Comparing the Activities of US11, A Herpes Simplex Virus Protein that Binds Double-stranded RNA and Inhibits PKR

By
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Submitted to the University of Manitoba, Faculty of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of

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Department of Medical Microbiology and Infectious Diseases
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Comparing the Activities of US11, A Herpes Simplex Virus Protein that Binds Double-stranded RNA and Inhibits PKR

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

MASTER OF SCIENCE

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<th>Description</th>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CD4+</td>
<td>Cluster of differentiation 4</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy terminal</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl Pyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Mixture of four deoxynucleotides</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF-Tu</td>
<td>Bacterial elongation factor Tu</td>
</tr>
<tr>
<td>eIF2-α</td>
<td>Eukaryotic elongation initiation factor α</td>
</tr>
<tr>
<td>EMSA(s)</td>
<td>Electrophoretic Mobility Shift Assay(s)</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>G+C</td>
<td>Guanine and Cytosine</td>
</tr>
<tr>
<td>gD2</td>
<td>Glycoprotein D2</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HP</td>
<td>High performance</td>
</tr>
<tr>
<td>HSV1</td>
<td>Herpes Simplex Virus type 1</td>
</tr>
<tr>
<td>HSV2</td>
<td>Herpes Simplex Virus type 2</td>
</tr>
<tr>
<td>HVP2</td>
<td>Herpes Virus papio, type 2 (Cercopithecine herpesvirus 16)</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early</td>
</tr>
<tr>
<td>IFNα</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>KCL</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>LATs</td>
<td>Latency-associated transcripts</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>M</td>
<td>Molar concentration</td>
</tr>
</tbody>
</table>
MgCl₂  Magnesium Chloride  
MOI  Multiplicity of Infection  
mRNA  Messenger ribonucleic acid  
MW  Molecular weight  
MWM  Molecular weight marker  
NaCl  Sodium chloride  
NaPO₄  Sodium phosphate  
NCBI  National Center for Biotechnology Information  
NF-kB  Nuclear factor-kappa B  
Ni  Nickel  
NK  Natural killer  
NML  National Microbiology Laboratory  
N-terminal  Amino terminal end  
OAS  2'-5' oligoadenylate synthetase  
OD  Optical Density  
ORF  Open reading frame  
PAGE  Polyacrylamide Gel Electrophoresis  
PBS  Phosphate Buffered Saline  
PBS-T  Phosphate Buffered Saline with 0.1% Tween-20  
PCR  Polymerase Chain Reaction  
PKR  Protein kinase R  
PP₁α  Protein phosphatase 1 type alpha  
RBM  RNA binding motif  
RCF  Relative centrifugal force  
RNA  Ribonucleic acid  
RPM  Rotations per minute  
RRM  RNA recognition motif  
R-X-P  Arginine-X-Proline, where X is often an uncharged amino acid  
S200  Superdex™ 200  
SA8  Simian Agent 8 (*Cercopithecine herpesvirus* 2)  
SDS  Sodium Dodecyl Sulfate  
TBE  Tris borate EDTA buffer  
TE  Tris EDTA buffer  
TEMED  N,N,N',N'-Tetra-methy-ethylenediamine  
UL  Unique long region  
US  Unique short region  
UV  Ultra violet
Abstract

US11 is the only protein found in all members of the genus Simplexvirus that is able to inhibit the cessation of protein synthesis by targeting protein kinase R (PKR). US11 is a ribosome-associated dsRNA binding protein with a novel RNA-binding motif. US11 has been shown to bind dsRNA via its C-terminal end through a series of arginine-X-proline repeats, where x is often an uncharged amino acid. The US11 protein is highly conserved within HSV1, HSV2, B virus, SA8 and HVP2, however the number of R-X-P repeats is different within the protein from each virus. We set out to investigate whether there is a difference in the US11 proteins from these viruses in its ability to bind dsRNA. The ability of US11 to bind dsRNA may be important in its method of inhibiting the activation of PKR by sequestering dsRNA or using it to get into closer proximity to PKR for inactivation.

We have cloned and expressed US11 from HSV1, HSV2, B virus and HVP2. We have purified US11 via affinity chromatography and have shown that the protein remains active using electrophoretic mobility shift assays (EMSAs). Through filter binding assays we have shown that US11 binds dsRNA of 42 base pairs with an affinity of 2.6nM, independent of its viral source. Therefore at the dsRNA binding level, there is no comparable difference in the US11 proteins within simplexviruses. The next step of this project is to investigate the direct inhibition of PKR by US11 from these viruses in order to gain further insight into the pathogenicity between the simplexviruses.
1.0 Introduction

1.1 Herpesviridae

The *Herpesviridae* are a family of large double stranded DNA viruses. Herpesviruses are ubiquitous in nature and infect a wide range of primate and non-primate hosts. To date, over 130 different herpesviruses have been identified. Typically, herpesviruses are 120-220 nm in diameter and structurally complex consisting of the virus core, the capsid, the tegument and outer envelope. The virus core consists of the toroidal shaped linear viral DNA surrounded by a proteinaceous layer. The herpesvirus capsid is complex and it consists of pentameric capsomeres at the vertices and longitudinal hexameric capsomeres. Recent literature shows that the herpesvirus capsid is related in structure to the large DNA bacteriophages. Between the capsid and envelope lies a proteinaceous layer referred to as the tegument. The thickness of the tegument varies depending on the location of the virus in infected cells. The tegument is largely unstructured and contains viral proteins, some of which will be discussed in more detail later. Identified proteins found in the tegument of herpes simplex are listed in table 1. The herpesvirus envelope is made of a membrane derived from the host cells, in which viral glycoproteins are embedded. The virion structure is highly conserved among herpesviruses and they are indistinguishable in electron micrographs. Herpesviruses are characteristically able to cause both lytic and latent infections.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Essential (E) or Nonessential (N)</th>
<th>Possible Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL14</td>
<td>N</td>
<td>Aids in virus spread from cell to cell. Null mutants unable to spread efficiently.</td>
</tr>
<tr>
<td>UL17</td>
<td>E</td>
<td>Required for cleavage packaging of DNA and transport within nuclei.</td>
</tr>
<tr>
<td>UL36</td>
<td>E</td>
<td>Phosphoprotein, in temperature-sensitive mutants DNA is not able to be released from capsid at nuclear pores at nonpermissive temperatures.</td>
</tr>
<tr>
<td>UL41</td>
<td>N</td>
<td>Phosphoprotein that causes nonspecific degradation of mRNA and shutoff of macromolecular synthesis after infection.</td>
</tr>
<tr>
<td>UL46</td>
<td>N</td>
<td>Phosphoprotein thought to modulate the activity of UL48.</td>
</tr>
<tr>
<td>UL47</td>
<td>N</td>
<td>Phosphoprotein, thought to modulate the activity of UL48, associate with the nuclear matrix and bind DNA.</td>
</tr>
<tr>
<td>UL48</td>
<td>E</td>
<td>Induces α genes by interacting with cellular proteins Oct-1 and HCF.</td>
</tr>
<tr>
<td>UL49</td>
<td>E</td>
<td>Phosphoprotein transported from infected to uninfected cells by direct extension.</td>
</tr>
<tr>
<td>US10</td>
<td>N</td>
<td>Unknown</td>
</tr>
<tr>
<td>US9</td>
<td>N</td>
<td>Unknown</td>
</tr>
<tr>
<td>US11</td>
<td>N</td>
<td>Abundant protein, binds to the 60S ribosomal subunit and localizes to the nucleolus; if expressed early in infection, it will inhibit PKR from shutting off protein synthesis.</td>
</tr>
<tr>
<td>ICP22</td>
<td>N</td>
<td>Nucleotidylated regulatory protein, required for optimal expression of a subset of γ proteins.</td>
</tr>
</tbody>
</table>
1.1.2. Taxonomy

Herpesviruses are divided into three subfamilies based on their biological properties. These subfamilies include the alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. Alphaherpesvirinae members were grouped together based on their ability to infect a broad range of hosts, their relatively short reproductive life cycle, and ability to establish latency in sensory ganglia\(^3\). This subfamily is further comprised of different genera: *Simplexvirus, Varicellovirus, Marek's disease-like virus* and *Infectious laryngotracheitis-like virus*. The simplexviruses are rich in the G+C content of their genomes and generally cause orogenital and neuronal infections. Members of this genus include the human simplex viruses types 1 and 2 (HSV 1 and HSV 2), B virus (*Cercopithecine herpesvirus 1*), SA8 (*Cercopithecine herpesvirus 2*) and herpes virus papio type 2 (*Cercopithecine herpesvirus 16*) among others. Members of *Simplexvirus* are listed in table 2. Theses viruses generally infect mucosa and replicate in epithelial cells.

**Table 2:** Species in the Genus *Simplexvirus*

<table>
<thead>
<tr>
<th>Species</th>
<th>%(G+C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ateline herpesvirus 1</td>
<td>72</td>
</tr>
<tr>
<td>Bovine herpesvirus 2</td>
<td>64</td>
</tr>
<tr>
<td>Cercopithecine herpesvirus 1</td>
<td>74.5</td>
</tr>
<tr>
<td>Cercopithecine herpesvirus 2</td>
<td>68</td>
</tr>
<tr>
<td>Cercopithecine herpesvirus 16</td>
<td>76.5</td>
</tr>
<tr>
<td>Human herpesvirus 1</td>
<td>68.3</td>
</tr>
<tr>
<td>Human herpesvirus 2</td>
<td>69</td>
</tr>
<tr>
<td>Macropodid herpesvirus 1</td>
<td>53</td>
</tr>
<tr>
<td>Macropodid herpesvirus 2</td>
<td>50</td>
</tr>
<tr>
<td>Saimiriine herpesvirus 1</td>
<td>67</td>
</tr>
</tbody>
</table>

1.2 Simplexvirus

1.2.1. Clinical Manifestations of the Genus Simplexvirus

HSV 1 and HSV 2 naturally infect humans and the viruses target mostly mucosal tissue. Herpes infections can be asymptomatic (at least 75%) but symptomatic infections are marked by painful, watery blisters that may be recurrent. HSV 1 is predominantly responsible for oral lesions while HSV 2 most often causes genital lesions. However HSV 1 is now the cause of about 50% of primary genital infections in North America and HSV2 has been isolated from oral infections. HSV infections are contagious and spread through direct contact such as sexual contact and through saliva. In addition, herpes can be transmitted via the neonatal route and is among the most severe perinatal infections. It was been estimated that 50-80% of American adults are infected with oral herpes, while 13-40% are infected with genital herpes. A recent seroprevalence study performed in two Canadian clinics found that the prevalence for HSV 1 infection was 56% while HSV 2 prevalence was at 19%. In some cases HSV 1 and 2 can disseminate and cause encephalitis and meningitis respectively. In most cases HSV 1 is responsible for encephalitic infections, however 4-6% of cases are caused by HSV2. HSV infections are the most common cause of encephalitis in children older than 6 months and in adults.

The simian simplexviruses typically cause the same clinical manifestations as HSV1 and 2 in their natural hosts. The viruses are transmitted in the same manner with the addition of biting as a means for virus spread. SA8 infection in baboons is mostly sexually transmitted and asymptomatic. In symptomatic cases, the infection causes oral and genital lesions, with disease being more prevalent in female than in male baboons.
Severe SA8 infections have been recorded in newborns and can cause large scale morbidity in baboon colonies. HVP2 symptoms are again similar with 90% prevalence of infection in baboons. B virus causes mild or asymptomatic infection in Asian macaques, with a prevalence of 80-100% in adult captive macaques. Some simplexviruses have the ability to infect and cause disease in non natural hosts. In the case of B virus, once this virus is transmitted to humans, most often via a bite from an infected macaque, severe encephalitis ensues. In humans, B virus infection has a mortality rate of >70% if the infection is not treated with antivirals early enough. In addition, it has been documented that HSV 1 can be transmitted to non human primates in which the outcome may be a severe infection. In addition, the simplexviruses have different abilities to cause disease in mice, which is marked by the onset of encephalitis. Interestingly, HVP2 which is not known to cause disease in humans can cause encephalitis in mice. Following intramuscular injection in mice, the virus invades the peripheral nervous system causing death. Table 3 summarizes the clinical manifestations and ability to cause disease in non-natural hosts of simplexviruses.

### Table 3: Simplexvirus Characteristics

<table>
<thead>
<tr>
<th>Virus</th>
<th>Natural Primate Host</th>
<th>Clinical Manifestations</th>
<th>Severe Disease in Humans</th>
<th>Virulence in Mice: Encephalitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpes Simplex Virus 1 (HSV1)</td>
<td>Humans</td>
<td>Cold sores</td>
<td>Encephalitis, disseminated infections</td>
<td>Yes</td>
</tr>
<tr>
<td>Herpes Simplex Virus 2 (HSV2)</td>
<td>Humans</td>
<td>Genital sores</td>
<td>Encephalitis, disseminated infections</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Cercopithecine herpesvirus</em> I (B virus)</td>
<td>Macaques</td>
<td>Genital sores</td>
<td>Deadly encephalitis</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Cercopithecine herpesvirus</em> 2 (SA8)</td>
<td>Baboons</td>
<td>Genital and oral sores</td>
<td>None</td>
<td>No</td>
</tr>
<tr>
<td><em>Cercopithecine herpesvirus</em> 16 (HVP2)</td>
<td>Baboons</td>
<td>Genital and oral sores</td>
<td>None</td>
<td>Yes</td>
</tr>
</tbody>
</table>
1.2.2. Treatment

Currently there is no cure for herpesvirus infections. However, there are antiviral treatments to reduce herpes recurrences. Acyclovir, valacyclovir, gancyclovir and penciclovir are nucleoside analogs used for treatment. The nucleoside analogs reduce viral replication due to their similarity to natural nucleotides and therefore are incorporated in the virus and inhibit the generation of infectious virus. These antivirals are effective in reducing the duration of symptoms. Other antivirals include fusion inhibitors such as docosanol that inhibit the fusion of the virus envelope to the cell membrane. Helicase-primase inhibitors and topical ointments are also other means of treatment, however oral treatment is thought to be more effective. Mutant viruses that exhibit drug resistance have been isolated from immunocompetent patients; however their prevalence is still very low (0.1%-0.7%) \(^{19}\). All antivirals are limited due to their inability to target latency-established virus.

1.2.3. Vaccine Development

Currently vaccines against HSV 1 and 2 infections are being investigated. The latest vaccine in phase III clinical trials is Herpevac by GlaxoSmithKline. This is a recombinant vaccine in which the gD2 (glycoprotein D2) has been truncated and it is administered with adjuvant, alum. This vaccine has shown to be effective in women but not in men against infection by HSV2. In addition, the vaccine is only effective in women who have not been previously infected with HSV1 \(^{15}\). The vaccine is still under study and was shown to elicit a strong type 1 CD4+ response in the women. Other vaccines with attenuating mutations in the virus are under study. One obstacle in replication-competent strains used for vaccine development is the ability to, on occasion,
establish latency. Whole inactivated virion vaccines and subunit vaccines are also under investigation in animal models\textsuperscript{34}. A vaccine that could elicit both neutralizing antibodies and a strong T-cell response that is safe and unable to establish latency would be ideal.

Vaccines against B virus are also under investigation. A vaccine against B virus in monkeys will reduce the prevalence of infection in non human primates as well the transmission to humans. In 1960, a formalin-inactivated B virus was developed to which an antibody response was elicited, however antibody titres were very low and therefore protection required frequent boosters\textsuperscript{12}. In addition, a vaccinia vector that delivered the gD envelope protein was shown to elicit protection from B virus in rabbits and a DNA vaccine is currently being explored. Nevertheless, a vaccine against HSV 1 and 2 is most important and will probably precede a vaccine to B virus infection.

1.2.4. Overview of Replication: HSV 1 Prototype

Herpes simplex virus 1 attaches to the cell membrane via its glycoprotein interactions with several cell receptors. Fusion of the viral envelope with the host membrane occurs followed by the release of the nucleocapsid into the cytoplasm. At this point the tegument proteins are released. Some proteins such as US11 and UL41 (vhs) remain in the cytoplasm where they may aid in converting the cell into a more conducive environment for viral replication. The vhs protein is thought to cause the nonspecific degradation of cellular mRNA and subsequently the shut off of macro-molecular synthesis. While the US11 protein inhibits the activation of PKR (a mammalian antiviral response enzyme) when expressed later in the viral lifecycle, its role in the initial stages of infection, like many other tegument proteins is not yet known. Other tegument proteins such as Vp16
(UL48) are transported into the nucleus where they initiate transcription of viral genes while others remain associated with the capsid and may aid in release of viral DNA into the nucleus. The nucleocapsid is transported to the nucleus via nuclear pores and the viral DNA is released. Upon entry into the nucleus, the viral DNA circularizes and is transcribed by the host RNA polymerase II. Replication is thought to occur via a rolling circle mechanisms in which long concatemers of DNA are generated and are later cleaved. Gene expression involved in replication is tightly regulated and occurs in a cascade fashion. The immediate early genes or alpha mRNA’s are first transcribed and give rise to regulatory genes such as ICP0 and ICP4. These genes regulate the transcription of early or beta mRNA’s. Early genes are involved in the replication of the viral genome. Viral replication then stimulates the expression of late or gamma genes. The late genes are involved in assembly of the capsid and egress of the virus upon which the viral envelope is acquired. The nucleocapsid acquires its tegument and envelope while budding from the inner nuclear membrane to the Golgi. It is unclear whether the envelope is acquired and maintained after nuclear export or whether the virus de-envelopes at the outer nuclear membrane and re-envelopes at the Golgi.

As mentioned above, all herpesviruses have the ability to cause both lytic and latent infections in their natural hosts, however not much is known about the latent lifecycle. In HSV infections, the virus latently infects neuronal ganglia, upon which the viral genome circularizes. The virus persists here however viral transcription is limited and includes the expression of LATs (latency associated transcripts). Reactivation of the virus is not well understood but is thought to be associated with stress, UV light and
immune suppression. Upon reactivation, the virus migrates to the original site of infection where viral replication and shedding takes place.

**Figure 1:** Schematic diagram of the replication cycle of HSV. The virus binds to the cell and undergoes membrane fusion. The capsid and tegument proteins are released into the cytoplasm. The vhs protein degrades mRNAs and the viral capsid migrates to the nucleus where the viral DNA is transported through the nuclear pores. The viral DNA circularizes and the host RNA polymerase II transcribe the immediate early (IE) mRNAs. The VP16 protein acts to initiate IE gene transcription. Further gene expression occurs in a cascade fashion, where IE (α) genes transactivate the early (β) genes and the late (γ) gene expression is stimulated by DNA replication. Available in: Roizman B and Knipe DM. 2001. Herpes Simplex Viruses and their Replication, p. 2410, Fig 3. In Knipe DM and Howley PM (ed.), Fields Virology. Lippincott Williams and Wilkins, Philadelphia.
1.2.5. Viral Genome and Gene Expression

The HSV 1 genome is approximately 152 kilo base pairs in size, about 68.1% G+C rich and encodes over 80 proteins. The dsDNA genome is linear and consists of two segments referred to as the unique long (UL) and unique short (US) regions. These segments are separated by inverted repeats referred to as RL and RS, flanking the UL and US segments, respectively (figure 2) 33. This genome arrangement is referred to as “type E” and as is typical of simplexviruses and other alpha and beta herpesviruses. The genome contains three palindromic origins of replication. These include the two oriS origins in the inverted repeat regions and one oriL origin in the unique long region in HSV1. Viral replication is achieved by seven essential proteins expressed by the virus along with other viral and cellular proteins. The essential proteins include a DNA binding protein referred to as ICP8, a two subunit DNA polymerase, a three subunit helicase and an origin binding protein (UL9) that are found in all human and animal herpesviruses 52.

Figure 2: Schematic representation of the organization of the herpes simplex virus genome. Highlights the typical “type E” arrangement including the unique long (UL) unique short (US) regions together with inverted repeats (RL and RS).

After recent sequencing, the simian viruses were found to contain the same basic arrangement as the HSV1 genome 30,50,51. SA8 has a 150 kbp genome with 76% G/C, B virus has a 157 kbp genome with 74.5% G/C, HVP2 has a 156 kbp genome with a highest G/C content of 76.5%. Similarly, HSV 2 has a genome size of 155 kbp and a G/C
content of 70.4%. All open reading frames (ORFs), except one (see below), are homologous and colinear. SA8 has 85% genome homology with HVP2 and 83% homology with B virus while HVP2 is 79% homologous to B virus. Although HVP2 is overall more homologous to SA8 there are segments in the virus that are much more homologous to B virus than to SA8. Interestingly, some of these segments of homology with B virus encode for virulence-determining genes such as UL36, an essential protein that is thought to be responsible for capsid release of viral DNA into the nucleus. B virus, HVP2 and SA8 are 79%, 60.1% and 68% homologous to HSV1, respectively. Figure 3A represents the phylogenetic relationship between these viruses. One difference found in B virus is the presence of 2 copies of each of the three origins of replication mentioned above; however this may be a strain specific feature (Eberle, personal communication). A major difference between the simian and human simplexvirus genomes is the absence of the RL1 open reading frame in the simian viruses (please see figure 3B). RL1 codes for an important protein referred to as γ34.5 which is able to counteract the host interferon response to shut off protein synthesis and is also responsible for neurovirulence in mice 29.

As mentioned above, herpesvirus transcription and translation occurs in a cascade fashion with alpha, beta and gamma genes. The initiation of alpha or immediate early (IE) gene expression is controlled by Vp16, a protein packaged into the virion. Vp16 complexes with cellular enzymes (Oct1 and HCF) that activates the transcription of IE genes by binding to a common regulatory element (TAATGARAT). Five immediate early genes have been identified in HSV 1 and include ICP0 (RL2), ICP4 (RS1), ICP22
Figure 3: (A) Phylogenic relationship between the simplexviruses. Whole genome alignments of HVP-2, SA8, B virus, HSV 1 and HSV 2 were compared and the above dendrogram was generated in our laboratory. (B) Map of the open reading frames and features of the SA8 genome. Polyadenylation sites are indicated by single chevrons pointing in the predicted direction of transcription. Also highlighted is the position of where the RL1 open reading frame is situated in the HSV virus genomes only. The US11 protein is also highlighted by an oval.
(US1), ICP27 (UL54) and ICP47 (US12), whose roles are mainly in the regulation of gene expression. After the activation of alpha genes, the beta or early genes are activated. Early genes are regulated by an upstream promoter containing an essential TATA element and include the genes necessary for DNA replication and the thymidine kinase gene. Following the expression of early genes the onset of DNA replication begins. The expression of late genes is concurrent with viral DNA replication and the inhibition of DNA replication leads to the inhibition of late gene expression. Late genes are expressed while early gene expression is in general reduced. The late genes encode mostly structural proteins and like the immediate early and early genes, their promoters require a TATA element. Within the late gene sets there are “true late” genes or gamma2 genes which absolutely require DNA replication for expression as well as “leaky late” genes or gamma1 genes, the expression of which is increased by DNA synthesis.

1.3. Innate Immune Response to Viral Infection

The innate immune response is an organism’s first line of defense to pathogenic invasion. Innate immunity is comprised of NK (natural killer) cells, physical barriers, interferon and the complement system. The innate immune response is stimulated by several factors; it is non specific and is able to activate the more specific adaptive immunity. Upon infection with viruses, mammalian cells initiate a rapid and efficient antiviral response. One efficient trigger of this response is the presence of large amounts of dsRNA. Double stranded RNA activates the interferon response, a central arm of innate immunity, via toll-like receptors. The interferon response is grouped into type 1 interferons, regulated by alpha and beta interferon genes, type 2 interferons, regulated by
gamma genes and recently classified type 3 interferons consisting of IFN-lambda molecules. Viral infection elicits a type 1 interferon response. Interferons are secreted immunoregulatory proteins that once activated can initiate a cascade of events which induce antiviral proteins in neighbouring cells, for example, protein kinase R (PKR), which further take action to inhibit pathogen replication in neighbouring cells. The activation of enzymes like PKR further enhance the antiviral state by signaling additional proteins such as eIF2-α, FADD and NF-κB that can shut off protein synthesis, induce apoptosis or lead to other differences in the cellular environment.

PKR is a ubiquitously expressed serine-threonine kinase dimer that is activated by dsRNA. PKR is latent in uninfected cells and in response to type 1 interferon stimulation is produced in much larger amounts as a means to protect the host cell from further viral attack. PKR recognizes dsRNA via a pair of dsRNA binding motifs at its N-terminus. This recognition is thought to be based on the RNAs structure as opposed to specific nucleotide sequences. PKR is also thought to associate with ribosomes, specifically the 60S subunit. Once PKR bind dsRNA, it dimerizes and undergoes autophosphorylation. As mentioned above, phosphorylated PKR is then able to activate several other cellular mechanisms. One important and well characterized mechanism is the phosphorylation of the eukaryotic translation initiation factor, eIF2α. EIF2 becomes phosphorylated on the alpha subunit which leads to the inhibition of the exchange of eIF2-GDP (guanosine diphosphate) with GTP (guanosine triphosphate) from eIF2B, a critical step in translation initiation on the ribosome. Phosphorylated eIF2 alpha is therefore an inhibitor of eIF2B and leads to the shut off of protein synthesis in the cell and consequently the inhibition of viral replication. However many viruses have evolved mechanisms to inhibit
this response. For example the vaccinia E3L protein is able to bind to PKR and inhibit its phosphorylation while the reovirus sigma3 protein is thought to compete for dsRNA and thereby limiting PKR activation \(^\text{18}\). At least two herpes simplex virus proteins have been shown to counteract the interferon-induced antiviral response. These proteins are the \(\gamma 34.5\) gene product and the US11 gene product.

1.4. \(\gamma 34.5\)

\(\gamma 34.5\), also referred to as ICP34.5 or RL1, is an early gene that is expressed before the onset of DNA replication. The \(\gamma 34.5\) gene is located in the repeat sequence flanking the \(U_L\) region and therefore is present in two copies. The protein product of \(\gamma 34.5\) plays a crucial role in evading the innate immune response, specifically the interferon type 1 response. The protein does so by aiding in the dephosphorylation of eIF2-\(\alpha\), a translation elongation factor that is involved in protein synthesis. Specifically, the C-terminal, GADD34-like domain of \(\gamma 34.5\) is able to redirect the protein phosphotase 1 type \(\alpha\) (PP1 \(\alpha\)) to dephosphorylate eIF2\(\alpha\), thereby increasing pools of unphosphorylated eIF2\(\alpha\) and allowing protein synthesis to occur. Studies have shown that \(\gamma 34.5\) has the trafficking ability to pass between the cytoplasm, nucleus and nucleolus and is thought to be found in the nucleus early in infection and in the cytoplasm at later times \(^\text{37}\).

\(\gamma 34.5\) plays a critical role in HSV infections as \(\gamma 34.5\) null mutant viruses show a strong attenuation of growth both \textit{in vivo} and \textit{in vitro} \(^\text{29}\). In a recent study by Pasieka et al. through transcriptional profiling, the authors were able to show that there was a maximal difference in the genes up and down regulated between wild-type and \(\gamma 34.5\)-null mutant virus infected cells. Specifically in the \(\gamma 34.5\) null virus infected cells, upregulation
occurred in a majority of antiviral genes including greater amounts of beta-interferon than in wild-type infected cells \textsuperscript{29}. In addition, \(\gamma\)34.5 mutant viruses are nonneurovirulent when injected intracranially in mice. These mutated viruses progress to the onset of DNA replication and accumulate late mRNAs however viral protein synthesis is shut off due to host cell PKR activation and consequent inhibition of protein synthesis. However, the wild type phenotype can be restored if the US11 protein is expressed as an immediate early gene \textsuperscript{9,24,26}. The restoration of growth through mutants in which US11 is expressed as an immediate early gene releases US11 from being under the control of \(\gamma\)34.5 and is thought to be primarily due to the ability of US11 to also inhibit the phosphorylation of eIF2\(\alpha\) thereby allowing the virus to once again gain control of the cell to allow replication to proceed, as explained in detail below. It is important to note that the lack of \(\gamma\)34.5 leads to the inhibition of the US11 gene because US11 is a late gene that requires DNA synthesis, which cannot occur in \(\gamma\)34.5 null mutants. As a result, protein synthesis is shut off in \(\gamma\)34.5 mutants as a result of the loss of both US11 and \(\gamma\)34.5 \textsuperscript{25}. Other roles of \(\gamma\)34.5 include eIF2\(\alpha\)-dependant amino acid starvation, facilitation in viral egress, and the blockage of the MHC class II surface expression. The \(\gamma\)34.5 gene is only found in HSV 1 and HSV 2 of the simplexvirus genus.

1.5. US11

US11 is a 21 kDa ribosome-associated protein found in all members of the simplexvirus genus \textsuperscript{42}. US11 is produced as an abundant late gene in herpes simplex viruses 1 and 2 and is thought to be same for the other simian herpes simplexviruses. US11 is an RNA-binding protein that binds duplex RNAs of 39 bp or higher \textsuperscript{14}. This protein contains a unique RNA-binding motif distinctive from any known motif. As
mentioned above, US11 also has the ability to inhibit the interferon induced antiviral response. US11 has been shown to bind dsRNA via its C-terminal end which consists of a series of R-X-P repeats (arginine-X-proline), where X is often an uncharged polar or acidic amino acid. These R-X-P are conserved in all simplexviruses but are present in different numbers. Figure 4A shows the amino acid sequence alignment for US11 in simplexviruses. Areas highlighted in yellow indicate sequence of high homology. Briefly, HSV1 contains 20-24 copies of R-X-P repeats in the US11 protein, the number of repeats has been shown to vary among viral strains but is not a strain specific feature (figure 4B). HSV2 contains 19-20 repeats and this also is thought to vary slightly among strains. In regards to US11 from the non-human primate viruses; B virus contains 20 R-X-P repeats, SA8 contains only 10 while HVP2 has the longest number of 32 R-X-P repeats.

A)
Figure 4: Amino Acid sequence alignment of US11. (A) US11 protein sequence aligned from HSV 1, HSV 2, B virus, HVP 2 and SA8. Yellow regions indicate sequence of high homology, blue regions indicate moderate homology and green regions indicate low homology among proteins. The yellow boxes highlight the R-X-P repeats at the C-terminal end of the protein. (B) US11 sequence alignment from various strains of HSV 1.

The protein product of US11 has a physical association with PKR (presumably near the 60S ribosome) and is thought to bind dsRNA in the cellular environment, preventing the activation of PKR \(^\text{8,31}\). In turn, eIF2-\(\alpha\) remains dephosphorylated and virus replication and protein synthesis continues. In addition to inhibiting PKR activation by dsRNA binding, US11 has also been shown to inhibit PKR activation through the PACT
enzyme both in vivo and in vitro. PACT is an enzyme that is able to activate PKR in an RNA-independent manner and subsequently lead to apoptosis. US11 does not block the binding of PACT to PKR, but is able to block its activation. In addition, US11 is also able to counteract OAS (2'-5' oligoadenylate synthetase), another host defense, in part by sequestering dsRNA through its C-terminal domain. It is important to note that this C-terminal domain is unique and does not resemble any known RNA binding motif.

RNA-binding proteins have been grouped into several families based on their shared common RNA-binding motifs. They often bind to structures as opposed to specific RNA sequences. Some of these families include RRM (RNA recognition motif) proteins that bind primarily single stranded RNA species, zinc finger motif proteins that bind to duplexed nucleic acid, arginine-rich motifs that bind RNA via a short arginine rich peptide sequence and dsRNA-binding motif (dsRBM) that bind to highly structured RNA species. Double stranded RBM proteins often have an α-β-β-α structure that is implicated in the binding of dsRNA. The R-X-P RNA binding domain of US11 does not fit into any of the designated RNA binding protein families. The presence of a proline at every third residue at the C-terminal end of the protein suggests the formation of a poly-L-proline II helix. This structure has been found in some globular proteins and regions of neuropeptides but there has not yet been any evidence of RNA-binding activities associated with this structure.

US11 is packaged into the tegument containing about 600-1000 copies of the molecule. As the virus uncoats, US11 is released into the cytosol before immediate early gene expression begins. The role of US11 here is undefined and may have activities that are not related to the PKR system. Thus far, US11 is not thought to be necessary for
the onset of protein synthesis or DNA replication, however it has been found that the
HSV 1 virus relies more heavily on US11 in later stages of its lifecycle to maintain an
anti-antiviral state. A study by Mulvey et al. showed that regulation of eIF2α occurs
in discrete phases of the HSV 1 lifecycle via γ34.5 and US11. By designing several
panels of virus with combination mutations in the γ34.5 and US11 genes, they were able
to show that before the onset of DNA synthesis, the virus relies on γ34.5 to maintain
pools of unphosphorylated eIF2α. However in later times in infection, cells infected with
a US11 null mutant virus that still expresses γ34.5, showed viral translation rates 6-7 fold
lower than in cells infected with wild-type virus or a virus in which the US11 mutation
was repaired. In this case, viral replication was reduced 13-fold in the US11 null mutants.
Therefore the proposed model is that earlier in infection, γ34.5 via its interaction with
PP1α, dephosphorylates eIF2α before the onset of DNA synthesis. Later in infection
transcription of late (gamma) genes generates large amounts of dsRNA such that if US11
was absent, would activate PKR and phosphorylate eIF2α to amounts that would be too
much for γ34.5-PP1α to handle. Therefore US11 may act specifically and necessarily late
in the herpesvirus lifecycle to inhibit PKR and allow protein synthesis to occur.

US11-null mutants are extremely sensitive to interferon α while protein synthesis
in cells infected with a γ34.5 null mutant expressing US11 as an immediate-early gene
and wild-type virus infected cells are virtually unaffected. Additionally, in cells
infected with a virus in which the US11 null mutation is repaired, translation in cells is
resistant to IFN α once again. Interestingly in the virus carrying the US11 null mutant,
there is still the presence of two copies of the γ34.5 gene and expression of wild type

levels of the γ34.5 protein supporting the idea that the shut off of protein synthesis in γ34.5 null mutants is due to the combined loss of both γ34.5 and US11.

1.6. Rationale

Given that US11 is thought to compensate for the loss of the critical γ34.5 protein in mutant viruses if expressed early, further investigation into its role in herpesvirus pathogenicity has become increasingly interesting. In addition, the γ34.5 is only present in the human herpes simplex viruses while US11 is present in all simplexviruses. The simplexviruses have a wide range of pathogenicity between them, with some virus being extremely severe in the non natural host and in their ability to cause disease in mice. In addition, these viruses are genetically similar and therefore any subtle differences between them can lead to answers in the difference in virulence members of this genus encompass.

US11 from HSV1 has been shown to bind dsRNA via its carboxy-terminal end through a series of R-X-P repeats. This dsRNA motif is not homologous to any other known RNA-binding motif and has lead to research that investigates the relationship between HSV1 US11 protein, dsRNA and PKR. The active C-terminal end of US11 is highly conserved in HSV1, HSV2, B virus, SA8 and HVP2 but the number of R-X-P repeats is different. Based on these findings, several questions to the role of US11 in the non-human primate viruses arise. Does US11 do the job of γ34.5 in these viruses? Is γ34.5 newly evolved in the human viruses and therefore US11 is not relied on as much? Additional questions on whether US11 from the simian viruses is more or less effective in evading the immune response and overall if the US11 gene product in human and non-
human simplexviruses is different needs to be investigated. We have access to US11 from SA8, B virus, HVP2 and HSV2 from which US11 has not been investigated. Given that an intrinsic ability of US11 is to bind dsRNA, it is rational to start with dsRNA binding in our exploration of this protein. Therefore this project proposes to compare the activities of US11 from five simplexviruses with respect to its dsRNA binding capabilities.

We tested the hypothesis that US11 proteins from different simplexviruses have different binding affinities for dsRNA. The binding affinity might correlate with the number of repeats or with the different virulence displayed by theses viruses in mice or primates. Since HSV 1 and 2 and B virus have a similar number of R-X-P repeats on their US11 protein, we hypothesized that the activity of the protein, i.e. the RNA binding affinity and PKR inhibition maybe be similar, while SA8 and HVP2 having a much lower or higher number of repeats and lower pathogenicity may have a lower affinity for RNA and ability for inhibiting PKR. However, because the simian simplexviruses only posses US11 as opposed to the human viruses that also have γ34.5, an alternative hypothesis may be that the activity of US11 from theses viruses is stronger and compensates for the absence of γ34.5 in suppressing the interferon induced response. A subsequent step, not approached in this thesis, would be to try to compare the PKR inhibition by US11 from theses viruses with the ultimate goal of gaining further insight to the diverse pathogenicity between them.
1.7. Objectives

1. To clone, express and purify in an active state the US11 protein from 5 simplexviruses: HSV 1, HSV 2, SA8, B virus and HVP 2.

2. To measure the equilibrium binding constant (Kd) for these proteins and to evaluate if any differences in their ability to bind RNA correlates with the number of RNA-binding repeats or the neurovirulence of the viruses.

3. For the proteins obtained in objective 1 to be available for further studies of their ability to inhibit PKR *in vitro* and *in vivo* assays.
2.0. Material and Methods

2.1. Cloning

2.1.1. Polymerase Chain Reaction (PCR)

Template DNA for PCR reactions was retrieved from infected Vero (monkey kidney) cells. Cells were infected with the simplexvirus with a multiplicity of infection (MOI) of 1 and allowed to reach a cytopathic effect (CPE) of 100%. Infected cells were detached and spun-down at 1500 rotations per minute (rpm) for 5 minutes. The pellet was resuspended in lysis buffer (10mM Tris pH8.0, 10mM EDTA and 1% SDS). Proteinase K was added to a final concentration of 200µg/ml and incubated overnight with gentle mixing. DNA was extracted with a 1:1 phenol/chloroform solution and precipitated with 100mM NaCl and 2 volumes of ethanol at 4°C. The DNA was washed in 70% ethanol, dried and resuspended in TE buffer with gentle mixing at 37°C. The DNA was purified via a sodium iodide gradient. A large bore needle was used to remove the viral DNA after sedimentation. The DNA was dialyzed at 4°C against TE buffer plus 100mM NaCl. Purified DNA was stored at -20°C in TE buffer.

The PCR was performed on template DNA from HSV1, HSV2, B virus, SA8 and HVP2. Several primers were used for each DNA template (table 4) and were based on US11 sequences taken from the NCBI website, http://www.ncbi.nlm.nih.gov. Each primer sequence included additional nucleotides responsible for restriction digestion sites for NdeI and HindIII. In addition, supplementary nucleotides were added to improve amplicon digestions. Primers were designed using the Vector NTI sequencing program version 9 (Invitrogen, CA, USA). All PCR reactions used 1 ng DNA template per reaction, 200 µM dNTPs (Invitrogen), 1 mM MgSO₄ and 2.5 units per reaction of Pwo
polymerase (Roche, QC, Canada). Annealing temperatures varied based on primers (table 4). The Gene Amp® PCR System 9700 from Applied Biosystems (CA, USA) was used to carry out PCR amplifications.

Table 4: Primer Sequences and Annealing Temperatures

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<th>Viral Template DNA</th>
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\(^1\) Italized sequence indicates added 5'end sequence to improve digestion, underlined sequence is recognized by restriction digest enzymes.

\(^2\) US11 sequences are according to the published complete genome sequences: HSV 1 strain 17 (NC_001806), HSV2 strain HG52 (NC_001798), B virus strain E2490 (C_004812), SA8 (NC_006560), HVP2 (NC_007653).

2.1.2. DNA Purification

After the PCR, amplified DNA was purified either from PCR reactions or from isolated amplified bands that had been run on 1% agarose gels. In the case of PCR mixture purification, the amplified DNA was trapped on a membrane while remaining contaminants were filtered out using micron filter columns (Ambion, ON, Canada). For gel purification, the DNA fragment was excised and purified using Qiagen QIAquick gel extraction kits (Qiagen, ON, Canada). The kit allows for the gel slice to be melted, the DNA is trapped on a membrane while other contaminants are washed away and finally the DNA is eluted in Tris buffer, pH 7.
2.1.3. DNA Ligation to the pET 28 a (+) Expression Vector

The pET 28 expression vector was chosen because of the presence of the strong T7 promoter and the coding region for a hexa-histidine tag which would aid in protein purification. Both the US11 insert and pET 28 a (+) were digested for three hours to overnight at 37°C using 20 units of NdeI and HindIII restriction enzymes (New England Biolabs, ON, Canada). One times restriction enzyme buffer, NEB2 (10 mM Tris-HCL, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, pH 7.9) was added to the 30 µl digestion reactions. Ligation of insert to vector was done in a 3:1 ratio using 1000U of T4 DNA ligase (New England Biolabs) and 1X DNA ligase buffer (50 mM Tris-HCL, 10 mM MgCl₂, 1 mM ATP, 10 mM Dithiothreitol, 25 µg BSA, pH 7.5) in a total volume of 20 µl. Ligation reactions were incubated at room temperature overnight. Using these restriction sites allowed US11 to be inserted into the vector downstream of the hexa-histidine site and upstream of a stop codon (see figure 5, Results section).

2.1.4. Transformation

Ligated vectors were transformed into BL21 (DE3) competent Escherichia coli cells (Novagen, CA, USA). Frozen competent cells were thawed on ice for approximately 4-7 minutes and 1 µl of ligation reaction was added to 20 µl of bacterial cells. The mixture was incubated on ice for 5 minutes and then heat-shocked at 42°C for 45 seconds. The tubes were then placed on ice for another 2 minutes and 80 µl of SOC (20 g/L bacto tryptone, 5g yeast extract, 0.5g NaCl, 2.5 mM KCl, pH 7.0) media was added. Cell recovery was allowed to take place at 37°C for 3 hours with mixing at 300 rpm in an Eppendorf Thermomixer (Fisher, ON, Canada). Transformed cells (50 µl) were
plated on warmed Luria-Bertani (LB) kanamycin medium (0.01 g/ml tryptone, 0.005 g/ml yeast extract, 0.01 g/ml NaCl, 50 µg/ml kanamycin) and incubated at 37°C overnight. Individual colonies were picked and grown in 5ml LB broth kanamycin (Kan) at 37°C with vigorous agitation. The bacteria were pelleted by centrifugation at 5000 rcf at 4°C for 5 minutes. Plasmids were purified using Qiagen QIAprep spin kits. Purified clones were sequenced for conformation by the DNA Core Facility (National Microbiology Laboratory, Winnipeg). Vector and insert sequences were aligned with known NCBI (National Centre for Biotechnology Information) sequences using Vector NTI (Invitrogen). Successful clones were stored in 80% glycerol at -80°C.

2.2. General Protein Techniques

2.2.1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

15% SDS-PAGE gels were most often used with protein studies and were hand-cast in our lab. Minigel cassettes (7.5cm x 8.2cm) contained 4% acrylamide/bisacrylamide solution in the stacking gel and 15% acrylamide/bisacrylamide solution in the resolving gel. N,N'-methylenebisacrylamide (bisacrylamide) was used as across linker, gels also contained 375 mM Tris-HCL, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate (APS) and 0.05% TEMED (N,N,N',N'-Tetra-methy-ethylenediamine, Biorad). Protein samples were mixed with an equal volume of SDS PAGE gel loading buffer (6X stock: 3% glycerol, 0.6 g/ml bromophenol blue, 0.6% SDS, 10 mM Tris p 6.8 and 50 µl/ml β-mercaptoethanol) and boiled for 3 minutes at 95°C. Samples were loaded onto the gel and were run in 1X Tris-glycine buffer (25mM Tris, 192mM glycine and 0.01% SDS, pH 8.6) at 140 volts for approximately 1 hour. In some
case, commercially purchased (Biorad, ON, Canada) 15% Tris gels were used. In addition 20% acrylamide gels were also used, in which the resolving gel contained 20% acrylamide/bisacrylamide solution.

2.2.2. Gel Staining

Protein bands that had been resolved on SDS-polyacrylamide gels were visualized using GelCode Blue stain reagent (Fisher). Gelcode Blue utilizes the colloidal properties of Coomassie G-250 dye for protein staining. Staining was performed according to the manufacturer’s protocols.

2.3. US11 Recombinant Protein Expression

A small amount of bacterial culture was scraped from frozen stocks of clones placed on ice and it was inoculated into 30 ml of LB kanamycin broth and allowed to grow for 5 hours to overnight at 37°C to an \( \text{OD}_{600} \) of 0.6 to establish a starter culture. The culture was added to 1 L of Kanamycin LB media and allowed to grow for an additional 4 hours. The production of US11 was induced with a final concentration of 1 mM isopropyl-\( \beta \)-thiogalatopyanoside (IPTG, Novagen) and allowed to grow overnight at 25°C. Cells were harvested by centrifugation at 10 000 rpm for 10 minutes. The fraction containing the protein was obtained by re-suspending the pellet in 4.7 ml of lysis buffer (20 mM Tris-HCL, 10 mM imidazole, 1% triton x-100, 10 mM \( \beta \)-mercaptoethanol, pH 8.0) with the addition of protease inhibitor cocktail (Sigma, Ontario, Canada). Cells were sonicated using a microson ultrasonic cell disrupter (Mandel Scientific, ON, Canada). Soluble and insoluble fractions of protein were separated by centrifugation at 14000 rpm.
for one and a half hours. Most protein was found in the soluble fractions and these
fractions were stored at -20°C. In the case of US11 from SA8, Rosetta BL21 (DE3) E.
coli cells and BL21 (DE3) pLysS E. coli cells were also used to check for expression of
the targeted protein. For these cells, chloramphenicol was also included in the kan LB
growth medium at a concentration of 1μl/ml.

2.4. Purification of US11

2.4.1. Affinity Chromatography using Nickel as the Ligand

A separation column was prepared using 2ml of Profinity™ IMAC Ni resin
(Biorad, ON, Canada). The Ni resin was equilibrated with 1X binding buffer (20 mM
Tris-HCL pH 7.4, 10 mM imidazole, 1M NaCl, 1% triton x-100, 10 mM β-
mercaptoethanol). Soluble fractions of protein were combined and mixed with an equal
volume of 2X binding buffer (20 mM Tris-HCL pH 7.4, 10 mM imidazole, 2M NaCl, 1%
triton x-100, 10 mM β-mercaptoethanol) and applied to the affinity column. Unbound
protein was washed with wash buffer 1 (20 mM Tris-HCL, 10 mM imidazole, 1M NaCl,
1% triton x-100, 10 mM β-mercaptoethanol pH 7.4) and wash buffer 2 (20 mM Tris-HCL
pH 7.4, 10 mM imidazole, 300 mM NaCl, 1% triton x-100, 10 mM β-mercaptoethanol).
Bound US11 was then eluted with elution buffer (20 mM Tris-HCL pH 7.4, 300 mM
imidazole, 300 mM NaCl, 1% triton x-100, 10 mM β-mercaptoethanol). Fractions
containing US11 were confirmed by sodium dodecyl sulphate polyacrylamide gel
electrophoresis (SDS-PAGE) on a 15% SDS gel.
2.4.2. FPLC using Heparin as the Ligand

Fast protein liquid chromatography (FPLC) was used as a second means of purification. The apparatus used was the AKTAfplc system (GE healthcare, QC, Canada). Eluted protein fractions from the Ni column were loaded onto a 1 ml HiTrap heparin HP column (GE Healthcare) on the FPLC using phosphate binding buffer A (10 mM NaPO₄ pH 7.4). The US11 protein was eluted using phosphate buffer B (10 mM NaPO₄ pH 7.4, 1.5 M NaCl). The column was run over a 20 ml gradient with a target concentration of 100 % buffer B and with an average flow rate of 1 ml/minute. Fractions containing US11 were confirmed by SDS-PAGE on a 15% SDS-PAGE gel.

2.4.3. Gel Filtration Column Purification

A gel filtration column was used to try to further remove contaminating bands from the targeted US11 protein. Samples off the heparin column were pooled and loaded onto an S200 (Superdex™ 200) HP gel filtration column (GE Healthcare). The 50 ml S200 column separates proteins or peptides based on size in the molecular weight range of 10,000-600,000. The column was run in PBS buffer over 1.5 ml column volumes at a flow rate of 0.5-1 ml/min on the FPLC.

2.5. Obtaining the Concentration of US11

The ReDc Protein Assay kit (Biorad) was used to obtain the concentration of each protein after purification. Concentrations were taken based on the manufacturer’s protocol. Bovine gamma globulin was used as a protein standard. Absorbances were read
at 700 nm using a Beckman DU® 650 spectrophotometer (Beckman Coulter, ON, Canada).

2.6. Identification of US11

2.6.1. Western Blot Analysis

As there is no commercial antibody against US11, a monoclonal anti-polyhistidine tag antibody (Sigma) was used to confirm the presence of recombinant US11 through western blot analysis. In this case, proteins ran on 15% SDS gels were transferred on to nitrocellulose membranes (Trans-Blot® transfer medium, Biorad) cut to size. Transfers took place overnight in a mini-blot system apparatus (Biorad) with transfer buffer (25 mM Tris-HCL, 192 mM glycine, 20% methanol) at a constant current of 90 mAmps per gel. Non-specific binding sites were blocked with PBS containing 0.1% tween-80 (PBS-T) and 5g of skim milk for 2 hours. The membrane was then rinsed three times with PBS-T. Incubation with the primary antibody took place for an additional two hours using a mouse anti-histag antibody (Sigma) at a dilution of 1:6000. The membrane was once again rinsed three times for 5 minutes each with PBS-T. The secondary antibody incubation took place for one hour. The secondary antibody used was an antimouse IgG antibody conjugated to horse radish peroxidase (Sigma) at a dilution of 1:80,000. The membrane was once again washed three times with PBS-T. For chemiluminescent visualization of protein bands the Amersham ECL™ western blotting detection kit was used. The kit required the membrane to be covered with equal amounts of solution A and B for one minute and then stored in a clean piece of plastic wrap. The membrane was then exposed to hyperfilm ECL™ film (Amersham Biosciences, NJ,
USA) for 30 seconds to 10 minutes and film was developed using the Feline™ 14 x-ray
film processor (Fisher).

2.6.2. Protein Identification using Mass Spectrometry

Samples for mass spectrometry were run on 15% SDS-PAGE gels and stained
with Gelcode Blue (Fisher) for visualization of the protein. Stained bands were excised
using sterile scalpels in a biological safety cabinet to minimize keratin contamination.
The bands were further cut into smaller pieces of about 1mm x 1mm in size. The gel
pieces were placed in siliconized tubes containing 100 µl of sterile water. The samples
were sent directly to the Proteomics Core facility of the National Microbiology
Laboratory, Winnipeg, Manitoba. Protein samples under went tryptic digestion to
produce peptides. The mass spectrometry instrument (qQ-TDF, QSTAR-XL, ABI-
SCIEX, ON, Canada) first measured the molecular weight of the peptides and generated a
peptide mass fingerprinting spectrum. Peptides with the highest peaks were selected for
sequencing and an ion spectrum was obtained. The sample peptides mass values were
compared with calculated ones in a specific database and a scoring algorithm was used to
match the sample peptides to already known peptides in a known protein. Protein
identification from the generated mass spectrometry data was done under the NCBI
protein database using Mascot Daemon (Matrix Science, UK). The search was done
against all species with a mass tolerance of ± 0.4 Da.
2.7. **Confirmation of US11’s Activity – Electrophoretic Mobility Shift Assays**

The electrophoretic mobility shift assay (EMSA) protocol used was adapted from previously published protocols. The RNA used for confirming protein binding was a 42 bp duplex sequence containing a random sequence that was produced by Invitrogen (table 5). Binding reactions were prepared using varying concentration of purified US11 protein (10-100 ng) and 20 ng of RNA in 1X binding buffer (150 mM KCl, 0.1 mM DTT, 0.1 mM EDTA and 50 mM Tris, pH 7.4) in a final reaction volume of 20 μl. US11 and dsRNA were allowed to bind at room temperature for 30 minutes without agitation. In addition, control reactions were also prepared consisting of protein only, RNA only, protein and DNA and DNA only. The DNA used was also a randomly designed 42 base pair duplex sequence. The separation of reaction mixtures was done on 10% non-denaturing polyacrylamide gels using 10μl of binding reaction and 1.6 μl of gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll-type 400). The non-denaturing gels were run at 100 volts in TBE buffer (0.89M Tris, 0.02M EDTA, 0.89M boric acid, pH 8). Bound complexes were visualized by staining with 0.5 μg/ml of ethidium bromide for one hour and exposure to UV light using the Quantity One® Gel doc XR (Biorad) system.

**Table 5:** 42 Base Pair Sequence of RNA and DNA used in Affinity Experiments.

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<th>Source</th>
<th>Sequence</th>
<th>Length</th>
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<td>RNA (5'-3')</td>
<td>UUCUCAAGUGAAGUCUGCUGAAGUACGUAACCUUAGAUACAU AUGUAUCUAAGGUUACGUACUUCAGCAGACUUCACUUGAGAA</td>
<td>42 base pairs</td>
</tr>
<tr>
<td>DNA (5'-3')</td>
<td>TTCTCAAGTGAAAGTCTGCTGAAGTACGTAACCTTAGATACAT ATGTATCTAAGGTTACGTACTTCAGCAGACTTCACTTGAGAA</td>
<td>42 base pairs</td>
</tr>
</tbody>
</table>
2.7.1. Modified EMSA using SYBER® Green and SYPRO® Ruby Staining

To visualize both nucleic acid and protein in shift assays, an Invitrogen EMSA kit was used. The preparation of samples for the binding reaction was carried out the same as above. In this case, the loading buffer for running samples on a non-denaturing gel was supplied by Invitrogen (6X gel loading buffer, ingredients not listed). The gel was run under the same conditions as above. The nucleic acid bands were visualized first by staining the gel with 50 ml of 1X SYBR green solution (Invitrogen) for 20 minutes with agitation. The gel was rinsed once with water and complexed bands were visualized by UV transillumination using the Quantity One® Gel doc XR system. The gel was then stained with 100ml of SYPRO Ruby solution (Invitrogen) for 3 hours to overnight with agitation. The gel was rinsed twice with water for 10 seconds. The gel was destained with a solution of 10% methanol and 7% acetic acid for 1-2 hours. The gel was rinsed again with water and complexes containing protein were visualized by UV transillumination using the Quantity One® Gel doc XR system.

2.7.2. Radioactive EMSA

A radioactive EMSA was performed to try to quantify bound complexes. The sample preparation and gel conditions were carried out same as above. The only modification was the addition of RNA previously labeled with gamma $^{32}$P radioisotope to binding reactions. Bound complexes in this case were visualized using a typhoon phosphoimager (Amersham Biosciences).

RNA radioactive labeling was carried out as follows. Labeling reactions included 1 µl of 20 µM stock RNA, 1X T4 kinase buffer (70mM, Tris-HCl, 10 mM
MgCl₂, 5mM dithiothreitol pH 7.6), 1 unit of T4 polynucleotide kinase (New England Biolabs) and 50 pico moles of γ-³²P- ATP (500μCi, Amersham Biosciences) and brought to a final reaction volume of 50 μl with DNase/RNase free water (Invitrogen). The reaction was incubated for 30 minutes at 37°C. The labeled RNA was purified on Sephadex™ G-50 separation media (Amersham Biosciences). Briefly, the reaction containing the labeled RNA was loaded onto a column containing 2 ml of Sephadex™ and washed approximately 50 times with 50 μl of DEPC (diethyl pyrocarbonate) treated TE buffer (10 mM tris, 1 mM EDTA pH 7.5). The 50 μl fractions eluted off the column were collected separately and radioactivity was counted using a LS 6500 multi-purpose scintillation counter (Beckman Coulter, ON, Canada). Fractions containing the highest counts per minute (CPM) values were run on a 10% non-denaturing gel to further confirm which fractions contained the labeled RNA. Fractions containing the labeled RNA were pooled and became the working stock for protein/RNA binding reactions. Radioactive RNA was added to binding reactions in a ratio that allowing at least 1 million counts of radioactivity.

2.7.3. Preparation of Non-denaturing-PAGE

For protein-nucleic acid studies, 10% non-denaturing polyacrylamide gels were used. These gels were prepared in our lab using the same format as in section 2.2 above. In this case however, the resolving gel contained 10% acrylamide/bisacrylamide solution and no SDS was added to the gel. In addition, non-denaturing gels were run in TBE buffer (0.89M Tris, 0.02M EDTA, 0.89M boric acid, pH 8) at 100 volts for approximately 1 hour.
2.8. Filter Binding Assays

The filter binding assay protocol was adapted from a protocol taken from RNA: Protein Interactions, A Practical Approach. Filter binding assays were performed using a reaction volume of 1 ml. These reactions consisted of varying concentrations of RNA and a fixed concentration of US11 protein. Each reaction also contained 5% Ni resin (Biorad), 1X binding buffer (150 mM KCl, 0.1 mM DTT, 0.1 mM EDTA and 50 mM Tris, pH 7.4) and was brought to a final volume of 1 ml with DNase/RNase free water (Invitrogen). The Ni resin was washed twice with binding buffer to remove its storage solution of 20% ethanol. Blank reactions were also prepared as above however there was no protein added. The RNA was initially prepared at a concentration of 31 nM containing at least one million counts of radioactive RNA and serially diluted down to the required concentrations. RNA was kept on ice and added last to reactions. Each reaction containing RNA and protein as well as blanks was prepared in duplicate. The binding reactions were allowed to occur for 25 minutes at room temperature with gentle mixing to keep reaction components in suspension. Blank reactions were treated under the same conditions. After 25 minutes, reactions were loaded on to glass microfibre filters (VWR) which had been placed on a vacuum manifold (VWR) and had been presoaked in 1X binding buffer. The reactions were filtered using a vacuum for approximately 10 seconds and washed 3 times with 1X binding buffer with vacuuming. The filters were carefully placed in scintillation vials, 10 ml of scintillation fluid (Fisher) was added and the radioactivity remaining on the filters was counted using a LS 6500 multi-purpose scintillation counter.
2.9. Determining Kd Values

The affinity of a ligand to a protein is measured by the dissociation constant, Kd, which is defined as the ratio of Koff/Kon, where Koff is the rate constant of dissociation of the ligand-protein complex and Kon is the rate constant of complex formation. The equation used to determine the Kd values was derived from basic protein and ligand kinetics where the binding of a protein to a ligand can be described as an equilibrium equation: 

\[ [P\cdot RNA] = [P][RNA]/(Kd+[RNA]), \]

where \([P\cdot RNA]\) is the protein and RNA complex amount, \([P]\) is the total concentration of binding sites and Kd is the dissociation/equilibrium constant. The dissociation constant quantitatively describes the binding of a protein to a ligand to form a specific complex. Kd can also be defined as the molar concentration of ligand at which there is 50% of the maximum binding of the protein to the ligand. The equation above has a function of a rectangular hyperbola, if the concentration of binding sites is fixed. In this case the amount of bound complex is a hyperbolic function of the concentration of RNA at equilibrium. The calculated bound complex (in pmoles) was plotted against the [RNA] nM using DeltaGraph software, version 5.6 and the Kd values were extrapolated from these graphs. We used the equation describing binding as the equilibrium equation above: 

\[ y = (k_1 x)/(kd+x), \]

where \(k_1\) is the maximum binding, \(y\) is the bound complex and \(x\) is the concentration of free RNA. We performed non linear regression analysis of our data using GraphPad Prism® and DeltaGraph software. The specific activity was calculated by dividing the radioactive counts of \(\gamma^{32}\)P-RNA by the number of pmoles of \(\gamma^{32}\)P-RNA. We then divided the bound CPMs by the specific activity to establish the total amount of pmoles of bound complex and plotted this against the total concentrations of RNA. We used non linear regression
analysis on the equilibrium binding equation instead of generating a Scatchard plot to
examine the data in a linear fashion because this would generate a larger error since in a
Scatchard plot the dependent variable subjected to experimental variability (bound RNA)
appears on both Y and X axes.

2.10. Statistical Analysis

Several experiments were performed to determine the Kd of each US11 protein. A
weighted average of the kd values and standard deviation of the weighted average was
calculated. The following formulas were used ³:

Weighted mean

\[
\bar{x} = \frac{\sum_{i=1}^{n} w_i x_i}{\sum_{i=1}^{n} w_i}
\]

Where, \( w_i = 1/\text{standard error on the Kd} \)
\( x_i = \text{Kd value in each experiment} \)

Weighted sample variance

\[
s^2 = \frac{\sum_{i=1}^{N} w_i}{\left(\sum_{i=1}^{N} w_i\right)^2} - \sum_{i=1}^{N} w_i \left( x_i - \mu \right)^2
\]

Where, \( w_i = 1/\text{standard error on the Kd} \)
\( x_i = \text{Kd values in each experiment} \)
\( \mu = \text{weighted mean} \)
Standard deviation of the weighted mean

\[ SD = \sqrt{\frac{s^2}{N-1}} \]

Where, \( s^2 \) = weighted population variance
\( N \) = number of samples

In addition, statistical analysis of the variance of each protein’s Kd value was carried out. A one-way ANOVA test was performed to establish whether the Kd values were significantly different by generating a P value summary. GraphPad Prism® software was used for this analysis.
3.0. Results

3.1. Cloning of the US11 insert into the pET 28 a(+) Vector

We performed directional-cloning to express the US11 protein. The pET 28 a(+) vector was chosen because of the presence of a hexa histidine tag and the control of the T7 promoter. US11 from HSV1, HSV2, HVP2, SA8 and B virus was cloned downstream of a hexa histidine tag using restriction enzymes NdeI and HindIII (figure 5). All clones were sequenced for confirmation.
Figure 5: (A) Schematic representation of the pET 28 a(+) expression vector (Novagen). (B) The sequence downstream of the T7 promoter is magnified to show relevant features such as the position of the histidine tag and thrombin cleavage site. Also highlighted in red is the targeted position of US11 within the vector, restricted enzymes used are circled and the start codon (methionine) for expression is underlined in yellow. The figure was extracted from: The pET System Manual, Novagen, http://www.emdbiosciences.com
3.2. Expression of US11 in E. coli

Transformed E. coli cells were first grown to 100 ml culture volumes to check for the expression of each of the US11 proteins. The cultures were induced with 1 mM IPTG. To scale up protein levels, culture volumes were increased to one litre. A control expression system was also used which included a pET vector without an insert cloned into bacteria. Control cells were extracted in the same manner as US11. Figure 6A shows the results for US11 expression from HSV2. Lane 4 shows that most of the protein was found in the soluble induced fraction and therefore this fraction was used for all US11 purification and RNA binding work described in this thesis. Expression patterns of US11 of the other viruses were similar to the one showed for HSV 2. Figure 6B shows the extracted soluble induced fractions of recombinant US11 from HSV1, HSV2, HVP2 and B virus. Note here that we did not include US11 from the SA8 virus (discussed below).
Figure 6: (A) Expression of US11 from HSV2 from extracted cellular fractions. Fractions were run on a 15% SDS-PAGE gel and stained with GelCode Blue protein stain. Lanes (1) molecular weight protein ladder (MWM), (2) Total cell protein from induced fractions carrying HSV2 US11, (3) Total cell protein from un-induced fractions, (4) Soluble protein from induced fractions, (5) Soluble protein from un-induced fractions, (6) Insoluble protein from induced fractions, (7) Insoluble protein from un-induced fractions, (8) Control induced fraction and (9) Control un-induced fraction. (B) Soluble protein from induced fractions from different clones containing recombinant US11. Lanes (1) MWM, (2) Recombinant US11 from HSV1, (3) US11 from HSV2, (4) US11 from HVP2, (5) US11 from B virus, (6,7) Controls induced and un-induced. The actual molecular weights of HSV1, HSV2, B virus and HVP2 are 18.7 kDa, 17.2 kDa, 17.5 kDa and 21 kDa respectively.
The expression of US11 from the SA8 virus proved to be problematic. The cloned vector including the US11 insert was sequenced and the presence of US11 from SA8 was confirmed. However, once we tried to express it in the same manner as US11 from the other viruses, there was no visible protein band present at the estimated 12 kDa weight. Figure 7A shows the extraction from 100 ml bacterial culture containing the SA8 US11 clones. We also performed western blot analysis on these samples, using anti-his tag antibodies. Figure 7B shows the appearance of a slight band in lanes 2, 3 and 4 (corresponding to total cell protein induced, total cell protein un-induced and soluble induced fractions), however these bands are probably non-specific and even if they did indicate US11 from SA8, it is hardly significant expression. Other bacterial strains were also used for expression of SA8 US11, however without success. The other bacterial cells used included the Rosetta strain and BL21 (DE3) pLysS strain of E. coli cells. The Rosetta E. coli strain expresses tRNAs for a number of mammalian codons that are rare in E. coli. The BL21 (DE3) pLysS strain has the ability to suppress the T7 promoter activity prior to the addition of IPTG, in case the protein was toxic to the cells. However no expression of SA8 US11 could be obtained. In addition we transformed our plasmid containing the US11 insert from HSV1 into these cells as a control; the US11 protein from HSV1 was strongly expressed.
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**Panel A:**
- Expected US11 Protein from SA8
- US11 Protein from HSV1

**Panel B:**
- MWM (kDa)
Figure 7: Lack of expression of US11 from SA8 in BL21 (DE3) cells. (A) All fractions extracted from BL21 (DE3) E. coli cells were run on a 15% SDS-PAGE gel and stained with GelCode Blue. Lanes (1) molecular weight protein ladder (MWM), (2) Total cell protein from induced fractions, (3) Total cell protein from un-induced fractions, (4) Soluble protein from induced fractions, (5) Soluble protein from induced fractions, (6) Soluble protein from un-induced fractions, (7) Insoluble protein from induced fractions, (8) Insoluble protein from un-induced fractions, (9) Control induced fraction, (10) Control un-induced fraction and (11) HSV1 US11 extracted from soluble induced fractions (for comparison purposes). (B) Western Blot, samples from part (A) were transferred onto a nitrocellulose membrane and subject to western blot analysis with anti-His tag antibodies. Lane 1 in figure B contains MagicMark™ XP Western blot protein standard (Invitrogen), a protein ladder different from the pre-stained protein ladder (Invitrogen) used in lane 1 figure A.
3.3. Purification of US11

3.3.1. Round One – nickel affinity

The first step of purification of US11 was performed using a nickel (Ni²⁺) column. Ni forms coordination bonds with the imidazole groups on 2 histidine molecules. Imidazole was used as the competitor ligand to eluted bound US11 after contaminating proteins were washed out. Figure 8 shows a SDS-PAGE gel of the eluted fractions containing US11 from HSV 1, HSV 2, HVP 2 and B virus.

Earlier experiments in our laboratory had shown that Ni column purified US11 preparations had considerable ethidium bromide fluorescence. The fluorescence was decreased by treatment with RNase and therefore it was likely due to RNA binding to the US11 protein during expression and extraction (data not shown). In order to remove bound nucleic acids from US11, 2M NaCl was added to the column binding buffer before the protein extract was applied to the column. In addition, the protein bound to the column was washed with wash buffer containing 1M NaCl to remove any residual RNA. The protein was then eluted in a lower amount (300mM) NaCl as recommended by the Ni resin manufacturer.

As shown in figure 8, the first round of purification with the Ni column achieves considerable purification of the US11 protein compared to the crude extract (figure 6A). However there were still a few contaminating bands of higher molecular weight and a second round of purification was needed.
Figure 8: Fractions of US11 Eluted by Ni Affinity Chromotography. Eluted fractions were run on a 15% SDS-PAGE gel and stained with GelCode Blue stain™ (Fisher). Lane (1) molecular weight marker, (2) Recombinant US11 from B virus, (3) US11 from HVP2, (4) US11 from HSV1, and (5) US11 from HSV2.
3.3.2. Round Two – heparin affinity purification

In the second step of purification, heparin was used as the affinity ligand and the purification procedure was carried out using FPLC. Heparin was chosen because nucleic acid binding proteins have a strong affinity for this substrate and it is typically used for purifying these types of proteins. Eluted fractions containing US11 off the Ni-affinity column were pooled and diluted in 10 mM phosphate buffer. The protein was diluted 25-fold before applying it onto the heparin column to reduce the 300 mM NaCl to 12 mM NaCl. The protein was loaded on the FPLC and eluted over a 20 ml gradient (0-100% NaCl), where the final concentration of NaCl was 1.5 nM. A typical chromatogram profile for the purification by heparin affinity is shown in figure 9A. Peak fractions, as seen on the chromatograms were run on a 15% SDS-PAGE gel. Figure 9B shows elution fractions off the FPLC of US11 from B virus. The purple peaks on the chromatogram indicate a rise in absorbance, i.e. an increase in protein presence, the green line indicates the gradient of salt concentration while the orange line represents the rise in relative conductivity. The protein peaks are numbered for discussion purposes. The peaks coincide with a salt concentration of approximately 75%. Specifically, the protein starts detaching from the heparin column at 1.1M NaCl (peak 1) and continues to elute to until the gradient has reached 100%, that is 1.5M of NaCl. The peaks (1,2,3) on the chromatogram overlap and US11 is eluted in multiple fractions. Roughly, on the SDS-PAGE gel (fig. 9B), peak number 1 corresponds to fractions A11 and A12, peak 2 contains fractions B1, B2 and B3 and peak 3 corresponds to fractions B4-B6.
Figure 9: (A) Typical chromatogram profile of recombinant US11 eluted off a heparin column using an FPLC system. The purple peaks indicate a rise in absorbance, the green line indicates the gradient of salt concentration while the orange line represents the rise in relative conductivity. The protein starts detaching from the heparin column at around 1.1M NaCl and continues to elute as the gradient has reaches 1.5M of NaCl. The peaks on the chromatogram overlap and US11 is eluted in multiple fractions. (B) Peak fractions were run on a 15% SDS-PAGE gel. Lane (1) molecular weight marker, lane (2) US11 from B virus eluted off a nickel affinity column, lanes (3-14) fractions eluted off a heparin affinity column. Peak number 1 corresponds to fractions A11 and A12, peak 2 contains three fractions B1, B2 and B3 and peak 3 corresponds to fractions B4-B6. The blue arrow indicates a contaminating protein band of approximately 60 kDa.
3.3.3. Gel Filtration

Of note in the purification profile for US11 from the heparin affinity column, is the presence of a contaminating protein of higher molecular weight, approximately 60 kDa (indicated by a blue arrow). The protein band appears from fractions A12 to B4 (lanes 8-12) and also seems to intensify as the later fractions are eluted off the column. Also of note here, is that the approximate amount of US11 in these fractions does not vary much, in contrast to the higher MW protein. An attempt to further purify to try to remove the upper protein band was made by applying the samples from the heparin column on to a gel filtration column. The column used was a gel filtration S200 column, using PBS as the running buffer. Fractions (A11-B2) containing US11 were pooled off the heparin column, loaded on to the 50 ml S200 column and eluted over 1.5 volume lengths at a flow rate of 0.5-1 ml/minute. Peak fractions were run on a 15% SDS-PAGE gel and the resulting fraction containing US11 can be seen in figure 10.

As seen in figure 10, there is still the presence of a higher molecular weight band in the US11 fraction. The band under US11 is most likely a degradation product of the protein, as they react with anti-his tag antibodies on Western blots. Since gel filtration separates according to size, and this band is clearly larger than US11, it was curious that we were not able to remove this band. Therefore our next step was to try to identify the higher molecular protein band by mass spectrometry.
Figure 10: US11 purification on a S200 gel filtration column. Fractions of US11 from B virus from a heparin column were pooled and loaded on to a 50 ml S200 gel filtration column. (A) Chromatogram profile of recombinant US11 eluted off a gel filtration column using an FPLC system. (B) Peak fractions (A6, A7, A12 and B1, indicated by an asterisk) were run on a 15% SDS-PAGE gel and the fraction containing US11 is shown. Lane (1) protein molecular weight marker, (2) eluate containing US11 from the S200 column.
3.3.4. Mass Spectrometry

The fractions containing purified US11 protein off the heparin column were sent for mass spectrometry analysis. We excised both US11 and the approximate 60 kDa molecular weight band out of a 15% SDS-PAGE gel which had been stained with GelCode blue™. We included control pieces of the gel collected between the protein bands to reduce the probability of carry-over from protein to protein during the mass spectrometry analysis. We did not excise the bands below US11 because these bands are most likely degradation products of US11. The mass spectrometry results confirmed that the 21 kDa protein was US11. There were two proteins identified by mass spectrometry on the 60 kDa band. Mass spectrometry identified peptides that corresponded most likely to the US11 protein and also identified peptides that were most probably from a prokaryotic elongation factor (EF-Tu). The mass spectrometry results from 3 independent sets of samples were identical. US11 from the heparin column from all viral sources were sent and the results were the same as stated above. Figure 11 shows the identified peptides and protein sequence by mass spectrometry for the two protein bands. EF-Tu was identified with a score of 179 with scores >50 being considered significant at p<0.05. US11 identified in the 60 kDa spot was identified with a score of 141 and US11 at the 21 kDa spot was identified with a score of 220.
Figure 11: Mass spectrometry results. This figure highlights the mass spectrometry results for two protein samples from recombinant US11 from B virus expressed in E. coli cells and purified using nickel and heparin affinity chromatography. Generated peptides were matched to known proteins using the NCBI database and Mascot Daemon. Red sequences indicate the generated peptide sequences from samples and black peptide sequences indicate that of a known protein.
The EF-Tu protein contained in the 60 kDa band is an elongation factor from *E. coli* with a molecular weight of 43 kDa. EF-Tu is known to be a ribosome-associated protein \(^7,13\). In bacterial protein synthesis, EF-Tu is responsible for the transport of aminocylated tRNAs to the mRNA:ribosome complex. US11 is also a ribosome-associated protein and hence it is possible that the two proteins may form a strong bond on the ribosome. The 43 kDa EF-Tu factor and the 21 kDa US11 could conceivably form a ~60 kDa complex. In an attempt to remove the 60kDa band, strong denaturants and detergents such as urea (8M) and NP40 (1%) were added during the nickel purification step to try to break the hypothetical bond between US11 and the 60 kDa protein. Boiling of protein samples for extended periods in SDS buffer was also done to try to break any residual disulfide bonds in protein interactions. Neither method had any effect on removing the contaminant. At this point, we decided to isolate the cleanest fractions of US11 from the heparin column and move a head with our studies. The fractions were those in the first US11 peak off the heparin column that did not contain the 60 kDa contaminant (see fig 9B).

In order to investigate if the 60 kDa contaminant had an effect on the binding characteristics of US11, we measured the Kd value of US11 by filter binding assays, using equal protein amounts of “clean” (fraction of US11 off the heparin column containing no or very little of the ~60 kDa protein) and “dirty” (fraction of US11 off the heparin column containing the ~60 kDa protein) fractions of US11 off the heparin column. Referring to figure 9B above, the fractions used were A11 and B2. The results are graphically presented in figure 12 along with the calculated Kd values. As seen in the figure, the Kd values of 3.2 nM and 2.7 nM are not significantly different. There is
however a visible difference in the maximum binding (plateau), which was 2.0 pmoles and 0.5 pmoles for fraction B2 and A11, respectively. This suggests that there is a difference in the amount of active protein in these fractions, nonetheless this does not affect the Kd value. From this data we conclude that the 60 kDa does not affect the affinity of US11 for dsRNA, but that it is either required for maintaining the activity of US11 or, it may be that the first peak out of the heparin column may represent misfolded US11 protein.

Successful purification of US11 from B virus and its minimal degradation lead to the further purification of the other US11 proteins. Figure 13A shows a 15% SDS-PAGE gel containing the cleanest fraction off the heparin columns for each US11 protein. To confirm that this was in fact our recombinant protein, a western blot was performed on these fractions (figure 13B).
Affinity Assay using B virus US11 Fractions Post FPLC Purification

Figure 12: Filter binding assay using a clean and dirty fraction of US11 from B virus after purification by FPLC on a heparin column. The solid square indicates the binding profile of dsRNA by US11 from fraction A11 (clean fraction) and the solid triangle indicates the binding profile of dsRNA by US11 from fraction B2 (dirty fraction).
Figure 13: Fractions of US11 eluted by nickel and heparin affinity chromatography. (A) Eluted fractions were run on a 15% SDS-PAGE gel and stained with GelCode Blue stain™ (Fisher). Lane (1) molecular weight marker, (2) Recombinant US11 from HSV1, (3) US11 from HSV2, (4) US11 from HVP2 and (5) US11 from B virus. (B) Western blot of fractions of US11 after nickel and heparin affinity chromatography using an anti-his tag antibody. Lane 1 in figure B contains MagicMark™ XP Western blot protein standard (Invitrogen), a protein ladder different from the pre-stained protein ladder (Invitrogen) used in lane 1 figure A.
3.4. RNA Binding Activity by US11 using EMSA

Double stranded RNA binding is an intrinsic ability of US11. In order to confirm dsRNA binding activity of the purified US11 proteins we employed electrophoretic mobility shift assays. We designed a 42 base pair random sequence of RNA (see table 5 in Material and Methods section for sequence). We chose this length based on previous work which showed that US11 from HSV1 binds a minimum of 30 to a maximum of 300 bp of dsRNA efficiently. Optimization experiments were carried out to establish the most suitable amount of RNA to be used in these experiments as well as a suitable length of time for binding. We concluded that 20ng of RNA with increasing concentration of protein for a binding time of 30 minutes was most appropriate for our experiments. Figure 14A displays a shift assay using HSV1’s US11 from nickel and heparin column purification. At lower concentrations of protein (lanes 4-7; 4.3ng, 6.4 ng, 9.7 ng and 14.5 ng US11) only one shifted band is visible by ethidium bromide staining. A second band of higher molecular weight (MW) appears at higher concentrations of protein (lanes 8-10; 21.8 ng, 32.6 ng and 48.98 ng US11) and some RNA forms a high MW smear or remains trapped in the gel wells at the highest protein concentration (lanes 11-12; 73.5 and 110 ng US11). This result indicates that more than one molecule of US11 protein can bind to one molecule of dsRNA.

To ensure that this was in fact US11 that was binding to and retarding the movement of the RNA, we employed two means for visualization of the protein. The first was staining the gel in figure 14A with SYPRO Ruby™ luminescent protein stain (Invitrogen). Figure 14B shows that the protein pattern visualized is the same as the shift pattern of the RNA in part A. The second means for confirming the presence of US11
was by performing a Western blot on the protein using an anti-histidine tag as the primary antibody (figure 14C). Of note is that in figure 14C, lane 7, there is not a strong visible band of protein as in figure 14B lane 7, all though there was the same amount of protein added in these lanes. Firstly, SYPRO® Ruby staining is not quantitative and therefore the amount of protein in lane 7 figure 14B can look deceivingly high. In addition it may be that there was a technical issue in figure 14C where an air-bubble could have occurred in the spot during membrane transferring or during antibody binding that prevented the protein band from developing in the Western blot.
Figure 14: Electrophoretic mobility shift assay with dsRNA and US11. (A) US11 from HSV1 post-purification was added in varying concentrations to 20ng of dsRNA. Lane (1) DNA ladder, (2) protein only, (3) RNA only, (4-12) 20 ng of RNA with increasing concentrations of US11 ranging from 4.3-110 ng. Samples were run on a 10% non-denaturing gel and stained with ethidium bromide. (B) Gel from part (A) stained with SYPRO® Ruby protein stain. Staining of gels in part (A) and (B) was visualized using the Gel Doc XR (Biorad) UV transilluminator. (C) Western Blot analysis of the gel in part (A) is shown using an anti-his tag antibody. Lane 1 in part C contains MagicMark™ Western blot protein standard (Invitrogen).
As part of our shift assays, we decided to incorporate DNA as a control for binding of US11 to RNA based on the fact that in recent literature, US11 has been described predominantly as an RNA binding protein. However once we combined our 42 bp random dsDNA (of same sequence as the RNA used above) together with the highest concentration of protein in the shift assay, we noticed that US11 also seemed to bind the random dsDNA as well. This is in agreement with previous reports in the literature that show that US11 is also able to bind dsDNA \(^{21,41}\). Therefore we performed an electrophoretic mobility shift assay using the same concentration of DNA and protein as we had used in the RNA shift assays. Figure 15A shows that US11 does bind dsDNA but with a lower affinity. For example, in lane 6 (figure 14A) close to half the RNA is bound by 21.8 ng of US11 where as in figure 15A, lane 6, the dsDNA does not seem to be bound by the same concentration of protein. In addition, the pattern of bands does not seem to be as clear cut as for the RNA binding (see fig. 14A), suggesting that US11 may bind to DNA with a different mechanism. US11 probably binds DNA more randomly as in smaller concentrations the protein does not bind but when there is a lot more protein present, US11 binds most of the DNA (lanes 8-12, figure 15A). The presence of US11 was also confirmed in figures 15B and C, similarly to what was done in figure 14B and C.
Figure 15: Electromobility shift assay with dsDNA and US11. (A) US11 from HSV1 post-purification was added in varying concentrations to 20ng of random dsDNA. Lane (1) DNA ladder, (2) protein only, (3) DNA only, (4-12) 20 ng of DNA with increasing concentrations of US11 ranging from 4.3-112 ng. Samples were run on a 10% non-denaturing gel and stained with ethidium bromide. (B) Gel from part (A) stained with SYPRO® Ruby protein stain. Staining of gels in part (A) and (B) was visualized using the Gel Doc XR (Biorad) UV transilluminator. (C) Western Blot analysis of the gel in part (A) using an anti-his tag antibody. Lane 1 in part C contains MagicMark™ Western blot protein standard (Invitrogen).
3.5. Affinity Assays

3.5.1. Radioactive Gel Shift Assay

In order to measure the affinity of US11 for dsRNA, a quantitative assay was needed. This first assay we employed was a radioactive gel shift assay. In this case we radio-labeled the dsRNA with $\gamma^{32}$P-ATP and performed the assay the same as the shift assays we used above. The concentration of $^{32}$P RNA was negligible and was not taken into account in determining the RNA concentration in the reactions. Therefore only 20 ng of cold RNA is what was accounted for. Reactions were done in a 20 µl volume with varying concentrations of protein and samples and were run on a 10% non-denaturing gel. The gel was then scanned using a typhoon phosphoimager and the intensity of the radioactive bands was quantitated. Figure 16 shows an example of a typhoon image of a gel shift experiment.

In order to calculate the apparent Kd the intensity of the free RNA band was measured as a fraction of the total RNA, and the bound RNA was calculated as $[\text{RNA}]_{\text{total}}- [\text{RNA}]_{\text{free}}$ and plotted against the protein concentration. Non linear regression analysis of the equilibrium binding equation was performed as shown in section 2.9. The apparent Kd value calculated from this experiment shown in figure 16 was 170 nM. This value is similar to a Kd calculated by Bryant et al. in which they calculated Kd value for US11 and a 81 bp dsRNA sequence to be 70 nM using a similar technique $^6$.

However both these values are quite different from the range of Kds calculated by filter binding assays, as shown below. Calculation of the Kd of US11 binding to dsRNA using a radioactive gel shift assay presents several problems. First, the amount of US11-dsRNA complex could not be measures with confidence because of the presence of
multiple bands and smearing of the US11-dsRNA complex. Therefore the amount of complexes could only be calculated from the intensity of the bands of the free RNA. The binding of multiple protein molecules to RNA cannot be quantified. A second problem was that RNA was not in excess with respect to the protein and therefore the free RNA concentration had to be calculated as [total RNA]-[bound RNA]. Since the bound RNA could not be measured directly, as explained above, we were not confident that this type of experiment could produce a valid Kd. Another problem is that the fraction of active protein is not known and cannot be determined by this technique. The measured Kd value would be overestimated if the fraction of inactive protein is substantial.

Therefore in order for the inactive protein to be irrelevant, according to laws of equilibrium kinetics the RNA must be in excess in the binding reactions. Since gel shift assays can only handle a small reaction volume that would not allow for our RNA to be in excess, we decided to abandon the gel shift assay as a means for quantifying the amount of dsRNA bound by each US11.
Figure 16: Radioactive EMSA. $^{32}$P-labeled RNA (20 ng) was run on a 10% non-denaturing polyacrylamide gel alone, lane (1) or mixed with varying concentrations of US11 (6.25 ng, 12.5 ng, 25 ng, 50 ng and 100 ng), lanes (2-6). The gel image was visualized using a typhoon phosphoimager (Amersham Biosciences).
3.5.2. Filter Binding Assays

Filter binding assays (FBAs) are an alternative way to measure the affinity of two molecules. For an easier measurement of the Kd, in equilibrium kinetics, the ligand should be in excess to the protein. In addition, we found that two or more molecules of protein can bind to one molecule of RNA (figure 14A) which complicates the quantitation of US11-RNA complexes. With excess RNA, binding of multiple US11 molecules to one RNA molecule becomes negligible, if we assume that the US11 binding to RNA is not cooperative. This biochemical assay is advantageous because a large reaction volume can be used instead of the 20μl maximum volume that can fit in the well of a gel in an EMSA assay. In addition, theses assays are fast and fairly simple. The principle involves incorporating a way to quantify how much of a substrate is bound to a ligand during varying concentrations. In order to quantify the nucleic acid bound, a 32P labeled RNA was used as a tracer. The protein was initially allowed to bind to Ni resin added to the reaction mixture, radioactive RNA was then added and allowed to bind to the protein for 30 minutes. The binding reaction was applied to glass filters that retained the resin with bound protein complexed with labeled RNA. Unbound material is washed away with repeated washes of binding buffer. Therefore only protein-RNA complexes bound to the Ni resin remained on the filter. The filters were placed into a scintillation counter to measure the amount of radioactivity and hence the amount of RNA trapped on the filter after washing excess RNA away. The pmoles of US11-RNA complexes were plotted against each concentration of RNA which generated a binding curve from which a binding constant could be extrapolated (see section 2.9 for details).
The conditions of the assay were optimized by determining the appropriate range of RNA concentration as well as the amount of washing that was needed to remove excess unbound radioactive RNA. We initially used an RNA concentration of 250 nM – 0.4 nM, and a fixed protein concentration of 2 nM. These concentrations were chosen based on the fact that the protein concentration should be in the range of the Kd in molar concentration and the range of RNA should include concentrations lower than that of the protein as well as in great excess of the protein and anticipated Kd 4. We suspected that the Kd would be low, possibly in the range of 1-70 nM based on previous research done on HSV1 US11 and dsRNA binding 6,14. However after experimentation, we realized that it was not necessary to use such high RNA concentrations of 250, 125 and 62.5 nM and decided on a range or 31.25 nM – 0.24 nM instead with 0.7 nM of US11. The 0.4 and 0.24 nM concentrations of RNA were not in excess of the protein in molar concentration, however we have found that the protein only binds ~0.01 nM of the 0.24 nM of RNA that was added and therefore the bound amount was negligible. The 31 nM concentration still allowed the RNA to be in excess of 44-fold over the protein and also to be higher than the Kd value.

We performed the affinity assay for each US11 protein and compared their Kds for dsRNA. We performed filter binding assays for US11 from HSV1, HSV2, HVP2 and B virus side by side. We carried-out these assays over 6 times and took the weighted average of the Kds. Figure 17 shows a typical experiment for all four US11 proteins. Table 6 summarizes the weighted mean Kds for each US11 protein together with the variance and standard deviation of the weighted mean. The difference between Kd values for different US11 proteins were not statistically significant, as determined by a one-way
ANOVA test. We then calculated the average of all the individual Kds for US11 from HSV1, HSV2, B virus and HVP2 and dsRNA to be approximately 2.6 nM with an average standard deviation of 1.0 nM.
**FBA for all US11 Proteins**

![Graph depicting binding assay results for US11 from HSV2, HSV1, B virus, and HVP2. The graph shows the concentration of RNA (nM) on the x-axis and the number of pmol bound on the y-axis. Different symbols represent different viruses: square for HSV2, triangle for B virus, cone for HSV1, and diamond for HVP2. The concentration of RNA used was 31.25-0.24 nM and the protein concentration was fixed at 0.7 nM.]

**Figure 17:** Representative experiment of a filter binding assay for US11 from HSV2, HSV1, B virus, and HVP2. The solid square, triangle, cone, and diamond indicate the binding profile of US11 for dsRNA from HSV2, B virus, HSV1, and HVP2, respectively. The concentration of RNA used was 31.25-0.24 nM and the protein concentration was fixed at 0.7 nM.
Table 6: Summary of the Affinity of US11 for dsRNA from HSV1, HSV2, B virus and HVP2.

<table>
<thead>
<tr>
<th>US11 Source</th>
<th>Weighted Mean of Kds (nM)</th>
<th>Weighted Sample Variance</th>
<th>Standard Deviation of the Weighted Mean (nM)</th>
<th>Kd Range (nM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV1</td>
<td>1.602</td>
<td>2.055</td>
<td>0.585</td>
<td>0.46 - 8.9</td>
<td>7</td>
</tr>
<tr>
<td>HSV2</td>
<td>1.067</td>
<td>0.859</td>
<td>0.463</td>
<td>0.29 - 4.27</td>
<td>6</td>
</tr>
<tr>
<td>B Virus</td>
<td>1.819</td>
<td>3.723</td>
<td>0.788</td>
<td>0.35 - 6.1</td>
<td>7</td>
</tr>
<tr>
<td>HVP2</td>
<td>1.855</td>
<td>3.268</td>
<td>0.808</td>
<td>0.42 - 6.1</td>
<td>6</td>
</tr>
</tbody>
</table>

1A statistical significance report of the Kd values listed in Table 6 using a one-way ANOVA Test was performed in which the P value calculated was 0.2336, where P < 0.05 is considered to be significantly different.

The Kd values varied between experiments, but within each experiment the Kd was similar for all the US11 proteins. It is likely that the variation between the experiments was due to differences in the preparation of the RNA substrate, the degradation of RNA or other uncontrolled factors. In fact, experiments performed with the same RNA preparation showed similar Kd values. For example, the Kd values for four separate filter binding assays using two RNA preps are shown in table 7.

Table 7: Kd Values for Four Filter Binding Assay Experiments using Two Different Preparations of 32P-RNA.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HSV 1 US11 Kd (nM)</th>
<th>HSV 2 US11 Kd (nM)</th>
<th>B virus US11 Kd (nM)</th>
<th>HVP 2 US11 Kd (nM)</th>
<th>RNA Prep</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.340</td>
<td>N/A</td>
<td>6.071</td>
<td>6.277</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>2.597</td>
<td>N/A</td>
<td>4.361</td>
<td>6.097</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>1.842</td>
<td>1.377</td>
<td>1.692</td>
<td>1.134</td>
<td>B</td>
</tr>
<tr>
<td>4</td>
<td>1.252</td>
<td>1.657</td>
<td>2.226</td>
<td>2.579</td>
<td>B</td>
</tr>
</tbody>
</table>

1The Kd values for US11 from HSV 2 were not available in these experiment because at the time of the experiment the protein had become inactive.
We were not able to quantify the exact amount of RNA that became radiolabeled but we know that the maximum amount of $^{32}$P-RNA added to the first concentration of cold RNA (31 nM) was 5-10% of the RNA. This ratio is probably a lot lower, as this estimate is assuming all the RNA is recovered after labeling with $\gamma^{32}$P-ATP and purification. Also of note, is that initial experiments were done in two days, i.e. filter binding assays were done with two proteins on one day, the batch of RNA was frozen overnight and used for affinity assays for another two proteins the following day. However we noticed that whatever proteins were done on the second day of experiments (varied order of proteins) seemed to have a higher affinity for RNA, that is a lower Kd value. Therefore we suspected that by taking two days to do the filter assay experiments, this allowed for the dsRNA to be degraded and therefore the proteins assayed on the second day appeared to have a higher affinity because there was actually less dsRNA to bind to than for the first set of proteins. Therefore, to resolve this, we performed the filter binding assays for all four proteins in one day, storing the dsRNA on ice to minimize degradation.

It should also be noted that the maximum binding in the above experiment (fig. 17) was approximately 0.2 nM, considering we put in 0.7 nM of protein the maximum binding is lower than the total amount of US11. The first reasoning for this lower maximum binding is that not all of the protein put into the experiment is active. This is to be expected in recombinant protein assays. Another factor is that our protein needed to be diluted several times in order to reach a concentration to which the RNA could be in excess in the binding experiments. These dilutions could have lead to loss of some of the protein during the procedure, for example on the sides of the plastic tubes or in pipette
tips used in the dilutions. In the future the loss of protein could be prevented by the addition of BSA or glycine to the protein stocks to prevent the protein from adhering to laboratory consumables.

In order to confirm that the binding observed was due to specific interactions between US11 and RNA, we performed an experiment in which the amount of protein was varied instead of the RNA with the intent that the Kd would not change. The protein used was US11 from HSV1. In this case, a protein concentration range of 100 nM-1.5 nM was mixed with a fixed dsRNA concentration of 1nM and allowed to bind. A graphical representation of the results from the experiment is shown in figure 18. The Kd value calculated by non-linear regression was 0.7 nM, a value that is in the range of the Kd value for HSV1 listed in table 6. Thus, this experiment further solidifies that the Kd values in table 6 are accurate.
Figure 18: Filter binding assay using a varied concentration of US11 from HSV1 and a fixed concentration of dsRNA. Protein concentrations used: 100, 50, 25, 12.5, 6.25, 3.125 and 1.5 nM, RNA concentration was fixed at 1nM. The Kd value was calculated in the same manner as in figure 17 using nonlinear regression analysis as shown in section 2.9.
4.0. Discussion

In this work we have expressed in bacteria and purified the US11 proteins from 4 closely related simplexviruses, the human HSV1 and HSV2 and the simian B virus and HVP2. We then developed a filter binding assay to correctly measure their Kd of binding to a 42bp long molecule of dsRNA. Repeated measurement of the Kd showed that the value is approximately 2.6 nM, with no significant difference among US11 from the 4 viruses.

We have successfully cloned and expressed US11 with a His tag from HSV 1, HSV 2, B virus and HVP2 and we have purified by a 2-step method involving a nickel column and heparin affinity purification to a degree of purity that should be sufficient for an unbiased determination of RNA binding, as shown in this thesis, and for future experiments on PKR inhibition activity. Unfortunately we were unable to express US11 from SA8. The reasons for this still remain unclear. In the future, expression of SA8 US11 may be attempted using a eukaryotic system such as yeast or baculovirus.

A contaminating band of approximately 60 kDa was however visible on the gels after purification with Ni and heparin affinity columns. Several attempts to remove the contaminant by additional gel filtration chromatography and treatment with detergents failed. Analysis by mass spectrometry showed that the 60 kDa band contained both US11 and EF-Tu, a 43 kDa ribosomal protein from *E. coli*. The 60 kDa MW is consistent with a complex consisting of US11 and EF-Tu. The fact that the 2 proteins could not be separated by detergents and gel filtration is puzzling and may indicate covalent binding. US11 from HSV1 has been expressed in bacteria before but to our knowledge, no one has shown or discussed the SDS-PAGE profile for the protein.\textsuperscript{6,14,31}
However, US11 without the 60 kDa contaminant can be obtained from the initial fractions from the heparin column (see figures 9B and 13A). This is suggestive of different species of the US11 protein and in fact the “clean” fractions showed lower activity than the “dirty” fraction with the 60 kDa band (see figure 12). Both “clean” and “dirty” fractions had the same Kd of binding to RNA, suggesting that the contaminant did not bind significantly to RNA in our assay and did not influence the binding affinity of US11 for dsRNA. The difference in maximum binding is suggestive that the activity of these proteins is different. It is possible that the first fraction of US11 off the heparin is misfolded protein and is therefore less active. Also, since both EF-Tu and US11 are ribosome associated protein, it is a possibility that the ~60 kDa band that we identified to contain EF-Tu is necessary for the full function of US11. EF-Tu could be supportive for US11 to bind dsRNA at the ribosome in vivo. This hypothesis however, seems unlikely because EF-Tu appears present in much lower amounts than the abundant US11 (see figures 9B and 10B) and the EF-Tu in this case is of prokaryotic origin. However it may be that US11 associates with RNA on the ribosome in the prokaryotic system and since EF-Tu is a ribosome protein the two proteins are somehow linked and are co-purified through nickel and heparin chromatography.

From our RNA-shift assays we were able to show that post-purification, US11 remained active and had a high affinity for dsRNA. In addition, we were able to show that US11 binds dsDNA of the same length and sequence but with lower affinity and probably less specificity. Further experimentation would be necessary, such as competitive binding assays to see if this is the case. The thought arises then that even though US11 does not seem to recognize specific sequences, it can distinguish between
nucleic acid containing one-less hydroxyl group and those containing 2' hydroxyl groups. From the shift pattern of two bands of dsRNA shown in our assays, it is suggestive that US11’s binding to dsRNA is not co-operative. In co-operative or simple binding there is the presence of one shifted band of nucleic acid when protein is added, however in non-co-operative binding there is often the presence of multiple shifted bands. This hypothesis would need to be further investigated but the binding is more complex and may consist of multiple binding sites that may be sequentially filled as the protein concentration is increased.

However gel shift assays are not suitable for a correct measurement of the Kd of US11 and RNA interaction because it is difficult to accurately measure the amount of US11/RNA complex due to the presence of many bands and smears. In addition, it is difficult to work under conditions of excess RNA in particular, which favors the formation of complexes with only one molecule of US11. For these reasons we decided to measure the Kd using a filter binding assay. This assay allowed us to directly measure the amount of US11 and RNA complexes bound to the filters under conditions of excess free RNA.

Through our filter binding assays we conclude that US11 from HSV1, HSV2, B virus and HVP2 binds dsRNA with the same high affinity, independent of the number of R-X-P repeats within the protein and we calculated an average Kd of approximately 2.6nM. Other papers have suggested that US11 binds different conformational isomers of dsRNA of a specific primary sequences with different affinities \textsuperscript{6,14}. These observations were made through gel-shift assay profiles. Therefore it would be interesting to investigate these isomers in detail and use our filter binding assays to look at each
US11’s affinity for more complex dsRNA sequences. It could be that the dsRNA in our study was not complex enough to see a difference in the affinities of US11 protein from each of the simplexviruses.

Both Bryant et al. and Khoo et al. have reported Kd values for US11 for different dsRNA species$^{6,14}$. Both papers also showed that the affinity for dsRNA varied and was impacted by the length of the nucleic acid. Bryant et al. reports a Kd of 70 nM in the case of US11 for a 67 bp dsRNA fragment which was achieved by electrophoretic mobility-shift assays. They were also able to map the binding site of US11 to within a 46 base pair segment of the RNA. On the other hand Khoo et al. reported an apparent Kd value of 12.6 nM for a 81 bp dsRNA species using a filter binding assay. Clearly the Kd values obtained thus far for US11 from HSV1 and dsRNA is highly dependant on the size of the dsRNA fragment and probably also on the techniques used to establish the Kd value.

Compared to other Kd values listed for US11 the variation as mentioned above, depends on the dsRNA species, specifically in its length and in the techniques used to measure the Kd value. We feel that the filter binding assay allows for a less biased result as it allows the ligand to be in excess over the protein and takes into account the free RNA in the reaction which is an important parameter in equilibrium kinetics. In the case of the Bryant et al. study, this rule was not achieved and therefore their Kd value of US11 for dsRNA is mistakenly high. In addition, our experiments were repeated over six times for each protein with slight variations. In the end, we took the weighted mean of the Kds which makes for a more accurate measure than one or two repeated experiments.

The exact role(s) of US11 during the course of herpesvirus infection still remains unclear. It is however evident that US11 binds dsRNA of various lengths and binds
RNAs of higher structure more efficiently. Exactly what species of dsRNA US11 binds still needs to be elucidated. US11 may bind and sequester dsRNA produce by its virus as a means to prevent the activation of PKR. This theory is supported by binding of in vitro transcribed RNA complementary to the US11 open reading frame and segments derived from the HSV 1 UL12, UL13 and UL14 mRNAs and a truncated version of the UL32 mRNA. US11 may bind RNA as a means of homing to get closer to PKR and physically inhibit the enzyme. This is most likely the case, as studies have shown that US11 forms a physical association with PKR. It is also clear that US11 inhibits PKR. In addition, US11 could also bind dsRNA within the cellular environment as a means to regulate gene expression. US11 has been shown not to be necessary for viral replication in vitro. However there is evidence surfacing that the cell lines used in these studies were permissive for the growth of viruses that are inhibited by interferon. Therefore the role of US11 remains to be investigated and proves to be an appealing factor in answering questions to the complex pathogenicity of simplexviruses. The cloning, expression and purification of US11 in our laboratory leads to the availability of the proteins for PKR inhibition studies. Our study presents the idea that the dsRNA binding of US11 is a basic ability that is not affected by the length of its dsRNA binding motif. However it raises the possibility that the R-X-P domain and the length of its repeats is more important in the physical association and inhibition of PKR.
4.1. Future Work

We have established that all US11 proteins from the different simplexviruses have the same affinity for RNA, the next step would be to measure their ability to inhibit PKR *in vitro*. This can be achieved by preparing PKR containing extract from human cell lines and measuring the phosphorylation activity of PKR in the absence or presence of dsRNA and in the absence and presence of the various US11 proteins. In addition, considering several papers have reported different Kd values for dsRNAs of different length, it would be appealing to test our method of the filter binding assays on US11 and different lengths of dsRNA to establish whether the Kd will change. It would also be beneficial to express US11 from SA8 since this protein posses such a low number of R-X-P repeats. It could show whether there is a minimum number of repeats required for dsRNA binding and or PKR inhibition. Another interesting future project would be to make deletion mutations in the C-terminal region to investigate this same question. It could also be that the binding of dsRNA US11 is not dependent on the number of R-X-P because this step is a superficial step in US11’s function and is used to get into closer proximity to PKR.
Reference List


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