.

**Induction of PKR production in HeLa cells with IFN-α (Sigma).** HeLa cells were plated into 24-well plates. At 24 h post plating, the cell monolayers were treated with different amounts of IFN-α (0, 125, 250, 500, 1000, 2000, and 4000 units/ml). At 18 h post-treatment, the cell monolayers were washed with cold isotonic buffer and scraped into the buffer. The cell pellet was collected by centrifugation and dissolved in 20 µl of 2X SDS sample loading buffer, followed by boiling for 5 minutes. The samples were fractionated using 10% SDS-PAGE and analyzed by immunoblotting for PKR.

**Preparation of IFN-α induced HeLa cell S10 extracts.** S10 extracts were prepared from IFN-α (500 units/ml) induced HeLa cells as described previously (Mulvey
et al., 1999). Briefly, sets of 6 confluent T75 flasks of HeLa cells were treated with 500 units/ml of human IFN-α (Sigma). At 18 h post-treatment, the cell monolayers were washed with cold phosphate-buffered saline (PBS) twice, and the cells were scraped into 3 mls of PBS. After centrifugation for 4 min at 800 × g, the cell pellets were resuspended in 2.5 mls of cold Dounce buffer A (10 mM HEPES-KOH, pH 7.4, 15 mM KCl, 1.5 mM Mg(Oac)2, 1 mM DTT). After centrifugation for 4 min at 800 × g, the cell pellets were resuspended in 2.5 volume of cold Dounce buffer A. After incubation on ice for 10 min, the solution was homogenized with a tight-fitting pestle using about 30 strokes. The 0.1 volume of 10× Dounce buffer B (100 mM HEPES-KOH, pH 7.4, 1050 mM KCl, 35 mM Mg(Oac)2, 10 mM DTT) was added, followed by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 100 μM, and the extract was mixed by gentle inversion. After centrifugation at 10000 × g for 4 min at 4°C, the S10 supernatant was recovered and aliquots were quick-frozen at -80°C.

**Pull-down assay with recombinant B virus Us11 protein.** The expression and purification of the fusion protein T7 gene 10-Us11 were described previously. The T7 gene 10-Us11 protein binds anti-V5 agarose beads (Sigma) and Ni-NTA agarose beads (Qiagen), by its V5 epitope and histidine tags, respectively. For anti-V5 pull-down assays, V5-tagged T7 gene 10-Us11 protein or control protein P40 (2 μg) was mixed with 20 μl of anti-V5 agarose beads in PBS. The mixture was placed in a rotating wheel for 1 hour at room temperature for potential binding. The agarose beads were washed with PBS three times and resuspended in 20 μl storage buffer (50 mM Hepes, pH 7.4, 150 mM
NaCl, 5 mM DTT, and 10% v/v glycerol). To block non-specific binding, 2 μl of BSA (10 mg/ml) was added to the beads and the mixture was incubated for 5 min at room temperature. The mixture was then centrifuged at 5,000 rpm for 5 min at 4°C and the supernatant was removed. The 10 μl of IFN-α induced HeLa cell lysates (source of PKR), 150 μl of binding buffer (20 mM Tris-Hcl pH 7.5, 100 mM NaCl, 1% Triton X-100, 20% glycerol, 100 U aprotinin/ml, 0.2 mM PMSF), and 40 μl of BSA (10 mg/ml) were added to the blocked agarose beads, and the mixture was rotated for 2 h at 4°C. The beads were then washed eight times with 500 μl of binding buffer, centrifuged, and boiled with 20 μl 2X SDS sample loading buffer for 5 min. The protein samples were fractionated using 10% SDS-PAGE and analyzed by immunoblotting for PKR.

For Ni-NTA pull-down assays, histidine-tagged T7 gene 10-U11 protein or control protein, T7 gene 10 (2 μg), was mixed with 20 μl of Ni-NTA agarose beads in Buffer B (0.1 M NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0). The mixture was placed in a rotating wheel for 1 hour at room temperature for potential binding. The agarose beads containing potential protein were washed with PBS three times and resuspended in 20 μl storage buffer. The subsequent steps were identical to those in the anti-V5 pull-down assay.

**PKR activation assay and PKR activation inhibition assay in vitro.** IFN-α induced HeLa cell S10 extracts (described previously) served as a source of inactive PKR. The PKR activation assay and PKR activation inhibition assay were performed as described elsewhere with some modifications (Mulvey et al., 1999). Different amounts of
reovirus dsRNA (a kind gift from Jing Song) were used to activate PKR. Activation reactions were prepared with 15 μl of IFN-α induced HeLa cell S10 extracts, 30 μM ATP, 5 mM Mg(OAC)₂, 100 mM KCl, 20 mM HEPES-KOH(pH 7.4), 1.5 mM DTT, 100 μM PMSF, and different amounts of reovirus dsRNA (0, 1.5 ng, 0.75 ng, 0.375 ng). The volume of the reaction mixture was brought to 25 μl with distilled water. After incubation at 30°C for 30 min, the 25 μl of 2X SDS sample loading buffer was added into each reaction and boiled for 5 min. Each sample was analyzed by immunoblotting for total PKR, phospho-PKR, and GAPDH, respectively. For PKR activation inhibition assay, purified T7 gene 10-Us11 protein or control protein T7 gene 10 was dialyzed in a buffer containing 20 mM Hepes-KOH, pH 7.4, 100 mM KCl, and then 0.5 mM DTT was added to the reaction mixture in increasing amounts (0.4, 0.8, and 1.3 μg), followed by the addition of 0.75 ng of reovirus dsRNA. The following procedures were the same as that described in PKR activation assay. The anti-total PKR antibodies (#2766), specific for the N-terminus of PKR was used in the immunoblotting analysis.

**Multicycle growth curves.** U373 cells or B virus Us11 stable expression cell lines were seeded onto 6 well plates. Twenty-four hours later the cell monolayers were infected with WT or Δγ134.5 HSV-1 at a MOI of 0.001. At 1, 2, 3, and 4 days post infection, the supernatants were harvested and titrated in Vero cells.

**Plaque assay.** Confluent Vero cells were infected with a tenfold serial dilution of virus in a volume of 500 μl in 6 well plates. After a 1 h adsorption, the inoculum was removed and 2 ml of maintenance media with 1% methylcellulose was added to each well. After incubation for 48 h at 37°C, the cells were washed with PBS, fixed with 100%
methanol for 15 min at room temperature, and stained with Giemsa for 5 min at room temperature.
SECTION III. RESULTS

PART 1. B VIRUS GENOME TERMINI LACK \( \gamma_1 34.5 \) HOMOLOG OF HSV-1

B Virus Genome Termini Contain a Gene to Counteract the Action of PKR

Because a \( \gamma_1 34.5 \) deletion mutant of HSV-1 fails to block PKR activation, it replicates dramatically less efficiently in neuroglial cells. The observation that B virus replicates efficiently in neuroglial cells suggests B virus encodes a protein, which inhibits the action of PKR. In addition, B virus shares extensive homology with HSV-1 genetically. The \( \gamma_1 34.5 \) gene (RL1) of HSV-1, located in the long terminus of the genome plays a major role in counteracting PKR activity. Taken together, these data suggest that B virus genome termini contain a \( \gamma_1 34.5 \) gene homolog of HSV-1 that counteracts PKR activity. In order to test this hypothesis, the termini of B virus genome were cloned and sequenced to identify and map sequences corresponding to the \( \gamma_1 34.5 \) gene homolog of HSV-1.

The B virus genome, schematically represented in Figure 2, consists of two sets of unique sequences, each flanked by inverted repeats. The long unique sequence is flanked by 9-kbp inverted repeats designated as \( ab \) and \( b'a' \), respectively. The complete B virus genomic sequence was assembled according to the HSV-1 prototype genome structure. The \( \gamma_1 34.5 \) gene product of HSV-1 has at least two known, distinct, functional activities. One of the functions as described above, encoded by the \( \gamma_1 34.5 \) carboxyl-terminal domain, circumvents the antiviral effect of induced PKR by redirecting the host protein phosphatase 1\( \alpha \) to dephosphorylate translation initiation factor eIF2\( \alpha \), preventing protein
synthesis shutoff in infected cells. Another function, mapped to both the $\gamma_{134.5}$ amino-terminal and carboxyl-terminal domains, enables the virus to replicate in the peripheral and central nervous systems of experimentally infected animals. Deletion of $\gamma_{134.5}$ leads to complete neuro-attenuation of highly neurovirulent HSV-1 strains.
Figure 2. Organization of the genome of B virus. The linear double-stranded DNA is represented. The unique portions of the genome (UL and US) are shown as heavy solid lines, and the major repeat elements (TRL, IRL, IRS, and TRS) are shown as open boxes. For each pair of repeats the two copies are in opposing orientations. As indicated, the L region is composed of TRL, UL, and IRL, and the S region is composed of IRS, US, and TRS.
Cloning the Termini of the B Virus Genome into puc19 Plasmids and Confirmation of Recombinant Plasmids using Southern Blot.

To sequence the termini of B virus lab strain (E2490) genome, termini were cloned, and the first and last nucleotides in the B virus genome were determined. Initially, the locations of the internal \( a \) sequence and the joint between the B virus long and short internal repeat regions (L/S joint) were estimated after aligning the HSV-1 and B virus IR\(_L\)-IR\(_S\) sequences. Two \( SphI \) restriction sites were located approximately 1.8 kb (in the IR\(_L\) region) and 1.5 kb (in the IR\(_S\) region) from the predicted internal \( a \) sequence. Since the \( R_L \) and \( R_S \) internal and terminal copies in all alphaherpesvirus genomes are identical, \( SphI \) restriction sites were expected to be located \( \sim 1.8 \) kb and \( \sim 1.5 \) kb from the B virus L and S genomic termini, respectively, and therefore were used for cloning the B virus genomic ends.

The strategy of cloning B virus genomic termini is schematically represented in Figure 3. Blunt-ended B virus genomic DNA was digested with \( SphI \) and ligated into \( SmaI \)-and \( SphI \)-digested vector pUC19. All internal genomic fragments had \( SphI \) sticky ends on both sides, and consequently, only terminal genomic fragments, that had one blunt end and the other a \( SphI \) digested end, were compatible with the prepared vector and successfully cloned by this procedure. As predicted, two sets of recombinant clones were generated with inserts of the estimated sizes. To confirm that the clones isolated included viral DNA ends, plasmid DNA from clones containing 1.5-kb and 1.8-kb inserts was used to probe \( SphI \)-digested B virus genomic DNA on Southern blots. Theoretically,
if these clones indeed contain genomic ends, they will hybridize to the two SphI genomic fragments of 1.5 kb or 1.8 kb (terminal) and 3.1 kb (internal from the L/S junction). These fragments and two additional 2.1-kb and 3.3-kb fragments of lower intensity were detected (Figure 4). The observed cross-hybridization patterns indicated that the two genomic ends had common sequences. The additional fragments are most likely L-terminal (2.1 kb) and junction (3.3 kb) fragments containing an extra copy of a sequence. The variable number of a sequence repeats was previously described at the L terminus and L/S junction in HSV-1 (Roizman and Knipe 1990).
Figure 3. The strategy of cloning the B-virus genomic termini. The structural organization of the B virus genome is shown, with the U_L and U_S regions represented by solid lines and the TR_L, IR_L, TR_S, and IR_S regions represented by open boxes. The 1.8 kb long and 1.5 kb short terminal fragments generated by digesting the blunt-ended genomic DNA with *Sphi* I were cloned into pUC19 predigested with *Sma*I and *Sphi* I.
Figure 4. Southern blot analysis of 1.5 kb and 1.8 kb Sph I fragments to confirm their terminal locations in the B virus genome. SphI-digested genomic DNA was transferred to nylon membrane and hybridized with digoxigenin-labeled plasmids containing 1.5 kb (lane 1) and 1.8 kb (lane 2) terminal fragments.
DNA Sequencing and Gene Identification

DNA sequencing was performed as described in Materials and Methods. Once the sequence of the termini of B virus genome was determined, both major approaches to gene identification, extrinsic and intrinsic, were used to identify and characterize B virus genes. Extrinsic methods, such as Blast™, identify protein-coding genes by detecting similarity of translated protein sequences to the primary structure of a known protein. The intrinsic approach, an ab initio statistical method such as GeneMark™, identifies protein-coding regions by detecting specific frequency patterns in nucleotide order, including the codon usage pattern. These two types of methods have complementary strengths in terms of sensitivity and specificity. Extrinsic, similarity search methods have high specificity but may miss some genes, up to 30 to 40%, that would be recovered by the intrinsic, statistical method. The B virus genomic sequence was efficiently analyzed by similarity search methods due to extensive knowledge of the closely related virus species, HSV-1 and HSV-2. Nonetheless, to confirm that unique virus-specific genes were not missed, ab initio methods, GeneMark and GeneMark.hmm were trained on the set of genes confirmed by similarity search. These methods were modified to allow a noncanonical translation initiation codon, GTG, which could be used in this highly G+C-rich genome.

Long Termini of B Virus Genome Lack HSV-1 γ134.5 (RL1) Homolog

A unique discovery of the work of this dissertation was the absence of an HSV-1 γ134.5 (RL1) homolog in the long termini of the B virus genome as shown in Figure 5. This conclusion was reached after repeated searches with computational genome analysis tools. To determine that this observation was not limited to the laboratory strain (E2490)
of B virus, two low-passaged clinical isolates were cloned and sequenced. One isolate (MR7) was derived from a rhesus macaque and the other (A5), post mortem, from a zoonotically infected human patient. Sequence comparison of the fragments derived from these isolates with the corresponding fragment of the laboratory strain did not reveal any significant differences: only single nucleotide substitutions and variations in the number of copies of short reiterations were found in the clinical isolates relative to the laboratory strain. Since the absence of the $\gamma_{134.5}$ gene homolog was verified in three independent B virus strains, data suggested that this was a genuine and consistent property of B virus.
Figure 5. L-termini of B-virus genome lack $\gamma_{134.5}$ gene homolog of HSV-1. Both major approaches to gene identification, extrinsic and intrinsic, were used to search for a $\gamma_{134.5}$ gene homolog in the B virus genome long terminus. As indicated, there is no $\gamma_{134.5}$ gene homolog between the $\alpha$ sequence and ICP0 in the long terminus of B virus genome.
PART 2. B VIRUS, ALTHOUGH LACKING A $\gamma_{134.5}$ GENE HOMOLOG OF HSV-1, IS ABLE TO CIRCUMVENT ACTIVATION OF PKR

The observation that B virus replicates efficiently in neuroglial cells suggests that B virus encodes a protein, which can inhibit the action of PKR. However, the sequencing result of the long termini of the B virus genome revealed that B virus lacks a $\gamma_{134.5}$ gene homolog of HSV-1, the product which plays a major role in counteracting PKR activity in HSV-1 infected cells. These findings prompted an investigation of whether B virus was still able to circumvent PKR activity in the absence of $\gamma_{134.5}$ gene homolog of HSV-1.

To examine this, U373 cells were infected with B virus, WT or $\Delta\gamma_{134.5}$ HSV-1. The virus infected cell lysates harvested at 2h, 4h, 6 h post infection were analyzed by quantifying for total PKR, phospho-PKR, phospho- eIF2$\alpha$, and GAPDH. No phospho-PKR or phospho- eIF2$\alpha$ was detected in B virus or WT HSV-1 infected cells by immunoblotting, whereas, significant levels of phospho-PKR and phospho- eIF2$\alpha$ were detected in $\Delta\gamma_{134.5}$ HSV-1 infected U373 cells (Figure 6).

These data indicate that the B virus genome encodes another protein that compensates for the lack of $\gamma_{134.5}$ gene homolog and prevents PKR activation and subsequent eIF2$\alpha$ phosphorylation. Identification of the gene product in B virus that prevents PKR activation and subsequent eIF2$\alpha$ phosphorylation was the next goal. The genes of HSV-1 that counteract PKR phosphorylation have been studied extensively. It has been shown that Us11, if expressed at early times of infection as a result of mutation, can effectively
substitute for the missing ICP34.5 function in HSV-1 $\gamma_1$34.5 null mutants and helps to sustain near wild-type translation rates (He et al., 1997; Cassady et al., 1998). Although B virus genome lacks a $\gamma_1$34.5 gene homolog, it contains all other homologs of HSV-1, including HSV-1 Us11 (Ohsawa et al., 2002; Perelygina et al., 2003). The B virus Us11 polypeptide contains 20 copies of the RXP repeats in its carboxyl-terminal domain, whereas the carboxyl-terminal domain of the HSV-1 Us11 protein contains 24 copies of the RXP repeats. These repeats are critical for Us11 functionality. Therefore, the next hypothesis examined in this study was that B virus Us11 protein is responsible for compensating for the lack of a $\gamma_1$34.5 gene homolog and counteracting PKR activity.
Figure 6. B virus, as well as HSV-1, can prevent PKR activation and subsequent eIF2α phosphorylation in U373 cells. The infection procedure was described in materials and methods. (A) The infected cell lysates harvested at different times post infection were analyzed by immunoblotting for total PKR. (B) The lysates described as above were analyzed by immunoblotting for phospho-PKR. The anti-phospho-PKR antibody used recognizes the phosphorylation site of PKR on threonine (Thr) at position 446. (C) The lysates described as above were analyzed by immunoblotting for phospho-eIF2α. (D) The lysates described above were analyzed by immunoblotting for GAPDH.
PART 3. TEMPORAL KINETICS OF US11 PROTEIN EXPRESSION SUGGESTS THAT B VIRUS US11 PROTEIN CAN COMPENSATE FOR THE LACK OF A $\gamma_1$34.5 GENE HOMOLOG OF HSV-1

Chou et al. (1992) showed that PKR activation occurs at early times of infection (5 h pi) and prior to late gene synthesis in HSV-1. In addition, Cassady et al. (1998) showed that Us11, if expressed at early times of infection as a result of mutation, effectively substituted for the missing ICP34.5 function in HSV-1 $\gamma_1$34.5 null mutants and helps to sustain near wild-type translation rates. However, HSV-1 Us11, if expressed as a true late protein in its natural context of the virus, would not be effective in counteracting early PKR activation. These observations collectively suggest that in order to counteract early PKR activation, B virus Us11 protein must be expressed at early times of infection and not as a true late gene. To examine this hypothesis, the kinetics of B virus Us11 protein expression was studied in this part of dissertation.

Expression and Purification of B Virus Us11 Recombinant Protein in a Bacterial System

To study the kinetics of Us11 protein expression in B virus infected cells, polyclonal Us11-specific antibody was produced in rabbits by immunization with the Us11 recombinant protein. Initial attempts to produce Us11 without a fusion partner in bacteria by using the pBAD-TOPO vector resulted in low protein yield most probably due to intensive degradation of the recombinant protein. To minimize degradation, the Ncol-Pmel fragment containing Us11, V5 epitope, and polyhistidine region from Us11-pBAD was subcloned into pSCREEN-1bT vector, resulting in the expression of a fusion protein,
T7 gene 10-Us11 (Figure 7). Upon induction, T7 gene 10-Us11 was produced in large quantities and formed inclusion bodies. After extraction from inclusion bodies, this protein was further purified by affinity chromatography on heparin agarose to more than 90% homogeneity as estimated by SDS-PAGE. Us11 production and purification were monitored by SDS-PAGE followed by Coomassie blue staining (Figure 8A) and immunoblotting with V5 Mab (Figure 8B). Purified T7 gene 10-Us11 migrated in a gel as a single band of approximately 60 kDa and strongly reacted with anti-V5 monoclonal antibodies. The observed protein mass corresponds well to the predicted value of 57 kDa for the T7 gene 10-Us11 fusion protein.
**Figure 7. Construction of the recombinant plasmid Us11-pScreenT.** The Us11 gene amplified by PCR was initially cloned into a pBAD-TOPO vector in frame with V5 epitope and histidine tags, resulting in Us11-pBAD. Then the Us11-V5-His module from Us11-pBAD was subcloned into a pScreen-1b T vector in frame with the T7 gene 10, resulting in Us11-pScreenT.
**Figure 8. Purification of B virus Us11 fusion protein, T7 gene 10-Us11.** Us11 fusion protein was extracted from the bacterial cells transformed with the Us11-pScreenT expression construct, and then purified on a heparin-agarose affinity column. The protein marker and the peak fraction from the heparin affinity column were separated by SDS-10% PAGE, followed by either Coomassie blue staining (A) or immunoblotting with anti-V5 Mab (B). Positions of molecular mass markers are indicated on the left.
Confirmation of the Specificity of Rabbit Us11 Antiserum

To study the kinetics of Us11 protein expression in B virus infected cells, polyclonal Us11-specific antibody was subsequently produced in rabbits (New Zealand white rabbits, Myrtle’s Rabbitry) by immunization with the Us11 recombinant protein as described in Materials and Methods. The Us11 antiserum induced by the purified fusion protein reacted on immunoblots with two bands of molecular masses of 20 kDa and 40 kDa from SDS-solubilized B virus E2490-infected cell lysates, whereas no reactivity was observed with proteins from uninfected cell lysates (Figure 9). The pre-immune serum did not react with either any B virus-infected or uninfected antigens (data not shown). These observations suggest that the 20-kDa protein is a product of the Us11 gene, since this protein was recognized specifically by the Us11 antiserum and its molecular mass is close to the estimated value of 16.6 kDa based on the predicted amino acid sequence of B virus Us11. The discrepancy between predicted and observed molecular masses was most probably due to posttranslational phosphorylation of Us11, as HSV-1 Us11 homolog was shown to be a phosphoprotein (Diaz et al., 1993) and six phosphorylation sites were predicted to be present in B virus Us11 by the NetPhos 2.0 server. The NetNGlyc 1.0 server predicted no glycosylation sites in the Us11 sequence. The second immunoreactive protein, which was twice the size of the Us11 protein and recognized by Us11 antisera, was likely a dimer of Us11.
Figure 9. Western blot analysis of the rabbit antiserum produced by immunization with recombinant Us11. Lysates of Vero cells, either mock infected (M) or infected with B virus E2490 were fractionated on SDS-10% PAGE, transferred onto a nitrocellulose membrane and then probed with rabbit antiserum against Us11 fusion protein (a dilution of 1: 6400 in Blotto). Positions of molecular mass markers are indicated on the left.
**B Virus Us11 Expression is Independent of DNA Replication**

To find out whether the B virus Us11 gene belongs to the same $\gamma_2$ kinetic class as its HSV-1 homolog, the effect of viral DNA synthesis on the accumulation of Us11 protein in Vero cells infected with B virus E2490 was examined in the presence and absence of phosphonoacetic acid (PAA), an inhibitor of viral DNA replication. For comparison, the accumulation of gC, the product of a B virus true late gene, was examined in the same infected cells by immunoblotting with the B virus polyclonal rabbit anti-gC serum. In the absence of PAA, large quantities of both Us11 and gC proteins were detected in the infected cells at 17 hpi (Figure 10). The PAA treatment of cells completely prevented gC synthesis, whereas Us11 synthesis and accumulation was only slightly affected. These results indicate that, in contrast to the Us11 gene of HSV-1, the B virus homolog is not expressed with the kinetics of the $\gamma_2$ proteins. Its expression is independent of DNA replication.
Figure 10. Sensitivity of Us11 accumulation in Vero cells treated with PAA, an inhibitor of viral DNA replication. Vero cell monolayers, either untreated or treated with PAA, were infected with B virus lab strain E2490 at an MOI of 3 and incubated at 37°C. Mock infected (M) and infected cell lysates collected at 0 hpi and 17 hpi were analyzed by immunoblotting with rabbit anti-Us11 antibody (A). Immunoblotting with rabbit antiserum against B virus gC served as a positive control for the efficacy of PAA treatment (B). Arrows indicate bands representing Us11 and gC proteins.
Temporal Kinetics of Us11 Protein Expression during B Virus Infection in Vero Cells

To study the detailed kinetics of Us11 protein accumulation in B-virus infected cell cultures, Vero cell monolayers were infected at a MOI of 3 with each of the E2490, A4, A5, and MR7 B virus strains. Next, infected cell lysates were harvested at 2-h intervals and immunoblotted using the Us11 antiserum (Figure 11A). The 20-kDa Us11 protein was first detected at 3 hpi in all infected cultures, increasing steadily to a plateau at 9 hpi in cultures infected with clinical strains (MR7, A4, A5), or at 13 hpi in E2490-infected cells. The appearance and accumulation of the 40-kDa protein paralleled the 20-kDa protein in SDS solubilized infected cell lysates, but was observed only in E2490-and A5-infected cells. In A4- and MR7-infected cells, this 40-kDa protein was not detected. No immunoreactive proteins were detected in mock-infected cell lysates. Interestingly, after long exposures of the blots (data not shown), both Us11 bands were also detected in lysates at 0 hpi suggesting that, similar to the HSV-1 homolog (Roller and Roizman 1992), Us11 is an abundant virion tegument protein of B virus. For comparison, the expression kinetics of gC were also assessed in the same samples of infected cells by immunoblotting using rabbit gC antiserum. The results for all isolates were identical as shown in representative western blots (Figure 11B). Glycoprotein C was first detected at 9 hpi and reached maximum intensity at 15 hpi. These results indicate that the pattern of Us11 expression differs clearly from the late kinetics of gC synthesis in B virus-infected cell cultures.
A. Immunoblots with US11 antisera

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<td>19</td>
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</tr>
</tbody>
</table>

- E2490 (rhesus)
- MR7 (rhesus)
- A5 (human)
- A4 (human)

B. Immunoblots with gC antisera

- A5
- A4

**Figure 11. Temporal kinetics of Us11 protein expression in Vero cells infected with different B virus strains.** Vero cells, either mock infected or infected at an MOI of 3 with either the B virus lab strain E2490, or low passage isolates MR7, A4, or A5. At the indicated times post infection, infected cell lysates were harvested with SDS buffer and analyzed by immunoblotting with rabbit anti-Us11 antibodies (A). For comparison, the same samples were analyzed by immunoblotting for the true late protein gC (B). Arrows indicate bands representing Us11 and gC proteins.
Comparison of the Us11 Expression Pattern in Vero Cells versus LLC-MK2 Cells

In order to test whether Us11 has the same expression pattern in its natural host cells, Vero cells (African green monkey origin) and LLC-MK2 (macaque origin) cells were infected in parallel with each of the four B virus isolates (MOI of 3). Infected cell lysates were collected at 0 hpi and at hourly intervals between 3 and 7 hpi, and analyzed by western blot. The results demonstrated that Us11 temporal expression was similar in infected Vero and LLC-MK2 cells. The 40-kDa protein was observed only in E2490-and A5-infected cells. In A4- and MR7-infected cells, this 40-kDa protein was not detected (only results for E2490 and MR7 are shown in Figure 12). In MR7-infected cells, the Us11 protein band at 5 h post infection was not detected due to a technical problem. Notably, the same B virus isolates (E2490 or A5) that produced the 40-kDa protein in Vero cells also produced this large molecular mass protein in LLC-MK2 cells, suggesting the observed band is B virus isolate-specific rather than host cell-specific.
Figure 12. Comparison of Us11 expression kinetics in B virus infected Vero and LLC-MK2 cells. Vero and LLC-MK2 cells, either mock infected or infected at a MOI of 3 with B virus E2490 or MR7 were harvested with SDS buffer at the indicated times post infection and analyzed by immunoblotting for Us11.
Determination of the Nature of the Large Molecular Mass Protein (40 kDa) that Reacted with Us11 Antiserum

To investigate whether the 40 kDa protein observed on western blot was a Us11 protein or a cellular or another B virus protein cross-reactive with the Us11 antibody, the expression construct E2490Us11-pcDNA was obtained by amplifying the Us11 ORFs and cloning the PCR fragments into a pcDNA3.1 vector as described in Materials and Methods. Vero cells were transfected with this construct or an empty vector followed by immunoblotting analysis of the transfected cell lysates for Us11. If this large molecular mass protein is Us11 specific, it would be expected to also appear in E2490Us11-pcDNA transfected cells. Otherwise it would be absent in transfected cells, since there was no virus infection or other virus proteins in transfected cells. The Us11 antibodies (Figure 13) reacted with two protein bands of 25 kDa and 50 kDa in E2490Us11-pcDNA transfected cells. No reactivity was observed in cells transfected with empty vector (Figure 13). The molecular masses of both protein bands (25 kDa, 50 kDa) was slightly higher in transfected cells than in infected Vero cells (20 kDa, 40 kDa) because of the addition of the V5-His tag to recombinant Us11 protein. In addition, anti-V5 Mab (not shown) reacted with two protein bands (25 kDa, 50 kDa) in E2490Us11-pcDNA transfected cells. These results strongly suggest that the larger molecular mass protein reactive with rabbit polyclonal anti-Us11 serum is Us11 specific and not a cross-reactive cellular or virus protein.
Figure 13. Us11 protein forms in Vero cells transfected with Us11 expression constructs. Vero cell cultures were transfected with either empty vector pcDNA3.1 (lane 1) or expression plasmids E2490Us11-pcDNA (lane 2) or MR7Us11-pcDNA (lane 3). The cell lysates harvested at 48 h post transfection were analyzed by immunoblotting for Us11. Positions of molecular mass markers are indicated on the left.
In the next series of experiments, to determine why some isolates of B virus produce the large molecular mass protein (40 kDa) reactive to Us11, while other isolates do not, a hypothesis was formulated and tested that alternative splicing events of a primary Us11 transcript gave rise to two Us11 forms. The Us11 gene of HSV-1 does not contain introns (Rixon and McGeoch 1984) and no introns were predicted by sequence analysis in the homologous gene of B virus (Perelygina et al., 2003). Two representative strains, E2490 and MR7, were selected to investigate this hypothesis. Vero cells were mock infected or infected with either E2490 and MR7 at a MOI of 0.1. At 20 hpi, total RNA was extracted from the cells and subjected to northern blot analysis with the radiolabeled Us11 probe. Two transcripts, ~1.0 kb and ~1.8 kb, were detected in both E2490- and MR7-infected cultures, but not in mock infected cells (Figure 14). These bands most likely represent Us10 (1.0 kb) and Us11, Us12 (1.8 kb) transcripts, which like the HSV-1 homologs (Rixon and McGeoch 1984) overlap and share a polyadenylation signal at the common 3’ end (Ohsawa et al., 2002) (Perelygina et al., 2003) (Figure 15). Since the transcript patterns did not differ in E2490- and MR7-infected cultures, these data suggest that the 40-kDa protein was not synthesized from an alternatively spliced Us11 transcript in E2490-infected cells.
Figure 14. Comparison of the Us11 transcription pattern in Vero cells infected with B virus strains. Vero cells were mock infected or infected at an MOI of 0.1 with B virus strains E2490 or MR7. At 20 h post infection, total RNA was extracted and analyzed by Northern blot using radiolabeled Us11 probe. The sizes of the RNA markers are indicated on the left.
Figure 15. Diagram of B virus genome **Us12,11,10 region**. **Us12, Us11, Us10** coding regions are shown as black, red, and orange arrow bars, respectively. The potential **Us12** promoter and **Us10** TATA box region are shown as green arrow bar and green bar, respectively. **Us11** may share the promoter of **Us12**. The common 3’ polyadenylation site shared by these 3 genes is shown as black bar.
To investigate whether the polymorphism in the Us11 gene is linked to specific B virus isolates, the Us11 gene was amplified from the genomic DNA of each of four B virus isolates and sequenced. Comparison of the deduced amino acid sequences showed that A4 Us11 contains three A4-specific amino acid substitutions (Table 1). Remarkably, the Us11 protein from E2490 and A5 isolates, that contained two Us11 forms, had threonine (Thr) at position 6, whereas the Us11 protein from MR7 and A4 isolates, that produced only the 20-kDa protein, and had alanine at this position. To determine whether the single amino acid substitution, alanine to threonine, was responsible for an Us11 dimer formation, and consequently, the appearance of the additional 40-kDa protein in E2490 and A5-infected lysates, transient expression of the Us11 ORFs of the E2490 and MR7 isolates in Vero cells was utilized. If the Us11 polypeptide with Thr at position 6 indeed formed a stable dimer in the context of a virus infection, it might also form this dimer when transiently expressed in the absence of a virus infection. Vero cells were transfected with the E2490Us11-pcDNA or MR7Us11-pcDNA constructs or an empty vector. Transfection was followed by immunoblot analysis of the transfected cell lysates. Interestingly, Us11 antibodies (Figure 13) and anti-V5 Mab (not shown) reacted with two protein bands of 25 kDa and 50kDa not only in E2490Us11-pcDNA transfected cells but also in MR7Us11-pcDNA transfected cells. Since no difference was detected upon expression of the two Us11 alleles, the data suggested that Thr residue at position 6 was not responsible for the appearance of the 40-kDa isoform in B virus infected cells. It is presently unknown why this isoform appeared only in cells infected with only some of the B virus isolates.
**TABLE 1.**
Comparison of Us11 amino acid sequences among different isolates of B virus

<table>
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<tr>
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<td>T</td>
<td>R</td>
<td>E</td>
<td>H</td>
</tr>
<tr>
<td>A5</td>
<td>T</td>
<td>R</td>
<td>E</td>
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</tr>
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<td>MR7</td>
<td>A</td>
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<td>Q</td>
<td>Y</td>
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</table>

*Isolates producing 40-kDa isoform of Us11 are indicated in bold print.
In the transfected or infected cells, the size of the high molecular mass isoform was always twice the size of the low molecular mass isoform, suggesting that 40-kDa isoform is an Us11 dimer. This 40-kDa protein, however, was not denatured and reduced when boiled in standard 1x Laemmli buffer containing 1% SDS and 1% β-mercaptoethanol (β-ME) for 5 min. To examine this point further, infected cell lysates were either pre-treated with 8M urea at 37°C overnight and then boiled in 1x Laemmli buffer or boiled in Laemmli buffer supplemented with 8M urea. The samples were then resolved in 10% SDS-PAGE and immunoblotted for Us11. The intensities of the 40-kDa bands were not decreased in the protein samples after the urea treatment (data not shown). These data indicate that the 40-kDa protein was highly stable to denaturation by both ionic detergents (SDS) and chaotropic agents (urea). Moreover, this protein does not form disulfide bonds, since treatment with two disulfide-reducing agents, DTT and β-ME did change the migration of the band. These data suggest that the 40-kDa band represents a dimeric form of Us11 protein that is irreversibly covalently linked.
PART 4. B VIRUS US11 PROTEIN PREVENTS PKR ACTIVATION IN VITRO AND IN VIVO.

Previously, data presented in this dissertation demonstrated that the Us11 protein (20 kDa) of 4 different B virus isolates was expressed early following infection (3 h post infection) in Vero cells and rhesus macaque kidney cells. Expression of B virus Us11 protein was not blocked by PAA, an inhibitor of DNA replication, confirming Us11 is not a “true late” gene of B virus as it is in HSV-1 and HSV-2 and suggesting B virus Us11 protein compensates for the lack of a B virus γ134.5 counteracting cellular anti-viral mechanisms mediated by PKR. These findings prompted an investigation to determine whether B virus Us11 protein could prevent the action of PKR. Initially, IFN-α-treated HeLa cells S10 extracts served as the source of inactive PKR to test whether B virus Us11 protein prevented PKR activation in vitro. Subsequently, a B virus Us11 stable expression cell line was utilized to test whether a B virus Us11 protein prevented PKR activation and subsequent eIF2 phosphorylation in Δγ134.5 HSV-1 infected U373 cells.

Optimization of IFN-α treatment to Induce PKR Production in HeLa Cells and Preparation of IFN-α Induced HeLa Cell S10 Extracts.

The experiments used HeLa cells because IFN-α induces a relatively larger amount of PKR in HeLa cells compared with other cell lines tested (data not shown). In order to determine the optimal dose of IFN-α required to induce PKR production, HeLa cell monolayers were treated with different doses of IFN-α, and the cell lysates were collected at 18 h post-treatment and analyzed by immunoblotting. The results revealed that both 250 and 500 units/ml of IFN-α induced peak amounts of PKR production in HeLa cells (data not shown). Thus, 500 units/ml of IFN-α were used to induce PKR in
the subsequent experiments. S10 extracts were prepared from IFN-α induced HeLa cells as described in Materials and Methods. The protein concentration in these S10 extracts was 3 mg/ml. The S10 extracts from IFN-α treated HeLa cells served as the source of inactive PKR in the following experiments.

**B Virus Us11 Protein Prevents Activation of PKR Kinase in Vitro.**

To assess the effect of B virus Us11 protein on PKR activation, a cell-free system derived from IFN-α-treated HeLa cell S10 extracts was prepared as described in Materials and Methods to serve as the source of inactive PKR. First, different amounts of reovirus dsRNA were used to activate PKR. Upon the addition of different amounts (0, 1.5 ng, 0.75 ng, and 0.375 ng) of reovirus dsRNA, activation of cellular PKR kinase was evaluated by monitoring phosphorylation using immunoblotting analysis to detect phospho-PKR. Both 1.5 ng and 0.75 ng of reovirus dsRNA caused cellular PKR phosphorylation (Figure 16A). Thus, 0.75 ng of reovirus dsRNA was used in the following PKR activation inhibition assay. In this assay, increasing amounts of T7 gene 10-Us11 fusion protein or control protein, T7 gene 10, were added to the HeLa S10 extracts. Following the addition of reovirus dsRNA and incubation, the reaction mixtures were analyzed for total PKR, phospho-PKR, and GAPDH, respectively, by immunoblotting. While addition of increasing amounts of purified T7 gene 10 protein had no measurable effect on PKR phosphorylation, addition of increasing amounts of the T7 gene 10-Us11 fusion protein resulted in inhibition of PKR phosphorylation (Figure 16B). Thus, B virus Us11 protein is sufficient to prevent activation of the cellular PKR kinase in vitro.
Figure 16. B virus Us11 protein inhibits PKR activation in vitro. (A) PKR activation assay. IFN-α-induced HeLa cell S10 extracts served as the source of cellular PKR. Different amounts of reovirus dsRNA (0, 1.5 ng, 0.75 ng, and 0.375g) were added to HeLa S10 extracts containing 30 μM ATP. The reaction mixtures were incubated at 30°C for 30 min. The reaction samples were analyzed by immunoblotting for PKR, phospho-PKR, and GAPDH respectively. (B) PKR activation inhibition assay. Increasing amounts of purified B virus Us11 fusion protein T7 gene 10-Us11, or control protein, T7 gene 10 were added to HeLa S10 extracts containing 30 μM ATP. Following the addition of 0.75 ng of reovirus dsRNA, the reactions were processed as described above.
**B Virus Us11 Protein Counteracts the Action of PKR In Vivo**

**Construction of U373 cells constitutively expressing B virus Us11 protein**

Although B virus Us11 has been previously shown to inhibit PKR phosphorylation, all of these studies were performed in a cell-free system derived from IFNα-treated HeLa cell S10 extracts. Up to this point, there had been no efforts to assess the capacity of B virus Us11, expressed constitutively in U373 cells, to inhibit PKR activation. It was previously reported that Δγ134.5 HSV-1 induced PKR activation, subsequent eIF2α phosphorylation, and protein synthesis shutoff in U373 cells, and the Δγ134.5 HSV-1 could not replicate effectively in U373 cells (Mulvey et al., 1999; Mulvey et al., 2003). To test the effects of B virus Us11 on PKR activation, eIF2α phosphorylation status and the replication of Δγ134.5 HSV-1 in U373 cells, B virus Us11 stable expression U373 cell lines (U373-BVUs11) were first generated. U373 cells were transfected with E2490Us11-pcDNA plasmid DNA and Geneticin® was added for selection. The colonies resistant to Geneticin were picked using sterile cloning cylinders. The cell lysates from these colonies were analyzed for B virus Us11 protein expression by immunoblotting. The fourteen cell lines expressed Us11 protein, albeit at various levels (data not shown). Clone 12, one of the fourteen cell lines, was found to consistently express higher levels than others. Analysis of immunoblot results showed that both clone 12 and clone 4 expressed 25 kDa B virus Us11 protein, whereas wild type U373 cells did not express any Us11 protein (Figure 17). Furthermore, the analysis of band densities showed that clone 12 expressed 3 times the levels of B virus Us11 protein than clone 4. In addition, Us11 expression levels in clone 4 and clone 12 were compared with that in B virus infected U373 cells. U373 cells were infected with the B virus E2490 isolate at a MOI of 5, then infected cell lysates were harvested
at 2h, 4h, and 6h post infection and immunoblotted using the Us11 antiserum. Us11 expression levels in clone 4 of U373-BVUs11 cells were similar to those in BV infected cells at 2 h post infection. However, Us11 expression levels in clone 12 of U373-BVUs11 were similar to those in BV infected cells at 4h or 6h post infection (Figure 18). The molecular mass of Us11 protein (25 kDa) was slightly higher in stable expression cell lines than in infected U373 cells (20 kDa) due to the addition of the V5-His tag to the recombinant Us11 protein. After clone 12 and clone 4 were selected, they were maintained in medium containing Geneticin® by serial passages. Production of Us11 was stable and undiminished in medium containing drug. For comparison, an HSV-1 Us11 stable expression U373 cell line (U373-HSVUs11) was also constructed. In addition, U373 cells stably transfected by empty vector pcDNA3 (U373-pcDNA) were established and used as a negative control in the subsequent experiments.
Figure 17. Establishment of B virus Us11 stable expression U373 cell lines. The lysates of control U373 cells and U373-BVUs11 cell lines, clone 4 and clone 12 were analyzed by immunoblotting for B virus Us11 protein and GAPDH, respectively. The position of B-virus Us11 protein is shown.
Figure 18. Comparative analysis of Us11 expression level between B virus Us11 stable expression cell lines and B virus infected cell lysates. The lysates of control U373-pcDNA cells, U373-BVUs11 cell lines, clone 4 and clone 12, and B virus infected U373 cell lysates harvested at different hours post infection were analyzed by immunoblotting for B virus Us11 protein (A) and GAPDH (B). The positions of B-virus Us11 protein were shown. The same amount of the protein that was adjusted according to GAPDH was loaded into each lane.
**B virus Us11 protein prevented PKR activation and subsequent eIF2α phosphorylation in vivo.**

To investigate the effect of B virus Us11 protein on PKR and subsequent eIF2α phosphorylation induced by Δγ134.5 HSV-1 infection in U373 cells, the U373-pcDNA and U373-BVUs11 clone 4 and clone 12 cells were infected with WT or Δγ134.5 HSV-1. The mock infected or virus infected cell lysates harvested at 6 h post infection were analyzed for total PKR, phospho-PKR, total eIF2α, phospho-eIF2α, and GAPDH by immunoblotting. The band intensities were analyzed by Fuji film MultiGauge program. The phospho-PKR and phospho-eIF2α level was normalized by the total PKR and total eIF2α level, respectively. As expected, no phospho-PKR was detected in mock infected U373-pcDNA cells or U373-BVUs11 cell lines. In WT HSV-1 infected cells, very low levels of phospho-PKR were detected (Figure 19B). Greater amounts of phospho-PKR were detected in Δγ134.5 HSV-1 infected U373-pcDNA cells, whereas phospho-PKR levels were decreased in Δγ134.5 HSV-1 infected U373-BVUs11 cells (Figure 19B). If the phospho-PKR level in Δγ134.5 HSV-1 infected U373-pcDNA cells was considered as 100%, the phospho-PKR level in Δγ134.5 HSV-1 infected U373-BVUs11 clone 4 and clone 12 cells was 87% and 42%, respectively (Figure 19D). These data suggest that B virus Us11 prevents PKR activation in dose-dependent manner when it is expressed constitutively in U373 cells.
**Figure 19.** B virus Us11 protein prevents PKR phosphorylation in U373 cells. U373-pcDNA cells and U373-BVUs11 clone 4 and clone 12 cells were mock infected or infected with Δγ134.5HSV-1 or WT HSV-1. (A) These lysates harvested at 6 h post infection were analyzed by immunoblotting for total PKR. (B) The lysates described as above were analyzed by immunoblotting for phospho-PKR. (C) The lysates described as above were analyzed by immunoblotting for GAPDH, (D) The band intensities were analyzed by Fuji film multigauge program. The phospho-PKR level was normalized by total PKR level. The phospho-PKR level in Δγ134.5HSV-1 infected U373-pcDNA cells was considered 100%, and the values for other treatments are presented as percentages of that value.
As expected, no phospho-eIF2α was detected in mock or WT HSV-1 infected U373-pcDNA cells or U373-BVUs11 cell lines. Greater levels of phospho-eIF2α were detected in Δγ134.5 HSV-1 infected U373-pcDNA cells than in Δγ134.5 HSV-1 infected U373-BVUs11 cells (Figure 20B). Assuming the phospho-eIF2α level in Δγ134.5 HSV-1 infected U373-pcDNA cells as 100%, then the phospho-eIF2α levels in Δγ134.5 HSV-1 infected U373-BVUs11 clone 4 and clone 12 cells was 90% and 49%, respectively (Figure 20D). These data suggest the hypothesis that B virus Us11 protein, expressed constitutively in U373 cells, prevents eIF2α phosphorylation in dose-dependent manner.
Figure 20. B-virus Us11 protein prevents the subsequent phosphorylation of eIF2α in U373 cells. The procedures of infection of cells were exactly the same as figure 19. (A) Cell lysates harvested at 6 h post infection were analyzed by immunoblotting analysis for total eIF2α. (B) The cell lysates described as above were analyzed by immunoblotting for phospho-eIF2α. (C) The cell lysates described as above were analyzed by immunoblotting for GAPDH. (D) The band intensities were analyzed by Fuji film multigauge program. The phospho- eIF2α level was normalized by total eIF2α level. The phospho- eIF2α level in Δγ34.5HSV-1 infected U373-pcDNA cells was considered as 100%, and the values for other treatments were presented as percentages of that value.
The previously shown data suggested that B virus Us11 protein inhibited PKR and the subsequent eIF2α phosphorylation induced by Δγ1,34.5 HSV-1 infection in U373-BVUs11 clone 12 cells. Since eIF2α phosphorylation was inhibited by B virus Us11 protein in Δγ1,34.5HSV-1 infected U373-BVUs11 cells, the subsequent protein synthesis shutoff would also be prevented in these cells, and as the consequence, the protein accumulation of Δγ1,34.5 HSV-1 would continue in U373-BVUs11 cells.

To investigate whether B virus Us11 protein restored virus protein synthesis in HSV-1 infected U373 cells in addition to prevention of PKR and eIF2α phosphorylation, we evaluated HSV-1 ICP5 protein accumulation using immunoblotting analysis. ICP5 protein was picked as a marker since it is a γ1 protein of HSV-1, whose expression will be affected by protein synthesis shutoff induced by PKR phosphorylation and it appears much early than HSV-1 Us11 protein which might be confused with B virus Us11 protein stably expressed in cells. The U373-pcDNA cells and U373-BVUs11 clone 4 and clone 12 cells were mock infected or infected with WT or Δγ1,34.5 HSV-1 at a MOI of 5. Cell lysates harvested at 9 h post infection were analyzed for HSV-1 leaky late protein ICP5 by immunoblotting as shown in Figure 21 A. The band intensities of ICP5 9 h post infection were analyzed by Fuji film multigauge program. As shown in that figure, the ICP5 level in Δγ1,34.5 HSV-1 infected U373-BVUs11 cell lines is much higher than that in Δγ1,34.5 HSV-1 infected U373-pcDNA cells (Figure 21A). Considering the ICP5 level in WT HSV-1 infected U373-pcDNA cells as 100%, the ICP5 level in Δγ1,34.5 HSV-1 infected U373-pcDNA cells was only 37%, suggesting protein synthesis shutoff occurs at
some point in Δγ134.5 HSV-1 infected U373-pcDNA cells (Figure 21C). However, the accumulation of ICP5 in Δγ134.5 HSV-1 infected U373-BVUs11 clone 4 and clone 12 increased compared with that in Δγ134.5 HSV-1 infected U373-pcDNA cells (Figure 21C). The ICP5 level increases to 49% and 74%, respectively, in Δγ134.5 HSV-1 infected U373-BVUs11 clone 4 and clone 12 cells (Figure 21C), suggesting that B virus Us11 protein can overcome the protein synthesis shutoff in Δγ134.5 HSV-1 infected cells and restore the leaky late gene synthesis of Δγ134.5 HSV-1 in U373 cells in a dose-dependent manner. Interestingly, at 6 h post infection, the ICP5 levels in U373-BVUs11 cell lines was only slightly greater than that in Δγ134.5 HSV-1 infected U373-pcDNA cells (data not shown). This result suggests that the protein synthesis shutoff in Δγ134.5 HSV-1 infected U373-pcDNA cells occurs around 6 h post infection.
Figure 21. B virus Us11 protein restores the synthesis of Δγ134.5HSV-1 leaky late gene product ICP5 in U373 cells. Control U373-pcDNA cells, U373-BVUs11 cell lines clone 4 and clone 12 were mock infected, or infected with WT or Δγ134.5HSV-1 at MOI of 5. The lysates of these cells were harvested at 9 h post infection. The 9 h post infection lysates were analyzed by immunoblotting for HSV-1 ICP5 (A) and for GAPDH (B). The band intensities of ICP5 9h pi were analyzed by Fuji film multigauge program. The ICP5 level in WT HSV-1 infected U373-pcDNA cells was considered as 100%, and the values for other treatments were presented as percentages of that value (C).
B virus Us11 protein restores the replication abilities of Δγ134.5 HSV-1 in U373 cells.

It has been reported that low levels of phosphorylated eIF2α are critical for virus replication, and the ability of HSV-1 to replicate correlates with phosphorylated eIF2α level (Ward et al., 2003). Previously discussed experiments revealed that B virus Us11 protein inhibited PKR, subsequent eIF2α phosphorylation in Δγ134.5 HSV-1 infected cells in a dose-dependent manner. In addition, these data suggest that B virus Us11 protein overcomes protein synthesis shutoff and restores the virus protein accumulation in Δγ134.5 HSV-1 infected cells. Therefore, it was reasonable to speculate that B virus Us11 protein restores the replication abilities of Δγ134.5 HSV-1 in U373 cells.

To test this hypothesis, growth curves of WT or Δγ134.5 HSV-1 in U373 cells and U373-BVUs11 cell lines clone 4 and clone 12 were compared. Indicated cell lines were infected at a low MOI of 0.001. The supernatants were collected for titration at 1, 2, 3, and 4 days post infection. The replication of Δγ134.5 HSV-1 in U373 cells was the most impaired, and virus titers in supernatant were between 10^4 - to 10^6 - fold reduced compared to those for WT HSV-1 in U373 cells (Figure 22). However, the replication abilities of Δγ134.5 HSV-1 were restored in U373-BVUs11 clone 12 (Figure 22A). The titers of supernatant from Δγ134.5 HSV-1 infected U373-BVUs11 clone12 were 10- to 10^3 fold increased compared to those for Δγ134.5 HSV-1 infected U373 cells (Figure 22A). These results suggest B virus Us11 protein restores the replication abilities of Δγ134.5 HSV-1 in U373 cells.
It was interesting to compare the titers in supernatants between WT HSV-1 infected U373 and U373-BVUs11 cell lines. The titers of supernatant from WT HSV-1 infected U373-BVUs11 clone 12 were 10- to 10^2 fold decreased compared to those in infected U373 cells, whereas the titers of supernatant from WT HSV-1 infected U373-BVUs11 clone 4 were similar to those in infected U373 cells (Figure 22B). These results suggested that overexpression of B virus Us11 protein in U373 cells inhibits WT HSV-1 replication in U373 cells.
Figure 22. B virus Us11 protein restores the replication of Δγ134.5 HSV-1 in U373 cells, whereas overexpression of B virus Us11 protein in U373 cells inhibits replication of WT HSV-1. U373 cells and B virus Us11 stable expression cell lines clone 4 or clone 12 were infected with WT HSV-1 or Δγ134.5 HSV-1 at an MOI of 10^3. At 1, 2, 3, 4 days postinfection, the supernatants were collected for titration in permissive Vero cells. (A) Comparison of infectivity of supernatants from Δγ134.5 HSV-1 infected U373 cells and B virus Us11 stable expression cell line clone 4 and clone 12 cells. (B) Comparison of supernatants from WT HSV-1 infected U373 cells, B virus Us11 stable expression cell line clone 4 and clone 12 cells.
Comparison of B Virus and HSV-1 Us11 Proteins in Counteracting PKR Activity.

Construction of U373 cells constitutively expressing HSV-1 Us11 protein.

Comparative analysis of Us11 protein amino acid sequence between B virus and HSV-1 revealed that in the carboxyl domain of the HSV-1 and B virus Us11 protein all contained RXP repeats, indicating B virus and HSV-1 Us11 have similar function. To compare the effects of B virus and HSV-1 Us11 protein on PKR activation, eIF2α phosphorylation status, and the replication of Δγ134.5 HSV-1 in U373 cells and HSV-1 Us11 stable expression U373 cell lines (U373-HSVUs11), these lines generated as described as Materials and Methods. Forty-three colonies resistant to Geneticin® were picked using a sterile cloning cylinder. The cell lysates from these colonies were analyzed for expressed HSV-1 Us11 protein. Since HSV-1 Us11 protein strongly cross-reacted with rabbit anti-B virus Us11 antibody (data not shown), anti-B virus Us11 antibodies were used in an immunoblotting analysis to measure the HSV-1 Us11 expression level in U373-HSVUs11 clones. The thirty cell lines tested expressed HSV-1 Us11, albeit at various levels (data not shown). Clone 10, one of the thirty cell lines, was found to consistently express relatively greater levels of Us11 than other clones. Analysis of immunoblotting results revealed that the Us11 expression levels in U373-HSVUs11 clone 10 was similar to that in U373-BVUs11 clone 4 and were much lower than that in U373-BVUs11 clone 12, whereas the other U373-HSVUs11 clones expressed even lower levels of Us11 protein (Figure 23). The reason that no U373-HSVUs11 clone with expression levels of Us11 similar to that in the U373-BVUs11 clone 12 were detected is not known. One possibility is that overexpression of the HSV-1 Us11 protein was toxic to
the cells and those clones in which Us11 was overexpressed to the same levels as in the U373-BVUs11 clone 12 were not able to divide or survive. Therefore, the U373-HSVUs11 clone 10 was picked to compare with BV Us11 stable expression cell lines.
Figure 23. Comparative analysis of Us11 expression level of B-virus and HSV-1 Us11 stable expression cell lines. The lysates of control U373-pcDNA cells, U373-BVUs11 cell lines, clone 4 and clone 12, and U373-HUs11 cell lines, clone 10, clone 43, clone 1, clone 20, clone 24, and clone 34 were analyzed by immunoblotting for Us11 protein (A) and GAPDH (B). The positions of Us11 protein are shown.
B virus Us11 protein has similar effects on PKR phosphorylation as HSV-1 Us11 protein when expressed at a comparable level

The efficacy of the B virus and HSV-1 Us11 proteins stably expressed in U373 cells for preventing PKR activation and subsequent eIF2α phosphorylation induced by Δγ134.5 HSV-1 infection was compared in U373-pcDNA cells, U373-BVUs11 clone 4 and clone 12, and U373-HSVUs11 clone 10 infected with WT or Δγ134.5 HSV-1. The mock infected or virus infected cell lysates harvested at 6 h post infection were analyzed for total PKR, phospho-PKR, phospho-eIF2α, and GAPDH by immunoblotting. The cell lysates harvested at 9 h post infection were analyzed for ICP5. As predicted, the amount of B virus Us11 protein expressed in U373-BVUs11 clone 12 prevented PKR activation and subsequent eIF2α phosphorylation induced by Δγ134.5 HSV-1 infection (Figure 24 B and C). In addition, Us11 significantly restored the synthesis of ICP5 protein in Δγ134.5 HSV-1 infected U373 cells at 9 h post infection. The amount of Us11 protein in U373-BVUs11 clone 4 and U373-HSVUs11 clone 10 was comparable and this amount of Us11 protein did not significantly prevent PKR activation and subsequent eIF2α phosphorylation induced by Δγ134.5 HSV-1 infection. However, Us11 partially restored synthesis of ICP5 protein in Δγ134.5 HSV-1 infected U373 cells (Figure 24D). These results suggested that B virus and HSV-1 Us11 proteins have similar functions when they are expressed at comparable levels.
Figure 24. Comparison of the effects of B-virus and HSV-1 Us11 protein on PKR activation, subsequent eIF2α phosphorylation and restoration of ICP5 protein synthesis in Δγ134.5 HSV-1 infected U373 cells. U373-pcDNA cells, U373-BVUs11 clone 4 and clone 12 cells, and U373-HSVUs11 clone 10 were mock infected or infected with Δγ134.5HSV-1 or WT HSV-1. (A) These lysates harvested at 6 h post infection were analyzed by immunoblotting for total PKR. (B) The lysates described as above were analyzed by immunoblotting for phospho-PKR. (C) The lysates described as above were analyzed by immunoblotting for phospho-eIF2α. (D) The lysates harvested at 9 h post infection were analyzed by immunoblotting for ICP5. (E) The lysates described as above were analyzed by immunoblotting for GAPDH.
**B Virus Us11 Protein Interacts Physically with Cellular PKR in Vitro.**

Previous data showed that B virus Us11 protein prevents PKR phosphorylation in vitro and in vivo. The mechanism by which B virus Us11 protein prevented PKR phosphorylation remains unknown. It was shown that the RXP repeats in the C-terminal domain of HSV-1 Us11 protein bind to PKR and prevent PKR phosphorylation (Peters et al., 2002). In addition, the C-terminal domain of B virus Us11 protein also contains these RXP repeats (Figure 1). Thus experiments were designed to test the hypothesis that B virus Us11 protein prevents PKR phosphorylation by binding to it. V5-tagged T7 gene 10-Us11 fusion protein was used in an anti-V5 pulldown assay to test for interaction with PKR. IFN-α-induced HeLa cell S10 extracts served as the source of inactive cellular PKR. S10 extracts were mixed with anti-V5 agarose beads prebound with V5-tagged T7 gene 10-Us11 or control protein P40 for potential interactions. As predicted, cellular PKR was not retained in anti-V5 agarose beads prebound with control protein P40. However, it was retained in the anti-V5 agarose beads prebound with T7 gene 10-Us11 protein (Figure 25A). These results clearly showed that T7 gene 10-Us11 fusion protein interacts physically with cellular inactive PKR.

To investigate whether the T7 gene 10 part or Us11 part of the fusion protein interacts with cellular PKR, interaction between T7 gene 10 protein and PKR was tested using Ni-NTA pulldown assays. The purification of T7 gene 10 protein was described previously (Perelygina et al., unpublished data). IFNα-induced HeLa cell S10 extracts were mixed with Ni-NTA agarose beads prebound with protein for potential interactions. As expected, cellular PKR was not retained on the Ni-NTA beads prebound with T7 gene 10
protein. In contrast, cellular PKR was retained on the Ni-NTA agarose beads prebound with T7 gene 10-Us11 protein (Figure 25B). These results demonstrate that it is the Us11 part, not the T7 gene 10 part of the recombinant Us11 protein that interacts physically with cellular PKR.
Figure 25. B virus Us11 protein interacts physically with inactive PKR in vitro. (A) Anti-V5 pull-down assay. IFN-α induced HeLa cell S10 extracts (the source of PKR) were incubated with anti-V5 agarose beads pre-bound with purified V5-tagged T7 gene 10-Us11 fusion protein or a control protein, V5-tagged P40 protein. The bound proteins were analyzed by immunoblotting for PKR. Lane 1, beads bound with T7 gene 10-Us11 fusion protein; lane 2, beads bound with control protein P40. (B) Ni-NTA pull-down assay. IFN-α induced HeLa cell S10 extracts (the source of PKR) were incubated with Ni-NTA agarose beads pre-bound with purified histidine-tagged T7 gene 10-Us11 fusion protein or histidine-tagged T7 gene 10 protein. The following procedures used were the same as described above. Lane 1, beads bound with T7 gene 10-Us11 fusion protein; lane 2, beads bound with T7 gene 10 protein; lane 3, beads only.
PART 5: B VIRUS US11 PROTEIN COUNTERACTS THE INNATE IMMUNE RESPONSES MEDIATED BY TYPE I IFN.

Previously, we demonstrated that B virus Us11 protein binds to PKR and prevents PKR phosphorylation \textit{in vitro} and \textit{in vivo}. Furthermore, PKR has been shown as one of the key players in the function of type I interferon (Langland et al., 2006). These findings prompted the investigation of whether B virus Us11 protein counteracted the innate immune responses mediated by type I IFN as a result of preventing PKR phosphorylation.

\textit{IFN-\(\alpha\) Pretreatment of U373 Cells Inhibited HSV-1 Replication and B Virus Us11 Protein Counteracted this Inhibition Effect}

\textit{IFN-\(\alpha\) was shown to be a good inducer of PKR in several cell lines (Mulvey et al., 2003; Mulvey et al., 2004). Therefore, in the following experiments, IFN-\(\alpha\) was utilized to pretreat U373 cells to induce PKR activation. IFN-\(\alpha\) pretreatment of U373 cells resulted in the inhibition of virus replication (Mulvey et al., 2004). To ascertain the ability of B virus Us11 protein to overcome the inhibitory effect of type I IFN on virus replication, U373 and U373-BVUs11clone 12 cells were treated with 0 or 1400 U IFN-\(\alpha\) for 16 hours. The cells were then infected with HSV-1 (Patton strain) at a MOI of 0.1. At 24 hpi, the virus titers in the supernatant were determined by a plaque assay. As predicted, the replication of HSV-1 in U373 cells was significantly inhibited by IFN-\(\alpha\) treatment (1400 U), as evidenced by 10,000- fold reduction of viral titers in the supernatants of IFN-treated cells compared to the untreated control. On the contrary, we observed only a 10- fold reduction of the HSV-1 titer in IFN-treated U373-BVUs11 cells.
compared to the untreated control, thus suggesting HSV-1 replication was only slightly inhibited by IFN-α treatment if Us11 protein was present (Figure 26). These results indicate that B virus Us11 protein can overcome the inhibiting effect of type I IFN on HSV-1 replication.
Figure 26. Plaque assay of supernatants from HSV-1 infected U373 and U373-BVUs11 clone 12 cell lines treated or untreated with IFN-α (1400 U/ml). U373 and U373-BVUs11 clone 12 cells treated or untreated with IFN-α were infected with HSV-1 at MOI of 0.1. At 24 h post infection, the supernatants were collected and were subjected to plaque assay.
This result was also confirmed by western blot. Both U373-pcDNA and U373-BVUs11 clone 12 cell lines were treated with increasing concentrations of IFN-α (0, 1.4, 14, 140, or 1400 U/ml) for 16 hours. The cells were then infected with HSV-1 strain Patton at MOI of 5. At 6 hpi, the cell lysates were collected and analyzed by immunoblotting for ICP5, the product of the HSV-1 γ1 gene. In the absence of IFN-α pretreatment, large quantities of ICP5 proteins were detected in the infected U373-pcDNA and U373-BVUs11 cells at 6 hpi. Using increasing amounts of IFN-α for pretreatment of U373-pcDNA cells resulted in progressive prevention of ICP5 accumulation. If the ICP5 levels in IFN-α−untreated HSV-1 infected U373-pcDNA cells were considered as 100%, the ICP5 levels in 1.4, 14, 140, and 1400 U/ml of IFN-α−treated infected U373-pcDNA cells were 66%, 59%, 42%, and 27%, respectively, suggesting IFN-α pretreatment is able to inhibit HSV-1 replication in a dose dependent manner. However, IFN-α pretreatment of U373-BVUs11 cells slightly reduced the ICP5 synthesis and accumulation (Figure 27). If the ICP5 level in IFN-α−untreated HSV-1 infected U373-BVUs11 clone 12 cells was considered as 100%, the ICP5 levels in 1.4, 14, 140, and 1400 U/ml of IFN-α−treated infected U373-BVUs11 cells were 100%, 81%, 85%, and 57%, respectively, suggesting that the HSV-1 replication is only slightly inhibited by IFN-α treatment if Us11 protein is present. This result also indicated that the B virus Us11 protein can overcome the inhibitory effect of type I IFN on HSV-1 replication.
Figure 27. Western blot analysis for ICP5 of IFN-α pre-treated and HSV-1 infected U373-pcDNA and U373-BVUs11 cell lysates. U373-pcDNA and U373-BVUs11 clone 12 cells were treated with increasing amounts of IFN-α (0, 1.4, 14, 140, and 1400 U/ml) for 16 hours and were then infected with HSV-1 at a MOI of 5. At 6 h post infection, the cell lysates were then collected and analyzed by immunoblotting for ICP5 and GAPDH, respectively. The band intensities of ICP5 were analyzed by Fuji film multigauge program. The ICP5 level in IFN-α-untreated WT HSV-1 infected cells was considered 100%, and the values for other treatments were presented as percentages of that value.
B Virus Us11 Protein Counteracts IFN-α Inhibitory Effects on HSV-1 Replication by Preventing PKR Phosphorylation.

Previous data showed that IFN-α pretreatment inhibited HSV-1 replication and that B virus Us11 protein counteracted this inhibitory effect of IFN-α on HSV-1 replication. In order to investigate whether B virus Us11 protein counteracted this inhibitory effect of IFN-α by preventing PKR phosphorylation, U373-pcDNA and U373-BVUs11 clone 12 cell lines were treated with 0, 1.4, 14, 140, or 1400 U/ml IFN-α for 16 hours. The cells were then infected with HSV-1 (Patton strain) at MOI of 5. The lysates of these cells were harvested at 6 h post infection and then analyzed by immunoblotting for total PKR, phospho-PKR, and GAPDH. Increasing concentration of IFN-α pretreatment of U373-pcDNA cells resulted in progressive phosphorylation of PKR, whereas in U373-BVUs11 clone 12 cell lines, the phosphorylation of PKR induced by IFN-α was significantly inhibited by B virus Us11 protein present in the cells (Figure 28). In U373-pcDNA cells, 1.4, 14, or 140 U/ml IFN-α treatment induced 5.7, 10.3, 9.6 fold PKR phosphorylation, respectively, whereas in U373-BVUs11 cells, 1.4, 14, or 140 U/ml IFN-α treatment resulted in 1.8, 3.5, or 5.8 fold phosphorylated PKR induction, respectively. The results suggested that B virus Us11 protein can overcome the IFN-α-block of HSV-1 replication by preventing PKR phosphorylation.
Figure 28. Western blot analyses for total, phospho-PKR and GAPDH of IFN-α pretreated and HSV-1 infected U373-pcDNA and U373-BVUs11 cell lysates. U373-pcDNA and U373-BVUs11 clone 12 cells were treated with increasing amount of IFN-α (0, 1.4, 14, 140, or 1400 U/ml) for 16 hours and were then infected with HSV-1. At 6 h post infection, the cell lysates were then collected and analyzed by immunoblotting for total PKR (A), phospho-PKR (B), and GAPDH (C), respectively. The band intensities of phospho-PKR were analyzed by Fuji film multigauge program. The phospho-PKR level in IFN-α-untreated WT HSV-1 infected cells was considered 1, and the values for other treatments were presented as folds of that value (D).
SECTION IV. DISCUSSION

Cellular type I IFN-induced anti-viral mechanism and virus counterdefense strategies

Interferon α/β was the first of the anti-viral innate immune modulators to be characterized. One of the key players in the interferon α/β system is the IFN inducible enzyme, PKR (Langland et al., 2006). A significant role of PKR in the innate response to virus infection is observed with a large number of different virus infections, including DNA and RNA viruses that encode PKR inhibitors. Viruses have developed mechanisms to inhibit the PKR response at virtually every stage in the eIF2α activation pathway, including binding to the dsRNA activator, blocking PKR activation, enhancing PKR degradation, and inhibiting PKR substrate phosphorylation. The γ134.5 gene product of HSV-1 plays a major role in counteracting PKR activation. It can complex with protein phosphatase 1α and promote the dephosphorylation of eIF2α (He et al., 1997).

The simplexvirus, B virus was shown in this dissertation to have no γ134.5 gene. The central hypothesis of these studies was that B virus Us11 gene product compensated for the absence of the γ134.5 gene. The function of HSV-1 Us11 protein in its natural context as a true late protein still remains unknown. Cassady et al. (1998) showed that Us11, if expressed as a true late protein in its natural context, prevented PKR activation ineffectively, whereas Mulvey et al. (2003) showed that Us11 protein of HSV-1 prevented PKR activation during the late phase of infection.
The long termini of the B virus genome lack a $\gamma_1$34.5 gene homolog of HSV-1

The observation that B virus replicates efficiently in neuroglial cells suggests that B virus may encode a protein that prevents PKR activation. In addition, B virus shares extensive homology with HSV-1 genetically. Because the $\gamma_1$34.5 gene product of HSV-1 plays a major role in counteracting PKR activation, it was logical to ask whether B virus encodes $\gamma_1$34.5 gene homolog of HSV-1 to prevent PKR activation. In order to answer this question, the B-virus genome termini were sequenced and the $\gamma_1$34.5 gene homolog of HSV-1 was mapped. To our surprise, the HSV $\gamma_1$34.5 homolog was observed to be absent in B virus. This conclusion was reached after repeated searches with state-of-the-art computational genome analysis tools. In addition, this result was confirmed by sequencing two additional low-passage clinical isolates.

The product of the $\gamma_1$34.5 gene inhibits the antiviral action of PKR by complexing with cellular protein phosphatase 1$\alpha$ and redirecting it to dephosphorylate eIF2$\alpha$, thus preventing premature protein synthesis shutoff and allowing the virus replication to continue. The $\gamma_1$34.5 deletion mutant of HSV-1 caused PKR phosphorylation, and subsequent eIF2$\alpha$ phosphorylation in infected U373 cells and could not replicate efficiently because of premature protein synthesis shutoff. The sequencing result showed that B virus lacks an HSV-1 $\gamma_1$34.5 homolog. The finding that B virus lacks a $\gamma_1$34.5 homolog prompted us to investigate whether B virus still has the ability to prevent PKR and eIF2$\alpha$ phosphorylation in infected neuronal cells.
B virus is able to prevent PKR activation and subsequent eIF2α phosphorylation in U373 cells.

Data shown in the “Results” section support that B virus infection is similar to infection caused by WT HSV-1 in neuroglial cells. And further, analysis revealed that B virus prevented PKR and eIF2α phosphorylation despite the absence of the γ134.5 protein. Chou et al. (1995) showed that PKR kinase was activated to the same extent in WT and Δγ134.5 HSV-1 infected HeLa cells, although the phosphorylation level of eIF2α was lower in WT HSV-1 infected HeLa cells compared with that in Δγ134.5 HSV-1 infected HeLa cells. In the studies for this dissertation, phospho-PKR levels in WT HSV-1 infected U373 cells were much lower than those in Δγ134.5 HSV-1 infected U373 cells. One reason for this contradiction could be that different cell lines and different MOI were used to infect cells, or different methodology to measure PKR activity. The low phospho-PKR level observed in WT HSV-1 infected U373 cells at 6 h post infection was puzzling. This low PKR activity observed at 6 h post infection was not due to HSV-1 Us11 protein, which prevents PKR activity, since HSV-1 Us11 protein was expressed until at least at 8 h post infection (He et al., 1997). This low PKR activity was not due to HSV-1 Us11 protein of virion, because the function of virion Us11 might be distinct from the previously described function: preventing PKR activity (Chou and Roizman 1992). This leads to the hypothesis that the γ134.5 gene product of WT HSV-1 complexes with PP1α and redirects it to dephosphorylate not only eIF2α but also PKR. This hypothesis remains to be tested.
Because PKR is one of the key players in the function of type I IFN, the fact that B virus is able to prevent PKR activation suggests that B virus has an interferon-resistant phenotype. Maybe this interferon-resistant property of B virus can partially explain why this virus is highly pathogenic in human. Which protein of B virus prevents PKR activation still remained unknown at this point.

**B virus Us11 protein counteracts the action of PKR.**

Us11 is the other HSV-1 protein that can prevent PKR activation at late times of infection. In addition, the HSV-1 Us11 protein can inhibit PKR activation and compensate for the absence of the ICP34.5 function in deletion mutants if expressed early in infection as a result of mutation (He et al., 1997; Cassady et al., 1998). The functionality of HSV-1 Us11 protein depends on the 24 RXP repeats in the C-terminal domain (Roller et al., 1996; Peters et al., 2002). Except for the γ134.5 gene, B virus contains all the other gene homologs of HSV-1, including Us11. Comparison of amino acid sequences between the B virus and HSV-1 Us11 proteins showed that the C-terminal domain of B virus Us11 protein contains 20 RXP repeats (Perelygina et al., 2003), suggesting B virus Us11 protein may have a function similar to HSV-1 Us11 protein. PKR activation was shown to occur at early times of HSV-1 infection and HSV-1 Us11 protein can prevent PKR activation effectively only when expressed prior to the activation of PKR. Therefore, to inhibit the action of PKR successfully, B virus Us11 protein should be expressed with early kinetics.

**B virus Us11 protein is expressed at early times of infection.**
This early Us11 appearance was not a result of B virus adaptation in cell culture, since we also detected this protein in cells infected with low passage clinical isolates at the same time post infection as in lab strain-infected cells. Moreover, the kinetics of Us11 expression in LLC-MK2 cells derived from natural B virus host was indistinguishable from the Us11 kinetics in infected Vero cells. Most interestingly, newly synthesized Us11 appeared in B virus infected cells notably earlier (3 hpi) than the homologous protein in cells infected either with wt HSV-1 (9 hpi) or even with recombinant HSV-1 (4 to 6 hpi) expressing the Us11 gene under the immediate early promoter of the α47 gene (He et al., 1997; Cassady et al., 1998). Remarkably, Us11 protein was also detectable by immunoblotting at 0 hpi, and most likely was delivered into the cytoplasm of B virus infected cells by virions. HSV-1 Us11 is a tegument component and estimated to be present at 600 to 1000 copies per virion (Roller and Roizman 1992). Although quantitative analysis was not performed, we believe that B virus tegument contains more Us11 molecules per virion than HSV-1 tegument based on the fact that this protein was easily detectable in cells immediately after infection with B virus at an MOI of 3 while tegument-derived Us11 has never been seen in HSV-1-infected cells on immunoblots.

The expression of B virus Us11 protein was not blocked by PAA, an inhibitor of DNA replication.

We have demonstrated that viral DNA replication is not required for the appearance and accumulation of the B virus Us11 protein in infected cells, and, therefore, unlike the HSV-1 Us11 gene (Johnson et al., 1986), the B virus homolog is not expressed as a true late gene. This feature of B virus Us11 is critical, as this protein can inhibit PKR only if
present in a cell prior to PKR activation (Mulvey et al., 1999), the latter which was shown to occur after the onset of viral DNA synthesis in HSV-1 infected cells (Chou and Roizman 1992). B virus DNA synthesis begins between 4 and 5 hpi (Hilliard et al., 1987), when B virus infected cells have already accumulated notable amounts of Us11, and this might be sufficient to bind all inactive PKR molecules blocking their activation. Another essential characteristic of B virus is its capability to maximize Us11 protein production by 9 hpi, rather than later by 12-16 hpi as in HSV-1 infections (Johnson et al., 1986). This enables production of copious amounts of Us11 before massive production of late mRNAs and dsRNA in B virus infected cells. Although RNA-binding activity of B virus Us11 has not been verified experimentally, this protein contains C-terminal conserved RXP elements, which were shown in HSV-1 Us11 to interact with both dsRNA and inactive PKR and thus, prevent PKR activation (Poppers et al., 2000). Therefore, due to its early and abundant expression, B virus Us11 was tested for its ability to block PKR activation in B virus infected cells, by both direct physical association with PKR and competition for dsRNA, which would prevent protein synthesis shutoff.

While early expression of Us11 can supply one function of ICP34.5 in HSV-1 null mutants, i.e., blocking shutoff of protein synthesis, it cannot fully restore this mutant to wild type replication properties (Mohr et al., 2001; Cheng et al., 2003). ICP34.5 is a multifunctional protein, and additional functions essential for efficient viral replication and neuronal spread have been mapped to both the N terminal and C terminal domains (Chou et al., 1990; Cheng et al., 2003; Harland et al., 2003; Jing et al., 2004). It is
presently unclear whether the Us11 gene supplies these additional ICP34.5 functions for B virus and other alphaherpesviruses lacking the \( \gamma_1 \),34.5 gene homolog (Tyler et al., 2005) or whether they have evolved alternative mechanisms to control their replication in neurons.

**B virus Us11 protein exists as 2 forms: 20-kDa monomer and 40-kDa dimer.**

Interestingly, unlike HSV-1 protein, B virus Us11 exists in infected cells in two forms as observed by the presence of 20-kDa and 40-kDa bands. The estimated mass of the fast migrating form of B virus Us11 was slightly smaller than that of 21-kDa Us11 protein of HSV-1 (Rixon and McGeoch 1984; Johnson et al., 1986), which is most likely attributable to the 14-residues difference between the corresponding predicted polypeptides. However, a Us11 band predicted to have a molecular mass less than 20-kDa in B virus infected cells has never been observed despite the fact that this is often seen in HSV-1 infected cells and believed to reflect incompletely phosphorylated Us11 precursors (Simonin et al., 1995).

There are several possible explanations for the nature of the 40-kDa band: (1) a cellular or B virus protein cross-reactive with Us11 antibody; (2) protein translated from a longer, alternatively spliced Us11 transcript; (3) posttranslational modification of Us11; (4) covalently linked stable complex between Us11 and a cellular protein or another viral protein; (5) covalently linked stable Us11 dimer.

Several lines of evidence argue strongly against the first possibility. Us11 antiserum did not react with proteins in mock infected, or HSV-1 infected cell lysates, or lysates of a number of cell cultures transfected with an empty expression vector. Most importantly,
the 40-kDa form protein was not observed in cells infected with 2 out of 4 B virus isolates tested. In addition, two forms of V5-tagged recombinant Us11, 25 kDa and 50 kDa, were also detected in cells transfected with the Us11 recombinant construct. The recombinant 50-kDa form of Us11 migrated in an SDS gel slower than its natural isoform and was recognized by Us11 and V5 antibody. These data support the Us11-specificity of the 40-kDa band.

Because the large Us11 isoform was detected in cells transfected with the Us11 plasmid, and no other viral sequences contained unidentified introns, alternative splicing does not explain the observed isoform in the transfected cells. Unfortunately, a Us11-specific probe for northern blot analysis could not be designed, because of the predicted extensive overlap of Us12, Us11, and Us10 transcripts, as seen in Figure 15, and, consequently, unequivocal identification of the B virus Us11 transcript was not possible. Nevertheless, northern blot analysis of cells infected with different isolates with a probe containing the Us11 ORF revealed that, regardless of the presence of the 40-kDa band in cell lysates, the transcript patterns in the infected cells were identical, suggesting that hypothetical alternative splicing of the Us11 transcript could not be responsible for appearance of 40 kDa protein.

The 20-kDa increase in the molecular mass of B virus Us11 is unlikely to be the result of posttranslational modifications in Us11 precursor. The predicted molecular mass of B virus Us11 is 16.6 kDa. And like HSV-1 Us11, the 20-kDa protein is most likely a fully phosphorylated, mature form of Us11. A significant 20-kDa increase of protein molecular mass is typically caused by glycosylation, but since no glycosylation sites were
predicted in Us11, Us11 modification by glycosylation appears to be highly unlikely, leaving the observed 40 kDa immunoreactive band unexplained.

Formation of irreversible Us11 homodimers was considered as the most probable alternative explanation of the 40 kDa band, since the exact twofold difference in molecular mass was observed in both infected (20 kDa vs. 40 kDa) and transfected (25 kDa vs. 50 kDa) cell cultures. The capability to form heterogeneous oligomers was also noted for HSV-1 Us11, but oligomers were not linked irreversibly (Diaz et al., 1993). On the contrary, B virus Us11 dimers were extremely stable, since 8 M urea treatment or boiling with 1% SDS/1% β-ME could not disrupt them. It was demonstrated that the ability of certain B virus isolates to form Us11 dimers was not related to the Us11 gene polymorphism detected, since the recombinant proteins produced from different Us11 alleles formed dimers in transfected cells, although at considerably lower levels. The functional significance of its appearance in infected cells remains unknown. Us11 dimers might possess novel biological properties and perform unique functions in the infected cells. Alternatively, irreversible dimerization of Us11 could be a cellular counter mechanism to inactivate this protein during B virus infection. Furthermore, Us11 itself might inactivate some cellular proteins by forming stable complexes with them. Further investigations are needed to resolve these issues.

**B virus Us11 protein prevents PKR activation in vitro and in vivo.**

This study demonstrated that B virus Us11 protein prevents PKR activation in vitro. When increasing amounts of B virus Us11 fusion protein were added to the reaction, PKR phosphorylation was inhibited, whereas the addition of control protein had no effect.
on PKR phosphorylation. In addition, B virus Us11 protein inhibited PKR and subsequent eIF2α phosphorylation in Δγ134.5 HSV-1 infected U373 cells. In Δγ134.5 HSV-1 infected U373-pcDNA cells, relatively higher levels of phospho-PKR and phospho-eIF2α were detected by immunoblotting analysis, while in Δγ134.5 HSV-1 infected U373-BVUs11 clone 4 and clone 12 cell lines, lower levels of phospho-PKR and phospho-eIF2α were detected, especially in clone 12 cells. As a consequence of preventing PKR and subsequent eIF2α phosphorylation, B virus Us11 protein complements Δγ134.5 of HSV-1 in U373 cells. Two lines of evidence indicate that B virus Us11 protein complements Δγ134.5 of HSV-1 in U373 cells. Specifically, the accumulation of protein ICP5 in Δγ134.5 of HSV-1 infected U373-BVUs11 clone 4 and clone 12 at 9 h post infection increased compared with that of Δγ134.5 of HSV-1 infected U373-pcDNA cells. And furthermore, the virus titers in supernatant from Δγ134.5 HSV-1 infected U373-BVUs11 clone 12 were 10- to 10^3 fold increased compared to those for Δγ134.5 of HSV-1 infected U373 cells. Lastly, analysis of data revealed that B virus Us11 protein binds PKR in vitro as determined by using pulldown assays, supporting the hypothesis that Us11 protein prevents PKR activation by binding to it. However, these results also raise some very interesting issues.

First, two B virus Us11 stable expression cell lines, clone 4 and clone 12, were established. The Us11 protein expression level in clone 4 was similar to that in BV infected cells at 2 h post infection, whereas the Us11 expression level in clone 12 was similar to that in BV infected cells at 4h or 6h post infection. In addition, the amount of B virus Us11 protein expressed in clone 12 significantly reduced PKR activation and
subsequent eIF2α phosphorylation in Δγ134.5 HSV-1 infected U373 cells, whereas the amount of B virus Us11 protein expressed in clone 4 did not demonstrate this effect. This is quite understandable. At 2 h post infection, when PKR is not phosphorylated, it is not surprising to see that Us11 protein in BV infected cells at this time is not present in sufficient concentrations to prevent PKR phosphorylation significantly. However, at 4h or 6h post infection, when PKR is either about to be phosphorylated or phosphorylated already, Us11 protein levels in BV infected cells at this time are sufficiently great enough to inhibit PKR phosphorylation, and subsequent eIF2α phosphorylation.

Second, the accumulation of protein ICP5 in Δγ134.5 HSV-1 infected U373-BVUs11 clone 4 and clone 12 at 9 h post infection was much greater compared with that in Δγ134.5 HSV-1 infected U373-pcDNA cells. Surprisingly, at 6 h post infection, the accumulation of protein ICP5 in Δγ134.5 HSV-1 infected U373-BVUs11 clone 4 and clone12 was only slightly higher than that in Δγ134.5 HSV-1 infected U373-pcDNA cells (data not shown). ICP5, a major capsid protein of HSV-1, is a γ1 protein, suggesting it is expressed around 4 h post infection and its expression does not totally depend on DNA replication. Chou and Roizman (1992) showed that at 5 h post infection, viral DNA synthesis or tightly linked events, such as accumulation of γ2 transcripts, trigged PKR, subsequent eIF2α phosphorylation, and protein synthesis shutoff. Therefore, ICP5 in Δγ134.5 HSV-1 infected U373-pcDNA cells was synthesized before the protein synthesis shutoff, which occurs at about 5 h post infection. When protein synthesis shutoff occurs, probably at 5 h post infection, ICP5 synthesis stops in Δγ134.5 HSV-1 infected U373-
pcDNA cells, whereas ICP5 synthesis continues in Δγ134.5 HSV-1 infected U373-BVUs11 cells because B virus Us11 protein stably expressed in the cells prevents PKR activation, subsequent eIF2α phosphorylation, and protein synthesis shutoff. Therefore, at 6 h post infection, there was no significant difference in the accumulation of protein ICP5 in Δγ134.5 HSV-1 infected U373-BVUs11 and U373-pcDNA cells. However, at 9 h post infection, the accumulation of protein ICP5 in Δγ134.5 HSV-1 infected U373-BVUs11 clone 12 was significantly greater than that in Δγ134.5 HSV-1 infected U373-pcDNA cells. In Δγ134.5 HSV-1 infected U373-BVUs11 clone 12 cells, HSV-1 Us11 protein just started to appear at 9 h post infection and the amount of HSV-1 Us11 protein was relatively low at this point. The accumulation of ICP5 in Δγ134.5 HSV-1 infected U373-BVUs11 clone 12 cells at 9 h post infection is associated with stably expressed B virus Us11 protein rather than HSV-1 Us11 protein, because of the following two reasons: 1) HSV-1 Us11 protein, when expressed after PKR phosphorylation, prevented PKR phosphorylation ineffectively. 2) the levels of HSV-1 Us11 protein were very low at 9 h post infection and not sufficient for preventing PKR activation.

B virus Us11 protein expressed in U373 cells supported Δγ134.5 HSV-1 replication in these cells in dose dependent manner. The reason was apparent, since in Δγ134.5 HSV-1 infected U373-pcDNA cells, PKR and subsequent eIF2α were found to be phosphorylated, whereas in Δγ134.5 HSV-1 infected U373-BVUs11 cells, B-virus Us11 prevented PKR and subsequent eIF2α phosphorylation by binding PKR, thus keeping proper translation rates necessary for virus replication. However, the amount of
Us11 protein expressed in U373 cells was inversely proportional to the replication rates of WT HSV-1. In WT HSV-1 infected U373-pcDNA cells, the γ134.5 gene product of HSV-1 complexes with PP1α and redirects it to dephosphorylate eIF2α, keeping phosphorylated-eIF2α at low levels. Therefore, Us11 expressed in U373 cells does not provide any advantage for WT HSV-1 to replicate. Roller and Roizman (1994) reported that a HSV-1 Us11–expressing cell line was resistant to HSV-1 infection, but the exact step at which the infection was blocked was not known. U373-BVUs11 clone 4 and clone 12 grow much slower than control U373 cell lines. Therefore, it is reasonable to speculate that B virus Us11 protein, the DNA and RNA binding protein, may have some side effects on U373 cell growth. This low growth rate in B virus Us11 stable expression cell line may be responsible for the low virus yield.

Lastly, this study demonstrated that B virus Us11 protein binds to PKR and prevents PKR activation \textit{in vitro} and \textit{in vivo}. Whether Us11 protein is the only protein of B virus to prevent PKR activity remains unknown. Construction of a Us11 deletion mutant of B virus will be helpful in answering this question.

\textbf{B virus Us11 protein, when expressed at the comparable levels to HSV-1 Us11 protein levels in U373 cells, has a similar function to HSV-1 Us11 protein.}

In order to compare the function of B virus and HSV-1 Us11 protein in U373 cells, HSV-1 Us11 stable expression U373 cell lines were also established. Interestingly, clone 10, which expressed the most abundant levels of HSV-1 Us11 among U373-HSVUs11 clones only expressed similar levels of Us11 protein as that in U373-BVUs11 clone 4. However, Mohr et al. conducted similar experiments, but were unsuccessful at
generating HSV-1 Us11 stable expression in U373 cells (personal communication). The reason that none of the U373-HSVUs11 clones expressed equal amounts of Us11 protein to the U373-BVUs11 clone 12 still remains unexplained. Because HSV-1 Us11 protein is shown to bind to ribosomal 60S subunit (Roller and Roizman 1992), overexpression of this protein in cells might affect protein translation and have toxic effects on the cells. The observations that the morphology of some clones of U373-HSVUs11 cells were abnormal and that they never divided and grew supports this possibility.

Even though the amount of Us11 protein expressed in U373-BVUs11 clone 4 and U373-HSVUs11 clone 10 was not sufficient to prevent PKR and eIF2α phosphorylation significantly in Δγ134.5 HSV-1 infected U373 cells, it did partially restore ICP5 protein synthesis in Δγ134.5 HSV-1 infected U373 cells. Perhaps the methods used to detect the level of phospho-PKR and phospho-eIF2α was not sufficiently sensitive to detect subtle changes. Us11 protein of B virus or HSV-1 prevents PKR activation, subsequent eIF2α phosphorylation, and restoration of virus protein synthesis in Δγ134.5 of HSV-1 infected U373 cells in a dose-dependent manner. It is not surprising that B virus and HSV-1 Us11 protein share similar functions, since they each contain RXP repeats in the C-terminal domain, which is critical for functionality. However, the N-terminal domain of B virus Us11 protein has little homology to the corresponding part of the HSV-1 homolog. No information on the function of N-terminal domain of B virus or HSV-1 Us11 protein is currently available. We speculate that lack of success in producing an HSV-1 Us11 stably expressing cell line is because of its unique N-terminal domain.
B virus Us11 protein counteracted IFN-α inhibitory effect on HSV-1 replication by preventing PKR phosphorylation.

WT HSV-1 which contains the $\gamma_134.5$ gene is more resistant to interferon pretreatment when compared with $\Delta\gamma_134.5$ HSV-1 since the $\gamma_134.5$ gene product complexes with protein phosphotase 1 $\alpha$ and dephosphorylates eIF2$\alpha$ (Mulvey et al., 2004). However, the increasing amounts of IFN-α used for pretreatment of U373-pcDNA cells did inhibit WT HSV-1 replication indicating that the HSV-1 interferon resistance is limited. Assuming the ICP5 levels in untreated U373-pcDNA cells as 100%, ICP5 protein levels were 42% and 27%, respectively, in 140 U/ml and 1400 U/ml IFN-α-treated U373-pcDNA cells, However, if ICP5 levels in untreated U373-BVUs11 cells were assumed as 100%, ICP5 protein levels were 85% and 57%, respectively in 140 U/ml and 1400 U/ml IFN-α-treated U373-BVUs11 clone 12 cells. These results suggested that B virus Us11 protein, when constitutively expressed in U373 cells provided the extra capacity for HSV-1 to counteract IFN-α.

Next, the demonstration that B virus Us11 protein counteracted type I IFN inhibitory effect by preventing PKR phosphorylation was accomplished experimentally. This provided the first evidence that the B virus Us11 protein inhibits the innate immune response mediated by type I IFN. In addition to Us11, other proteins of B virus may be also involved in resisting the effects of type I IFN, for example ICP0. However, the Us11 protein of B virus at least partially explains the interferon-resistant phenotype. It raises the possibility that the interferon-resistant function of B virus Us11 protein could play a critical role in viral pathogenesis.
Overall discussion

The experiments performed for this dissertation have revealed for the first time that the B virus genome lacks a homolog of HSV-1 γ134.5 gene, which has been shown to be critical for HSV-1 to counteract PKR activation. Also, from the experiments, it is clear that B virus is able to prevent PKR activation and subsequent eIF2α phosphorylation in the absence of a γ134.5 gene homolog. Furthermore, B virus Us11 protein is expressed at early times of infection as evidenced from the data presented, and Us11 is not a true late gene in B virus. Early expression of B virus Us11 protein during infection is the likely determinant responsible for counteracting PKR activation. The presented studies demonstrate that B virus Us11 protein prevented PKR activation and subsequent eIF2α phosphorylation in vitro and in vivo by binding to PKR. Lastly, the data support the hypothesis that B virus Us11 protein counteracts the innate immune response mediated by type I IFN in U373 cells by preventing PKR phosphorylation.

These observations have led to the formulation of the model shown in Figure 29. It is known that PKR is expressed constitutively at a basal level in cells, and its expression is induced several fold by treatment of type I IFN or viral infection. In addition, constitutively expressed or induced PKR normally exists as a latent form having the DRBM-II interacting with its catalytic domain and inhibiting kinase activity (Wu and Kaufman 1997). Although usually inactive, PKR can be activated, usually by dsRNA. It is known that PKR activation is achieved by a conformational change of the protein. Binding of two PKR molecules to the same dsRNA leads to formation of stable dimers and dissociation of the inhibitory interaction between DRBM-II and the kinase domain.
Formation of PKR dimer also facilitates trans-autophosphorylation of multiple sites by the two kinase moieties in a PKR dimer (Huang et al., 2002). Besides dsRNA, type I IFN induces tyrosine phosphorylation of PKR through activation of JAK1 and TYK2. The activated PKR can cause phosphorylation of eIF2α, which results in the global protein synthesis shutoff in cells and stop of virus replication. At an early phase of infection, PKR activated by dsRNA not only inhibits viral protein synthesis, but also induces IFN-β gene expression through the activation of NF-κB. At a later phase of infection, the produced IFN activates JAKs and induces PKR phosphorylation. However, binding of the B virus Us11 protein to PKR would prevent the conformational switch of PKR, keeping it in a closed inactive conformation. Therefore, PKR activation would be blocked and the downstream pathway would also be blocked and virus replication would continue.
Figure 29. Model of B virus Us11 inhibition of cell anti-viral activity mediated by PKR.

Inactive PKR

Us11

Kinase domain

DRBM-IDRBMI-II

IFN + IFNR

Active PKR

Us11

Activate Jak1

Inhibition of protein synthesis

Viral replication blocked

eIF2

dsRNA

IFN

P

P

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This study demonstrated that B virus Us11 protein prevented PKR phosphorylation induced by 2 molecules: dsRNA and type I IFN. In addition, B virus Us11 protein was shown to interact physically with PKR. Other investigators demonstrated that HSV-1 Us11 protein binds to PKR and dsRNA through its RXP repeats in the C-terminal domain and prevents PKR activation by dsRNA and PACT. Since B virus Us11 protein prevents PKR activation induced by type I IFN and HSV-1 Us11 protein prevents PKR activation induced by PACT in dsRNA independent manner, the Us11 protein is likely to result in PKR activation mainly by directly binding to it. By physical interaction with PKR, B virus Us11 protein prevents PKR conformation switch, which can be induced by dsRNA, JAK or PACT.

Although B virus shares extensive homology with HSV-1, B virus and HSV-1 have apparently have selected for different mechanisms to counteract the action of PKR. Selection implies that these mechanisms are advantageous to the respective virus hosts. HSV-1, a human virus, predominantly utilizes the $\gamma_1$34.5 gene product to dephosphorylate eIF2\(\alpha\), whereas B virus, a macaque virus, maintains a primitive strategy by using the early expressed Us11 to prevent PKR activity. The carboxyl terminus of the $\gamma_1$34.5 gene product is homologous to the corresponding terminus of murine protein known as MyD116 (Lord et al., 1990) or a Chinese hamster protein known as GADD34 (Fornace et al., 1989), and its function can be substituted by the carboxyl terminus of the MyD116 protein (He et al., 1996). Therefore, HSV-1 “borrowed” part of a host gene to complex with PP1\(\alpha\) and dephosphorylate eIF2\(\alpha\), in order to maintain proper translation rates.
Besides PKR, mammalian cells contain three additional eIF2α kinases (PERK, GCN2, HRI), each of which inhibits translation in response to a different physiological stress. While the accumulation of dsRNA activates PKR, the accumulation of unfolded proteins in the ER lumen activates PERK, nutrient deprivation triggers GCN2 and reduced levels of heme induce HRI activity (Mulvey et al., 2007). HSV-1 γ134.5 protein binds to PP1α to maintain adequate active eIF2α. By virtue of acting downstream of eIF2α phosphorylation, it has the potential to counteract multiple eIF2α kinases. B virus Us11 is a PKR-specific antagonist that physically interacts with PKR and prevents its activation by dsRNA and PACT. It seems that the γ134.5 protein of HSV-1 is more efficient at keeping the levels of phosphorylated eIF2α low than does B virus Us11. HSV-1 Us11 protein was shown to inhibit OAS activity by sequestering dsRNA through C-terminal RXP repeats (Sanchez and Mohr 2007). Therefore, the early expressed B virus Us11 protein would not only prevent PKR activity, but also inhibit 2’-5’ OAS activity, thereby counteracting the type I IFN response.

Sequence data showed that the macaque genome also contains a GADD34 homolog (Gibbs et al., 2007). The reason why B virus did not “borrow” part of GADD34 homologous gene to create a γ134.5 type viral protein to counteract PKR activity and instead uses only Us11 to prevent PKR activity remains unknown.

In summary, studies done as part of this dissertation reveal that two very similar viruses have selected distinctly different mechanisms to arrive at the same conclusion within their respective hosts. The possible selective advantages of these different mechanisms can only be understood by further study of the benefits of these mechanisms.
within the microenvironments of cells from each species. B virus infection in the natural hosts, macaque monkeys, very closely resembles HSV-1 infections in humans. When B virus, however, infects humans, the outcome of infection is dramatically different than what is seen in macaques. Morbidity and mortality of B virus infections in the foreign host, viz., humans, is accompanied by a lack of significant levels of antibody induction, profound regional cell death, and ultimately loss of a viable host system in which the virus can replicate. The studies presented in this dissertation, for the first time, reveal a glimpse of the different mechanisms each virus utilizes to maintain the host cell in a state that permits virus replication. Perhaps these different mechanisms reflect each virus’ tactical approach to ensure replication. However, these differences may ultimately be linked to the inability of a foreign host to constrain virus replication in a manner, which allows survival of both host and virus.
Section V. References


unrelated polypeptide one of which has highly reiterated amino acid sequence."


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