THE ROLE OF HSV-2 PROTEINS ICP0 AND US3 IN COUNTERACTING CELLULAR ANTIVIRAL DEFENCE

by

Stephanie Wan

A thesis submitted to the Microbiology and Immunology Graduate Program within the Department of Biomedical and Molecular Sciences in conformity with the requirements for the degree of Master of Science

Queen’s University
Kingston, Ontario, Canada
(January, 2014)

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Abstract

In response to viral infection, host cells activate various antiviral defence mechanisms to inhibit virus replication. Therefore in order for a virus to replicate efficiently, it must counteract cellular antiviral defence. Promyelocytic leukemia protein (PML) is a cellular protein involved in intrinsic immunity. It inherently forms nuclear bodies (PML-NBs) that assemble at the site of viral genomes. Proteins related to epigenetic regulation are recruited to PML-NBs, and silence viral gene transcription. This study focuses on the role of two herpes simplex virus type 2 (HSV-2) proteins, ICP0 and Us3, in disrupting PML-NBs and counteracting cellular antiviral defence.

*En passant* mutagenesis was used to create recombinant HSV-2 viruses lacking ICP0, Us3, or both ICP0 and Us3. Growth analysis of these recombinants indicates no growth defects for the ICP0Δ virus, while the Us3Δ virus grows to one log lower titres than wild type virus (WT). By contrast, the ICP0Δ virus displays a delay in PML-NB disruption, but the Us3Δ virus is as efficient as WT. However, Us3 is still important for PML-NB disruption, since the ICP0Δ/Us3Δ double mutant exhibits a greater delay than the ICP0Δ single mutant.

Although PML is a mediator of the interferon (IFN) response and it was predicted that ICP0 and Us3 interfere with the IFN response through disruption of PML-NBs, my results show that only some HSV-2 Us3Δ clones are hypersensitive to the effects IFN, and others are resistant. Us3 affects more than one cellular pathway, and those cellular pathways are affected by more than one viral protein. I conclude that the activities of multiple viral proteins create a fine balance between activating cellular pathways to promote virus replication, and inhibiting cellular antiviral defence.
Co-Authorship

HSV-2 186 Us3Δ and repair viruses were constructed by Dr. Valerie Le Sage.
Acknowledgements

First and foremost I would like to thank my supervisor, Dr. Bruce Banfield, for all his support and guidance during my Master’s studies. His office was always open for questions and discussions, and he provided helpful supervision while allowing me to learn and work independently. Thank you as well to my committee, Dr. Eric Carstens and Dr. Nancy Martin, for their advice and encouragement.

I also could not have completed this thesis without the help of my lab members. Thank you to Dr. Renee Finnen for introducing me to the lab and for her continuous support throughout these two years. Thanks to Dr. Valerie Le Sage for passing on her expertise in en passant mutagenesis and for helping me with all my troubleshooting. Thank you to Dr. Linda Kang for sharing her experiences and in particular for helping me with the immunoprecipitation protocol. A big thank you to Masany Jung who is always willing to discuss my project with me, and for her insight and knowledge when nothing seemed to make sense. A special thank you to Jake Alter for sharing this Master’s experience with me, and for all the fun times at the lab.

I would also like to express my appreciation for the staff members in the department who ensure that everything is running smoothly. In particular, thank you to Jerry Dering for washing all our glassware and for sharing his music and smiles, and to Alana Korczynski for her helpfulness in booking rooms and equipment.

Outside the lab, I am lucky to have the unconditional support of all my family and friends, and I have to thank them for their well wishes and moral support.
Finally, thank you to the Microbiology and Immunology program for providing me with an excellent learning environment. It has been a wonderful experience completing my Master’s degree here at Queen’s University.
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<th>Description</th>
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<tbody>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CoREST</td>
<td>RE1-silencing transcription factor corepressor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocytes</td>
</tr>
<tr>
<td>E</td>
<td>Early gene</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
</tr>
<tr>
<td>GAS</td>
<td>Gamma activation site</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetylase</td>
</tr>
<tr>
<td>HCF</td>
<td>Host cell factor 1</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>HD</td>
<td>Histone demethylase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HFV</td>
<td>Human foamy virus</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>HPI</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>HHV</td>
<td>Human herpesvirus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatin protein 1</td>
</tr>
<tr>
<td>HSV-1/2</td>
<td>Herpes simplex virus type 1/2</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon stimulated response element</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi's sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>L</td>
<td>Late gene</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>MCP</td>
<td>Major capsid protein</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal/sequence</td>
</tr>
<tr>
<td>Oct-1</td>
<td>Octamer binding transcription factor</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia protein</td>
</tr>
<tr>
<td>PML-NBs</td>
<td>Promyelocytic leukemia protein nuclear bodies</td>
</tr>
<tr>
<td>PRV</td>
<td>Pseudorabies virus</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>SCP</td>
<td>Smallest capsid protein</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin like modifier</td>
</tr>
<tr>
<td>TRI-1/2</td>
<td>Triplex monomer protein/trplex dimer protein</td>
</tr>
<tr>
<td>TRIM</td>
<td>tripartite motif</td>
</tr>
<tr>
<td>VHS</td>
<td>Virion host shutoff protein</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatis virus</td>
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<tr>
<td>VZV</td>
<td>Varicella zoster virus</td>
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Chapter 1

Introduction

The *Herpesviridae* family of viruses are enveloped, linear double stranded DNA viruses. There are three subfamilies, consisting of *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae*. Herpes simplex virus type 2 (HSV-2) is an alphaherpesvirus that causes genital herpes infections, devastating neonatal infections, and is fuelling the epidemic transmission of HIV in Sub-Saharan Africa [1]. Therefore it is important to develop new strategies for limiting the consequences of infection and preventing transmission of virus. In order to do so, we must understand the interaction between viruses and the cells they infect.

After HSV-2 infection, the viral genome is delivered to the cell nucleus and becomes associated with cellular histones [2]. An intrinsic cellular antiviral response to this incident is to repress viral gene expression by recruiting chromatin modifiers and chromatin remodelling proteins that aim to impart a transcriptionally inaccessible, heterochromatin-like, state on the viral genome. This antiviral defence mechanism is mediated by cellular structures called promyelocytic leukaemia nuclear bodies (PML-NBs).

Promyelocytic leukemia protein is a member of the tripartite motif (TRIM) family of proteins. Many TRIM proteins play an important role in cellular antiviral defences. PML is of particular interest because it has been shown to inhibit DNA viruses, RNA viruses, and retroviruses [3]. PML interacts with itself and a variety of other proteins to form punctate structures in the nucleus called PML-NBs. Although numbers are cell type
dependent, there are on average 15 PML-NBs per cell, and they have been associated with many different functions, including transcriptional regulation, protein modification, and involvement in the DNA damage response and apoptosis [4-6]. This thesis deals with the role of PML-NBs in antiviral defence.

PML-NBs are a part of the cellular intrinsic antiviral defence mechanism. Intrinsic immunity refers to the presence of pre-existing cellular factors that target and inhibit virus infection. Since intrinsic factors do not require infection to become activated, their response is immediate, making them part of the first line of defence against infections [7]. As opposed to indirectly activating response pathways, intrinsic antiviral factors directly inhibit virus replication, for example by preventing uncoating of the virus, inhibiting viral DNA transcription, or preventing virion release [8]. Therefore in order for a virus to replicate in host cells, they must circumvent the antiviral properties of PML-NBs. ICP0 and Us3 are examples of two herpesvirus proteins that help the virus evade the antiviral activities of PML-NBs.

ICP0 is a herpesvirus immediate early protein and it is required for early and late viral gene expression. It inhibits histone deacetylase (HDAC) activity, by dissociating the chromatin repressive complex formed by HDACs 1 and 2 [9]. HDACs are enzymes that deacetylate histones, forming more compact nucleosomes that prevent access of transcription factors to the DNA. Therefore repressing HDAC function promotes viral gene expression. ICP0 has largely been studied in HSV-1, where it targets PML for proteasome dependent degradation, resulting in PML-NB dispersal [10]. Our lab has shown that HSV-2 ICP0 also causes disruption of PML-NBs (Finnen and Banfield, unpublished).
Us3 is a serine threonine kinase that is conserved amongst the alphaherpesviruses. Us3 functions include preventing the host cell from undergoing apoptosis and promoting nucleocapsid egress from the nucleus. It also hyperphosphorylates HDAC 1 and 2, which inhibits HDAC function [11]. In HSV-2, Us3 has also been shown to disrupt PML-NBs in a proteasome and kinase activity dependent manner. However it is unknown which protein Us3 phosphorylates or how it disrupts PML-NBs [12].

In addition to being part of the intrinsic immune response, PML-NBs are also part of the innate immune system, as the synthesis of PML and other key components of PML-NB are induced by interferon. In the presence of interferon, mRNA levels of PML are elevated, resulting in an increase in the size and number of PML-NBs per cell [13]. Underscoring the role of ICP0 in disruption of PML-NBs, HSV-1 ICP0 mutants are hypersensitive to the effects of interferon [14]. Unpublished work by Masany Jung has shown that Us3 also plays a key role in the resistance of cells to the antiviral effects of interferon and that this Us3 activity is mediated through PML.

The goal of this project was to study the relative functions of ICP0 and Us3 in disrupting PML-NBs, in order to gain further insight into how these viral proteins counteract intrinsic cellular antiviral defences.
Chapter 2
Literature Review

2.1 Herpesviridae

The Herpesviridae family of viruses are large viruses with linear, double stranded DNA genomes that can cause lifelong latent infections in their hosts (Figure 2.1). Depending on the virus, the genome is 120kbp to 250kbp long, coding for 70-200 genes [15-17]. The DNA is encased in a T=16 icosahedral nucleocapsid, which is 100-130nm in diameter [15, 16]. Four main viral proteins make up this capsid, including the major capsid protein (MCP), triplex monomer protein (TRI-1), triplex dimer protein (TRI-2), and smallest capsid protein (SCP) [16]. The capsid is surrounded by a viral envelope, composed of cellular phospholipids acquired through budding into trans-Golgi network derived vesicles [16, 18]. In addition, the envelope contains many viral proteins and glycoproteins, which play important roles in viral attachment and entry, and also facilitate the envelopment of capsids during virus assembly [16, 17]. Between the capsid and envelope exists a proteinacious layer, called the tegument. Tegument proteins are released directly into the host cell after infection, therefore they can impact host cell function even before viral gene transcription occurs. This means tegument components can play very important roles in establishing an effective infection, even though the specific functions of most tegument proteins are currently unclear. The tegument is discussed in more detail in Chapter 2.1.3. Figure 2.1 shows a 3D cryo-electron structure of a herpes simplex virion and the structure of the herpes simplex virus (HSV) genome.
Figure 2.1: 3D cryo-electron structure of a herpes simplex virion. A. The herpes simplex virus is composed of a capsid (light blue) containing the linear double stranded genome, surrounded by a layer of tegument proteins (orange), and an envelope (dark blue) embedded with glycoproteins (yellow). pp: proximal pole, dp: distal pole. Scale bar: 100 nm. Figure adapted from Grunewald et al, 2003 [19]. B. The HSV genome contains a unique long region (UL) and a unique short region (US). These are flanked by direct and inverted repeat regions, RL and RS. The genome also contains a few hundred base pairs of redundant sequences at the genome termini and between RL and RS (black boxes). Arrows are used to indicate the orientations of the repeat regions. A segment of the RL2 gene, coding for the ICP0 protein, and Us3 gene are shown. The bases highlighted in red indicate the region where two stop codons were introduced using en passant mutagenesis to create ICP0Δ, Us3Δ, and ICP0Δ/Us3Δ recombinant viruses.
2.1.1 *Herpesviridae* Subfamilies

The *Herpesviridae* are classified into three subfamilies: *alphaherpesvirinae*, *betaherpesvirinae*, and *gammaherpesvirinae* (Table 2.1). These subfamilies can be distinguished by their host range, length of reproductive cycles, and the types of cells that they establish latent infections in. Alphaherpesviruses are neurotropic viruses, have a broad host range, relatively short reproductive cycles, spread rapidly in cell culture, and are able to establish latent infections in neurons of sensory ganglia [15, 16]. Betaherpesviruses can infect many different cell types [16, 17] but have a more restricted host range than the alphaherpesviruses, and demonstrate a long reproductive cycle and slow spread of infection in cell culture. Betaherpesviruses establish latent infections in lymphoreticular cells and possibly in secretory glands, kidneys, and other tissues [15]. Gammaherpesviruses infect lymphoblastoid cells and establish latency in lymphoid tissues. They are the most limited in terms of the types of cells and hosts they can infect [15-17].

**Table 2.1: The human herpesviruses.** Humans are the natural hosts for eight different herpesviruses, which can be divided into three subfamilies of *alpha*, *beta*, and *gammaherpesvirinae*. HHV: human herpesvirus, HSV: herpes simplex virus, VZV: varicella zoster virus, CMV: cytomegalovirus, EBV: Epstein-Barr virus, KSHV: Kaposi’s sarcoma-associated herpesvirus.

<table>
<thead>
<tr>
<th>Alphaherpesvirinae</th>
<th>Betaherpesvirinae</th>
<th>Gammaherpesvirinae</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1 (HHV-1)</td>
<td>CMV (HHV-5)</td>
<td>EBV (HHV-4)</td>
</tr>
<tr>
<td>HSV-2 (HHV-2)</td>
<td>HHV-6</td>
<td>KSHV (HHV-8)</td>
</tr>
<tr>
<td>VZV (HHV-3)</td>
<td>HHV-7</td>
<td></td>
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</table>

6
2.1.2 Herpesvirus Replication Cycle

Replication of herpesviruses begins with the attachment of the viral envelope to cellular membrane receptors triggering membrane fusion between the virus and the cell (Figure 2.2). In certain cell types, the viral envelope fuses with the cell plasma membrane, whereas in other cell types it fuses with endosomal membranes instead, entering the cell via a pH dependent endocytic pathway [20]. It is unclear what dictates the different methods of entry in different cell types.

Three essential and conserved glycoproteins, gB, gH, and gL, play important roles in fusion [17]. However, the different subfamilies of herpesvirus require various additional proteins. These other interactions may be determining factors of the differing cell and tissue tropisms between the subfamilies [17]. In HSV, binding of gD to its receptor induces conformational changes in the gH/gL heterodimer [21], which becomes activated and interacts with gB to initiate membrane fusion [22].

After fusion and entry, the capsid traffics along microtubules towards the nucleus, where it docks at a nuclear pore complex and injects the genome into the nucleus (Figure 2.2) [23]. Herpesvirus genes are then expressed in a temporal manner, divided into three kinetic classes: immediate early (IE), early (E), and late (L). IE gene transcription requires the gene transactivator VP16. VP16 interacts with two cellular proteins, the octamer binding transcription factor (Oct1) and host cell factor 1 (HCF-1), to form the VP16-complex. This complex binds to “TAATGARAT” regulatory regions in IE promoters, and activates transcription [25]. Initiation of IE gene expression then
Figure 2.2: **Herpesvirus entry into host cells.** Interactions between herpesvirus glycoproteins and cell surface receptors initiate fusion of the viral envelope with the cellular membrane. (An alternative method of entry is by a pH dependent endocytic pathway where the virus fuses with endocytic vesicles instead). Glycoproteins including gB, gH, and gL are important for the fusion process, which releases the viral capsid and tegument into the cytoplasm. The capsid is trafficked along microtubules to the nuclear membrane where viral DNA is injected into the nucleus. Some tegument proteins, such as the virion host shutoff (vhs) protein, function in the cytoplasm, while others, including VP16, ICP0, and Us3, are shuttled to the nucleus via interactions with cellular proteins or nuclear localization signals (NLS). Some tegument proteins remain bound to the capsid. *Figure adapted from Mettenleiter, 2002* [24].
regulates E and L gene expression. For example, the IE protein ICP4 is required for the transcription of E and L genes by increasing the formation of transcription preinitiation complexes [26, 27]. Another IE protein, ICP0, is able to regulate transcription from all three kinetic classes [28-32]. E gene products are important for viral DNA replication, and L gene products are mostly structural proteins. The resulting level of viral gene expression is dependent on the balance between cellular proteins that try to repress viral gene transcription, and viral proteins that counteract the function of those cellular proteins.

After DNA replication is complete and the viral genome is packaged into capsids, viral assembly and egress occurs. This is a multistep process, with the capsid first gaining a primary tegument and envelope by budding at the inner nuclear membrane, followed by a de-envelopment step at the outer nuclear membrane that releases the capsid into the cytoplasm. Tegument assembly begins in the cytoplasm, and then the virus buds into trans-Golgi vesicles for secondary and final envelopment. Lastly, the virus is released into the extracellular space through a vesicular secretory pathway. This process of viral assembly and egress is driven by a large network of protein-protein interactions, many of which involving the herpesvirus tegument.

2.1.3 Herpesvirus Tegument

The tegument can make up approximately 40% of virion protein mass, and is composed of approximately 20 viral proteins and many cellular proteins [16, 33]. Although originally believed to be an amorphous layer of proteins, the structure of the tegument is actually important for virus assembly. Inner tegument proteins interact with the capsid, while outer tegument proteins interact with the envelope. The interaction
between inner and outer teguments proteins work together to assemble the virion, and mutants of various tegument proteins often show defects in cytoplasmic transport of capsids [34, 35] and secondary capsid envelopment [36-40].

In addition, the role of tegument proteins in establishing efficient virus replication is becoming clearer. Since they are immediately available to the virus after infection, they function to ensure that virus replication can proceed successfully. After entry into the cell, some tegument proteins remain bound to the capsid and are shuttled to the nucleus, while others function in the cytoplasm (Figure 2.2) [41]. An example of a tegument protein is VP16, an important gene transactivator that forms a complex with two cellular proteins, Oct1 and HCF-1, to induce transcription of immediate early viral genes. This activates the cascade of early and late viral gene expression. It is thought that the VP16-complex also plays a role in determining whether the virus establishes a lytic infection, or a latent infection [25]. Another tegument protein is the virion host shutoff protein (vhs). During herpesvirus lytic infection, the host cell RNA polymerase machinery switches from transcribing host cell genes to transcribing viral genes. Vhs is an mRNA specific RNase that degrades cellular mRNAs and shuts off cellular protein synthesis [42].

Since the network of tegument proteins play significant roles in virus replication, it is important to gain insight into their specific functions. This thesis looks at the role of two inner tegument proteins, ICP0 (Chapter 2.5) and Us3 (Chapter 2.6), in counteracting cellular antiviral defences.

2.1.4 Herpes Simplex Virus 1 and 2

Two human alphaherpesviruses are herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2). Although HSV infections are rarely fatal, they
affect billions of people in both developed and developing countries, and result in lifelong infections in their hosts. Primary and recurrent infections occur in mucosal membranes, but the virus is also able to travel down sensory neurons in a retrograde manner to latently infect sensory ganglia. When establishing latent infections, the viral genome interacts with cellular histones and circularizes to form episomes. During latency, the viral genome can be replicated by cellular DNA polymerases, but viral gene expression is mostly silenced by epigenetic factors [43]. However, latent viruses can be periodically reactivated by various stimuli such as physical or emotional stress, fever, ultraviolet light, and tissue damage [44]. These external factors activate host stress response pathways, leading to linearization of episomes and activation of lytic genes [43, 45]. Mothers shedding the virus can transmit the virus to neonates during delivery. HSV-1 infection in the eye can cause herpes stromal keratitis, which is the leading infectious cause of blindness in developed countries [46]. Encephalitis is a rare occurrence of HSV-1 or 2 causing severe and frequently lethal infections in the brain. Although HSV-1 is usually the cause of oral infections, including herpes labialis (cold sores), while HSV-2 causes genital infections, increasing numbers of genital infections are also being attributed to HSV-1. However, these are less severe and less likely to recur [44, 47]. Viruses are transmitted through close contact of mucosal surfaces or abraded skin with infected individuals, with HSV-2 usually transmitted through sexual contact. Since HSV infection can occur asymptomatically, many people are unaware that they carry the virus, and spread it unknowingly to others. This is extremely problematic as HSV-2 is also associated with a 2-3 fold increase in risk of HIV acquisition. In areas with high HSV-2 prevalence, it may account for 40-60% of new HIV infections [48]. Therefore it is
important to understand the methods HSV viruses use to counteract cellular antiviral defences in order to establish effective lytic and latent infections.

2.2 Intrinsic Immunity

Cells and organisms have many ways to defend themselves against invading pathogens. In addition to innate and adaptive immune responses, a third line of defence is called intrinsic immunity. While innate and adaptive immunity require signals from an infected cell in order to activate an antiviral response, intrinsic immunity refers to the presence of pre-existing cellular factors that target and inhibit the viral life cycle. Since intrinsic factors do not require infection to become activated, their response is immediate, making them part of the first line of defence against infections [7]. Pathways of the viral life cycle that are targeted by intrinsic antiviral factors include reverse transcription (of retroviruses), virus uncoating, transcription, translation, and release of viruses (Figure 2.3).

APOBEC3G is a cytidine deaminase enzyme, which can inhibit HIV-1 reverse transcription and integration of the viral genome by converting cytidine to uridine in the cDNA [49-51]. TRIM5α inhibits virus uncoating by recognizing viral capsids and causing its disassembly [52, 53]. Mx1 is a mouse protein that has antiviral properties against various influenza viruses, including H5N1 and H1N1, by inhibiting viral polymerase activity [54, 55]. Its human homologue, MxA, also inhibits influenza virus replication, although it impedes a subsequent step that occurs after transcription [56]. Protein kinase R (PKR) is activated by double stranded RNA and phosphorylates the translation initiation factor eIF2α, shutting off viral protein synthesis. It can also induce
Figure 2.3: Intrinsic immunity involves constitutively expressed proteins that inhibit various stages of the virus replication cycle. Pathways of the viral life cycle that are targeted by intrinsic antiviral factors include reverse transcription (APOBEC), virus uncoating (TRIM5α), transcription (Mx1), translation (PKR), and release of enveloped proteins (tetherin). Although HIV and influenza are shown in this figure, many of the intrinsic factors described have broad specificity against other viruses as well, including herpesviruses. Figure adapted from Yan and Chen, 2012 [8].
signal transduction pathways, inhibiting viral replication at the transcriptional level [57-59]. Tetherin is a transmembrane protein that binds to enveloped viruses and prevents their release from the host cell. This has been shown for various retroviruses, filoviruses, arenaviruses, and herpesviruses [60-62].

An important family of genes related to intrinsic immunity is the TRIM gene family, including TRIM5α mentioned above. TRIM genes contain a conserved N-terminal RBCC motif, which includes a “really interesting new gene” (RING) domain, one or two B-boxes, and a coiled-coil region [3]. RING domains are mainly involved in protein-protein interactions [63], and many also possess E3 ubiquitin ligase activity [64, 65]. B-boxes are zinc finger motifs that are found exclusively in TRIM proteins, although a specific function for them has not been identified [3, 66]. The coiled-coil domain can interact with other coiled-coil domains, forming homo and hetero-oligomers and leading to the development of high molecular weight cellular compartments [3, 66]. The C-termini of TRIM proteins are more diverse, although many contain an immunoglobulin-like SPRY domain, which has been shown to play a role in protein-protein interactions [67], RNA binding [68], and viral capsid binding in HIV-1 [69].

There are at least 68 human TRIM proteins and each of them can interact with a variety of other proteins, resulting in a wide range of possible functions. Despite this great diversity, many TRIM proteins play important roles in antiviral defence [3]. In addition to TRIM5α, TRIM1, TRIM19, and TRIM22 have all been shown to possess antiviral effects, particularly against retroviruses [70-72]. TRIM19, also known as promyelocytic leukemia protein (PML), is the most well studied TRIM protein, as it has been shown to inhibit retroviruses, RNA viruses, and DNA viruses [3]. PML interacts
with itself and a variety of other proteins to form punctate nuclear structures called PML nuclear bodies (PML-NBs), discussed in more detail in Chapter 2.4.

2.3 Epigenetic Regulation

Through interactions with proteins, eukaryotic DNA is folded into a higher order structure called chromatin [73]. The study of how chromatin structure is important for regulating gene expression is called epigenetics. Specifically, modifiers or remodellers are able to alter the structure of chromatin, regulating the expression of specific genes. Therefore epigenetics is the study of changes in gene expression that do not involve changes in DNA sequence [74]. Although herpesvirus virions do not contain histones, they interact with cellular histones after infection [2, 75]. Therefore herpesviruses can take advantage of epigenetic regulation to control the expression of their own genes.

Interaction between DNA and histones form structures called nucleosomes. Approximately 146 base pairs (bp) of DNA are wrapped around a histone protein core. The core histones consist of an octomer of H2A, H2B, H3 and H4 dimers [73], with N-terminal tails that protrude from the nucleosome structure [76]. Nucleosomes are folded into an even more compact structure through interactions with the linker histone H1 (Figure 2.4). Loosely packed nucleosomes allow transcription factors to access gene promoters and initiate gene expression, whereas tightly packed nucleosomes form a compact chromatin structure that does not allow binding of transcription factors [78].

Modification of histones tails can alter chromatin structure and regulate gene expression [73]. Acetylation of lysines on histone tails neutralizes their positive charge, which reduces their interactions with the negatively charged DNA, resulting in a more open chromatin structure and induces gene expression [79]. Acetylation of histones is
Figure 2.4: Chromatin structure. Nucleosomes are formed by approximately 146 base pairs of DNA wrapped around a histone core octomer composed of H2A, H2B, H3, and H4 dimers. The N termini of the histones, called histone tails, protrude from the nucleosome (shown on one nucleosome as an example). Further interactions are mediated by linker histone H1, with helps form higher order structures of chromatin. Modifications to histones can affect chromatin structure. Figure adapted from Seitz, 2005 [77].
regulated by proteins called histone acetylases (HATs) or histone deacetylases (HDACs), which respectively add or remove acetyl groups from lysine molecules. Methylation of histones, mediated by histone methyltransferases (HMTs) and histone demethylases (HD), can lead to either activation or repression of transcription (Figure 2.5) [80].

Another way that nucleosome structures can be regulated is through chromatin remodeling, which alters the position of nucleosomes. This requires the activity of 2 main families of ATP dependent complexes, SWI/SNF, and ISWI [82]. Using the energy from ATP hydrolysis, chromatin remodeling complexes are able to alter the interaction of DNA with histones resulting in a change in the position of nucleosomes on the DNA. Histones can be translocated from one segment of the genome to another [83], or slid along the DNA to open a particular area for transcription (Figure 2.6) [84]. Chromatin remodeling complexes can also remove histones from a promoter in order to initiate gene transcription [85].

Because the herpesvirus genome interacts with cellular histones, the virus is able to actively modify histones and alter chromatin structure to induce viral gene expression. For example, the viral transcription activator VP16 recruits cellular factor SNF2H to viral gene promoters, where it functions to remove histone 3 from the promoter DNA. This leads to a more accessible chromatin structure for transcription, resulting in increased immediate early viral gene expression and viral replication [86, 87]. Another way to promote viral gene expression is to prevent cellular factors from silencing viral gene expression. Viral proteins HSV-1 ICP0 and Us3 have been shown to inhibit cellular HDAC function, which deacetylates histones and forms a repressive chromatin
Figure 2.5: Chromatin modification. Enzymes such as histone acetylases (HATs), histone deacetylases (HDAC), histone methyltransferases (HMT) and histone demethylases (HD) modify histones by adding or removing acetyl or methyl groups. These modifications can lead to a more open chromatin structure (euchromatin) or a more repressed chromatin structure (heterochromatin). Some examples are listed. Euchromatin contains fewer nucleosomes, leading to a more open structure conducive to transcription. Heterochromatin has a more condensed structure and is not conducive to transcription. Therefore regulation of gene expression can be controlled through chromatin modification. Figure from Knipe and Cliffe, 2008 [81].
Figure 2.6: Chromatin remodelling. ATP dependent remodelling complexes can bind to DNA and induce a loosening of the chromatin structure. Using energy from ATP hydrolysis, nucleosomes can be transferred to a different segment of DNA, or slid along the DNA to reveal a specific promoter. Therefore recruitment of chromatin remodelling complexes can regulate gene expression. *Figure adapted from Vignali et al., 2000* [82].
structure. These two proteins are discussed in more detail in Chapters 2.5 and 2.6. At the same time, cellular antiviral defence mechanisms aim to impart a repressive chromatin structure on the herpesvirus genome, inhibiting viral gene expression. One of the cellular proteins involved in this is PML.

2.4 Promyelocytic Leukemia Protein (PML)

As mentioned above, PML is a member of the TRIM gene family. Like all TRIM proteins, PML contains the conserved tripartite motif [3]. This tripartite motif, also called the RBCC motif, includes a RING domain, B-boxes, and a coiled coil domain. This motif is important for protein-protein interactions and promotes the formation of subcellular complexes [88].

PML is an essential protein for the formation PML-NBs, or ND10. On average there are approximately 15 PML-NBs per nucleus in a mammalian cell [4] although this is cell type dependent. They range in size between 0.1 and 1μm, containing many different proteins but typically not DNA or RNA [89]. It is believed that these structures are held together by the interaction between SUMO groups and SUMO interaction motifs (SIMs). SUMO stands for small ubiquitin-like modifier protein, and refers to a family of proteins approximately 100 amino acids in length that are important post-translational modifiers. Conjugation of SUMO to its target proteins can affect many different pathways, including cell cycle progression, protein localization, and transcription [90]. PML has three sumoylation sites in its RBCC motif, and the addition of SUMO is required for the formation of PML-NBs [91, 92]. Many of the proteins that are recruited to PML-NBs also contain sumoylation sites and SIMs [89], supporting the hypothesis that SUMO groups are important for maintaining the integrity of PML-NBs (Figure 2.7).
Figure 2.7: Sumoylation of PML is required for PML-nuclear body formation. The small ubiquitin-like modifier (SUMO) is conjugated to promyelocytic leukemia protein (PML) and other PML nuclear body (PML-NB) components such as Sp100 and Daxx. Interactions between SUMO and SUMO interaction motifs (SIMs) is important for maintaining PML-NB structure. 
*Figure adapted from Bernardi and Pandolfi, 2007 [5].*
The large variety of proteins that get recruited to these nuclear structures allows PML-NBs to affect many different pathways, including apoptosis, DNA damage response, senescence, oncogenesis, and antiviral defence (Figure 2.8) [5, 6]. In addition to being an important mediator of the intrinsic immune response, PML-NBs also play a role in the innate immune response, and even the adaptive immune response.

### 2.4.1 PML Nuclear Bodies and Antiviral Defence

Although nucleic acids are not usually found in PML-NBs, these structures have been shown to interact with viral genomes [93]. It was originally thought that viral genomes are deposited at PML-NBs [94], however it has now been shown that PML-NB components actually disassemble and reform at the site of viral genomes [95]. Viral genomes undergoing biological activity, such as transcription, seem to increase the probability of that DNA interacting with the nuclear bodies [96], suggesting that this biological activity is recognized by the cell as foreign activity, signaling PML-NBs to associate at the site of viral DNA. This association has a negative impact on virus replication, as the absence of PML results in increased viral proliferation. PML knockout mice are more susceptible to lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), encephalomyocarditis virus (EMCV), and rabies virus [97-99]. Primary human fibroblasts knocked down for PML also results in an increase in plaque forming ability of human cytomegalovirus (HCMV) [100].

PML has been shown to inhibit retroviruses, RNA viruses and DNA viruses, and the mechanism of how they inhibit virus replication differs based on the virus. In human
Figure 2.8: Functions of PML nuclear bodies. PML-NBs have been associated with many different pathways, including roles in DNA damage response, apoptosis, senescence, angiogenesis, and also antiviral defence. Figure adapted from Bernardi and Pandolfi, 2007 [5].
foamy virus (HFV) infection, overexpression of PML inhibits viral mRNA synthesis and DNA replication [101]. HFV is a retrovirus and contains a viral gene transactivator called Tas. Tas binds to the 5’ LTR region or an internal promoter of the HFV genome and initiates viral transcription. PML interacts directly with Tas, recruiting it to PML-NBs and preventing it from binding to the LTR or internal promoter. Therefore the physical interaction between PML and Tas results in repressed viral gene expression, inhibiting HFV infection [101].

Vesicular stomatitis virus (VSV) and influenza virus, both RNA viruses, are also inhibited by PML overexpression. VSV mRNA and protein synthesis were impaired in PML transfected cells [102]. Although the specific mechanism for PML function has not been identified in these cases, it is believed that a crucial viral protein required for viral replication is inhibited. Since VSV transcription occurs in the cytoplasm but influenza virus transcription occurs in the nucleus, PML may inhibit viral replication indirectly by modifying cellular proteins that can repress virus transcription in the relevant cellular compartment [102].

An effective way to inhibit virus replication is to repress viral gene transcription. Indeed, the majority of proteins that interact with PML-NBs have functions related to transcription regulation [103]. Sumoylation itself is typically associated with transcriptional repression, and the fact that PML and many of its interacting partners are sumoylated provides a strong link between PML-NBs and regulation of gene transcription [104, 105]. Specifically, PML-NB proteins Sp100, Daxx, and ATRX all have associations with chromatin remodeling function including histone deacetylases and SWI/SNF histone remodeling complexes.
Sp100 interacts with the heterochromatin protein 1 (HP1) family of non-histone chromosomal proteins, which are structural proteins of heterochromatin [106]. Experiments done with HCMV infected cells show that Sp100 plays a repressive role in virus replication, and that decreasing levels of Sp100 by RNA interference treatment enhances levels of acetylated histones at the major immediate early gene promoter [107]. This suggests that Sp100 plays a role in regulating gene expression through epigenetic pathways. Daxx is part of a chromatin remodeling complex with HDACs, and ATRX is a member of the SWI/SNF family. Daxx and ATRX have been shown to confer a repressive viral chromatin structure to the HCMV viral genome [108, 109]. The fact that these proteins are related to the chromatin complex suggests that PML-NBs may inhibit virus replication by modifying viral chromatin to prevent viral gene expression.

PML-NBs can also inhibit the spread of viral infection by trapping viral nucleocapsids of varicella-zoster virus (VZV), a DNA virus [110]. PML can interact with viral capsid proteins forming a cage-like structure around immature or mature nucleocapsids, preventing them from leaving the nucleus and forming mature virions [110].

In addition to affecting the actual virus, PML-NBs also limit virus spread by inducing apoptosis in infected cells. PML-NBs can interact with p53, an important regulator of the cell cycle. Under normal growth conditions, p53 levels are low due to degradation mediated by its negative regulator, Mdm2. However, recruitment to PML-NBs in response to infection leads to phosphorylation of p53 which alters its conformation and prevents Mdm2 binding. Without Mdm2 binding, p53 does not get degraded, and activates transcription of genes involved in apoptosis [111, 112].
Although PML-NBs are pre-existing structures in cells, they are also upregulated by interferons (IFNs), cytokines produced during virus infection. Therefore PML-NBs are not only part of the intrinsic immune response, they are also a part of the innate immune response. IFNs bind to cellular receptors and initiate a JAK/STAT signaling pathway which activates downstream interferon stimulated genes. Proteins encoded by these genes are mediators of the IFN response [113]. The PML and Sp100 genes both possess an interferon stimulated response element (ISRE) and an interferon gamma activation site (GAS). This allows them to be bound by transactivating proteins whose synthesis is stimulated by IFNα, β, or γ (Figure 2.9). As a result, mRNA and protein levels of PML and Sp100 are significantly enhanced in the presence of IFN, leading to an increase in number and size of PML-NBs [13]. Cells derived from PML knockout mice and human cells treated with silencing RNA targeting PML prevent IFN mediated apoptosis [114, 115]. This data suggests that PML is an important effector of the interferon response. This is further supported by the fact that IFN treatment of PML-null cells is unable to inhibit HSV-1 or HFV replication [101, 116].

PML also plays a role in adaptive immunity, by increasing the expression of LMP-2, LMP-7, TAP-1 and TAP-2, proteins required for efficient presentation of peptides by major histocompatibility complex (MHC) class I molecules [117].

2.4.2 PML Isoforms

PML, Sp100, and Daxx are three proteins that are consistently found in nuclear bodies. However there are over 100 proteins identified as PML-NB partners [103]. The large variety of proteins associated with PML-NBs allows them to have a wide range of
Figure 2.9: Induction of PML expression through the interferon signaling pathway. Binding of interferon (IFN) to its receptor activates the JAK/STAT signaling pathway. Under IFNα or IFNβ signal, the phosphorylated STAT1 STAT2 heterodimer, along with an interferon regulatory factor p48, binds to the interferon stimulated response element (ISRE) of target genes and induces transcription. IFNγ activates a slightly different JAK/STAT pathway that leads to STAT1 homodimers binding to gamma activation sites of target genes [113]. Both PML and Sp100 contain ISRE and GAS elements, allowing them to be upregulated by IFN.
functions [6]. Different PML isoforms may be important for determining which proteins get recruited to PML-NBs.

The human PML gene is 35kb long and has nine exons. Alternative splicing produces 7 major different isoforms, 6 of them localizing to the nucleus. These are named PML I-VI (Figure 2.10) [5, 118, 119]. Since the isoforms all share the N terminal RBCC motif, their unique C termini are important for directing protein-protein interactions, conceivably influencing the composition and function of PML-NBs. The different PML isoforms show varying subcellular localization patterns when expressed individually [119]. For instance PML IV forms smaller nuclear bodies while PML V forms larger, denser nuclear bodies. This suggests that the isoforms interact with different proteins, resulting in a varied appearance [119].

There are also multiple examples of PML isoforms possessing unique functions. As PML-NBs play a role in cellular antiviral defence, it is intriguing to determine whether the various PML isoforms play differing roles in this process. Studies have shown that PML-null cells are more susceptible to lymphocytic choriomeningitis virus (LCMV) and rabies virus infection [97, 99]. However, expressing PML III alone did not inhibit LCMV replication [120]. Similarly, only PML IV inhibited rabies virus infection [99]. At the same time, PML III can provide resistance to vesicular stomatitis virus (VSV), influenza A, or human foamy virus (HFV) infections in a p53 independent manner [101, 102], but requires p53 to inhibit poliovirus infections [112]. Collectively, this means that different PML isoforms can have different functions, and that this can be dependent on the virus.
Figure 2.10: Six of the PML isoforms localize to the nucleus. The PML gene contains 9 exons and produces at least 7 different isoforms. The first 6 contain a nuclear localization signal (NLS) and therefore are found in the nucleus. The N terminus of all the isoforms contain the RBCC motif, composed of the RING finger (R), two B-boxes (B), and a coiled coil domain (CC). Figure from Bernardi and Pandolfi, 2007 [5].
Current studies implicating PML in antiviral defence have mostly been done with PML III, IV, or VI [6]. It will be useful to also determine the roles of the more abundant isoforms, PML I and II [119]. Studying each of the PML isoforms may also provide more insight into the mechanisms of PML function.

In order to counteract antiviral properties that PML-NBs exhibit, viruses have developed mechanisms to disrupt PML-NBs. For HSV-2, this involves the viral proteins ICP0 and Us3.

2.5 Infected Cell Protein 0 (ICP0)

ICP0 is an immediate early herpesvirus protein encoded by the RL2 gene. With orthologs in many herpesvirus subfamilies, including varicella zoster virus and cytomegalovirus, ICP0 has been most extensively studied in HSV-1. ICP0 is a gene transactivator, capable of activating all three kinetic classes of herpesvirus genes: immediate early, early, and late. Although not an essential gene, ICP0 is necessary for efficient virus propagation, particularly at low multiplicities of infection (MOI). ICP0 also possesses E3 ubiquitin ligase activity, degrades cellular proteins, and is involved in counteracting cellular antiviral defences (Figure 2.1).

2.5.1 ICP0 Structure

HSV-1 ICP0 is 775 amino acids in length while HSV-2 ICP0 is 825 amino acids in length with a predicted molecular weight of approximately 82 kDa [121]. HSV-1 and HSV-2 ICP0 only have a 61.5% amino acid sequence identity, compared with over 80% identity seen for other HSV proteins [122-124]. However all alphaherpesvirus ICP0 orthologs contain a conserved N-terminal RING domain [125-128]. This zinc binding
**Figure 2.11: Functions of Infected Cell Protein 0 (ICP0).** ICP0 is an immediate early gene that plays important roles in establishing lytic infection. It transactivates other viral genes from all three kinetic classes, and counteracts cellular antiviral defences. It disrupts PML-NBs by targeting a variety of its components (including PML, Sp100, ATRX, hDaax) for degradation. It also disrupts the repressive REST/CoREST/HDAC1/LSD1 complex by dissociating HDAC1. As an E3 ubiquitin ligase, ICP0 polyubiquitinates various proteins, such as CENP-A, B, and C, RNF8, RNF168, and p53, and targets them for proteasome dependent degradation. ICP0 inhibits NFκB signaling by decreasing levels of MyD88 and Mal, and inhibits the interferon (IFN) signaling pathway by preventing the phosphorylation and dimerization of IRF3. ICP0 also inhibits cell surface expression of CD83, preventing T-cell priming and activation of adaptive immune responses.
RING domain is important for most ICP0 functions, including gene transactivation, inhibiting the interferon signaling pathway, and proteasome dependent degradation of PML and the PML-NB component Sp100 [128-132] Other important regions include the C-terminal PML localization signal, a dimer or multimer motif, and a binding site for ubiquitin-specific protease (USP7) (Figure 2.12) [133].

2.5.2 ICP0 Counteracts Antiviral Defence

ICP0 functions to counteract intrinsic, innate, and adaptive immune responses. Through its E3 ubiquitin ligase activity, ICP0 targets PML and other PML-NB components for degradation in a proteasome dependent manner. This disrupts PML-NBs, an important part of the intrinsic immune response. In HSV-1, ICP0 null mutants exhibit a growth defect, which is partially complemented by the decrease of PML and other PML-NB constituents (Sp100, ATRX, and hDaxx) by RNAi treatment [72, 134, 135]. Simultaneously decreasing levels of PML and Sp100 has a greater effect on ICP0 null virus growth than depleting either protein individually [134], meaning that PML-NB proteins work together to repress viral gene expression. At the same time, since this method only partially complements the ICP0 null virus growth defect, there must be additional cellular factors that play a role in inhibiting viral growth, and those are also targeted by ICP0. Although these studies have been done using HSV-1 ICP0, our lab has shown that HSV-2 ICP0 also disrupts PML-NBs (Figure 2.13).

As to the molecular mechanism of how ICP0 counteracts antiviral defences, it has been shown that ICP0 inhibits chromatinization of viral DNA. ICP0 decreases the amount of histones associating with viral genomes, and promotes acetylation of histones,
Figure 2.12: Important ICP0 domains. Notable domains of ICP0 include the really interesting new gene (RING) finger motif, the nuclear localization signal (NLS), a Usp7 binding site, a PML-NB binding domain, a region implicated in Co-REST interaction, and a dimerization motif.
**Figure 2.13: Herpesvirus protein ICP0 disrupts PML-NBs.** Vero cells were transfected with plasmids expressing EGFP, an HSV-1 ICP0 expression construct, or HSV-2 ICP0 expression construct. At 24 hours post-transfection, cells were fixed and stained with rat polyclonal antiserum specific for ICP0 (green), or rabbit polyclonal antiserum against PML (red). Nuclei were stained with Hoechst (blue). Positive nuclear staining for EGFP or ICP0 identified transfected cells. Cells transfected with EGFP plasmids contained similar number of PML-NBs compared to non-transfected cells. However, cells transfected with either HSV-1 or HSV-2 ICP0 expression constructs displayed a decreased number of PML-NBs per cell relative to non-transfected cells. Arrowheads indicate colocalization between ICP0 and PML. *R. Finnen, unpublished data.*
conferring a more open chromatin conformation and leading to an increase in viral gene transcription [136-139]. It is unclear whether this is a direct effect or a consequence of ICP0’s ubiquitin ligase activity. However ICP0 does interact with class II HDACs-4, 5, and 7, and is able to counteract repressive activities of HDAC 5 [140]. ICP0 has also been shown to interact with the RE1-silencing transcription factor corepressor (CoREST) [141]. CoREST forms a repressive transcription complex called the REST/CoREST/HDAC1/LSD1 complex. Studies have shown that ICP0 binds to CoREST and displaces HDAC1 from the complex, thereby inactivating it [9, 141]. However, this property of ICP0 has not been fully elucidated due to the fact that the identified CoREST binding region overlaps with many other important segments of ICP0, including the PML localization signal and the Usp7 binding site (Figure 2.12) [128]. In addition, mutations to the CoREST binding site have a much smaller effect on ICP0 activity compared to mutations in its RING finger domain, suggesting that interactions with CoREST may not be very important for ICP0 function [142, 143]. ICP0 also interacts with other proteins related to chromatin modification, such as ATRX and Daxx.

In addition to inhibiting intrinsic immune responses, ICP0 is also important for counteracting the innate immune response. After infection with HSV-1, the interferon regulatory factor IRF3 is activated by phosphorylation, allowing it to dimerize, translocate to the nucleus, and induce production of IFNβ. This further activates the IFN response pathway leading to production of IFNα and subsequently other interferon stimulated genes [144]. Although the exact mechanism is unknown, ICP0 inhibits nuclear localization of IRF3 by either preventing phosphorylation, or dephosphorylating IRF3 [145, 146]. As such, HSV-1 is considered to be relatively immune to the effects of IFN.
ICP0 also inhibits NF-κB signaling pathways. After infection, toll-like receptors (TLRs) in the host cell recognize viral components, and with the help of TLR adaptor proteins MyD88 and Mal, activate the NF-κB signaling pathway leading to upregulation of various proinflammatory cytokines. By decreasing levels of MyD88 and Mal, ICP0 prevents signaling through TLR-2 [147]. Furthermore, ICP0 recruits USP7 from the nucleus to the cytoplasm, where it removes polyubiquitin chains from TRAF6 and IKKγ, two other proteins involved in activating NF-κB [148]. Loss of polyubiquitin chains inhibits the ability of TRAF6 and IKKγ to cause translocation of NF-κB to the nucleus, where it is required to activate its target genes.

Another role that ICP0 plays is to degrade the cell surface marker CD83. CD83 is expressed in mature dendritic cells (DCs), which are the most potent antigen presenting cells. DCs are able to prime both CD4+ and CD8+ T cells, meaning they play an important role in activating the adaptive immune response. By degrading CD83, ICP0 reduces or prevents the activation of T cells and thus inhibits acquired immune responses [149].

Taken together, ICP0 is not only important for activating viral gene expression, it also plays a major role in counteracting host antiviral defences.

2.6 Serine/Threonine Kinase Us3

Us3 refers to the third gene encoded in the unique short region of the herpesvirus genome, and its orthologues are found in all alphaherpesviruses. Although amino acid sequences between the various orthologues are quite different, the functions of Us3 are relatively conserved. Us3 is a serine/threonine kinase, and has been associated with a wide range of functions including regulating viral gene expression [150], promoting
translation [151], aiding nuclear egress [152-156], rearranging the host cell actin cytoskeleton [157], inhibiting apoptosis [158, 159], interfering with MHC class I expression [160], and inhibiting the interferon pathway [161] (Figure 2.1).

2.6.1 Us3 Regulates Viral Gene Expression

Activity of histone deacetylases (HDACs) lead to chromatin condensation and inhibition of gene transcription. Therefore it is believed that in order to prevent transcriptional silencing of the viral genome, viruses inactivate HDACs. The fact that all Us3 orthologues hyperphosphorylate HDAC1 and HDAC2 indicate an important role for Us3 in this process [162-165]. Phosphorylation of HDACs can affect their enzymatic activity, cellular localization, and protein-protein interactions [166]. Walters et al. showed that inhibition of HDAC activity resulted in an increase in plaque forming abilities of Us3 null PRV and VZV viruses [165]. Thus it appears that Us3 mediated phosphorylation of HDACs impedes their function. Therefore Us3 may be important for regulating gene expression by influencing activity of HDACs. This is likely more complicated as species-specific observations have been made regarding how phosphorylation of HDACs affects virus replication. However, in PRV, Us3 was shown to increase levels of proteins UL29 and UL39, but decrease levels of UL42 [150], meaning that it is able to regulate gene expression at least to a certain extent.

2.6.2 Us3 Counteracts Antiviral Defence

In response to infection, host cells activate apoptotic pathways to prevent the spread of virus. One of the functions of Us3 is to protect cells from undergoing apoptosis [158, 159]. Since this property of Us3 is observed even in the absence of other viral
Figure 2.14: Multiple functions of the herpesvirus tegument protein Us3. Us3 is a serine/threonine kinase involved in many different pathways. It prevents transcriptional repression of viral genes by phosphorylating and inhibiting HDACs. It’s important for facilitating nuclear egress by recruiting the required proteins to the nuclear membrane, and protects the host cell from undergoing apoptosis by inhibiting multiple pro-apoptotic proteins. Us3 also counteracts antiviral defence by hindering the interferon induced antiviral state and decreasing cell surface expression of MHC Class I molecules. *Figure from Deruelle and Favoreel, 2011* [157].
proteins, it was hypothesized that Us3 phosphorylates and modifies the activities of a cellular protein. Further studies showed that HSV-1 Us3 phosphorylates and inactivates a member of the pro-apoptotic Bcl2 family of proteins, BAD [167-170]. However, Us3 also blocks apoptosis initiated by a BAD mutant that does not contain the relevant phosphorylation sites [168, 170], meaning phosphorylation may not be the only way Us3 inhibits apoptosis. Other groups have shown that Us3 can affect steps occurring downstream of BAD as well [170, 171], therefore, multiple mechanisms for the anti-apoptotic properties of Us3 exist. Us3 orthologues also share these anti-apoptotic functions to varying degrees [172-177].

Us3 may also be involved in counteracting adaptive immunity. In VZV and PRV, Us3 has been shown to decrease cell surface expression of major histocompatibility complex (MHC) class I receptors, a key component of the adaptive immune response. The exact mechanism is unknown, but in VZV it may be related to regulating the cellular location of MHC peptides [178-180]. There is also evidence suggesting that Us3 can inactivate cytotoxic T-lymphocytes (CTLs), which are important immune cells that target and kill infected cells [181]. However it is not known whether or not this is a direct effect of Us3. Finally, Us3’s phosphorylation of gB may not only affect nuclear egress as discussed previously, but may also downregulate gB cell surface expression, resulting in an impairment in the activation of immune responses [182].

It was observed that Us3 null HSV-1 virus was also much more sensitive to the effects of interferon than wild type virus, indicating a role for Us3 in counteracting the interferon induced antiviral state [161]. This may be explained by Us3’s ability to disrupt
PML-NBs. HSV-2 Us3 was shown to disrupt PML-NBs in a kinase and proteasome dependent manner [12]. PML-NBs are important mediators of the interferon response, as PML and a number of other PML-NB constituents are upregulated by interferon. Since PML-NBs have antiviral properties, upregulation by interferon further induces the antiviral state. By disrupting PML-NBs, Us3 counteracts the effects of interferon. In addition, like ICP0, Us3 also interferes with the IRF3 signaling pathway and inhibits IFNβ expression [183].

The goal of this thesis is to study the relative contributions of ICP0 and Us3 to counteracting cellular antiviral defences, specifically in terms of disrupting PML-NBs and overcoming the effects of interferon.
Chapter 3

Materials and Methods

3.1 Cell Lines

All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) (Thermo Scientific, Waltham, MA), 1000 units/ml penicillin and 1000 μg/ml streptomycin (Invitrogen, Burlington, ON), and 1% GlutaMAX (Invitrogen, Burlington, ON), in a 5% CO₂ environment. African green monkey kidney cells (Vero) were provided by the ATCC. Human osteosarcoma (U2OS) cells were a kind gift from Dr. K.L. Mossman, McMaster University. Human fibroblast T12 cells were a gift from Dr. W. Bresnahan (University of Minnesota, MN, USA).

3.2 Viruses

The HSV-2 BAC pYEcac373 encoding HSV-2 wild type strain 186 was kindly provided by Dr. Y. Kawaguchi and transferred into Escherichia coli (E. coli) strain GS1783 by Dr. Valerie Le Sage [184]. HSV-2 186 Us3Δ and Us3ΔR viruses were made by Dr. Valerie Le Sage using the en passant method to introduce two stop codons 3 nucleotides after the Us3 start codon [185]. All HSV-2 186 recombinant viruses were grown in U2OS cells. When unspecified, “HSV-2” refers to HSV-2 strain 186.

3.3 Antibiotics

Chloramphenicol (Fisher Scientific, Toronto, ON) was dissolved in 95% ethanol and used at a final concentration of 30μg/ml. Kanamycin (Fisher Scientific, Toronto, ON) was dissolved in sterile water and used at a final concentration of 50μg/ml.
3.4 Antibodies

Rat polyclonal antiserum against HSV-2 ICP0 was raised by Sue Johnston. Recombinant HSV-2 GST-ICP0 protein (exon 2) was expressed in *E. coli* strain Rosetta(DE3) after induction with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 37°C. Bacteria were lysed, and inclusion bodies were purified by using the B-Per protein purification kit (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions. Proteins in inclusion bodies were separated on preparative SDS-PAGE gels, and the band corresponding to the GST fusion was excised and sent to Cedarlane Laboratories (Burlington, ON, Canada) to immunize Wistar rats for polyclonal antiserum production. The ICP0 antibody was used at a dilution of 1:500 for Western blotting and 1:100 for indirect immunofluorescence microscopy (IF).

Rat polyclonal antiserum against HSV-2 Us3 was raised by Hui Zhang and has been described elsewhere [186]. It was used for Western blotting at a dilution of 1:500 and for IF at a dilution of 1:1000.

Mouse monoclonal antibody against HSV ICP27 (Virusys, Taneytown, MD) was used at a 1:1000 dilution for both Western blotting and IF. Mouse monoclonal antibody against β-actin (Sigma, St. Louis, MO) was used at a 1:2000 concentration for Western blotting. Rabbit polyclonal antibody against all isoforms of PML (Santa Cruz Biotechnology, Dallas, TX) was used at a 1:50 dilution for IF. Mouse monoclonal antibody against ICPP8 (Virusys, Taneytown, MD) was used at a dilution of 1:12,800 for IF. HRP conjugated secondary antibodies for Western blotting (Sigma, St. Louis, MO) were used at dilutions of 1:50,000 and 1:10,000 for α-rat and α-mouse antibodies.
respectively. Alexa-488 or Alexa-568 conjugated secondary antibodies (Invitrogen-Molecular Probes, Carlsbad, CA) were used for IF at 1:500 dilutions.

Nuclei were visualized by staining with 0.5μg/ml of Hoechst 33342 (Sigma, St. Louis, MO) diluted in phosphate buffered saline (PBS). All antibodies were diluted in Tris buffered saline with Tween 20 and 1% bovine serum albumin (TBST+BSA) for Western blotting, and in PBS + 1% BSA for IF.

3.5 Miniprep

Minipreps were used to isolate BAC DNA from E. coli cultures. 3ml of bacterial culture was grown in LB+30μg/ml Cm overnight at 32°C with shaking. Bacteria were pelleted by centrifugation at 14,000 x g for 1min. Supernatant was discarded and pellets were resuspended in 200μl of Solution I (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA). 200μl of Solution II (1% SDS, 0.2N NaOH) was added and tubes inverted gently to mix. Samples were incubated at room temperature for 5min then 200μl of Solution III (3M potassium acetate, 11.5% glacial acetic acid) was added. Inverted gently to mix then centrifuged at 14,000 x g for 10min to pellet cell debris. Supernatant was transferred to new tubes and 500μl of phenol/chloroform was added. Vortexed samples once very gently to mix aqueous and organic phases. Centrifuged at 14,000 x g for 5min to separate phases. Transferred aqueous phase, which contains the DNA, to new tubes and added 800μl of isopropanol. Samples were centrifuged at 14,000 x g for 30min at 4°C to precipitate DNA. Supernatant was discarded and DNA pellet washed with 70% ethanol. Ethanol was removed and pellets were air dried for 20-30min. DNA pellets were resuspended in 20μl of sterile water and stored at -20°C. For en passant mutagenesis, 15μl of the BAC DNA was used for restriction enzyme digest with EcoRI to confirm
correct digestion pattern. The remaining 5μl was used for transfection into Vero cells to reconstitute the virus (see Chapter 3.6).

3.6 En Passant Mutagenesis

The bacterial artificial chromosome (BAC) pYEbac373 encoding HSV-2 wild type strain 186 was kindly provided by Dr. Y. Kawaguchi. The E. coli strain GS1783 and Cre-expression plasmid pOG132 were gifts from Dr. G. Smith. Dr. Valerie Le Sage transferred pYEbac373 into GS1783 to facilitate the construction of HSV-2 recombinants. She also introduced a nonsense mutation into the Us3 gene to create a Us3Δ BAC. This BAC was used to create the ICP0Δ/Us3Δ mutant.

Appendix A provides a graphical summary of the en passant protocol, as well as a description of all the recombinant viruses made. The first step of en passant mutagenesis was to amplify a PCR product using specially designed primers containing homologous sequences to the ICP0 gene locus (RL2), with the introduction of two stop codons 3 nucleotides after the ICP0 start codon (see Appendix B Table B.1 for primer sequences). The template for the PCR reaction is a plasmid called pEP Kan S2, which contains an I-SceI restriction site and a kanamycin resistance gene (Kan cassette) (Figure A.2). The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen Cat. No. 28106, Toronto, ON) and digested with DpnI overnight at 37°C. The DNA was then separated on a 0.8% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen Cat. No. 28706, Toronto, ON).

GS1783 pYEbac373 Us3Δ was grown at 32°C in 100ml of LB broth containing 30μg/ml chloramphenicol (Cm) until an OD$_{600}$ of 0.6 to 0.8 was reached. The culture was transferred to a 42°C shaking water bath for 15 minutes to induce the Red recombination
system. To make the bacterial cells competent, the culture was pelleted at 3600xg for 15 minutes, and washed 4 times with 40ml of cold sterile water. The bacteria were then resuspended in approximately 2ml of sterile water.

40-500ng of the purified PCR product was transformed by electroporation into 60μl of competent E. coli GS1783 pYEbac373 Us3Δ, using settings of 1.8kV, 200Ω and 2.5μF on the Precision Pulse Electro Cell Manipulator ECM 630 (BTX Harvard Apparatus, Saint-Laurent, QC). Bacteria were plated onto LB agar plates containing 30μg/ml Cm and 50μg/ml Kan (LB+Cam+Kan), selecting for clones in which the PCR product had recombined into the BAC, imparting resistance to Kan. Positive clones were identified by performing PCR amplification using primers (see Appendix Table B.2) spanning the recombination site. Due to the presence of the Kan cassette in the PCR product used for recombination, clones in which recombination occurred produced a PCR product approximately 1kb larger than clones that did not undergo recombination. Positive clones were then grown in LB+Cam+Kan until an OD$_{600}$ of 0.4 to 0.6 was reached. 1% L-arabinose was added to the medium to induce the expression of the enzyme I-SceI which cleaves the insert at the unique I-SceI site. The culture was transferred to a 42°C shaking water bath for 15 minutes to induce the expression of Red recombination enzymes. This second recombination event removes the Kan cassette from the BAC, creating a markerless mutation. Bacteria were incubated at 32°C for one hour to enable time for recombination to take place and then plated onto LB+Cm+1% L-arabinose agar plates at 1:500 and 1:5000 dilutions. Colonies arose in approximately 48 hours and were grid plated in duplicate onto LB+Cm and LB+Kan plates. Those that were resistant to Cm but sensitive to Kan were analyzed by PCR and subsequent DNA
sequencing to confirm the absence of the Kan cassette and the presence of the mutation. Primers flanking the site of recombination (see Appendix Table B.2) were used to amplify part of the BAC DNA, which was then sent for sequencing. Clones with a confirmed single copy mutation in RL2 were subjected to a second round of \textit{en passant} mutagenesis to mutate the other copy of RL2. Since the recombination process was non-directional, clones with two mutated copies of RL2 were screened again by PCR and DNA sequencing.

Once both copies of RL2 were mutated and confirmed by DNA sequencing of the relevant loci, the next step was to reconstitute virus from the mutated BAC. BAC DNA was isolated using the mini-prep protocol described in Chapter 3.5 and 5μl out of the 20μl of DNA was transfected into Vero cells along with 1μg of the Cre expression plasmid pOG231. Vero cells were prepared by trypsinizing a confluent 100mm dish of cells and pelleting and resuspending in 1ml of DMEM. 10μl of 1M BES buffer pH7.2 was added to the cells. 250μl of cells was added to the BAC DNA and Cre expression plasmid, and electroporated at settings of 210V, 200Ω and 950μF using a Precision Pulse Electro Cell Manipulator ECM 630 (BTX Harvard Apparatus, Saint-Laurent, QC). The transfected cells were added to a dish of U2OS cells, in 10ml DMEM supplemented with 5mM hexamethylene bisacetamide (HMBA) (Sigma, St. Louis, MO). Growing ICP0Δ viruses in U2OS cells with HMBA have been shown to increase viral titres by complementing the lack of ICP0 [187, 188]. After incubation at 37°C overnight, DMEM was replaced with 10ml of methocel. Plaques that formed were collected by pipetting up methocel and cells immediately surrounding the plaque and transferring this material to microfuge tubes. This material was then used to infect a single well of U2OS cells in a 6-
well dish to amplify the virus. 5mM HMBA was added to the DMEM when growing these viruses.

The same *en passant* protocol was used to construct the ICP0Δ recombinant by repairing Us3 in the ICP0Δ/Us3Δ BAC, and to repair RL2 in both of these BACS (see Appendix Table B.1 for mutagenesis primer sequences). The naming convention and definition of each recombinant virus is shown in Table 3.1, and the lineage for constructing the recombinant viruses is shown in Figure 3.1.

### Table 3.1: Name and definition of HSV-2 186 recombinant viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (WT)</td>
<td>None</td>
</tr>
<tr>
<td>Us3Δ</td>
<td>2 stop codons located 3 nucleotides after the Us3 start codon</td>
</tr>
<tr>
<td>Us3ΔR</td>
<td>2 stop codons repaired to original Us3 sequence</td>
</tr>
<tr>
<td>ICP0Δ</td>
<td>2 stop codons located 3 nucleotides after both copies of the ICP0 start codon</td>
</tr>
<tr>
<td>ICP0ΔR</td>
<td>2 stop codons repaired to original ICP0 sequence for both copies of the gene</td>
</tr>
<tr>
<td>ICP0Δ/Us3Δ</td>
<td>2 stop codons located 3 nucleotides after the Us3 start codon, and 3 nucleotides after both copies of the ICP0 start codon</td>
</tr>
<tr>
<td>ICP0ΔR/Us3Δ</td>
<td>Repaired stop codons for ICP0 gene but not for Us3 gene</td>
</tr>
</tbody>
</table>
*another Us3Δ clone, 5.11, was grown from a different Us3Δ BAC clone

**Figure 3.1: Lineage of recombinant viruses.** The HSV-2 186 wild BAC (pYEbac373) was transferred into the *E. coli* strain GS1783 by Dr. Valerie Le Sage. Viral clones obtained from this BAC include WT (SW) and WT (MJ). Dr. Le Sage then a) introduced a nonsense mutation into the wild type BAC after the Us3 start codon to create the Us3Δ BAC. Viral clones obtained from this BAC include SW, MJ, and 10.1. Clone 5.11 comes from a separate Us3Δ BAC clone. Dr. Le Sage also d) repaired the nonsense mutation in the Us3Δ BAC to obtain the Us3ΔR BAC. Us3ΔR clones include SW and MJ. b) To construct the ICP0Δ/Us3Δ double mutant, nonsense mutations were introduced into both copies of ICP0 in the Us3Δ BAC. c) To construct the ICP0Δ single mutant, the Us3 nonsense mutation was repaired in the ICP0Δ/Us3Δ BAC. Both copies of ICP0 were then repaired in the ICP0Δ/Us3Δ and the ICP0Δ BACs (e-f).
3.7 Immunoprecipitation and Western Blotting

Vero or U2OS cells were seeded in 150mm dishes and grown to 60-80% confluency. Cells were infected with HSV-2 wild type (WT), ICP0Δ, ICP0Δ/Us3Δ, ICP0ΔR, or ICP0ΔR/Us3Δ virus at very low MOI (less than 0.1), limited by the low stock titres of the recombinant viruses. At 24 hours post infection (hpi), cells were washed with PBS and lysed using 700μl of Immunoprecipitation Lysis Buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100, protease inhibitor cocktail, 20mM N-ethylmaleimide, 0.1% SDS, 250U/ml benzonase). Cells were scraped into microfuge tubes. Approximately 100μl was kept as a lysate control. The remaining volume was incubated with 5μl of ICP0 rat polyclonal antiserum overnight at 4°C on a Nutator (Clay Adams Brand). 50μl of Protein G (Thermo Scientific, Waltham, MA) was then added to the samples and incubated for an additional 2 hours at 4°C on a Nutator to allow it to bind to the antibody complex. Next, samples were centrifuged at 14,000 x g for 90 seconds to pellet the Protein G complex. Pellet was washed 3 times with PBS by resuspension and centrifugation, then finally resuspended in 70μl of 1X SDS PAGE loading buffer and boiled for 5min.

10μl of IP sample or 5μl of lysate control (diluted to 10μl using 1X SDS-PAGE loading buffer) was loaded in each lane of a 7.2% SDS-PAGE gel. Gel was run at 180V for approximately 90 minutes, or until gel front ran off the gel. Proteins were transferred onto PVDF membranes using a semi-dry apparatus, transferring at 15V for 30 minutes. Membranes were stored in blocking buffer (TBST+3% BSA) overnight at 4°C. Membranes were then incubated in ICP0, Us3, ICP27, or β-actin primary antibody for one hour, and secondary antibody for an additional hour. The antibodies were diluted in
TBST+1% BSA at dilutions described in Chapter 3.4. Three 5min washes with TBST were performed in between each incubation. ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA) was used to visualize the bound antibody. Images were developed on film.

3.8 Immunofluorescence Microscopy

Cells were seeded onto 35mm glass bottom dishes or coverslips in 6-well dishes, and infected at a low MOI (less than 1). At the indicated times post infection, cells were washed with PBS and then fixed with 4% formaldehyde for 10 minutes. Stored cells in PBS+1% BSA (PBS/BSA) until staining. Permeabilized with PBS/BSA+0.5% Triton X-100 for 3 minutes. When staining with the rabbit α PML antibody, a blocking step with 0.1% human IgG was performed for 15 minutes to prevent non-specific interactions between the antibody and viral immunoglobulins. Cells were then stained for 45 minutes with primary antibody, followed by 30 minutes with secondary antibody. To visualize the nucleus, cells were stained with Hoechst for 7 minutes. In between each incubation step, cells were washed 3 times with PBS/BSA. Depending on the experiment, different primary and secondary antibodies were used. Dilutions are indicated in Chapter 3.4. Cells in glass bottom dishes were stored in PBS/BSA, while coverslips were mounted onto microscope slides.

For confirming ICP0 and Us3 expression in recombinant viruses, U2OS cells were infected with HSV-2 WT, ICP0Δ, ICP0Δ/Us3Δ, ICP0ΔR, or ICP0ΔR/Us3Δ virus and then fixed at 3 or 7hpi for ICP0/ICP27 and Us3/ICP8 staining respectively. ICP27 was used as a marker for immediate early gene expression and ICP8 was used as marker for early gene expression.
3.9 Growth Curve Assay

T12 cells were seeded in 6 well dishes and infected with HSV-2 WT, Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus at an MOI of 0.1. An extra well of cells was trypsinized and counted prior to infection to determine the amount of virus required. After a 1 hour inoculation period, cells were washed with DMEM, then a low pH citrate buffer (40mM sodium citrate, 10mM KCl, 135mM NaCl, pH 3.0) to inactivate all extracellular virus. Cells were washed again with DMEM then kept in 2ml of DMEM at 37°C. This was defined as time 0, although later time points were calculated based on when the virus was initially added to cells. At 0, 12, 18, 24, 48, and 72hpi, cells and media were scraped and collected in microfuge tubes. Samples were frozen at -80°C and subjected to 3 cycles of freezing and thawing to ensure all viruses were released from the cells. After the third thaw cycle, samples were sonicated for ten one second pulses using a Sonic Dismembrator Model 100 (Fisher Scientific, Toronto, ON) at setting 8. Samples were centrifuged at 1600xg for 3 minutes to pellet cell debris. The supernatant was collected and a plaque assay performed on U2OS cells to determine viral titres.

3.10 Measuring Plaque Sizes

U2OS or Vero cells were seeded in 6 well dishes and infected with HSV-2 WT, Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus. Based on known titres, enough virus was used to form a desirable number of plaques (aiming for 50-100 plaques per well). After a 1 hour inoculation time, the inoculum was replaced with 2ml of methocel supplemented with 2% FBS, 1000 units/ml penicillin, and 1000 μg/ml streptomycin. At approximately 48hpi, methocel was removed from each well and cells were washed with PBS. The cells were then fixed with 4% formaldehyde and stained for
ICP8 following the IF protocol described in Chapter 3.8. Plaques were visualized at 4X or 10X magnification on the Nikon Eclipse TE200 fluorescent microscope and photos taken using the Metamorph program. The diameters of 40 plaques from each sample were measured using Metamorph, taking the largest possible measurement for each plaque.

3.11 PML Nuclear Body Disruption

T12 cells were seeded onto glass coverslips in 6 well dishes and infected with HSV-2 WT, Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus at a low MOI (less than 1). An extra well of cells was trypsinized and counted prior to infection to determine the amount of virus required. It was assumed that approximately half the cells in a well would be on the coverslip. 120μl of virus was added to the top of the coverslip and kept on ice for 1 hour to synchronize the infection, allowing virus to bind to cell surfaces but not enter the cell. After this 1 hour inoculation, the inoculum was replaced with 2ml of warm DMEM and placed in the 37°C incubator to allow the infection to proceed. At 2, 3, 4, and 6hpi, cells were fixed with 4% formaldehyde and stained for PML and ICP27 following the IF protocol described in Chapter 3.8. Coverslips were mounted onto microscope slides and visualized on the Nikon Olympus FV1000 confocal microscope. The number of PML-NBs per cell was counted for 40 infected cells in each sample, using ICP27 as a marker of infection.

3.12 Interferon Assay

The interferon (IFN) assay was performed in tandem with the growth curve assay described in Chapter 3.9. T12 cells were treated with 1000U/ml of an IFNα hybrid (Universal Type I Interferon, PBL Interferon Source) or a sterile PBS+0.1% BSA carrier
18 hours prior to infection. Virus was collected at 0, 12, 18, 24, 48, and 72hpi. Results from the carrier treated cells correspond to the growth curve assay results. Viral titres in IFN treated cells were determined following the same procedures described in Chapter 3.9. Fold inhibition of virus growth due to IFN treatment was calculated as the titre in carrier treated cells divided by the titre in IFN treated cells.

3.13 Isolating BAC DNA for Sequencing

Bacterial cultures of pYEbac373 WT and Us3Δ were grown overnight in 20ml of LB+30μg/ml Cm, at 32°C with shaking. The bacteria were pelleted and supernatant removed. Bacterial pellet was resuspended in 3.5ml of Solution I (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA). 3.5 ml of Solution II (1% SDS, 0.2N NaOH) with 10μg/ml of RNase was added and mixed gently, then incubated at room temperature for 10 minutes. 3.5ml of Solution III (3M potassium acetate, 11.5% glacial acetic acid) was added and mixed gently. Sample was centrifuged at 8000 x g for 30 minutes to pellet cell debris. The clear supernatant was poured through a cheesecloth, and then purified using the Qiagen Plasmid Mini Kit (Qiagen Cat. No. 12123, Toronto, ON). DNA was resuspended in a final volume of 20μl and sent for genome sequencing.

3.14 Statistical Analysis

T-tests were used to conduct statistical analysis of data in Figures 4.6, 4.8, and 4.10. The two-tailed test was used, and equal variance was assumed.
Chapter 4

Results

4.1 En Passant Mutagenesis

Both ICP0 and Us3 are able to disrupt PML-NBs in HSV-2 infected cells. In order to study the relative activities of ICP0 and Us3, recombinant HSV-2 186 viruses lacking ICP0, Us3, or both ICP0 and Us3 were constructed using en passant mutagenesis as described in Chapter 3.6. Us3Δ and Us3ΔR viruses were made by Dr. Valerie Le Sage. Appendix A shows a diagram depicting the en passant protocol. Table 3.1 outlines which mutations are present in each recombinant virus, and Figure 3.1 shows a flowchart indicating which virus strains were made from which parental bacterial artificial chromosome (BAC).

After using en passant mutagenesis to introduce nonsense mutations into the ICP0 gene, RL2, DNA sequencing, Western blotting, and immunofluorescence microscopy were used to confirm the expression or lack thereof of ICP0 and Us3 in the respective null and repair virus strains.

Using BAC DNA as template, primers flanking the RL2 or Us3 gene mutation (see Appendix B) were used to amplify a PCR product that was subsequently sequenced. Figure 4.1 displays sequencing results demonstrating that the desired mutations and repairs were introduced at the specified locations. Panel A shows that two stop codons were introduced three nucleotides after both copies of the ICP0 start codon in the Us3Δ
Figure 4.1: Sequencing PCR products amplified from BAC DNA identified BACs with the desired mutations and repairs in ICP0 and Us3. BAC DNA was used as template. Primers flanking the ICP0 start codon (A, C, D) or the Us3 start codon (B) were used to amplify a PCR product for sequencing. The modified sequences are underlined in red. The wild type ICP0 sequence is CCCCGG while the mutation is 2 stop codons, TGATGA. The wild type Us3 sequence is TGTCGT, and the mutant is TGATGA. A. Two copies of ICP0 are knocked out in the Us3Δ BAC. B. Us3 is repaired in the ICP0Δ/Us3Δ BAC. C. Two copies of ICP0 are repaired in the ICP0Δ BAC. D. Two copies of ICP0 are repaired in the ICP0Δ/Us3Δ BAC.
BAC, creating a ICP0Δ/Us3Δ double mutant BAC. Panel B shows that Us3 was repaired to its wild type (WT) sequence in the ICP0Δ/Us3Δ BAC to create the ICP0Δ single mutant. Panels C and D show that both copies of ICP0 were repaired to WT sequences in the ICP0Δ and ICP0Δ/Us3Δ BACs, respectively.

Appendix C shows sequencing results from an intermediate BAC clone, where only one copy of RL2 was mutated. At the site of the mutation, two sets of peaks are observed. One set corresponds with the WT sequence (CCCCGG) while the other represents the mutated sequence (TGATGA). After the second copy of RL2 was mutated, the mixed sequence was no longer observed (Figure 4.1).

After sequencing results identified the BACs that contained the correct mutations or repairs, the BACs were transfected into mammalian cells to produce infectious virus. Viral stocks were subsequently prepared following procedures described in Chapter 3.6. ICP0 and Us3 protein expression were confirmed in these viruses by Western blotting and immunofluorescence microscopy. However, the ICP0 antibody is not ideal for Western blotting, producing high background signals and cross reacting with numerous proteins in all samples including uninfected controls. Various methods were used to try to optimize this antibody for Western blotting, including adding an immunoprecipitation step, pre-absorption of the antibody with ICP0Δ infected cell lysates to remove background reactivity, and filtering the antibody. None of these methods were successful. Figure 4.2 shows Western blotting results confirming that Us3 was successfully repaired in the ICP0Δ/Us3Δ double mutant resulting in the ICP0Δ single mutant.
Figure 4.2: Western blotting confirms Us3 expression in ICP0Δ viruses. Vero cells were infected with virus at a very low MOI (<0.01), dependent on the lowest titre virus. At 24hpi, cells were washed with PBS and lysed with immunoprecipitation (IP) lysis buffer. The lysates were run on a 7.2% SDS-PAGE gel, transferred to PVDF membranes, and probed with antibodies against Us3, ICP27, and β-actin. Us3 is detected in WT, ICP0Δ, and ICP0ΔR viruses, but not the double mutant or ICP0ΔR/Us3Δ. ICP27 was used as a control for viral gene expression, and is expressed in all infected samples. β-actin was used as a protein loading control. ICP0 results are not shown, as despite multiple optimization attempts, the antibody detects a cross reacting protein in both infected and uninfected cell lysates.
Although Western blotting with the ICP0 antibody was unsuccessful, immunofluorescence microscopy indicated that ICP0 was not detectable in ICP0Δ and ICP0Δ/Us3Δ viruses, but was expressed in the WT and repaired viruses (Figure 4.3). The expression of Us3 in the ICP0Δ and ICP0ΔR viruses was also confirmed (Figure 4.4).

Together, these experiments confirmed that the ICP0Δ virus strains did not express ICP0, but that when the mutation was repaired, ICP0 expression was restored. Once all of these recombinant virus strains were constructed, their phenotypes were documented using a variety of analyses.

4.2 Analysis of Virus Growth Kinetics

To determine if the HSV-2 ICP0Δ mutants exhibited a growth defect, multi-step growth analysis was performed. Human fibroblast T12 cells were infected at a multiplicity of infection (MOI) of 0.1 with WT, Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus. After a one hour incubation with the inoculum, cells were washed with a low pH citrate buffer to inactivate extracellular virus. Newly replicated virus was collected at 0, 12, 18, 24, 48, and 72 hours post infection (hpi) by scraping the infected cells into the medium and titrating the virus on U2OS cell monolayers. Figure 4.5 shows the kinetics of virus production from an average of two experiments. The ICP0Δ virus did not exhibit a growth defect, growing to similar titres as WT virus. On the other hand, viruses lacking Us3 demonstrated one log lower peak titres than WT virus.
Figure 4.3: Immunofluorescence microscopy confirms ICP0 knockout and repair. U2OS cells were infected with wild type, ICP0Δ, ICP0Δ/Us3Δ, ICP0ΔR, or ICP0ΔR/Us3Δ virus. Cells were fixed after 3 hours and stained with antibodies against ICP0 (green) and ICP27 (red) as a marker of infection. Nuclei were stained with Hoechst (blue). Cells infected with the wild type HSV-2 virus and the repair viruses display ICP0 staining, whereas the staining intensity of cells infected with the ICP0Δ viruses does not exceed background levels seen in uninfected cells (arrowheads).
Figure 4.4: Immunofluorescence microscopy confirms expression of Us3. U2OS cells were infected with wild type, ICP0Δ, ICP0Δ/Us3Δ, ICP0ΔR, or ICP0ΔR/Us3Δ virus. Cells were fixed after 7 hours and stained with antibodies against Us3 (green) and ICP8 (red) as a marker of infection. Nuclei were stained with Hoechst (blue). Cells infected with wild type HSV-2, ICP0Δ, and ICP0ΔR viruses show positive Us3 staining, while the ICP0Δ/Us3Δ double mutant and its ICP0 repair virus do not exceed background levels seen in uninfected cells (arrowheads).
**Figure 4.5: Analysis of Us3Δ and ICP0Δ mutants demonstrate no growth defects for ICP0Δ viruses.** Human fibroblast T12 cells were infected with wild type HSV-2 (WT), Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus at an MOI of 0.1. After one hour, cells were washed with a low pH citrate buffer to inactivate extracellular virus. Infected cells were scraped into the medium and collected at 0, 12, 18, 24, 48, and 72 hours post infection. Viral titres at each time point were determined by performing plaque assays on U2OS cells. Log values of viral titres are plotted against time after infection. Peak titres for viruses lacking Us3 (Us3Δ, ICP0Δ/Us3Δ, and ICP0ΔR/Us3Δ) are approximately 1 log lower than wild type viruses, but no difference is observed for the ICP0Δ virus. Results are an average of two experiments with error bars indicating standard deviation.
4.3 Plaque Formation

Another experiment performed to characterize the recombinant viruses was to measure the efficiency of cell-cell spread of virus infection by comparing plaque sizes. U2OS or Vero cells were infected with WT, Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus strains to produce plaques on a 6-well dish (approximately 50-100 PFU/dish). After a one hour incubation with the virus inoculum, medium containing 1% methocel was added to the cells to enable the formation of isolated plaques. Virus was allowed to replicate and spread between adjacent cells for 48 hours, and then the methocel was removed. Cells were washed with PBS, fixed with 4% formaldehyde, and stained with an antibody reactive against the viral protein ICP8. Plaques were imaged by epifluorescence microscopy and MetaMorph 7.1.2.0 software was used to measure their diameter. The plaque diameters of 40 plaques were measured for each strain and the results are shown in Figure 4.6.

Two WT clones, SW and MJ, were produced from the same BAC but are different viral clones (see Figure 3.1). In both U2OS and Vero cells, clone MJ formed significantly smaller plaques than clone SW, indicating that there can be differences that arise between viral clones produced from the same BAC. Figure 4.6 compares plaque sizes of the recombinant viruses with WT (MJ) since it was more similar to results for the repair virus strains and is therefore more consistent with the expected WT phenotype. In U2OS cells, viruses lacking Us3 (Us3Δ, ICP0Δ/Us3Δ, and ICP0ΔR/Us3Δ) formed significantly smaller plaques than WT, and repairing Us3 restored plaque sizes. The
Figure 4.6: Analysis of plaque formation of recombinant and parental virus strains. Two clones of wild type (WT) virus (SW and MJ) as well as Us3 and ICP0 recombinant viruses (Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, and ICP0ΔR/Us3Δ) were used to infect monolayers of U2OS or Vero cells. Based on known titres of the virus stocks, dilutions were prepared to ensure formation of isolated plaques. After 48 hours under methocel, cells were fixed with 4% formaldehyde, permeabilized with 0.1% TX-100 and stained with HSV-2 ICP8 antibody. Images of plaques were captured using an epifluorescence microscope equipped with a CCD camera at 4X magnification, and plaque diameters were measured using MetaMorph 7.1.2.0 software. Data shown is an average (±SE) from 40 plaques for each sample, except WT (SW) in U2OS cells, ICP0Δ in Vero cells, and ICP0Δ/Us3Δ in Vero cells, where only 34, 16, and 32 plaques were measured, respectively. Some of the smaller plaques were measured at 10X magnification. In both U2OS and Vero cells, the WT (MJ) virus forms significantly smaller plaques than the WT (SW) virus. The rest of the recombinant viruses were all compared to clone WT (MJ) since it was more similar to the repair viruses. In U2OS cells, viruses lacking Us3 form significantly smaller plaques than the other viruses but the absence of ICP0 has no effect on plaque size. In Vero cells, the ICP0Δ virus appears to form significantly smaller plaques than WT (MJ), but there is no difference between ICP0Δ and ICP0ΔR. **p<0.01; ***p<0.001
ICP0Δ virus produced plaques that were in similar in size to WT and therefore did not show a defect in cell-cell spread of infection. In Vero cells, all of the recombinant viruses formed similar sized plaques as WT. Although the ICP0Δ strain formed slightly smaller plaques than WT virus on Vero cells, there was no significant difference between ICP0Δ and ICP0ΔR, suggesting that lack of ICP0 did not actually affect plaque size. However, the ICP0Δ strain formed much fewer plaques in Vero cells than was expected, based on the known titre of these stocks derived from U2OS cells. Everett has shown that HSV-1 ICP0Δ mutants display a higher level of abortive infections in Vero cells than in U2OS cells [189]. In abortive infections, single cells become infected, but are unable to complete the virus replication cycle and therefore do not lead to plaque formation. To determine if HSV-2 ICP0Δ viruses also result in more abortive infections in Vero cells, viral stocks that had been previously titred on U2OS cells were re-titred on Vero cells, and the relative plating efficiencies of these strains were calculated. Results in Figure 4.7 indicate that with the exception of WT virus, which displayed a higher plaquing efficiency in Vero cells than in U2OS cells, all the viruses plated approximately 2 fold better in U2OS cells. The samples sizes were too small to perform T-test analysis, and some error bars were quite large, however the ICP0Δ virus did not consistently show a plating deficiency in Vero cells compared with the other recombinant viruses.

4.4 Disruption of PML Nuclear Bodies

After initial characterization of the ICP0 and Us3 recombinant viruses, their ability to disrupt PML-NBs was studied. The role of HSV-1 ICP0 in targeting PML for
Figure 4.7: Plaques of viruses in U2OS cells is approximately 2 fold higher than in Vero cells. WT (SW), Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, and ICP0ΔR/Us3Δ viruses were titrated on both U2OS and Vero cells. The fold difference in titres was calculated by dividing titres obtained on U2OS cells by titres obtained on Vero cell monolayers. Results are displayed as an average from 2-4 samples ±SD. The small sample size prevented statistical analysis from being performed, but the large standard deviation suggests that the ICP0Δ virus does not consistently show a greater growth inhibition than other viruses in Vero cells compared to U2OS cells.
proteasome dependent degradation is well documented [72, 133, 190-192] and our lab has shown that HSV-2 ICP0 also disrupts PML-NBs (Figure 2.13). Furthermore HSV-2 Us3 also plays an important role in PML-NB disruption [12]. To begin to define the relationship between the function of these proteins, the ability of my panel of recombinant viruses to disrupt PML-NBs was compared. T12 cells were infected with WT, Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus strains at a low MOI (less than 1). Cells were fixed at 2, 3, 4, or 6 hpi and stained with antibodies against PML, and the immediate early viral protein ICP27. ICP27 was used to identify infected cells, and the number of PML-NBs in these cells was counted. For most strains, the numbers of PML-NBs in 40 infected cells were counted. The average number of PML-NBs per cell for each of the viruses was compared. Figure 4.8 shows that at 3hpi, cells infected with the ICP0Δ/Us3Δ virus have a significantly higher number of PML-NBs remaining in the nucleus, compared to cells infected with WT virus. At 4hpi, both cells infected with ICP0Δ and ICP0Δ/Us3Δ strains have a significantly higher number of PML-NBs, although the absolute number of PML-NBs is smaller. No differences were observed at 2 or 6hpi. There was an average of 21.82±1.26 PML-NBs in an uninfected control.

Figure 4.9 is an alternate representation of the data in Figure 4.8, to compare the disruption of PML-NBs over time in each sample. Cells with less than five PML-NBs were defined as having “diffused PML-NBs” and the percentage of infected cells with diffused PML was graphed over time, demonstrating the temporal disruption of PML-NBs. The Us3Δ virus is able to disrupt PML-NBs with the same efficiency as WT virus,
Figure 4.8: The average number of PML-NBs in cells infected with ICP0Δ or ICP0Δ/Us3Δ virus is significantly higher than in cells infected with WT virus at 4hpi. Human fibroblast T12 cells were infected with wild type (WT), Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus strains at a low MOI (less than 1). At 2, 3, 4 and 6hpi, cells were fixed and stained with antibodies against PML and ICP27. The numbers of PML-NBs in 40[^a] infected cells (identified by positive ICP27 staining) were counted and plotted as an average ±SE. At 3hpi, cells infected with ICP0Δ/Us3Δ virus showed a significantly higher number of PML-NBs per cell compared to cells infected with WT virus. At 4hpi, cells infected with ICP0Δ or ICP0Δ/Us3Δ virus showed a significantly higher number of PML-NBs per cell. No difference was observed at 2 or 6hpi. *p<0.05; **p<0.01.

[^a]: PML-NBs could not be counted for WT 6hpi and Us3ΔR 2hpi samples due to the cells drying up on the coverslip (ND). For Us3Δ 2hpi and ICP0Δ/Us3Δ 2hpi samples, only 22 and 6 infected cells were counted respectively.
Figure 4.9: Cells infected with ICP0Δ or ICP0Δ/Us3Δ virus strains exhibit a delay in the disruption of PML nuclear bodies. Human fibroblast T12 cells were infected with wild type (WT), Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus strains at a low MOI (less than 1). At 2, 3, 4 and 6hpi, cells were fixed and stained with antibodies against PML and ICP27. The numbers of PML nuclear bodies (PML-NBs) in 40 infected cells (identified by positive ICP27 staining) were counted and those with less than 5 PML-NBs per cell were defined as having “dispersed PML-NBs”. The percentage of cells with dispersed PML-NBs was graphed over time. At 3hpi, the percentage of cells with dispersed PML-NBs was lower in cells infected with ICP0Δ or ICP0Δ/Us3Δ virus than in cells infected with WT, Us3Δ, or any of the repair viruses. A synergistic effect was seen in the ICP0Δ/Us3Δ double mutant. This delay in the disruption of PML-NBs was still observed at 4hpi, though by 6hpi even cells infected with ICP0Δ or ICP0Δ/Us3Δ virus showed almost complete disruption of PML-NBs.

PML-NBs could not be counted for WT 6hpi and Us3ΔR 2hpi samples due to the cells drying up on the coverslip. For Us3Δ 2hpi and ICP0Δ/Us3Δ 2hpi samples, only 22 and 6 infected cells were counted respectively.
while the ICP0Δ and double mutant exhibit a delay in PML-NB disruption. The delay in the double mutant is more pronounced than that of the ICP0Δ single mutant. However by 6 hpi even the ICP0Δ and ICP0Δ/Us3Δ viruses cause almost complete disruption of PML-NBs. Similar trends were observed in multiple experiments (also see Appendix D).

4.5 Sensitivity of Recombinant Viruses to Interferon

Although the Us3Δ strain disrupted PML-NBs at a rate comparable to the WT strain, our lab has shown that when cells are pre-treated with IFN, the Us3Δ virus displays a defect in PML-NB disruption relative to WT (Jung and Banfield, unpublished). Furthermore, the Us3Δ virus is hypersensitive to the effects of interferon (IFN) (Jung and Banfield, unpublished). PML is a mediator of the IFN antiviral response, and an increase in size and number of PML-NBs is observed in cells treated with IFN [13]. Because phenotypic differences between WT virus and the Us3Δ strain were only observed in the presence of IFN, it was hypothesized that without IFN treatment, alternative viral proteins were sufficient for PML-NB disruption, but that in the presence of IFN, Us3 would be required for efficient disruption of PML-NBs. To determine how ICP0 fit into this model, we studied the effects of IFN on recombinant viruses lacking ICP0, Us3, or both of these proteins. Since it was shown that the ICP0Δ and ICP0Δ/Us3Δ strains were defective in disrupting PML-NBs, it was hypothesized that they would also be more sensitive to the effects of IFN.

Figure 4.10 shows that treatment of three different cell types, U2OS, Vero, and T12 cells, with IFN resulted in an increase in the average number of PML-NBs per cell, confirming that the IFN was working as expected.
Figure 4.10: Treatment with interferon results in an increase in the average number of PML nuclear bodies per cell. Vero, U2OS, and T12 cells were treated with a carrier control or 1000U/mL of Universal Type I Interferon. After 18 hours, cells were fixed with 4% formaldehyde, permeabilized in 0.1% TX-100 and stained with anti-PML antibodies. The numbers of PML nuclear bodies (PML-NBs) in 40 random cells from each sample were counted. The average number of PML-NBs per cell (±SE) are shown in the graph. Cells treated with interferon showed a significant increase in the average number of PML-NBs per cell, compared to carrier treated cells. ***p<0.001.
For the IFN assay, T12 cells were treated with 1000 U/ml IFN or a carrier control for 18 hours prior to infection. Cells were infected at an MOI of 0.1, and newly synthesized virus was collected at 0, 12, 18, 24, 48, and 72 hpi. Results from the carrier treated cells were used to generate the growth curves in Figure 4.5. Comparing titres in carrier treated cells with those from IFN treated cells were used to produce the graphs in Figure 4.11. Fold inhibition due to IFN treatment was calculated by dividing titres from carrier treated cells by titres from IFN treated cells.

The IFN assay was performed twice in independent experiments (replicates A and B). In replicate A, Us3Δ, ICP0Δ, and ICP0Δ/Us3Δ virus showed a higher fold-inhibition in IFN treated cells compared with WT virus at both 18 and 24hpi. A similar trend was seen for replicate B at 18hpi, although to a lower extent. At 24hpi, the Us3Δ and ICP0Δ viruses were no longer hypersensitive in replicate B. However, Appendix Figure E.1 shows results from an earlier IFN assay experiment, and demonstrates that at 24hpi, some Us3Δ virus clones are hypersensitive to IFN but others are not. Us3Δ clone MJ is more sensitive to IFN than WT, while Us3Δ clone SW is less sensitive. Us3Δ clones 5.11 and 10.1 are only marginally sensitive to the effects of IFN. Furthermore, the ICP0Δ and ICP0Δ/Us3Δ virus strains are no more sensitive to IFN than WT. It may be important to note that virus stocks were passaged during course of these experiments, and the viruses used in replicate A were from a later passage of viral stocks than those used in the experiment shown in Figure E.1. The viruses used in replicate B were also from a later passage than those used in replicate A. The discrepancies in sensitivity to IFN between these replicates suggested that passaging virus can introduce new mutations that affect
Recombinant viruses lacking Us3, ICP0, or both Us3 and ICP0 are hypersensitive to the effects of interferon. T12 cells were treated with a carrier control or 1000U/ml of Universal Type I Interferon (IFN). After 18 hours, cells were infected with wild type clone SW (WT (SW)), Us3Δ (SW), Us3ΔR (SW), ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus strains at an MOI of 0.1. Virus titres were determined at 0, 12, 18, 24, 48, and 72hpi. Fold inhibition was calculated by dividing viral titres in carrier treated cells by titres in IFN treated cells. Two independent experiments, replicates A and B, were performed. At 18hpi, Us3Δ (SW), ICP0Δ, and ICP0Δ/Us3Δ virus showed a higher fold inhibition in IFN treated cells compared with WT virus. However the difference in fold inhibition is higher in replicate A than in replicate B. At 24hpi, the Us3Δ (SW) and ICP0Δ viruses were still hypersensitive in replicate A, but not in replicate B. Results from 0 and 12hpi were not graphed. Fold inhibition observed at 48 and 72hpi was much lower than at 18 and 24hpi.
viral properties. Because we also observed differences between clones of the same virus, as mentioned in Chapter 4.3, we sequenced viral genomes to provide more information about how the viral genome sequence is affected when BAC DNA is transfected into mammalian cells, and when the virus is passaged in mammalian cells.

4.6 Genome Sequencing of BACs and Viruses

BAC DNA containing the HSV-2 186 wild type (WT) and Us3Δ genomes, along with viral DNA from viruses grown from those BACs (WT (MJ) and Us3Δ (MJ) clones), were sequenced (see Figure 3.1 for more detail regarding the BACs and virus clones grown from them). Contigs were assembled using the DNASTAR SeqMan Pro program and, because the complete HSV-2 186 genome has not been published, compared to the complete HSV-2 strain HG52 reference sequence from GenBank. Mutations were summarized by the SeqMan Pro program in the form of single nucleotide polymorphism (SNP) tables. Because two different strains of HSV-2 were being compared (i.e. 186 vs HG52), many SNPs were identified that are likely due to inherent differences between the two strains, rather than unintended mutations introduced during en passant mutagenesis. An additional BAC, containing an HSV-2 186 UL21 knockout virus genome, was also sequenced, and SNPs that were present in all three BACs were considered to be inherent to the 186 strain in the context of this analysis. SNPs with low depth (number of times that section of the genome was sequenced) or low SNP percentage (percentage of sequence runs in which the SNP was observed) were regarded as having low probability of impacting virus function, or as a possible result of errors in the sequencing process.

Table 4.1 shows a summary of the SNPs that may result in differences between the WT BAC and WT (MJ) virus, the WT BAC and Us3Δ BAC, or the Us3Δ BAC and
Us3Δ (MJ) virus. Row 1 identifies a SNP in the UL46 gene that was only found in WT virus. Rows 2-4 identify SNPs in the UL5, UL24, and Us8 genes respectively that were only found in the Us3Δ (MJ) virus. Rows 5 and 6 identify SNPs in the Us3 gene that were only found in the Us3Δ BAC or Us3Δ (MJ) virus. The likelihood of these SNPs affecting virus growth is discussed in Chapter 5.

Table 4.1: Select single nucleotide polymorphisms (SNPs) from HSV-2 186 WT virus, Us3Δ virus, and Us3Δ BAC, compared with the HSV-2 HG52 sequence. These SNPs were of interest due to relatively high depth (number of times the area was sequenced) and SNP% (percentage of sequence reads containing the SNP). The “Genome” column identifies the sample in which the SNP was present.

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<td>UL24</td>
<td>c.517G&gt;A</td>
<td>V173M</td>
</tr>
<tr>
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<tr>
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Chapter 5

Discussion

5.1 Stability of Recombinant Viruses Constructed Using En Passant Mutagenesis

*En passant* mutagenesis is a useful tool allowing manipulations of viral genomes to be performed in bacteria. Older methods of generating recombinant viruses included randomly mutating the viral genome and selecting for phenotypic differences in properties such as host range, plaque formation, drug resistance, and temperature sensitivity. This then required a cumbersome effort to map the positions of the mutations by marker rescue and complementation assays [193]. Homologous recombination methods allowed for the mutation of specific genes, by co-transfecting mammalian cells with the entire viral genome and a target specific vector containing a selectable marker and the desired mutation. Homologous recombination between the genome and the vector resulted in the production of mutant viruses that could be purified using the selectable marker. However this was troublesome since the amount of wild type virus produced was much higher than the amount of mutant virus [193]. Using cosmids eliminated the need to purify the mutant virus from a mixed population. Although entire viral genomes were too large to be encoded within a single cosmid, they could be encoded into four or five cosmids with overlapping sequences. The cosmids could be purified from *Escherichia coli* (*E. coli*) and transfected into mammalian cells, where recombinantation between the cosmids produced intact viral genomes and resulted in plaque formation. Mutations could be introduced directly into the cosmids prior to transfection into mammalian cells, therefore only the mutant virus would be produced. However, the multiple recombination
events required between cosmids resulted in a high rate of undesired mutations in progeny viruses, requiring extensive screening of mutants to ensure the correct mutation was introduced [194, 195]. This problem was solved by using bacterial artificial chromosomes (BACs), which are able to house the entire virus genome. *En passant* mutagenesis allows for precise manipulation of the herpesvirus genome within a BAC, contained within *E. coli*. Mutations can be introduced into the BAC, followed by transfection of BAC DNA into mammalian cells for reconstitution of the virus. Since the viral genome manipulation is done within a BAC and grown in bacteria, selective pressures that would affect the ability to construct recombinant viruses (such as mutations in essential genes) are eliminated [195].

However, although *en passant* mutagenesis theoretically allows us to predict exactly what and where mutations are introduced, it has not been confirmed whether recombination events at a specific site could affect unanticipated loci as well. In particular, the highly repetitive nature of the herpesvirus genome may result in mutations being targeted to unintended loci. When clonal differences between viruses grown from the “same” BAC were observed in my experiments (Figures 4.6 and E.1), it was hypothesized that unanticipated mutations may have occurred when the virus was reconstituted from BACs in mammalian cells, or during passaging of virus stocks between mammalian cells. Genome sequencing of HSV-2 186 BAC DNA and selective clones of their progeny viruses was performed in an attempt to identify any changes that might occur during the process of constructing recombinant viruses.

Row 1 of Table 4.1 identifies a SNP that was only found in WT (MJ) virus. The 273rd amino acid in the coding sequence of UL46 was changed from an arginine to a
cysteine. UL46 is a 722 amino acid (aa) tegument protein that activates the cellular PI3-kinase/Akt signaling pathway, and enhances virus replication [196]. UL46 also interacts with VP16 and enhances the VP16 mediated transactivation of immediate early genes [197]. Therefore mutations in UL46 may affect the ability of the virus to replicate efficiently. However, the SNP% was only 55.6% with a depth of coverage of 9, meaning that only 9 reads were completed for that section of the genome, and only 55.6% of those reads contained the mutation. This suggests that the presence of the SNP may only be due to errors during sequencing, or that a mixed population of viruses exists in the stock that was sequenced.

Rows 2-4 of Table 4.1 identify SNPs that were found only in the Us3Δ (MJ) virus. Although the SNP% were not particularly high (between 52.2% and 75%), the depth of coverage was up to 44. The genes affected were UL5, UL24, and Us8. UL5 is part of a helicase-primase complex and is required for viral DNA replication [198]. UL5 contains 6 important conserved motifs, but mutations in non-conserved regions do not have much of an effect on its function [199]. The observed SNP is at residue 497, which is in a non-conserved region of UL5 [199]. Therefore it is not expected that this mutation will have a profound impact on UL5 protein function. HSV-1 UL24 causes dispersal of the nucleolar proteins nucleolin and B23 throughout the nucleus [200, 201]. It has been suggested that this effect on nucleoli is related to the role of UL24 in promoting nuclear egress [201]. UL24 also affects the localization of multiple glycoproteins involved in membrane fusion, which may be the cause of HSV-1 UL24 mutant viruses forming syncytial plaques [202]. The C-terminal 100aa of UL24 (182-281) is important for its syncytial phenotype, while the conserved N-terminus is important for nuclear egress.
The observed UL24 SNP in the Us3Δ virus is at residue 173, which is in neither of those two regions. No syncytial plaque formation or nuclear egress defects were observed in the recombinant virus Us3Δ (MJ). Us8 codes for the glycoprotein gE, which is important for interactions between the viral envelope and tegument proteins, facilitating envelopment of virions [204-206]. gE mutants display a defect in cell to cell spread of virus, and exhibit a small plaque phenotype [207]. The C-terminal cytoplasmic tail of gE is crucial for its function [205, 206], however the observed SNP is at residue 73 in the ectodomain, therefore is unlikely to affect the cytoplasmic domain.

The SNP described in Rows 5 and 6 of Table 4.1 was only seen in the Us3Δ BAC and Us3Δ (MJ) virus, and corresponds with the mutation that was engineered into the Us3 gene. Many other SNPs were also identified as being present in some of the BACs or viruses, but missing from others. Although some of them had relatively high SNP% or depth, those with high SNP% tended to have low depth and vice versa. Furthermore, the SNPs were often in areas containing repeated nucleotide sequences (such as 6 or 7 consecutive cysteines); therefore it is more likely that the differences are due to sequencing limitations rather than the en passant mutagenesis.

Based on these findings, there does not appear to be any unintended mutations occurring during en passant mutagenesis or when viruses are reconstituted from the BACs that would impact the growth of the virus. However because of multiple repeated regions in the herpesvirus genome and its high GC content, not all regions of the genome were sequenced adequately. Therefore it is possible that there are mutations in repeated regions of the genome that were not identified but impact virus function. This may explain why viral clones from the same BAC exhibited different phenotypes in my results.
(Figures 4.6, E.1). As I currently only have genome sequencing results for one viral clone per BAC, it would be informative to compare genome sequences of multiple viral clones reconstituted from the same BAC, to determine what those differences are.

5.2 HSV-2 ICP0Δ Mutants Do Not Display a Defect in Growth Kinetics While Us3Δ Mutants Grow to One Log Lower Titres

HSV-1 ICP0 mutants typically show a growth defect, particularly at low multiplicities of infection (MOI) [32, 187, 189, 208-211]. To determine if growth defects would be observed for HSV-2 ICP0Δ mutants, growth analyses for WT, Us3Δ, Us3ΔR, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ strains were performed, with virus collected at 0, 12, 18, 24, 48, and 72 hours post infection (hpi). Since ICP0 is an important transactivator of viral genes, it was hypothesized that viruses lacking ICP0 would grow more slowly than WT. My results showed that the HSV-2 ICP0Δ mutant did not exhibit a growth defect in T12 cells when infected at an MOI of 0.1, and the HSV-2 ICP0Δ/Us3Δ mutant grew similarly to the Us3Δ single mutant, which displayed a one log lower end point titre relative to WT (Figure 4.5).

My experiments were performed using T12 cells, which are non-transformed human foreskin fibroblasts, but whose life span have been extended by telomerase expression [212]. Because transformed cell lines are immortalized and may not behave in the same way as cells in vivo, the use of non-transformed T12 cells is more reflective of a natural infection. Viruses collected from T12 cells were titred on U2OS cells, which is a human osteosarcoma cell line that has been shown to complement ICP0Δ viruses [187]. This was to ensure that observed differences between WT virus and any of the ICP0 mutants would be due to their ability to grow in T12 cells, and not their plaque forming
ability in a different cell line. Indeed, several other groups have reported that properties of HSV-1 ICP0Δ viruses are cell type dependent [187, 189, 208-210]. These cell type dependent effects make it difficult to compare results from the literature, since each group propagated and titred stocks of viruses using different cell lines. In general, the HSV-1 ICP0 mutants exhibited the largest growth defect in human fibroblast cells (up to 1000 fold lower titres than WT virus), intermediate defects in cell types such as Vero, baby hamster kidney (BHK), and HeLa cells (4 to100 fold lower than WT), and no growth defect in U2OS cells [187, 189, 208-210]. The reason for this is related to the ability of ICP0Δ mutants to establish productive infection in the different cell types (discussed more in Chapter 5.3). Furthermore, the effects of the ICP0Δ mutants are dependent on multiplicity of infection (MOI), and when higher multiplicities are used growth defects are not observed [32, 209]. For HSV-1 the threshold between low and high MOIs was defined by Everett et al to be less than 10 for fibroblast infections and less than 1 for Vero cell infections, using titres determined from U2OS cells [209].

My experiments were performed on T12 cells at an MOI of 0.1, and are therefore considered “low” MOI based on the threshold defined by Dr. Everett. However, inconsistent with the HSV-1 literature, I did not observe growth defects with HSV-2 ICP0Δ mutants. It would be informative to determine if such a threshold exists for HSV-2 ICP0Δ mutants. The multiplicity dependent results for HSV-1 suggest that growth deficiencies caused by the absence of ICP0 may be complemented by other viral factors when the MOI is high enough. For example, VP16 and ICP4 are two essential proteins also important for transactivating viral genes [25-27]. It is possible that for HSV-2, a lower MOI is enough to promote efficient replication even in the absence of ICP0,
resulting in similar growth curves for WT and ICP0Δ viruses. Contrarily, Halford et al did show that various HSV-2 ICP0 mutants showed growth defects in Vero cells at an MOI of 0.1 [213]. The different methods used to construct the recombinant viruses may account for these inconsistencies (discussed in Chapter 5.3).

Unlike the ICP0Δ mutants, the HSV-2 Us3Δ virus grew to end point titres that were approximately one log lower than WT virus (Figure 4.5). This is consistent with what others have seen for pseudorabies virus (PRV) and HSV-1 Us3Δ viruses [154, 214-218]. However, cell type dependent effects were also observed, and while some cell lines, including Hep2 (human epidermoid cancer cells) and SK-6 (swine kidney cells) show lower end point titres for Us3Δ viruses, Vero cells did not [154, 214, 217]. Therefore the Us3Δ growth defect is only observed in certain cells lines. MOI dependent results have not been reported. Since the ICP0Δ mutant did not show a growth defect, it is not surprising that the ICP0Δ/Us3Δ virus behaved like the Us3Δ single mutant virus in these assays (Figure 4.5).

5.3 ICP0Δ Mutants Do Not Show a Plaque Formation Defect, Us3Δ Mutants Exhibit a Small Plaque Phenotype in U2OS Cells

Everett showed that although the number of virus particles detected in virus stocks was similar between WT and ICP0Δ mutants, the percentage of particles that successfully elicited lytic infection in cells was much lower in the ICP0Δ mutants due to increased rates of abortive infections. This plaque formation defect resulted in ICP0Δ virus strains having much higher particle to pfu ratios than WT virus strains [189]. Depending on the cell type, the particle to pfu ratio for the ICP0Δ mutant ranged from 4.4 to 870 fold higher than WT virus [189]. A high particle to pfu ratio was observed in Vero
cells, but not in U2OS cells or cells that stably express ICP0 [187, 210, 211]. HSV-1 ICP0Δ mutants also formed smaller plaques than WT virus in Vero cells [32, 210]. Based on these findings with HSV-1, it was hypothesized that differences in plaque size would be observed for the HSV-2 ICP0Δ and ICP0Δ/Us3Δ mutants in Vero cells, but not in U2OS cells, and that plating efficiency of these viruses would be higher in U2OS cells.

Figure 4.6 shows that in U2OS cells, the ICP0Δ mutant formed similar sized plaques as WT (MJ), and the ICP0Δ/Us3Δ mutant formed similar sized plaques as the Us3Δ single mutant. However, there was also no difference in plaque size between WT (MJ) and the ICP0Δ mutant, or between the ICP0Δ/Us3Δ and Us3Δ strains on Vero cell monolayers. Furthermore, results in Figure 4.7 show that there was not a consistent plating defect of ICP0Δ virus in Vero cells relative to U2OS cells, and the highest deficiency observed was only a seven fold difference. Therefore my results indicate that the ICP0Δ virus strains do not exhibit a plating deficiency in in Vero cells, nor do they form smaller plaques than WT. These findings are inconsistent with the literature, however due to the small sample size and large standard deviation, these results require further study to determine their significance.

As mentioned above, growth defects observed for HSV-1 ICP0Δ viruses are dependent on MOI. This was also the case for plating efficiencies, and may be the reason why my results are inconsistent with the literature. ICP0Δ mutants are able to enter cells at a similar rate as WT virus and express the immediate early gene product ICP4, but expression of early and late proteins are inhibited, preventing spread of infection and presumably leading to abortive infection and concomitant reductions in plating efficiencies. Although HSV-1 ICP0Δ viruses typically resulted in a higher particle to pfu
ratios than WT virus, the ratio of cells expressing ICP4 (measured using fluorescent focus assays) was similar in both strains [32, 209, 211]. Conversely, the ratio of cells expressing the early protein ICP8 and late protein ICP5 was much lower in cells infected with ICP0Δ strains [211]. Chen and Silverstein showed that mRNA accumulation of early and late genes in Vero cells infected with HSV-1 ICP0Δ mutant virus was much lower than in cells infected with WT virus [210]. Protein expression of early and late genes was also lower in cells infected with ICP0Δ virus than in cells infected with WT virus [32, 209, 210]. Importantly, this delay in early and late gene expression was only seen after low MOI infections. At high MOIs, the level of early and late protein expression in cells infected with ICP0Δ virus was indistinguishable from cells infected with WT virus [32, 209]. Therefore, although inhibition of early and late protein expression is observed for HSV-1 ICP0Δ infections at low MOI, it does not appear to be affected at high MOI, suggesting that the plating deficiency would also be abrogated at high MOI. As mentioned previously, it would be informative to determine if the “low MOI” threshold for HSV-1 is the same for HSV-2 ICP0Δ mutants. It would also be useful to compare levels of early and late protein expression in cells infected with HSV-2 ICP0Δ strains with that in cells infected with WT strains. Contrary to my results, Halford et al showed that HSV-2 ICP0 mutants formed fewer plaques on Vero cells than in an ICP0 complementing cell line [213].

A common feature of both HSV-1 and HSV-2 ICP0Δ mutants described in the literature is that in mutants where only a portion of ICP0 is mutated or deleted, viruses that encoded a longer ICP0 peptide behaved more like WT virus, while viruses that encoded very little of the protein showed greater defects [32, 210, 213]. It is important to
note that the latency associated transcripts (LATs) are coded on the opposite strand of RL2, and overlap with RL2 sequences. Therefore some ICP0 deletion mutants are actually LAT mutants as well [32]. Although it has been concluded that phenotypes observed in HSV-1 mutants are due to the absence of ICP0 and not due to LAT disruptions [32, 209], Cai et al also indicated that the growth defects of ICP0 mutants were only partially complemented by the ICP0 expressing cell line 0-28 [32]. It has not been confirmed whether or not mutations in LATs affects the phenotype of HSV-2 ICP0Δ mutants. My recombinant viruses were made by introducing a nonsense mutation three nucleotides after the start codon of RL2. This was a markerless mutation and therefore has minimal impact on the RL2 or LAT mRNAs. Mutants studied by other groups contain GFP tags, linker sequences, or deletions in the genome [32, 209-211, 213], therefore having a greater effect on the mRNA sequences of both RL2 and LAT. Therefore their observed phenotypes may be partially due to differences in the genome and transcript, and not just the absence of the ICP0 protein. These differences may be the reason for the discrepancies in my observations.

It may also be possible that in my ICP0Δ mutants, transcription is initiated from a downstream start codon of the RL2 gene, creating a truncated version of ICP0. This could explain why no difference in phenotype was observed in my mutants. Unfortunately, due to the poor performance of the ICP0 antibody in Western blotting experiments, I could not determine whether any truncated ICP0 products were made. However no evidence of ICP0 expression was observed in IF experiments.

Another factor that affects plating efficiency of ICP0Δ viruses is cellular stress. Cells deprived of glutamine, which is a signal for cellular stress, enhances plating
efficiency of ICP0Δ viruses [219]. Since glutamine is supplemented in DMEM growth medium, differences in medium may also influence the discrepancy observed between my results and those from the literature. Overall, my data shows that HSV-2 ICP0Δ mutants do not exhibit a plating deficiency in Vero cells relative to U2OS cells, and that plaque sizes are not affected in either cell line.

The Us3Δ viruses, on the other hand, formed smaller plaques than WT virus in U2OS cells, but not in Vero cells (Figure 4.6). While the small plaque phenotype is consistent with what others have seen [214, 220], the cell type specific effect is also not surprising [154, 214, 217, 218, 220]. Ryckman et al showed that the nuclear egress defect seen in Us3Δ viruses is not the cause of the small plaque phenotype, since the titres of Us3Δ virus was comparable to that of WT in Vero cells even though accumulation of perinuclear virions was observed [154]. It is possible that the small plaque phenotype is related to the role of Us3 in actin cytoskeleton rearrangement. Van den Broeke et al showed that inhibition of cellular factor PAK2, which is important for actin rearrangement and disruption of actin stress fibres, also leads to reduced viral spread of WT PRV [221]. This suggests that the ability to rearrange actin filaments is important for virus replication. Interestingly, PAK2, like Us3, is involved in anti-apoptotic pathways. However, Deruelle et al showed that growth defects of PRV Us3Δ virus are not due to the anti-apoptotic activity of Us3 [214].

A final point to note from these results is that of the two WT clones analyzed, WT (SW) formed significantly larger plaques than WT (MJ). Although genome sequencing results suggested that there are not many differences between the WT BAC DNA sequence and WT (MJ) viral DNA sequence, WT (SW) was not sequenced. It is possible
that another polymorphism in the WT (SW) genome results in the observed differences in plaque size. Furthermore, plaque sizes between the repair viruses and WT (MJ) were more similar than between repair viruses and WT (SW), therefore WT (MJ) was chosen as the reference for comparing plaque size. The observed differences between WT clones made it more difficult to interpret results from my other experiments, since WT (SW) was used as a reference instead of WT (MJ). However, since repair viruses were constructed for all the recombinant viruses, we were able to use those as a reference for the WT phenotype instead, allowing us to study the effect of the ICP0Δ, Us3Δ, and ICP0Δ/Us3Δ strains without worrying about the discrepancy between the WT clones.

5.4 ICP0, but Not Us3, is Required for Efficient Disruption of PML Nuclear Bodies

My main interest was to study the role of ICP0 and Us3 in counteracting cellular antiviral defences mediated by PML-NBs. Although no major growth defects were observed with any of the recombinant viruses, we wanted to determine the relative activities of ICP0 and Us3 in disrupting PML-NBs. The main constituents of PML-NBs, PML and Sp100, are targeted by the E3 ubiquitin ligase activity of ICP0 for proteasome mediated degradation, leading to the dispersal of PML-NBs. Our lab has shown that although the HSV-2 protein kinase Us3 also disrupts PML-NBs, it does not target PML protein for degradation [12]. Determining the relative function of Us3 and ICP0 will provide a more detailed picture of how HSV-2 counteracts intrinsic antiviral defences mediated by PML-NBs.

To begin to study the relative roles of Us3 and ICP0 in the disruption of PML-NBs, T12 cells were infected with WT, Us3Δ, ICP0Δ, or ICP0Δ/Us3Δ viruses and stained with antibodies for PML. The average number of PML-NBs in 40 infected cells
from each sample was determined at multiple time points, and the results are shown in Figures 4.8 and 4.9. Despite the ability of Us3 to disrupt PML-NBs, no difference was observed in the number of PML-NBs in WT and Us3Δ infected cells (also see Appendix Figure D.1). This was actually consistent with previous results which only showed a defect in PML-NB disruption in IFN treated cells infected with HSV-2 Us3Δ virus (Jung and Banfield, unpublished). At 3hpi, cells infected with the ICP0Δ and ICP0Δ/Us3Δ viruses had a higher number of PML-NBs than cells infected with WT virus, although the increase in ICP0Δ infected cells was very small. At 4hpi, the average number of PML-NBs for all infected cells was very low, but the difference between WT and ICP0Δ and WT and ICP0Δ/Us3Δ infected cells was still statistically significant (Figure 4.8). Repairing ICP0 in these viruses restored the number of PML-NBs to WT levels. Figure 4.9 further shows that cells infected with ICP0Δ virus were delayed in the disruption of PML-NBs, and that this delay was even more pronounced in ICP0Δ/Us3Δ infected cells. Appendix Figure D.1 shows further results that support the observation that there is no difference in PML-NB disruption between WT and Us3Δ infected cells, that there is a delay in PML-NB disruption in ICP0Δ infected cells, and that the delay is greater in ICP0Δ/Us3Δ infected cells. The synergistic effect in ICP0Δ/Us3Δ infected cells suggests that Us3 does indeed play a role in PML-NB disruption, even though there was no effect seen for the Us3Δ single mutant virus. In the absence of Us3, ICP0 is still able to disrupt PML-NBs at the same efficiency as WT, but in the absence of ICP0, Us3 is unable to completely complement this activity.

Our lab showed that in IFN treated cells, mRNA expression of the immediate early protein ICP0 was inhibited in the absence of Us3, even though Us3 itself is an early
protein (Jung and Banfield, unpublished). The importance of Us3 for immediate early
gene expression suggests that it is virion-associated Us3 that promotes viral gene
expression, and that this occurs prior to ICP0 expression. Therefore one hypothesis
regarding the relative functions of Us3 and ICP0 is that immediately after infection Us3
disperses PML-NB structures, making it easier for ICP0 to subsequently target PML and
other proteins for degradation (Figure 5.1). Since my results indicated that the Us3Δ virus
was able to disrupt PML-NBs at the same rate as WT virus, while the ICP0Δ/Us3Δ virus
exhibited a greater delay than the ICP0Δ single mutant, this would mean that the initial
dispersal of PML-NBs by Us3 is only important in the absence of ICP0.

Another hypothesis is that Us3 and ICP0 function through distinct pathways to
disrupt PML-NBs. This may occur due to selective targeting of the six different PML
isoforms, PML I - VI (Figure 5.2). Although it was shown that Us3 does not target PML
for degradation, it is possible that certain isoforms of PML are degraded but that this was
not detected in the assays used. ICP0 is able to induce degradation of all six PML
isoforms, however, PML I is the only isoform that can be targeted by ICP0 in a SUMO
independent manner [192, 222]. Although PML I is one of the more abundant isoforms of
PML [119], interaction between the other isoforms is also important for intrinsic
immunity. Cuchet et al. showed that no single PML isoform is able to form fully
functional PML-NBs, and constitutive expression of PML I and II only partially reverses
the improved growth of ICP0Δ virus in cells depleted of endogenous PML [223].
Therefore it will be informative to determine whether herpesviruses encode multiple
proteins that specifically target different isoforms of PML. This will require optimization
of the use of antibodies specific to each PML isoform, in order to measure their
Figure 5.1: Model of Us3 and ICP0 functioning within the same pathway to disrupt PML-NBs. One hypothesis is that a) Us3 disperses PML-NBs prior to viral gene expression, which b) makes SUMO conjugated PML and Sp100 more accessible to ICP0 mediated degradation (Figure modified from http://viralzone.expasy.org/all_by_protein/1676.html).
Figure 5.2: Model of Us3 and ICP0 functioning in distinct pathways to cause disruption of PML-NBs. Another hypothesis is that Us3 and ICP0 target different isoforms of PML (represented by blue and red colours). The only PML isoform that ICP0 is able to target independently of SUMO conjugation is PML I. Us3 has not been shown to target PML for degradation, however it is possible that it only targets certain isoforms, and that it was not detected in previous assays (Figure modified from http://viralzone.expasy.org/all_by_protein/1676.html).
individual degradation in cells infected with the recombinant viruses. If this hypothesis is true, my current results would indicate that those isoforms targeted for degradation by ICP0 would be more important for PML-NB disruption than the isoforms targeted by Us3.

Despite the delay in PML-NB disruption observed in ICP0Δ/Us3Δ virus infected cells, by 6hpi, the number of cells with disperse PML reaches comparable levels to WT, meaning that in addition to ICP0 and Us3, there are still other viral proteins that are able to disrupt PML-NBs. A large number of proteins, including HSV proteins Us10, UL14, and UL8.5, were identified by Salsman et al to disrupt PML-NBs [224]. Therefore it will be informative to also study their roles relative to ICP0 and Us3 in counteracting cellular antiviral defence.

5.5 ICP0 and Us3 Counteract the Effects of Interferon

As mentioned above, it was observed that the HSV-2 Us3Δ virus only showed a defect in PML-NB disruption when cells were pre-treated with IFN (Jung and Banfield, unpublished). On the other hand, ICP0Δ strains exhibited a delay in the disruption of PML-NBs even without IFN treatment (Figure 4.9). Since expression of PML and other PML-NB constituents are upregulated by IFN [13], it was hypothesized that in the absence of IFN, ICP0 is sufficient to disrupt PML-NBs, and that Us3 only plays a more prominent role when the number of PML-NBs increase in response to IFN. HSV-1 and HSV-2 ICP0 mutants have been shown to be hypersensitive to IFN [14, 225, 226]. The HSV-2 Us3Δ (MJ) virus strain is also hypersensitive to IFN (Jung and Banfield, unpublished). Therefore I sought to study the sensitivity of Us3Δ, ICP0Δ, and
ICP0Δ/Us3Δ mutants to IFN in order to further define the roles of these two proteins in counteracting cellular antiviral defence.

In replicate experiments A and B, T12 cells were treated with 1000U/ml IFN 18 hours prior to infection with HSV-2 WT (SW), Us3Δ (SW), Us3ΔR (SW), ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ at an MOI of 0.1. My results showed that the effect of IFN on Us3Δ (SW), ICP0Δ, and ICP0Δ/Us3Δ viruses were variable, but repairing Us3 or ICP0 restored the WT phenotype (Figure 4.11). In replicate A, Us3Δ (SW), ICP0Δ, and ICP0Δ/Us3Δ viruses were hypersensitive to IFN at both 18 and 24hpi, while in replicate B the effect of IFN on the recombinant viruses was lower and with the exception of the ICP0Δ/Us3Δ double mutant, was observed at 18hpi but not 24hpi (Figure 4.11). It is unclear what caused this variability. However since repairing ICP0 or Us3 restored the WT phenotype, my results indicate that these two viral proteins do play a role in counteracting the effects of IFN. The differing sensitivity to IFN suggests that IFN activity is greatly affected by external factors, such as confluence of cells during treatment and infection, or modest differences in MOI. Others have shown that IFN assays are highly dependent on MOI and cell type [227, 228], which may partly explain the inconsistent results observed in my experiments. Alternatively, the viruses themselves may be the cause.

As mentioned previously, one cause for concern regarding the recombinant viruses is whether or not mutations can accumulate during passaging of virus stocks. Although genome sequence results discussed in Chapter 5.1 indicate that there were no mutations in the Us3Δ (MJ) strain compared to the Us3Δ BAC that would result in differences in virus growth, there was obviously variability in IFN sensitivity between
Us3Δ (MJ) and the other Us3Δ clones (Appendix Figure E.1). Us3Δ viral clones MJ, SW, and 10.1 are all viruses reconstituted from the same BAC, while clone 5.11 was reconstituted from a different BAC clone. For the experiment shown in Appendix Figure E.1, Us3Δ clones 5.11 and 10.1 were freshly reconstituted from their respective BACs (protocol described in Chapter 3.6), and, excluding the amplification of the virus, the stock was not passaged. Both clones MJ and SW had been passaged multiple times since their reconstitution from the BAC. Results showed that Us3Δ clones 5.11 and 10.1 were resistant to the effects of IFN, clone SW was less sensitive than WT (SW), and clone MJ was more sensitive than WT (SW) (Appendix Figure E.1). These results point to the possibility that, due to multiple rounds of passaging, these virus clones are in fact different from each other, despite three of them originating from the same BAC.

Furthermore, Us3Δ (SW) was originally less sensitive to IFN than WT (SW) at 24hpi, (Appendix Figure E.1) but later, after being further passaged, showed hypersensitivity to IFN (Figure 4.11, replicate A). In replicate B, the even later passage of Us3Δ (SW) is again less sensitive to IFN than WT (SW) at 24hpi. Others have observed that passaging viruses can introduce changes to genome sequence, as early as one passage later [Finnen and Banfield, unpublished, 229]. Therefore I do not rule out the possibility that mutations in the virus can occur when they are being passaged, affecting their ability to counteract cellular antiviral defence. These mutations may occur in repeated regions of the genome, which are difficult to sequence with precision and may be why they were not detected during genome sequence analysis.

Interestingly, Us3Δ clones 5.11 and 10.1 show resistance to IFN, with only a fivefold growth inhibition in IFN treated cells (Appendix Figure E.1). This was surprising
since not only is Us3 important for counteracting antiviral defence, but the opposite phenotypes observed in different Us3Δ clones means that the differences between those clones completely alter the effect of IFN on infected cells. The hypothesis that Us3Δ mutants would be more sensitive to IFN than WT virus was based on observations that Us3 plays a role in counteracting antiviral defence, through disrupting PML-NBs [12] and inhibiting IFNβ production by hyperphosphorylating interferon response factor 3 (IRF3) [183, 230]. HSV-1 Us3Δ mutants were also shown to be hypersensitive to IFN [161]. A reason why Us3Δ clones would instead be resistant to IFN may be related to the role of Us3 in inhibiting NFκB signaling [231]. Viral infection activates the NFκB signaling pathway, which then activates pro-inflammatory cytokines to counteract the infection. However, although the NFκB pathway is an antiviral response, Wei et al showed that NFκB knockout cells actually exhibited higher activation levels of several IFN stimulated genes (ISGs) than in cells that expressed NFκB [232]. Therefore it may be possible that by inhibiting NFκB signaling, Us3 could be enhancing the IFN response instead. In the absence of Us3, ISGs would be less activated and the IFN response diminished, resulting in improved virus growth relative to the WT strain. HSV contains multiple proteins that regulate the NFκB pathway, some that activate and others that inhibit NFκB signaling. Therefore the balance between the activities of all those viral proteins determines the end result of NFκB signaling. The tipping of this balance may explain why some Us3Δ clones are hypersensitive to IFN, while others were resistant.

The next goal is to determine whether passaging Us3Δ and ICP0Δ recombinant viruses introduces secondary mutations that can affect virus phenotype. To test the stability of passaging the recombinant viruses, replicates of the IFN assay can be
performed using viruses from the same passage. If viruses from one passage display results which are consistently different from viruses of a different passage, it would confirm that changes to viral sequence can occur when passaging the virus, and that this affects the virus’s ability to counteract the effects of IFN. Currently, cells are infected at a low MOI of 0.01 for the creation of new stocks. Passaging the virus at a low MOI reduces the chance for accumulation of mutations, however it can also increase selective pressure. Therefore adjusting the MOI for passaging viruses may prevent unwanted mutations. Genome sequencing could be used to identify current mutations and provide information regarding other proteins that affect the properties of Us3Δ and ICP0Δ viruses. Genome sequencing could also help determine the cause of the different phenotypes observed between the Us3Δ viral clones. Currently, only the Us3Δ (MJ) clone has been sequenced. The ability of a virus to counteract cellular antiviral defence is dependent upon a complex network of proteins. Identifying secondary mutations in ICP0Δ and Us3Δ viruses will provide more insight into how each of these viral proteins contribute to counteracting cellular antiviral defence.
Chapter 6

Summary and Future Directions

Following HSV-2 infection, PML-NBs assemble around the viral genome in the nucleus and silence viral gene transcription through epigenetic regulation. This cellular antiviral defence mechanism is counteracted by multiple viral proteins, including ICP0 and Us3. Both these proteins are known to disrupt PML-NBs, and counteract the antiviral effects of IFN. In order to determine the relative contributions of ICP0 and Us3 in PML-NB disruption, recombinant viruses lacking ICP0, Us3, or both ICP0 and Us3 were constructed using \textit{en passant} mutagenesis. Experiments using different clones and passages of these recombinant viruses revealed phenotypic differences between viruses reconstituted from the same BAC (Figures 4.6, E.1). Comparing genome sequencing results of BAC and viral DNA did not expose any mutations that occurred through reconstitution of virus from the BAC. However, since only one viral clone from each strain has been sequenced to date, it will be necessary to sequence additional clones in order to determine the cause of the phenotypic differences observed between viral clones.

Growth analysis and plating efficiencies were used to characterize the ICP0Δ, Us3Δ, and ICP0Δ/Us3Δ recombinant viruses. Consistent with results seen for PRV and HSV-1 Us3Δ mutants, the HSV-2 Us3Δ mutant grew to one log lower titres than WT virus in human fibroblast cells (Figure 4.5), and formed smaller plaques than WT virus in U2OS cells (Figure 4.6). On the other hand, the HSV-2 ICP0Δ mutant did not possess a growth defect in human fibroblast cells (Figure 4.5), nor exhibit a small plaque phenotype or plating deficiency in Vero cells (Figures 4.6, 4.7). This is contrary to results
for HSV-1 ICP0Δ mutants. Because previous studies showed that HSV-1 ICP0Δ growth and plating defects were dependent on MOI, we hypothesize that alternative viral proteins are able to complement the lack of ICP0 when cells are infected above a certain MOI threshold, and that this threshold is lower for HSV-2 ICP0Δ mutants than for HSV-1 ICP0Δ mutants.

Since ICP0 and Us3 have both been shown to disrupt PML-NBs, we measured the amount of PML-NB disruption in cells infected with each of the recombinant virus strains. We found that in the absence of Us3, ICP0 and other viral proteins were sufficient for PML-NB disruption, however Us3 was not able to completely complement the activity of ICP0 (Figures 4.8, 4.9). My results confirmed that Us3 is important for PML-NB disruption, but it is still unclear how exactly this relates to the role of ICP0. The next goal is to determine whether or not ICP0 and Us3 target distinct isoforms of PML. Different PML isoforms are responsible for recruiting different proteins to PML-NBs, therefore identifying specific targets of ICP0 and Us3 will provide more details regarding their role in counteracting antiviral defence.

PML is a mediator of the IFN antiviral response, therefore it was predicted that by disrupting PML-NBs, ICP0 and Us3 counteract the effects of IFN. In line with this, HSV ICP0Δ and Us3Δ mutants have been shown to be hypersensitive to the effects of IFN. My studies revealed the complications involved in determining the effects of ICP0 and Us3 on the IFN pathway. I observed that some Us3Δ clones were hypersensitive to IFN while others were resistant, and that the level of sensitivity to IFN varied between experiments. Us3 affects more than one cellular pathway, and those cellular pathways are affected by more than one viral protein. I concluded that the activities of multiple viral proteins

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create a fine balance between activating cellular pathways to promote virus replication, and inhibiting cellular antiviral defence. Future aims are to determine the genomic differences between viral clones in order to elucidate the factors that tip this balance. Identifying the causes of the phenotypic differences between viral clones will also give more information regarding the specific function of ICP0 and Us3, and how this influences the overall ability of a virus to counteract cellular antiviral defence.
Appendix A

*En Passant* Mutagenesis

![Diagram of En Passant Mutagenesis](image)
Figure A.1: Using *en passant* mutagenesis to construct recombinant viruses. I) The black circle represents the original sequence at the site of the desired mutation. II) A PCR product containing a kanamycin selectable marker (sm), a unique restriction site called I-SceI, repeated sequences on either side of the marker that are homologous to the area of the mutation (a, b, c, d), and the desired mutation itself (grey circle) was transformed into *E. Coli* containing the HSV-2 186 BAC. Homologous recombination between BAC sequences and the PCR product results in its insertion into the BAC at the desired location. III) Kanamycin resistance was used to screen for clones that had undergone recombination. IV) L-arabinose-mediated induction of I-SceI then cleaves the BAC at the unique I-SceI restriction site. This allows a second recombination to occur between the repeated sequences on either side of the kanamycin resistance gene, which excises the kanamycin selectable marker. V) Clones were screened for loss of kanamycin resistance. These clones contained the BAC that has the desired mutation with no other changes, resulting in a markerless mutation. This process was repeated twice to mutate both copies of ICP0 in the HSV-2 genome, and twice more to repair both copies of ICP0.
Figure A.2: Plasmid pEP Kan S2 was used as a PCR template for *en passant* mutagenesis.

pEP Kan S2 contains a kanamycin resistance gene flanked by I-SceI restriction sites. One of these I-SceI restriction sites was incorporated into the homologous recombination PCR product for *en passant* mutagenesis.
Appendix B

Primer Sequences

Table B.1: *En passant* mutagenesis primers. These primers were used to introduce stop codons (bold) three nucleotides after the ICP0 start codons, or to repair the stop codons back to their original sequences (bold and italicized). The lower case letters indicate HSV-2 sequences upstream or downstream of the ICP0 or Us3 start codon (red) while uppercase letters indicate sequences homologous to the pEP Kan S2 template.

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP0Δ Forward Primer</td>
<td><code>5’agagagaccgcgggctcgtgatctgacgctacctacccggtcctcgggaagactgacgagccc</code></td>
</tr>
<tr>
<td>ICP0Δ Reverse Primer</td>
<td><code>5’ggggccggggtcggccgggatctgacgagctttgtcatcaacctcgggnt</code></td>
</tr>
<tr>
<td>ICP0Δ Repair Forward Primer</td>
<td><code>5’agagagaccgcgggctcgtgatctgacgctacctacccggtcctcgggaagactgacgagccc</code></td>
</tr>
<tr>
<td>ICP0Δ Repair Reverse Primer</td>
<td><code>5’ggggccggggtcggccgggatctgacgagctttgtcatcaacctcgggnt</code></td>
</tr>
<tr>
<td>Us3Δ Repair Forward Primer</td>
<td><code>5’ccggtgcgctcggggtgctcgttggttgcggaacgagcgcggaacgagcgcggaatgcgttgg</code></td>
</tr>
<tr>
<td>Us3Δ Repair Reverse Primer</td>
<td><code>5’ctttggtgcgctcggggtgctcgttggttgcggaacgagcgcggaacgagcgcggaatgcgttgg</code></td>
</tr>
</tbody>
</table>

Table B.2: *En passant* sequencing primers. Amplification primers were used to amplify a PCR product spanning the recombination site. Sequencing primers were used to sequence the PCR product to confirm the presence of the mutation. For Us3, the same primers were used for amplification and sequencing.

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>ICP0 Amplification Forward Primer</td>
<td><code>5’TGATCGGGCCCTATTTGCTCC</code></td>
</tr>
<tr>
<td>ICP0 Amplification Reverse Primer</td>
<td><code>5’CACTATCGGTCACCGGCACC</code></td>
</tr>
<tr>
<td>ICP0 Sequencing Forward Primer</td>
<td><code>5’CCCGGAGAGACAGAGAAGACTAAAACC</code></td>
</tr>
<tr>
<td>ICP0 Sequencing Reverse Primer</td>
<td><code>5’CCCGGAGAGACAGAGAAGACTAAAACC</code></td>
</tr>
<tr>
<td>Us3 Amplification/Sequencing Forward Primer</td>
<td><code>5’TCTGACGTTCGCGATATAGGG</code></td>
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<tr>
<td>Us3 Amplification/Sequencing Reverse Primer</td>
<td><code>5’GGGGGTATAAAAAGGTGCTCG</code></td>
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</table>
Appendix C

Mutating One Copy of ICP0 in the BAC

Figure C.1: Mutating one copy of ICP0 in the BAC results in mixed peak signals when sequencing BAC DNA. *En passant* mutagenesis was used to insert two stop codons three nucleotides after the ICP0 start codon. PCR was used to amplify BAC DNA surrounding the ICP0 start codon. When the *en passant* mutagenesis procedure is performed once, only one copy of ICP0 is mutated. However both copies are amplified in the PCR reaction, resulting in mixed peaks during sequencing (underlined in red). A combination of the wild type sequence (CCCCCGG) and the mutated sequence (TGATGA) is seen.
Appendix D

Disrupting PML-NBs

(A) Percentage of infected cells with dispersed PML-NBs over time after infection. 
(B) Percentage of infected cells with dispersed PML-NBs over time after infection with different strains.
Figure D.1: Cells infected with ICP0Δ or ICP0Δ/Us3Δ virus strains exhibit a delay in the disruption of PML-NBs. A. Human fibroblast T12 cells were infected with wild type (WT), Us3Δ, or Us3ΔR virus strains at low MOI (less than 1). At 2, 3, 4, and 5hpi, cells were fixed with 4% formaldehyde, permeabilized in 0.1% TX-100, and stained with antibodies against PML and ICP27. 40 infected cells from each sample (identified by positive ICP27 staining) were scored as having punctate or diffuse PML-NBs, and the percentage of infected cells with diffuse PML-NBs was graphed as a function of time. There was no difference observed between WT and Us3Δ virus strains in terms of PML-NB disruption. B. Human fibroblast T12 cells were infected with WT, Us3Δ, ICP0Δ, or ICP0Δ/Us3Δ virus strains at low MOI (less than 1). At 2, 3, 4, 6, and 8hpi, cells were fixed with 4% formaldehyde, permeabilized in 0.1% TX-100, and stained with antibodies against PML and ICP27. 40 infected cells from each sample (identified by positive ICP27 staining) were scored as having punctate or diffuse PML-NBs, and the percentage of infected cells with diffuse PML-NBs was graphed as a function of time. There was no difference observed between WT and Us3Δ virus strains in terms of PML-NB disruption. The ICP0Δ strain exhibited a delay in PML-NB disruption at 4hpi, but reached WT levels by 6hpi. The ICP0Δ/Us3Δ strain showed an even larger delay at 4hpi but also reached WT levels by 6hpi. Each time point is an average (±SD) from 1-3 replicates. C. Human fibroblast T12 cells were infected with WT, ICP0Δ, ICP0ΔR, ICP0Δ/Us3Δ, or ICP0ΔR/Us3Δ virus strains at a low MOI (less than 1). At 2, 3, 4, 6, and 8hpi, cells were fixed with 4% formaldehyde, permeabilized in 0.1% TX-100, and stained with antibodies against PML and ICP27. The numbers of PML nuclear bodies (PML-NBs) in 40 infected cells (identified by positive ICP27 staining) were counted and those with less than 5 PML-NBs per cell were defined as having “dispersed PML-NBs”. The percentage of cells with dispersed PML-NBs was graphed over time. At 3hpi, the percentage of cells with dispersed PML-NBs was lower in cells infected with ICP0Δ or ICP0Δ/Us3Δ virus than in cells infected with WT, Us3Δ, or any of the repair viruses. An additive effect was seen in the ICP0Δ/Us3Δ double mutant. This delay in the disruption of PML-NBs was still observed at 4hpi, though by 6hpi even cells infected with ICP0Δ or ICP0Δ/Us3Δ virus showed almost complete disruption of PML-NBs. Repairing ICP0 restored the WT phenotype.
Appendix E

Sensitivity of ICP0Δ and Us3Δ Viruses to IFN

Figure E.1: Some Us3Δ virus isolates are hypersensitive to interferon while others are insensitive. T12 cells were pre-treated with Universal Type I Interferon (IFN) or a carrier control at 18 hours prior to infection, then infected with wild type clone SW (WT (SW)), Us3Δ clones SW, MJ, 5.11, or 10.1, Us3ΔR clones SW or MJ, ICP0Δ, or ICP0Δ/Us3Δ virus at an MOI of 0.1. At 24hpi, infected cells were collected and titred on U2OS cells. Fold inhibition was calculated as virus titres in carrier treated cells divided by virus titres in IFN treated cells. Us3Δ (SW) was less sensitive to IFN than WT while Us3Δ (MJ) was more sensitive than WT. Us3Δ clones 5.11 and 10.1 were only marginally sensitive to IFN. No difference can be seen between WT and the ICP0Δ and ICP0Δ/Us3Δ viruses. Averages from 2 experiments (±SD) are shown.


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