Herpes Simplex Virus Type 1:
Interactions With the Cellular Proteasome and MicroRNA Pathways

by

Daniel J. Munson

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Abstract

Herpes Simplex Virus Type 1 (HSV-1) is a human pathogen that is estimated to have infected 60-80% of the population worldwide, causing oral lesions, encephalitis, and blindness. Infection results in life-long latency of the virus, with periods of reactivation and symptomatic disease. Early in infection, HSV-1 induces the formation of replication compartments and VICE (virus induced chaperone enriched) domains within the nucleus. Adjacent to VICE domains, we observed foci which contained Mss1, a cellular protein normally associated with the proteasome. Mss1 belongs to the AAA-ATPase family of proteins, which have been shown to function in DNA repair, transcription, translation, and the stress response. In ECP19 cells, which are naturally reduced in Mss1 protein and mRNA levels, HSV-1 does not replicate. Based on Mss1’s accessory functions and observed localization, we sought to determine if Mss1 played a role in HSV-1 infection. Addition of Mss1 to non-permissive ECP19 cells which have endogenously lower levels of Mss1 did not alter HSV-1 replication. Furthermore, siRNA knockdown of Mss1 in normally permissive cells did not alter HSV-1 replication. These results indicated that Mss1 was likely a dispensable accessory factor incorporated to VICE domains during HSV-1 replication.

Interferon gamma induces the expression of a number of genes. Among them are three alternate subunits of the 20S core of the proteasome, β1i, β2i, and β5i. Expression and incorporation of these subunits alters the functionality of
the proteasome, thereby creating the immunoproteasome. While it is known that proteasomal function is required for HSV-1 replication, the functional capacity of immunoproteasomes during HSV-1 infection is not known. We found that immunoproteasomes persist throughout infection and that the activity of immunoproteasomes purified from infected cells is higher than that of uninfected controls.

MicroRNAs (miRNAs) are small ~20 nt regulatory RNAs that alter gene expression by binding to target mRNAs, causing translational repression. Viruses have evolved the use of miRNAs which regulate cellular and/or viral gene expression. HSV-1 miRNAs have exhibited functionality during latency, but little is known about viral miRNA function during lytic infection. We evaluated the production of viral miRNAs from HSV-1 infected cells from 2-24 hours post infection. We found 19 novel RNA species, 6 of which formed predicted stem-loop structures indicative of microRNAs. Using RT-PCR and stem-loop PCR we were able to determine the kinetic profile and mature 5’ and 3’ ends of the novel HSV-1 miRNA miR-92944. We identified the precursor structures using Northern blotting. Mutational studies revealed a 3-4 log decrease in viral titer in both single and multi-step growth analyses, as well as a significant reduction in plaque size. Through these studies we discovered a novel HSV-1 miRNA produced during latency that appears to control a global regulation pathway for HSV-1 replication.
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CHAPTER I

The Herpesviridae – General Overview with Emphasis on Herpes Simplex Type 1
For the DNA viruses, the family Herpesviridae is perhaps the most widely studied due to the large number of human and veterinary pathogens contained within. Members of the Herpesviridae have large double-stranded DNA genomes (100 kb or more), containing 80-100 genes, encased in an icosahedral shell. Replication of the genome occurs in the nucleus of host cells and, in some Herpesviridae members, the viral genome can be maintained in a latent state where very few (if any) proteins are produced. Reactivation from the latent state is driven by a number of factors, which vary depending on the virus in question and cell type, but include signals like cellular and organismal stresses, cell cycle progression, immune pressure, and viral microRNAs (106, 195, 199).

The Herpesviridae are divided into three sub-families, the alpha, beta, and gamma herpesviruses. The alphaherpesviruses are generally considered to be the most important group in a veterinary setting, with pseudorabies virus being an extremely important viral infection in pigs, Marek’s disease virus in chickens, and bovine herpesvirus 1 a problem in cattle. However, there are significant human pathogens as well, including herpes simplex virus types 1 and 2 and varicella zoster virus (the causative agent of chicken pox and shingles). Lastly, the alphaherpesviruses tend to establish latency in neurons. Several human pathogens are categorized as Betaherpesviruses, including cytomegalovirus and human herpesviruses 6 and 7, which cause roseola in children and typically replicate slower than the alphaherpesviruses. Additionally, Betaherpesviruses establish latency in leukocytes. The final sub-family is the gammaherpesviruses, which encompasses the important human pathogens Kaposi’s sarcoma-
associated virus and Epstein-Barr virus as well as the extensively studied mouse gammaherpesvirus 68. Gammaherpesviruses are typified by their ability to establish latency in lymphocytes (typically B cells) and transform cells causing cancer (106).

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are responsible for a large number of human cases and can establish latent infections in neuronal ganglia. Initial HSV-1 infection occurs in the oropharynx, with fever and ulceration of the buccal and gingival mucosa lasting 2-3 weeks being the primary symptoms (199). The virus establishes a life-long latent infection in the trigeminal ganglia by traveling in a retrograde fashion via sensory neurons from the initial site of infection. Recurrent infections can occur following periods of physical and/or emotional stress, illness, UV light exposure, and/or tissue injury (199). Recurrent infections are localized to the orolabial surfaces and are preceded by a brief period of tingling and pain followed by the appearance of 3-6 vesicular lesions. The lesions become ulcerated and then crust over within 96 h and completely heal between 8-10 days after appearance of the initial pustules (199). HSV-1 infections are typically non-fatal, although more serious complications can arise. Approximately 300,000 cases of herpetic keratoconjunctivitis are diagnosed each year in the US and HSV-1 is the leading cause of viral blindness in the world (198). In immunocompromised individuals and neonates, HSV-1-induced encephalitis is fatal in 70% of untreated cases and can cause long-lasting neurologic defects in survivors (57, 169, 198).
Transmission of HSV-1 occurs via transfer of infected salivary secretions, most often during recurrent infections, however viral shedding persists up to 2 weeks after the disappearance of orolabial lesions (198). HSV-2 infections occur in the genital regions causing lesions, with latency being established in the sacral ganglia. Transmission of HSV-2 occurs during sexual contact and involves the exchange of infected mucosal secretions. Reactivation of HSV-2 is stimulated by similar triggers to those described for HSV-1. Although the two viruses differ with respect to their infection sites, HSV-1 can infect the genital region and vice versa for HSV-2 (106). Studies in the U.S. indicate that by early adolescence, the seroprevalence of HSV-1 is about 60% (205), with worldwide seroprevalence approaching 80% by adolescence (199). Currently, no cure for HSV-1 (or HSV-2) infection is available. However, therapies exist which suppress latent reactivation using guanine analogs that are converted into viral DNA polymerase inhibitors by the viral thymidine kinase (55).

**Herpes simplex virion structure**

The mature HSV-1 enveloped virion is approximately 150 nm in diameter, composed of an electron dense 152 kb dsDNA genome surrounded by an icosahedral nucleocapsid core ($T = 16$ symmetry), which is further encased in a layer of tegumentary proteins and a host-derived lipid envelope. HSV-1 has a linear genome organized with two “unique” sections each flanked by inverted tandem repeats. The two unique regions are differentiated by their relative
lengths, being termed the unique long (UL) and unique short (US) (172). The nucleocapsid is composed of 162 total capsomers of three distinct types: 150 UL19 hexons, which form the capsid faces and edges, 11 UL19 pentons found at 11 of the 12 vertices, and a portal formed at the 12th vertex composed of 12 UL6 proteins. The portal is a cylindrical structure with a central channel allowing for DNA to be packaged and extruded, with each UL6 monomer containing a leucine zipper allowing for adhesion to the other (24, 143, 147, 194). The capsomers are connected by triplex proteins, an essential complex of the UL18 and UL38 gene products (152). Surrounding the nucleocapsid is the tegument, an amorphous layer of multifunctional proteins, whose specific roles will be discussed in subsequent sections. Acquired from the host during virion egress, the lipid bilayer envelope is studded with 11 (possibly 12) different viral proteins involved in mediating the attachment and eventual entry into the host cell. The HSV-1 life cycle is depicted in Fig. 1-1 and is described in detail in the subsequent sections.

**HSV-1 attachment and entry**

Upon encountering a susceptible cell, attachment is mediated by the viral surface glycoproteins B (gB), gC, gD, gE/gl and the gH/gL heterodimer. The HSV-1 envelope glycoproteins gB and gC bind to heparin sulfate receptors on the cell surface and interact independently with heparin sulfate proteoglycans to promote initial attachment (91, 113). These glycoprotein-heparin sulfate interactions, although important, are not absolutely essential for viral entry, at
**Figure 1-1**: Replication cycle of Herpes simplex virus type 1. Mature virus particles initially bind to cell surface receptors via interaction with various glycoproteins embedded in the virion envelope. Attachment triggers membrane fusion and release of the nucleocapsid and tegument proteins to the cytosol. Proteins found in the tegument function to prime the cell for infection by degrading host mRNA, inhibiting host induced blockage of translation, and provide transactivation signals for viral gene transcription. Tegument proteins which remain associated with the capsid shuttle nucleocapsids along microtubules to the nuclear pore, where the viral DNA is injected into the nucleus. Once within the nucleus, viral transcription occurs in a sequential fashion, with immediate early (IE) gene transcription occurring first, accompanied by disruption of ND10 domains and chromatin marginalization, and the formation of VICE domains and replication compartments. Early (E) gene transcription follows and is induced by IE gene products, and the E products initiate viral DNA synthesis. Late genes code for most of the tegument and structural proteins and initiate the formation of progeny virions, which traffic out of the nucleus to the Golgi for envelopment and eventual egress out of the cell.
least not in the infection of cultured cells (166). Deletion of gB or gC individually reduces, but does not abrogate, viral attachment or entry, although a tandem deletion of gB and gC severely compromises viral entry (91). The gB/C interaction is followed by an interaction of gD with a host of potential entry mediators, including herpesvirus entry mediator, nectin-1 and -2, a TNFα-member receptor, and immunoglobulins related to the poliovirus receptor, allowing for entry into a wide variety of cell types (170, 184, 192). gD interacts next with gB and the gH/gL heterodimer to fuse the envelope to the cell membrane allowing for transfer of the tegument proteins and nucleocapsid core to the cytosol (184). gE and gl are important during the spread of the virus within epithelial and neuronal tissues, and gC provides an accessory function by binding to C3b, inhibiting complement neutralization of the virus (64, 127).

**Migration to the Nucleus**

Once the nucleocapsid and tegument proteins have been released to the cytosol, the viral genome must be transported to the nucleus in order for replication and transcription to occur. The majority of the 23 tegument proteins dissociate into the cytosol, a process driven by phosphorylation by viral (UL13 and US3) and cellular kinases (137, 138). The viral tegument proteins UL35, 36, and 37 remain associated with, and are involved in trafficking, the capsid along the microtubule network to the nuclear pore complex via association with the motor proteins dynein and dynactin (4, 46-48, 203). Once at the nuclear pore,
current data indicate that the capsid-associated UL25 protein associates with the nucleoporin CAN/Nup214 (171) and a coordinated cleavage of both the portal protein UL6 and the capsid protein UL36 is initiated, allowing for release of the viral DNA into the nucleus through the nuclear pore complex (100, 144, 151).

**Transcription Initiation and Inhibition of Cellular Processes**

Transcription of the viral genes occurs in a sequential manner, with the immediate-early (IE) genes preceding the early (E) genes, which are then followed by the late (L) genes. In general, the IE genes are found in the inverted repeat regions of the genome, the early genes in the UL, and the late genes are found in both the UL and US (172). IE gene transcription is activated by a complex containing the tegument protein UL48 and the cellular transcription factors Oct-1 and HCF-1 to produce the IE gene products infected cell protein (ICP) 0, ICP4, ICP22, ICP27, and ICP47 (102, 204). Also a tegument protein, the IE gene product ICP0 has two main functions within infected cells, blocking interferon γ (IFNγ) induced inhibition of viral gene expression and transitioning transcription from IE to E genes. ICP0’s anti-IFNγ functionality is governed by a ring-finger domain containing ubiquitin ligase activity, which targets several cellular proteins for proteasomal degradation (19, 85). The transition to E gene transcription is governed by a G/C rich C-terminal domain that dissociates histone deacetylase from the CoREST/REST repressor complex (83). The second IE gene found in the tegument is ICP4, the major transactivator of the E
and L genes along with the cellular transcription factors SP1, CTF, and USF (173). When phosphorylated, ICP4 binds to and activates E promoters initiating transcription of genes involved primarily in viral genome replication (162). Following expression, ICP27 also stimulates viral transcription by binding the viral promoters of early and late genes (177). Lastly, ICP22 recruits cellular RNA polymerase II, viral kinases, and cellular kinases in order to direct RNA polymerase II to preferentially transcribe viral genes (122).

Cellular gene transcription and translation are shut down by a number of tegument proteins and IE genes, namely UL41, US11, ICP34.5, ICP47, and ICP0. UL41 is a non-specific RNase that degrades host and viral RNAs throughout infection (109, 183, 185, 190). The rapid turnover of viral RNA may play an accessory role in immune evasion (183). The tegument protein US11 blocks IFNγ-induced inhibition of cellular protein synthesis by regulating the activity of 2'-5' oligoadenylate synthetase and protein kinase R, which promote mRNA decay and shut off protein synthesis, respectively (27, 153, 157, 176). Protein kinase R-activated host protein shutoff is further avoided by the complexing of ICP34.5 with cellular phosphatase 1α which dephosphorylates, and thus activates, cellular translation initiation factor 2 (34, 89). ICP47 plays a vital role in blocking cellular TAP (the transporter associated with antigen processing), inhibiting the loading and expression of MHC class I molecules on the cell surface for recognition by the adaptive immune system (67). Immune signaling is further compromised by ICP0-induced destruction of nuclear domain 10 structures, important IFNγ-regulated signal transduction domains in the
Figure 1-2: The HSV-1 genome. The linear 152 kb dsDNA HSV-1 genome is depicted above, with the two unique regions, $U_L$ and $U_S$, each flanked by inverted (TR signifies the terminal repeat) repeat regions (blue and green). The positions of the origins of replication, ori$L$ and ori$S$ (only one ori$S$ is shown) are indicated on pertinent regions of the genome which have been expanded (see text for gene descriptions). The immediate-early genes (black boxes) are shown and are transcribed as soon as 15 min after initial attachment. Early genes (grey boxes) are transcribed as early as 1 h after attachment and the late genes (white boxes) are transcribed around 3 h after attachment.
nucleus (123, 132, 142).

**HSV-1 Replication**

Replication of the HSV-1 genome (Fig. 1-2) is initiated by the transition to E gene transcription. HSV-1 has three origins of replication, one within the UL segment approximately at the midway point situated between the divergent UL29 and UL30 open reading frames (orfs), and two within the inverted repeats flanking the US segment between the divergent orfs of ICP4 and ICP22/47 (17). Replication initiates via binding of UL9 homodimers to an origin. UL9 encodes both a helicase and DNA binding domain which recognizes palidromic AT rich regions within the origin (16, 54, 148). UL9 helicase activity is enhanced by the binding of ICP8 (UL29) to the single-stranded regions of the unwound DNA (12). UL9 recruits the helicase-primase, formed by the gene products of UL5, UL8, and UL52, which synthesizes the primers for DNA replication and further unwinds the genome (37, 133). Replication is achieved through the action of the DNA polymerase (UL30) and UL42 which form a heterodimer (159). UL42 acts as a sliding clamp that simultaneously binds the genomic DNA and UL30, increasing processivity (45). Replication of the genome proceeds by initially using a theta mechanism and transitions into a rolling circle mode, which yields head-to-tail concatemers (17). During rolling circle replication, ICP8 and the alkaline nuclease UL12 are required for strand exchange (168), and cellular recombination proteins are also recruited to facilitate efficient replication (201,
Packaging and Egress

Concatemeric DNA is packaged into preformed capsids in a mechanism that resembles that of DNA bacteriophages (145, 198). Capsid formation likely begins in the nucleus of HSV-1-infected cells with the assembly of a dodecameric portal made of UL6 proteins (147). Capsid pentamers and hexamers formed from the major capsid protein UL19 then associate with the scaffolding protein/protease UL26/UL26.5 (198). Scaffold-bound pentamers and hexamers bind the triplex complex and initiate procapsid formation on a single portal, which is also known to bind the scaffolding protein complex (146). This intermediate is known as the procapsid. DNA packaging is achieved by a complex known as the terminase, formed by UL15, UL28, and UL33, which is assembled in the cytosol and enters the nucleus via the nuclear localization signal on UL15 (206). In the nucleus, the terminase binds to viral DNA and the procapsid portal. ATP is used to package the DNA into the procapsid, and cleavage of the concatemeric DNA occurs at repeated sequences at the viral DNA termini (16). As the DNA is packaged, the scaffolding proteins self-cleave and exit the procapsid (173). DNA-filled capsids exit from the nucleus after disruption of the nuclear lamin network. This is achieved by the tegument proteins UL31 and UL34, which recruit cellular and viral kinases to phosphorylate and destabilize the lamin network (141, 150). UL25 drives the association and
budding of the packaged capsid from the inner nuclear membrane into the intranuclear membrane space (105). Fusion and de-envelopment at the outer nuclear membrane causes the release of capsids to the cytosol; the proteins responsible for this process remain unclear (135). Movement of capsids to the Golgi complex for tegumentation and re-envelopment requires the action of UL36 and kinesin-directed positive end movement along microtubules (128). Tegumentation occurs during interaction with Golgi-derived vesicles, which also contain the membrane glycoproteins. In HSV-1, the tegument appears to concentrate at a single pole, possibly around the portal, where a number of tegument proteins associate during entry (4, 46-48, 82, 203). Capsids bud into Golgi-derived vesicles resulting in an enveloped virion within a cellular vesicle (135). Vesicles are transported to the cellular membrane where they fuse, and the mature enveloped virus is released (134).

**Additional HSV-1 Induced Cellular Changes**

As obligate intracellular pathogens, viruses often must overtake the host cell and inhibit normal cellular function in order to efficiently produce progeny for further infection and replication. HSV-1-induced changes that have been briefly introduced earlier include the use of the microtubule networks for virion movement, destruction of host mRNA by vhs, deactivation of the PKR response, activation of cellular transcription factors, blocking of TAP, and destruction of nuclear ND10 domains. Within the nucleus of HSV-1 infected cells, chromatin is
marginalized to the edges of the nucleus and the interior space filled by viral replication compartments (161). The chaperone proteins, normally involved in ensuring the proper folding of other host proteins, are localized to sites immediately adjacent to replication compartments known as VICE (virus induced chaperone enriched) domains (21, 22). VICE domains also contain the 20S core of cellular proteasomes and sit adjacent to foci of the chaperone Hsp27 (131).

The cellular proteasome

HSV-1 replication has been shown to be highly influenced by the activity of the cellular proteasome, with inhibition of proteasomal function correlating with an inhibition in viral replication (40, 43, 61, 110). The 26S proteasome is a multi-subunit protein complex whose primary function is the degradation of proteins for recycling and/or normal immune surveillance (11). However, additional functions of the proteasomal subunits outside the context of the intact complex include DNA nucleotide excision repair, transcriptional initiation and elongation, and chromatin remodeling (35, 165, 174). Structurally, the proteasome is composed of three domains; a core, base, and lid (Fig. 1-3). The core (20S core) is comprised of four axially stacked rings with 7 proteins per ring in an $\alpha_1\beta_1\gamma\beta_1\gamma\alpha_1$. $\gamma$ arrangement (11). Within the beta subunits, $\beta_1$, $\beta_2$, and $\beta_5$ contain the proteolytic sites that line the central lumen. The alpha rings form 13 Å pores which allow substrates to reach the catalytic core (191). Entry through the alpha ring is regulated by the 19S regulator, comprised of a base and lid. The base is
Figure 1-3: The 26S proteasome is a multi-functional protein complex composed of a 20S core containing the proteolytic subunits β1, β2, and β5, and a lid and base which comprise the 19S regulator. Interferon (IFN) γ induces transcription of alternate forms of the proteolytic subunits (β1i, β2i, and β5i) of the 20S core as well as PA28α/β, which form hexa- or heptameric rings that replace the 19S regulator with the 11S regulator. Proteasomes with the alternate 20S core subunits and at least one 11S regulator are known as immunoproteasomes. At any given time, a heterogeneous population of 20S, 26S, and immunoproteasomes exist within a cell.
a six-membered ring formed by distinct members of the AAA-family ATPases (Rpt1-6; Rpt1 is synonymous with Mss1), along with three accessory proteins, Rpn1, 2, and 13 (36). The remaining 11 Rpn subunits of the 19S regulator comprise the lid. Proteins destined for proteasomal degradation are typically polyubiquitinated by E2 and E3 ligases that may or may not be associated with the 26S proteasome (191). Ubiquitinated proteins are captured by the Rpn10 subunit of the lid, stripped of ubiquitin, and unfolded by the reverse chaperone activity of the base in an ATP dependent fashion (191). Unfolded proteins enter the 20S core where the proteolytic beta subunits produce peptide fragments that are between 7 and 12 amino acids in length (191).

The proteasome is also intimately tied to the innate immune response and normal homeostatic surveillance. 26S proteasomes associate with an ER transporter called the transporter associated with antigen processing or TAP (98). TAP complexes with tapasin and binds MHC molecules in order to load them with proteasome-derived peptides for expression on the cell surface (98). Although a normal homeostatic process, during times of stress or infection foreign and/or damaged proteins are degraded and expressed, eliciting an immune response. IFNγ signaling stimulates the transcription of a number of genes which modulate the proteasome compositionally and functionally. IFNγ stimulated transcription of the alternate beta subunits β1i, β2i, and β5i (Fig. 1-2), and subsequent exchange for the non-induced forms in the 20S core alters the repertoire of peptides released from the proteasome to preferentially load onto MHC class 1 (18, 65, 68, 108, 182, 196). Additionally, IFNγ stimulated
transcription of the PA28α and PA28β genes leads to the formation of an alternate regulator termed the 11S, a hexa- or heptameric ring that displaces the 19S regulator and further drives the bias of peptides released from the proteasome to MHC class 1 binding ligands (44, 74, 80). Moreover, due to the duplication of the catalytic subunits within the core, hybrid proteasomes having both IFNγ-induced and nascent 20S proteins and both a 19S and 11S regulator exist and provide dual functionality (90). Proteasomes with either one or both sets of beta subunits exchanged for the IFNγ-induced forms and containing at least one 11S regulator are termed immunoproteasomes (98).

**miRNA biogenesis and function**

In the early 1990’s, the first small RNA which could regulate gene expression was discovered in *C. elegans* (116, 200). Within these seminal works, the authors showed that binding of a 22 nucleotide (nt) RNA to the 3’ untranslated region (UTR) of *lin-14* repressed translation without reducing transcript levels. Additional experiments showed that the 22 nt functional RNA was processed from a 61 nt precursor RNA (*lin-4*) which formed a hairpin structure. Subsequently, miRNAs were found to exist in most higher eukaryotes and to regulate a number of processes, ranging from development to regulation of the immune system and apoptosis (8). miRNAs are regulatory RNAs derived from primary transcripts (pri-miRNA) normally found in intergenic regions and are produced by RNA Polymerase II (118) or RNA Polymerase III (155), but can also
Transcription

Pri-miRNA

Processing

Drosha

DGCR8

Splicing

Mirtron or other non-canonical pathway

Pre-miRNA

Nucleus

Exportin 5

Cytoplasm

Maturation and cleavage

Dicer

TRBP

AGO 1-4

AGO 2

CCR4-NOT

Endonucleolytic cleavage

Translational repression or deadenylation

Strand selection: RISC assembly

AA

AA

AA
**Figure 1-4**: The microRNA biogenesis and processing pathway. miRNAs arise from transcripts normally transcribed by Pol II or Pol III and form long imperfectly paired hairpin structures called pri-miRNAs. The nuclear RNase enzyme Drosha and the dsRNA binding protein DGCR8 recognize the pri-miRNA. Drosha cleaves the base of the stem, creating a 2 nt 3’ overhang. This cleaved hairpin is called the pre-miRNA and is transported to the cytoplasm by Exportin 5, where it is recognized by the dsRNA binding protein TRBP and acted upon by the cytoplasmic RNase Dicer. Dicer cleaves the looped end, creating the second 2 nt 3’ overhang. Following Dicer processing, the miRNA duplex is dissociated by a helicase (Gemin3 or 4, not shown) at the less thermodynamically stable 5’ end. The strand opposite the unstable 5’ end strand is loaded onto a member of the Argonaute family (AGO 1-4) of proteins. The miRNA, helicase, AGO, RNase, and other RNA binding proteins form a complex known as the RNA-induced silencing complex (RiSC). miRNA homology and the AGO variant which has been loaded determine whether cleavage (AGO2) or translational repression (any AGO) of the target mRNA(s) occurs.
arise from intron sequences called Mirtrons (2, 8, 9, 136). Following transcription, the pri-miRNA adopts a thermodynamically stable hairpin structure that contains imperfectly paired bases within the stem (Fig. 1-4). The base of the stem is cleaved by the nuclear RNase III enzyme Drosha, leaving a 5’ phosphate and 2 nucleotide (nt) 3’ overhang, thus defining one end of the mature miRNA (117). This new structure, the pre-miRNA, is shuttled to the cytoplasm by the nuclear export factor Exportin 5 (20, 208). In the cytoplasm, the pre-miRNA is acted upon by another RNase III enzyme, Dicer. Dicer cleaves the looped end of the pre-miRNA into a small interfering RNA (siRNA)-like duplex (15, 97) leaving a second 3’ 2 nt overhang, thus defining the other end of the miRNA. The less stable 5’ end is unwound by a helicase, and the strand opposite the 5’ end that was unwound is loaded on to the argonaute (AGO 1-4) protein of the RNA induced silencing complex (RISC) becoming the mature miRNA (8). The non-loaded, or star, strand is typically degraded but may have some functional relevance (87). The miRNA then directs the RISC to its target mRNA based on binding of the “seed region” (nt’s 2-8 from the 3’ end) to the target mRNA (9). Following RISC binding, the miRNA and AGO protein direct translational repression via a number of mechanisms (26, 52, 53, 63, 111, 154, 200, 211).

My thesis work has focused on several aspects of the host-pathogen interface. The focus of Chapter 3 is on the proteasomal ATPase protein Mss1. During infection, Mss1 localizes to VICE domains and interacts with viral proteins. Studies were conducted to address the role of Mss1 in infected cells. Infection of a neighboring cell can result in the formation of immunoproteasomes
prior to HSV-1 infection via the paracrine signaling of IFNγ. Chapter 4 describes the effect of HSV-1 infection on IFNγ induced production of the immunoproteasome subunits, as well as the stability of immunoproteasomes in infected cells. Chapter 5 explores the production of viral miRNAs during the lytic phase of infection, dissects the kinetics with which they are produced, and explores the effect of deletion of one miRNA on HSV-1 replication.
CHAPTER II

General Materials and Methods
Cells and Viruses. Human embryonic kidney (HEK) 293T (ATCC #CRL-11268), Vero (ATCC #CCL-81), and HeLa (ATCC CCL-2) cells were maintained in Dulbecco’s Modified Essential Medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. HSV-1 strain KOS was a kind gift from Dr. Sandy Weller and was propagated on Vero cells and frozen at -80°C as described previously (197). Virus infections were performed at the indicated multiplicity of infection (MOI) by adding a suspension of virus in a minimal volume of serum free medium to adherent cells. The virus was allowed to adsorb to the cell surface for 1 h with gentle rocking every 15 min at 37°C to evenly distribute the viral particles. Following the 1 h adsorption, the virus containing media was removed and the cells washed once with warm 1x PBS, and normal media replenished.

Western Blotting. Samples were lysed in 1x SDS PAGE loading buffer (50 mM Tris-Cl pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 100 mM DTT) and boiled before loading on to 12% SDS-PAGE gels in a BioRad Mini Protean 3 apparatus (BioRad, Hercules, CA). Proteins were then electroblotted onto PVDF membranes (Millipore, Billerica, MA), blocked at 4°C overnight in 4% Blocking Grade Blocker Non-Fat Dry Milk (Bio-Rad, Hercules, CA) in 1x TBST (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.2% Tween 20). Three 15 min washes were performed in 1x TBST before primary antibody application. Following primary antibody binding, 3x washes in 1x TBST for 15 min each were performed, followed by secondary antibody application using antibodies specific
for either mouse or rabbit heavy chain conjugated to HRP at a dilution of 1:10000 in 1x TBST (Assay Designs, Ann Arbor, MI). 3x washes for 15 min each in 1x TBST following secondary antibody binding was subsequently followed by development of the HRP signal using the ECL Western Blotting kit (Pierce, Rockford, IL.) and exposure to film.

**Software:** GraphPad Prism 5.0 and Adobe Illustrator CS4 were used for figure preparation. GraphPad Prism 5.0 was used for statistical analyses.
CHAPTER III

Mss1: A dispensable AAA-ATPase relocalized by HSV-1 during infection
Abstract

Virus-induced chaperone-enriched (VICE) domains are formed during infection with Herpes Simplex Virus Type-1 (HSV-1). These nuclear structures lie adjacent to replication compartments (RC), are detected early in infection, and are enriched for host chaperones and proteolytic machinery. Since the composition of the cellular proteasome changes in response to stress signaling or infection, we sought to identify the type(s) of proteasomes present at VICE domains to learn more about VICE domain function and proteasome activity during infection. By systematically evaluating the proteasome subunits during infection, we found that one of the six regulatory AAA+ ATPases, Mss1, localized to RCs and VICE domains. Mass spectrometry and immunoprecipitation studies revealed an association between Mss1 and the HSV-1 protein ICP6. Interestingly, Mss1 localization was not altered in infections with an ICP6-null mutant virus. To determine whether Mss1 provided an essential role during infection, we analyzed HSV-1 growth in cells depleted for Mss1 by siRNA. We detected no difference in HSV-1 yields in single or multistep growth assays conducted in cells depleted for Mss1. We also analyzed HSV-1 growth in ECP19 cells, which have reduced Mss1 protein levels and are not permissive to HSV-1 infection. We found no difference in HSV-1 yields in either single or multi-step growth assays between untreated ECP19 cells and ECP19 cells that contained vector-expressed wild type Mss1. It is possible that the Mss1 protein, which is known to have important functions when dissociated from the proteasome, is...
utilized during other infection conditions, in other cell types, or \textit{in vivo}, but it does not appear to be necessary for viral growth in standard tissue culture. Moreover, the lack of Mss1 is likely not the reason ECP19 cells fail to support HSV-1 replication.
HSV-1 replication and assembly is restricted to the infected cell nucleus, inducing destruction of the antiviral nuclear domains 10 (ND10) (132), remodeling of the nucleolus (160), reorganization of cellular splicing factors (33), and the formation of novel nuclear structures: replication compartments (RC) (161), Hsp27 foci (131), and VICE (virus-induced chaperone-enriched) domains (21, 22). VICE domains are enriched with several chaperone systems (Hsp/c70, Hsp40, and Hsp90), 20S proteasomes, ubiquitin-conjugated proteins (58), and are flanked by Hsp27 foci. VICE domains are dynamic sites of chaperone storage and active proteolysis (126) that form very early during infection (2-3 hours). Formation of VICE domains requires the activity of several HSV-1 immediate-early proteins (10, 22, 120). When VICE domains were first characterized (22), the localization of the 19S regulatory complex, which increases 20S proteasome degradation of ubiquitinated substrates, was not determined.

The 19S regulatory complex can be subdivided into two subcomplexes: lid and base (11, 92, 191). The non-ATPase proteins of the lid are essential for ubiquitin-dependent substrate acquisition. After ubiquitin-dependent capture, substrates are then unfolded and threaded into the core 20S particle, where they are cleaved by proteases within. The unfolding, or reverse chaperone activity, is likely performed by the base components of the 19S complex, which is
comprised of six conserved proteins, all members of the “AAA+” ATPase family (AAA: ATPase associated with cellular activities). Increasing evidence supports auxiliary roles for the ATPase subunits of the 19S base when they are in a free or uncomplexed state. When not associated with the 20S core particle, these ATPases are referred to as the APIS (ATPase proteins independent of the 20S proteasome) and can perform functions that are distinct from their activities when complexed with the proteasome. A seminal study demonstrating a non-proteolytic role for the APIS was performed in the yeast system, where mutants with two defective APIS proteins, Rpt4 and Rpt6, had phenotypes indicative of defects in RNA polymerase II-mediated transcription elongation that were independent of proteolysis (66). The authors also showed that antibodies against Rpt4 or Rpt6 inhibited in vitro transcription elongation, a block overcome by the addition of purified 19S complexes to this reaction. Most importantly, evidence for a direct interaction between the APIS proteins and RNA polymerase II was provided. Since the APIS is thought to possess chaperonin-like unfolding functions, the authors speculated that this component is recruited to stalled or otherwise challenged, elongation complexes for remodeling. APIS proteins have also been shown to associate with DNA repair factors, translation initiation complexes, and heat/oxidative stress-responsive promoters (66, 70, 129, 174, 186, 189). Given the important activities associated with these cellular proteins, it is not surprising that some viruses utilize the APIS during infection (164, 181).

During a systematic evaluation of the subcellular localization of APIS proteins during HSV-1 infection, we found that the human APIS subunit Mss1
localized to viral RCs and VICE domains. Mass spectrometry and immunoprecipitation studies revealed an association between Mss1 and the viral ICP6 protein. Intrigued by these findings, we conducted virus yield assays in permissive cells treated with siRNAs targeting Mss1. Surprisingly, HSV-1 yields were not affected by the absence of Mss1. Furthermore, when Mss1 was supplemented to non-permissive cells that have endogenously lower levels of Mss1, HSV-1 growth was not enhanced. These results indicate that, although Mss1 localizes to virus-specific structures and interacts with a viral protein, it is not required for efficient growth of HSV-1 in the standard tissue culture setting.

**Results**

**Subcellular localization of proteasome subunits during HSV-1 infection.**

Immunofluorescence studies revealed that one subunit (subunit 7 (S7), Mss1) of the six 19S AAA+ ATPases localizes to virus replication compartments in infected Vero cells (Fig. 3-1A, panels a-d). There was also significant signal for Mss1 in the cytoplasm of infected cells. The other five subunits had a punctate localization pattern and were predominantly localized to the cytoplasm (Fig. 3-1A, panels e-x). In uninfected cells, the 19S subunits gave a diffuse signal throughout the cell typical of that seen in Fig. 3-1B, panel a. Signal for the Mss1 protein colocalized with Hsc70, a known component of and marker for VICE domains (Fig. 3-1B, panels d-f). We went on to conduct Western Blot analysis
Figure 3-1. Localization of the AAA+ ATPase subunits in HSV-1-infected cells. 

A. Immunofluorescence analysis of the proteasome subunits (Mss1/S7, S4, S6b, S10b, S6a, and S8) in HSV-1 infected cells. Shown are staining profiles for DNA using 4'-6-diamidino-2-phenylindole (DAPI) (blue in panels and the merged images), the cellular AAA+ ATPase subunits (red in panels and the merged images) and the viral ICP4 protein (green in panels and the merged images). Composite images showing the three signals merged are shown in rightmost panels. Images were collected at 63x magnification. 

B. Immunofluorescence analysis of the Mss1/S7 subunit (green) and Hsc70, a marker for VICE domains (red), in uninfected (panels a-c) or HSV-1-infected (panels d-f) cells. Composite images showing the three signals merged are shown in panels c and f. Images were collected at 100x magnification.
Figure 3-2: Mss1 is a long-lived protein that is not degraded during HSV-1 infection. **A.** Western blot analysis of the viral protein ICP4, Mss1, and actin collected from uninfected (Mock) and HSV-1-infected cells between 2 and 6 hours post infection. **B.** Western blot analysis of Mss1 and L26 in untreated (UT) or Cycloheximde (CHX)-treated 293T cells. Densitometric quantification of Mss1 protein levels relative to L26 is shown in graph format above the image.
on lysates collected during infection in order to determine whether Mss1 was targeted for degradation. The levels of Mss1 were relatively constant indicating that it is not likely a target for degradation at VICE domains during this time (Fig. 3-2A). The viral protein ICP4 was used as a marker for infection (Fig. 3-2A). We observed no evidence of ubiquitinated forms of Mss1 (data not shown). These results indicate that the proteasomal ATPase Mss1 is localized to virus structures during infection and the 19S regulatory subunit may be uncoupled during infection.

**Mss1 association with ICP6 and localization in ICP6 mutant infections.**

Mass spectrometric analysis of material which immunoprecipitated with Mss1 in HSV-1 infected cells revealed that ICP6, the large subunit of the viral ribonucleotide reductase, co-purified with Mss1 (data not shown). This result was confirmed by Western blot analysis of immunoprecipitated material derived from infected cells (Fig. 3-3A). Because ICP6 can bind nucleic acid, we wanted to determine whether DNA or RNA mediated the association between ICP6 and Mss1 by treating the immunoprecipitates with either DNase or RNase. RNase treatment did not impair the ability of ICP6 to co-purify with Mss1 (Fig. 3-3A), indicating that the association was not indirect via RNA. DNase treatment resulted in a slight decrease in the amount of ICP6 detected in Mss1 immunoprecipitates (Fig. 3-3B). We then tested whether Mss1 localization was altered in cells infected with an ICP6 mutant virus (ΔICP6) (72). Growth of the ΔICP6 mutant can be complemented on the d14 cell line, whereas a functional copy of ICP6 is not provided during infection of Vero cells. Mss1 and Hsc70
Figure 3-3: Mss1 association with ICP6 and localization in ΔICP6-infected cells.

A. Mss1 Western blot analysis of immunoprecipitates recovered from uninfected (M) or infected cell (HSV-1) lysates with an anti-Mss1 antibody (upper panel) and ICP6 Western blot analysis of immunoprecipitates recovered by using an anti-Mss1 antibody (lower panel) before or after treatment with RNase. B. ICP6 Western blot analysis of immunoprecipitates recovered from uninfected (M) or infected cell (HSV-1) lysates with an anti-Mss1 antibody before or after DNase treatment. Dots are used to indicate ICP6 species recovered in the precipitation. Positions of molecular weight markers are indicated on the left in kDa. Darker bands that migrate near the 50 kDa molecular mass marker in the lower panel of A and panel B are presumed to be antibody heavy chain. C. Representative images showing localization of Mss1 (green) and Hsc70 (red) in ΔICP6-infected D14 (a-c) and Vero (d-f) cells. Composite images showing the two signals merged are shown in panels c and f. White ovals indicate the outline of the nucleus in images d-f. Arrows indicate Mss1 localization at VICE domains as shown by co-localization with Hsc70. Images were collected at 100x magnification.
localization was analyzed after ΔICP6 infection in d14 (Fig. 3-3C, panels a-c) or Vero cells (Fig. 3-3C, panels d-f). As expected, on the d14 cell line, replication compartments and VICE domains were observed. Mss1 signal was detected within replication compartments and at some VICE domains (Fig. 3-3C, panel A and Fig. 3-1) as observed with other experiments. The localization of Mss1 was not significantly changed by the absence of a functional ICP6 protein (Fig. 3-3C, panel d), indicating that the localization of Mss1 to virus-specific nuclear structures (RCs and VICE domains) is likely not dependent upon the activity or presence of the viral ICP6 protein.

**siRNA knockdown of Mss1 and HSV-1 infection.**

To determine whether Mss1 was necessary and sufficient for robust infection *in vitro*, we analyzed virus yields in 293T cells treated with siRNAs targeting Mss1. Mss1 protein levels were significantly reduced by 24 h post-siRNA transfection and continued to decrease over the next 24 h during infection (Fig. 3-4A and B). The Mss1 protein has a relatively long half-life of approximately 16 h (Fig. 3-2B), which may help to explain our inability to achieve complete knockdown. Single step growth analysis conducted in Mss1-reduced cells revealed that depleting Mss1 protein levels did not alter virus yields (Fig. 3-4C). To determine whether Mss1 was needed for several rounds of virus infection, a multi-step growth analysis was conducted in cells treated with siRNAs targeting Mss1. We found no difference in the amount of virus produced from untreated cells compared to cells that had received Mss1 siRNA (Fig. 3-4F). The levels of knockdown achieved in this experiment are shown in Fig. 3-4D and E.
**Figure 3-4:** Knockdown of Mss1 in normally permissive cells does not affect HSV-1 replication in a single step (MOI=3, **A-C**) or multi-step (MOI=0.01, **D-F**) analysis. **A.** Representative Western blot of Mss1 and L26 in untreated (UT) or siRNA-treated (siPSMC2) 293T cells at 24 hours post-transfection (0) and 8 or 24 hours post-infection. **B.** Densitometric quantification of Mss1 levels from siRNA-treated cells compared to untreated cells from three separate experiments. **C.** Single-step growth analysis of HSV-1 in siRNA-treated (gray squares) or untreated (black circles) 293T cells (n=3). **D.** Representative Western blot of Mss1 and L26 or siRNA-treated (siPSMC2) 293T cells at 24, 48, 72, or 96 h post-infection. **E.** Densitometric quantification of Mss1 levels from siRNA-treated cells compared to untreated cells from three separate experiments. **F.** Multi-step growth analysis of HSV-1 in siRNA-treated (gray squares) or untreated (black circles) 293T cells (n=3). Error bars for all panels represent the 95% confidence intervals.
A 50-80% reduction in Mss1 protein levels was observed at various time points post infection (Fig. 3-4E). We cannot dismiss the possibility that only minimal amounts of Mss1 are needed for robust replication. However, we can surmise that wild type levels of Mss1 are not required for multiple rounds of virus infection.

Proteasome subunit transcript levels and HSV-1 infection in ECP19 cells with and without exogenous Mss1.

Previous reports describing ECP19 cell biology have shown that these cells contain less Mss1 protein (181) and do not support HSV-1 replication (13). Given these features, this cell line was used to investigate the possible requirement of Mss1 during HSV-1 infection by using “add-back” experiments to explore whether additional Mss1 can enhance virus infection. We compared the Mss1 transcript and protein levels in ECP19 cells to other standard cell lines using quantitative RT-PCR (qRT-PCR) and Western blot, respectively, and confirmed the inability of ECP19 cells to support HSV-1 replication (Fig. 3-5).

Compared to the other five AAA+ ATPase subunits of the proteasome, the relative levels of Mss1 transcripts (PSMC2) were significantly reduced (Fig. 3-5A, black bars) in ECP19 cells. qRT-PCR analysis of two other murine cell lines (A9 and L929, grey and white bars respectively) revealed similar levels all six AAA+ ATPase subunits. Comparing between cell lines, the ECP19 cells contain fewer transcripts overall of each of the six subunits but strikingly lower amounts of PSMC2. Protein levels of Mss1, as judged by western blot analysis, in ECP19 cells were reduced compared to several other common laboratory cell lines (Fig.
**Figure 3-5**: ECP19 cells exhibit lower levels of Mss1 transcript and protein and do not support HSV-1 replication.  

**A.** Quantitative RT-PCR of the six AAA+ ATPase subunit transcripts from ECP19 (black), L929 (white) and A9 (gray) cells.  

**B.** Densitometric quantification of the relative levels of Mss1 from several cell lines compared from three separate experiments.  

**C.** Single-step growth analysis of HSV-1 in ECP19 (black box), L929 (dashed line), A9 (grey triangle), 293T (black circle) and Vero (gray diamond) cells. Error bars for all panels represent the 95% confidence intervals.
3-5B). Furthermore, the ECP19 cell line fails to support the replication of HSV-1 when compared to the same common cell lines (Fig. 3-5C). These studies confirm previous findings by others and indicate that the ECP19 cell line is a useful tool to measure how the expression of exogenous Mss1 protein may alter HSV-1 replication.

Since Mss1 is reduced in ECP19 cells, we hypothesized that addition of exogenous Mss1 may rescue HSV-1 replication. To test this hypothesis, we transiently transfected ECP19 cells with a plasmid containing wild type Mss1 or an AAA-ATPase mutant, each tagged with a V5 epitope. The mutant Mss1 protein contained a lysine to alanine mutation in the ATPase active site at position 222 (K222A). Western blot analysis confirmed the expression of V5-tagged Mss1 after transfection (Fig. 3-6A). In parallel, a plasmid containing GFP was used to determine transfection efficiency, which approached 85% as judged by GFP fluorescence (data not shown). Despite the presence of wild type or mutant Mss1 in the ECP19 cells, we did not observe any changes in HSV-1 yields either in single- (Fig. 3-6B) or multi-step (Fig. 3-6C) growth analyses. Functional epitope-tagged proteasome subunits have been previously used to study proteasome localization and activity (56). Although it is possible that the V5 epitope impairs the function of the wild type Mss1 protein, the epitope sequence is not in a region that would be expected to alter the ATPase activity or interrupt the association between Mss1 and the other AAA+ ATPases. Furthermore, if the V5-tagged Mss1 proteins were defective, due to their ability to oligomerize and act in a dominant-negative fashion, it would be expected that
**Figure 3-6:** Exogenous supplementation of Mss1 to ECP19 cells does not rescue HSV-1 replication. **A.** Western blot analysis of Mss1, the V5 epitope, and L26. Samples were collected from 293T cells (293T), normal ECP19 cells (ECP19), ECP19 cells containing a GFP vector (GFP), and vectors expressing wild type (WT) or mutant (K222A) Mss1. The ribosomal protein L26 is used as a loading control. **B.** Multi-step growth analysis of HSV-1 in 293T cells (dark grey inverted diamond), normal ECP19 cells (black diamond), or ECP19 containing a GFP vector (grey diamond) or vectors expressing wild type (WT, grey circle) or mutant (K222A, grey square) Mss1. Time is given in hours post-infection (MOI=0.01). **C.** Single-step growth analysis of HSV-1 in 293T cells (dark grey inverted diamond), normal ECP19 cells (black diamond), or ECP19 containing a GFP vector (grey diamond) or vectors expressing wild type (WT, grey circle) or mutant (K222A, grey square) Mss1. Time is given in hours post-infection (MOI=3). Error bars for all panels represent the 95% confidence intervals.
their expression would be toxic to cells over time. We never observed such toxicity, leading us to conclude that the presence of excess Mss1 does not enhance HSV-1 infection in ECP19 cells and the lack of Mss1 in the ECP19 cell line cannot explain the inability of these cells to support HSV-1 replication.

**Discussion**

We have shown that, unlike other proteasome regulatory AAA+ ATPases, the proteasome AAA+ ATPase Mss1 has a distinct localization during virus infection and associates with the large subunit of the HSV-1 ribonucleotide reductase, ICP6. Despite the ability to detect an association between Mss1 and ICP6, Mss1 localization was not dramatically altered in cells infected with an ICP6 mutant virus. In particular, localization of Mss1 to RCs or VICE domains was not altered in ICP6 mutant virus infections suggesting that the ICP6 protein did not direct this process. When Mss1 was artificially depleted from permissive cells using siRNA, virus infection was neither diminished nor enhanced. Moreover, in cells which are naturally reduced in Mss1 transcript and protein, HSV-1 replication is undetectable, and supplementing these cells with additional Mss1 protein did not improve virus yield. Taken together, we surmise that the proteasome subunit Mss1 is dispensable for HSV-1 replication in the standard tissue culture setting.
Several studies have shown that the cellular proteasome is essential for robust HSV-1 replication (40, 43, 61, 110). These studies employed chemical inhibitors that inactivate the protease subunits found within the interior of the core proteasome. From those studies, the contribution of the regulatory complex (19S and/or APIS) was not addressed, as the inhibitors act on the core protease in the presence or absence of the regulatory component. Given the importance of the cellular proteasome during infection, we were initially surprised by our results as they imply that regulatory components of the proteasome are uncoupled during virus infection and one subunit is completely indispensable. However, these observations are congruent with more recent studies from our lab that indicate that the auxiliary proteasomes, such as the immunoproteasome or 11S-regulated proteasome, may be more important for managing unfolded or oxidized proteins found in infected cells (130). In previous work, we have shown that some proteins damaged by oxidation during infection are degraded by the core proteasome while other damaged proteins aggregate in the nucleus of infected cells in close association with the redox-regulated chaperone Hsp27 (130). It is well established that proteins damaged by oxidation are preferentially degraded by the 20S proteasome in a ubiquitin-independent fashion (42). This study supports this notion as well and provides indirect evidence that the ubiquitin-independent degradation pathway may not be the primary method of proteolysis. However, more work is needed to provide direct evidence.

In summary, despite the fact that Mss1 has an interesting localization in infected cells and appears to associate with viral proteins during infection, it is
not essential for robust replication in tissue culture and its depletion does not enhance replication. Perhaps in other cell types, culture conditions or *in vivo*, Mss1 provides some essential role that we cannot detect using our current system. Notwithstanding, although we have not discovered an essential role for Mss1 during infection, these studies provide important evidence that the AAA+ ATPase components of the 19S regulatory particle are uncoupled during HSV-1 infection and one subunit appears completely dispensable. These studies also support the idea that auxiliary proteasomes may be more valuable for protein turnover during Herpesvirus infection.

**Materials and Methods**

**Cells and Viruses:** Murine A9 (ATCC #CRL-1811) and L929 (ATCC #CRL-2148) cells were maintained in D-MEM containing 10% fetal bovine serum. Embryonal carcinoma P19 cells (ATCC #CRL-1825) were maintained in α-MEM with 7.5% bovine calf serum and 2.5% fetal bovine serum. D14 cells were obtained from Dr. Sandra Weller and described previously (72). Vero and 293T cells were propagated and maintained as described in Chapter II. HSV-1 strain KOS was propagated and maintained as described in Chapter II. The ΔICP6 mutant virus was kindly provided by Dr. Sandra Weller (University of Connecticut Health Center), and was propagated and maintained in D14 cells as described previously (72).
Immunofluorescence: Vero cells were grown on sterile glass cover slips to 70% confluency. Cells were infected at a multiplicity of infection (MOI) of 5. After infection for 7 h, the media was removed by aspiration and the cells were washed three times with 1x phosphate-buffered saline (PBS) at room temperature. The cells were then fixed with 200 proof ice-cold methanol for 10 min, permeabilized with 1% NP40 (in PBS) for 10 min, and incubated in blocking solution (3% BSA in PBS) overnight. After each step cover slips were washed three times with 1X PBS. The cover slips were inverted and placed on 100 µl of primary antibody diluted 1:1000 in blocking solution for 1 h. Cover slips were then washed seven times with 1X PBS and inverted in 100 µL secondary antibody diluted 1:600. All secondary antibodies were highly cross-absorbed Alexa Fluor antibodies (Molecular Probes, Carlsbad, CA.). After 1 h of incubation in secondary antibody cover slips were washed as before, rinsed with distilled water, and mounted on glass slides using ProLong Gold with DAPI (Molecular Probes, Carlsbad, CA.). Clear nail enamel was used to seal the cover slips. Images were obtained using Openlab 4.0.4 software at 63X or 100x with a Zeiss Axiovert 200M inverted microscope.

Western Blotting: Samples electroblotted onto PVDF membranes were probed using the following antibodies and concentrations: mouse anti-Mss1/S7, 320 ng/mL (Affiniti, Exeter, UK); rabbit anti-L26, 1 µg/mL (Sigma, St. Louis, MO.); mouse anti-V5 1 µg/mL (Sigma, St. Louis, MO.); and rabbit anti-NC-1/VP5, 1:10000 dilution of stock (provided by Drs. D.H. Cohen and R.J. Eisenberg). Detection of bound antibodies was done as described in Chapter II.
Quantification of band densities was performed with ImageJ and the samples normalized to the levels of L26 in the same sample over at least 3 separate experiments.

**Immunoprecipitation:** Vero cells were grown to confluency in 225 cm$^2$ flasks and infected with HSV-1 at an MOI of 5 as described in Chapter II. At 6 h post infection, cells were collected by gentle scrapping into growth media. Cells were pelleted by centrifugation for 10 min at 600 x g and washed twice with 1X PBS. Lysis was performed in 1 mL Lysis buffer (50 mM HEPES pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% (v/v) Triton X-100, Complete Protease Inhibitor Tablets (Roche) and Protease Inhibitor Cocktail (Sigma, St. Louis, MO.) added just before use) for 30 min on ice. Cells were sonicated and insoluble material was removed by centrifugation at 15,000 x g for 10 min. Samples were then pre-cleared by incubation with 2.5 µL of normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and 50 µL Recombinant Protein G Agarose (Invitrogen, Carlsbad, CA.) for 1 h at 4°C. Beads were collected by centrifugation at 1400 x g for 1 min and Supernatant was combined with 2 µl primary antibody (either normal mouse IgG or αMss1). After a 2 h incubation at 4°C, 50 µL Protein G Agarose was added and incubated with gentle inversion overnight. Beads were collected as before and washed three times in Lysis Buffer. After the last wash, 50 µL 2X SDS-PAGE Buffer (100 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM DTT) was added and samples were boiled for 5 min. Immunoprecipitated proteins were visualized with Sypro-Ruby (Molecular Probes, Carlsbad, CA.) or subjected to Western Blot.
**Mass Spectrometry:** After separation by SDS-PAGE and visualization with Sypro-Ruby protein stain (Bio-Rad, Hercules, CA.), selected bands were excised from the gel and placed in microcentrifuge tubes prewashed with 0.1% Trifluoroacetic Acid (TFA) and 50% acetonitrile. All buffers and solutions were made with ultra-pure water (Riedel de-Haen, Selze, Germany). Protein stain was removed from the samples by eight washes with 200 µL destain (1:1 Acetonitrile: 200 mM NH₄HCO₃ pH 8.1). After the last wash, destain was removed and gel plugs were allowed to dry overnight. Protein was digested with 0.4 µg per 15 mm³ gel volume Mass Spectrometry grade trypsin (Promega, Madison , WI) suspended in 20 µL Digestion Buffer (10% Acetonitrile, 40 mM NH₄HCO₃ pH 8.1). After one hour an additional 50 µL digestion buffer was added and samples were agitated overnight. Supernatant was collected and the plugs were washed twice for 45 min with 50 µL 0.1% TFA. Samples were then subjected to mass spectrometry analysis by the Wadsworth Center Biological Mass Spectrometry Group. All incubation steps were carried out at 37°C.

**Quantitative real-time PCR:** Sub-confluent dishes of ECP19, L929, and A9 cells were lysed and total RNA was collected using TRIzol according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA.). 1 µg of total RNA was reverse transcribed using Superscript III RT and random primers (Invitrogen, Carlsbad, CA.). Real-time quantitative PCR was performed on cDNAs using an ABI9300 (Applied Biosystems, Carlsbad, CA) and primers (IDT, Coralville, IA.) specific for murine PSMC1 (FP-gtgacgggcccaaactgtc, RP-gtcccaatggcgtcagttc), PSMC2 (FP-tgaatccgctctcagttc, RP-
gcatcagtccgattggcaac), PSMC3 (FP- tgccagtgttgggttggtg, RP- ggcagggtctccagagtacag), PSMC4 (FP- gatgccctcttggctgtg, RP- aaactccgagcccaccacac), PSMC5 (FP- tggtggagaaggtgccagac, RP- acgggcagctcgatcacttc), PSMC6 (FP- tcaggaagtggctgactccaa, RP- tgtcatgcagccaccaaaacc). The calculated ΔΔCt values (210) were normalized to those acquired for murine GAPDH (FP- ggctgcccagaacatcatcc, RP- aggccatgccagctgtgcttc).

siRNA treatment: siRNAs targeting Mss1 (S7) (Santa Cruz Biotechnology, Santa Cruz, CA.) were transfected into 293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA.) according to the manufacturer’s protocols. 24 hours after transfection, cells were infected with HSV-1 strain KOS at an MOI of 3 or 0.01. At the specified times post infection, samples were collected by scraping the cell monolayer with the media and splitting the sample in two. One sample was frozen on dry ice for subsequent viral titering while the second sample was centrifuged briefly to collect the cellular pellet, which was then resuspended in 1x SDS-PAGE buffer and western blotted using the specified antibodies.

Mss1 transfection: Mss1 add back to ECP19 cells was achieved by cloning either wild type Mss1 or an ATPase-deficient mutant into the plenti6/V5-DEST vector using the Gateway recombination system according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA.). A control plasmid containing GFP was also generated using the same methods. Mutation of the catalytic lysine at amino acid position 222 to alanine was done using single overlap extension PCR to change the 222 codon AAG → GCG. Plasmids were transfected into ECP19
cells using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA.). 24 hours after transfection, the cells were infected with HSV-1 strain KOS at an MOI of 3 or 0.01. At the specified times post infection samples were collected by scraping the cell monolayer with the media and splitting the sample in two. One sample was frozen on dry ice for subsequent viral titering while the second sample was centrifuged briefly to collect the cellular pellet, which was then resuspended in 1x SDS-PAGE buffer and blotted using the specified antibodies.

**Cyclohexamide treatment:** Sub-confluent HEK293T cells were treated with cyclohexamide (Sigma Chemical, St. Louis, MO.) at a final concentration of 100μg/mL. The cells were collected in 1x SDS-PAGE buffer and subjected to SDS-PAGE electrophoresis. Western blotting was performed to assess the half-life of the Mss1 protein.

**Acknowledgements**

April Burch and Kate Millington performed the experiments described by Figures 3-1, 3-2A, and 3-3. Trevor Barnes performed the qRT-PCR analysis in Figure 3-4A. We acknowledge the excellent support of the Wadsworth Center Media and Tissue Culture core facility. D.J.M. was supported by the BD-EID training grant (T32 AI 055429). Studies were supported by Wadsworth Center New Investigator funds to A.D.B.
CHAPTER IV

Immunoproteasome Function and Composition are not Altered in HSV-1 Infected Cells
Abstract

Auxiliary proteases that are incorporated into the cellular proteasome (immunoproteasome) during interferon γ (IFNγ) signaling promote the generation of MHC-I preferred peptides and enhance the host immune response to pathogens. Immunoproteasome formation is altered during human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV) infection, but whether herpes simplex virus type 1 (HSV-1) infection influences immunoproteasome formation and/or function in epithelial cells is not known. Using real-time PCR, we showed that in epithelial cells, HSV-1 infection causes depletion of preexisting immunoproteasome transcripts. Time course analysis revealed that wild-type HSV-1 was not able to completely block IFNγ-stimulated transcription. Immunoproteasome transcript levels remained high in epithelial cells infected with an ΔICP0 mutant virus at high multiplicity. Cells induced with IFNγ before infection contained immunoproteasome subunits that persisted during HSV-1 infection and co-purified with functional immunoproteasomes. Thus, although transcript levels diminish, immunoproteasome stability and function are not impaired in HSV-1 infected epithelial cells.
The proteasome is a multi-subunit protein machine involved primarily in the degradation of cellular proteins for the purpose of homeostatic control, immune signaling, and recycling (191). Peptides produced by the proteasome are translocated into the endoplasmic reticulum by the transporter associated with antigen processing (TAP), loaded onto MHC, and expressed on the cell surface. The 26S proteasome contains three domains: a base, lid, and central core. The 20S core forms a barrel-like structure consisting of four stacked heptameric rings in a $\alpha_1\beta_1\beta_1\alpha_1$ configuration. The beta subunits delta ($\beta_1$), Z ($\beta_2$), and MB1 ($\beta_5$) contain chymotrypsin and trypsin-like activity, whereas the alpha subunits lack protease activity. Interferon gamma (IFNγ) stimulation activates interferon regulatory factor 1 (IRF-1), inducing expression of LMP2 ($\beta_1i$), MECL-1 ($\beta_2i$), and LMP7 ($\beta_5i$), which replace the uninduced subunits to form the immunoproteasome (18, 49, 68, 178). The induced subunits exhibit altered proteolytic specificity and produce peptides preferred by MHC class I (65, 108, 182, 196). IFNγ also stimulates transcription of PA28 $\alpha$ and $\beta$, which form a multimeric complex that associates with and opens the 20S core facilitating proteolysis (44, 80, 158).

The innate immune response to viral infection is initiated by the release of IFNs, both $\alpha/\beta$ and $\gamma$, following stimulation of TLR3 by dsRNA, and is important both on the cellular and organism level (73, 163). Viruses, including herpes
simplex virus type 1 (HSV-1), have evolved measures to counteract the IFN-induced antiviral state (163). The HSV-1 virion host shutoff (vhs) and US3 protein kinase act together to reduce levels of IFNγ-stimulated transcripts and promote turnover of the IFNγ receptor (29, 121). The HSV-1 immediate early protein ICP0 acts as an E3 ligase, enhancing degradation of sumoylated forms of promyelocytic leukemia protein (PML) (19, 85, 124, 139, 142). Loss of PML leads to the dispersion of ND10 domains, sites of IFNγ-induced protein assembly and signal transduction (30, 59). Deletion mutants of ICP0 have been shown to be more sensitive than wild-type HSV-1 to IFNγ pre-treatment of cultured cells (88, 140). Additionally, in mice with attenuated IFN responses, ICP0-null mutant viruses are more pathogenic than in normal mice (86, 119). Notwithstanding, the functions of ICP0 are complex and cell-line dependent, with recent studies supporting the inability of ICP0 to effectively block IFNγ signaling and deplete signaling molecules in certain cell types and at various concentrations of input virus (60, 62).

Numerous viruses have been shown to interfere with immunoproteasome formation and function. HIV-1 and Hepatitis C virus encode proteins that bind to LMP7, preventing its incorporation and thus decreasing cell surface expression of viral peptides on MHC class I (5, 104). Human cytomegalovirus (HCMV) inhibits transcription of LMP2 and LMP7 (103) and Epstein-Barr virus (EBV) encodes a protein that directly inhibits incorporation of LMP7 (167). HSV-1 ICP47 has been demonstrated to interfere with peptide translocation into the endoplasmic reticulum by blocking the transporter associated with peptide
loading (TAP) (1, 93, 209). However, conclusive evidence whether immunoproteasome function is impaired has not been shown. Primary infection with HSV-1 occurs in the oral or genital mucosa — the first barrier to HSV-1 infection. A recent report showed that LMP7 transcripts escape vhs-mediated degradation and that LMP7 is incorporated into immunoproteasomes in dendritic cells (51); however, it is unknown whether functional immunoproteasomes are present during infection of the epithelium. In this study, we evaluated the production and stability of immunoproteasome subunits in infected epithelial cells, as well as the functionality and stability of immunoproteasomes produced before HSV-1 infection.

**Results**

We investigated the stability of the immunoproteasome transcripts of LMP2 and LMP7 during HSV-1 infection of HeLa cells using real-time PCR. Cells induced with IFNγ alone had increased transcript levels for both LMP7 (Fig. 4-1A) and LMP2 (Fig. 4-1B) throughout the course of the experiment as compared to uninduced (mock) samples. In cells stimulated with IFNγ for 16h before HSV-1 infection, we found a reduction in transcript levels along the course of infection for LMP7 (Fig. 4-1A) and LMP2 (Fig. 4-1B), similar to previous findings for LMP7 in dendritic cells (51). Interestingly, when viral infection was followed by IFNγ treatment, LMP2 transcript levels increased steadily until 4
**Figure 4-1:** Transcript levels of the immunoproteasome subunits (A) LMP7, (B) LMP2, and (C) the viral immediate-early protein ICP8 during HSV-1 infection of IFNγ-induced or uninduced HeLa cells. Mock, uninduced uninfected; IFNγ, only induced; HSV-1, only infected; HSV-1:IFNγ, infected then induced; IFNγ:HSV-1, induced then infected. All infections were done using an MOI=3.
hours post infection (h.p.i.), although not to the levels seen in IFNγ-induced uninfected cells (Fig. 4-1B). Transcript levels of the viral ICP8 gene were not significantly different in the presence of IFNγ compared to untreated infected cells (Fig. 4-1C). Collectively, these data agree with previous findings in dendritic cells that LMP7 transcript levels decrease at a rate comparable to that in actinomycin D treated cells during HSV-1 infection of IFNγ-induced dendritic cells (51). Additionally, LMP2 transcript levels decrease at a similar rate to LMP7, suggesting that both transcripts are protected from vhs as proposed previously for LMP7 (51). Furthermore, the increase in LMP7 transcripts when virus infection precedes IFNγ treatment suggests that the blockade of IFNγ signaling is incomplete.

Because both transcripts appeared to be vhs resistant, we next wanted to evaluate whether ICP0 played a role in inhibiting transcription directly (19, 30, 59, 85, 124, 139, 142) or indirectly via ICP0’s function as a transcriptional activator (60, 62). We used a virus with both copies of the ICP0 gene removed (ΔICP0) (175) as well as wild-type HSV-1 to infect HeLa and U2OS cells then quantified LMP7 and LMP2 transcript levels using real-time PCR at 8 h.p.i., with the same treatment groups as above (Fig. 4-1A-C). U2OS are a human osteosarcoma cell line which allow ΔICP0 to replicate to similar titers as wild-type HSV-1 without having the protein present (207). Samples from ΔICP0-infected HeLa cells had increased levels of transcripts for both LMP7 (Fig. 4-2A) and LMP2 (Fig. 4-2C) when compared to transcript levels of HSV-1 infected HeLa cells in all treatment groups. On the other hand, when ΔICP0 was used to infect U2OS cells,
Figure 4-2: Transcript levels of LMP7 (A,B) and LMP2 (C,D) in HeLa (A,C) and U2OS (B,D) cells infected with HSV-1 and ΔICP0. All sample assays were performed in triplicate with an MOI of 3. Mock, uninduced uninfected; IFNγ, only induced; Virus, only infected; Virus:IFNγ, infected then induced; IFNγ:Virus, induced then infected. Virus refers to either HSV-1 or ΔICP0. $p \leq 0.05$
transcript levels for both LMP7 (Fig. 4-2B) and LMP2 (Fig. 4-2D) were below that observed for HSV-1 infection of the same cell line. The LMP7 and LMP2 transcript levels of HSV-1 infected HeLa cells were similar to the levels seen previously at 8 h.p.i. (Fig. 4-1A & B). The overall decrease of transcript levels, especially observed when virus infection precedes IFNγ stimulation, by ΔICP0 on U2OS cells suggests that in this experiment the function of ICP0 as a transcription activator of other HSV-1 genes is what is required to inhibit transcription of the immunoproteasome subunits LMP7 and LMP2, similar to what has been observed for other IFNγ regulated genes (60, 62).

The IFNγ inducible subunits of the proteasome are very stable, with half-lives around 30 h (191). Since LMP7 protein has been shown to persist during HSV-1 infection of dendritic cells (51), we sought to explore whether this observation held true in epithelial cells. HeLa cells were infected with HSV-1 and treated as before (Fig. 4-1). Total protein was collected at 8 and 24 h.p.i. and subjected to Western blot. At 8 h.p.i., levels of VP5 in infected cells pre-induced with IFNγ (Fig. 4-3A, lane 4) were slightly lower than in uninduced infected cells (Fig. 4-3A, lane 3) or cells infected with HSV-1 then induced with IFNγ (Fig. 4-3A, lane 5), congruent with what has been reported concerning HSV-1 sensitivity to IFN treatment (107). In cells pre-induced with IFNγ (Fig. 4-3A & B, lane 2) and then infected with HSV-1 (Fig. 4-3A & B lane 4), LMP2 and LMP7 were readily detectable at 8 and 24 h.p.i., suggesting that these proteins are long-lived and not actively degraded during HSV-1 infection. This finding is similar to results determined previously in dendritic cells for LMP7 (51). In cells that have been
Figure 4-3. Protein levels of the immunoproteasome subunits LMP2 and LMP7 in IFNγ treated and virus infected cells. Immunoblots of 8 (A) and 24 (B) h.p.i. samples. Lanes: 1 – mock; 2 – IFNγ induced; 3 – HSV-1 infected; 4 – IFNγ induced then HSV-1 infected. 5 – HSV-1 infected then IFNγ induced. VP5 was used as a marker for virus infection. γ-tubulin was used as the loading control. Virus infections were performed using an MOI=3.
infected with HSV-1 prior to IFNγ induction, low levels of LMP2 or LMP7 protein was detected at 8 or 24 h.p.i. (Fig. 4-3A & B, lane 5). This suggests that although LMP7 transcripts appear to be present in HSV-1 infected cells (Fig. 4-1A), translation may be blocked either by mechanisms that have been established (34, 75, 114) or perhaps post-translationally by another means.

We next sought to determine whether the functional capacity of immunoproteasomes present in infected epithelial cells is comparable to that of uninfected cells. HeLa cells were either uninduced or IFNγ induced (16 h, 500 U/mL) and then infected with either HSV-1 or ΔICP0. Proteasomes were purified at 8 h.p.i. and proteasome purity assessed by western blotting for the ribosomal subunit L26, which was negative (data not shown). Proteolytic activity was assessed by monitoring increases in fluorescence from the cleavage of the peptide Suc-LLVY-AMC. Proteasomes purified from cells induced with IFNγ demonstrated a significantly higher proteolytic activity than uninduced, HSV-1 infected, or ΔICP0 infected HeLa cells (Fig. 4-4). Interestingly, in cells induced with IFNγ and then infected with either HSV-1 or ΔICP0 the proteolytic activity was significantly higher than cells induced with IFNγ alone (Fig. 4-4).

Next we sought to determine whether immunoproteasome subunits present during infection of IFNγ-induced cells were incorporated into, or associated with, immunoproteasomes. Western blots of the purified proteasome preparations were performed and the composition and concentration of the preps confirmed by Coomassie staining (Fig. 4-5A). LMP2 and LMP7 proteins were present in proteasomes purified from cells induced with IFNγ (Fig. 4-5B, lane 2)
Figure 4-4. Proteasome activity in IFNγ treated and virus infected samples. Relative fluorescent signal intensities from purified proteasomes and immunoproteasomes from infected and uninfected cells acting on the fluorogenic substrate Suc-LLVY-AMC (n=3). Lanes: B, Buffer; 1, Mock; 2, IFNγ; 3, ΔICP0; 4, IFNγ:ΔICP0; 5, HSV-1; 6, IFNγ:HSV-1.
Figure 4-5. Proteasome composition in IFNγ treated, virus infected, or mock cells. (A) Coomassie stained SDS-PAGE gel of purified proteasomes from IFNγ-induced cells. (B) Western blot of the components of proteasomes and immunoproteasomes from IFNγ-induced cells. Lanes: B, Buffer; 1, Mock; 2, IFNγ; 3, ΔICP0; 4, IFNγ:ΔICP0; 5, HSV-1; 6, IFNγ:HSV-1. All infections were performed using an MOI=3.
as well as from cells induced with IFNγ and then infected with either ΔICP0 (lane 4) or wild-type HSV-1 (lane 6). As expected, levels of the 11S regulator protein PA28α increased in all samples induced with IFNγ (Fig. 4-5B). Similar to the results seen in Fig. 4-3, proteasomes purified from cells not treated with IFNγ did not contain any of the inducible subunits. The levels of two invariant proteins found in the 26S proteasome (members of the 20S core and the regulatory cap protein Mss1) are shown (Fig. 4-5B). Thus, it is reasonable to conclude that intact, active immunoproteasomes are present during infection in cells treated with IFNγ and infected with virus. Moreover, it appears that HSV-1 circumvents immune recognition not by blocking immunoproteasome activity, but by interfering with the translocation of immunoproteasome-derived peptides to the ER (1, 93, 209).

**Discussion**

In this report we found that during HSV-1 infection of epithelial cells transcripts of the immunoproteasome subunits LMP2 and LMP7 appear to be protected from vhs degradation and levels decline in a manner similar to that seen in actinomycin D treated dendritic cells (51). Additionally, in cells infected with HSV-1 prior to treatment with IFNγ, levels of the LMP7 transcript increase, although not to the pre-infection levels seen in IFNγ treated cells. However, although LMP7 transcripts appear to be present and, in fact, increase slightly
over time, no observable protein is detected following IFNγ signaling, suggesting that the inhibition of translation occurs post-transcriptionally. Our data also suggest that ICP0, although shown to degrade key IFNγ signal transduction centers in the nucleus (29, 31, 50), is not required as an E3 ligase in our model. Instead, the ability of ICP0 to transactivate early and late stage HSV-1 gene transcription is vital for blocking LMP2 and LMP7 transcription. IFNγ induction increases the activity of the proteasome and also alters its composition, increasing the number of 20S cores capped by 11S regulators. Immunoproteasomes are known to cleave a wider variety of substrates, as the 11S regulator allows damaged, unfolded, and modified (Ub, SUMO, etc.) proteins access to the proteolytic core (49, 191). Recent data from our lab indicates that proteolytic activity increases in order to clear oxidized proteins, which increase greatly during HSV-1 infection (130). It stands to reason that IFNγ signaling, which increases proteasome activity, would further enhance immunoproteasome degradation of oxidized (and other) proteins during infection. Additionally, because immunoproteasomes can degrade damaged proteins, expression of these proteins on the cell surface would be a signal of stress and apoptosis (41), which the virus blocks by inhibiting translocation of all peptides into the nucleus (67).

Materials and Methods
Cells and Virus: HeLa and U2OS (A kind gift from Dr. Priscilla Schaffer) cells were maintained in D-MEM with 10% FBS as described in Chapter II. The ΔICP0 HSV-1 was a kind gift from Dr. Priscilla Shaffer and was propagated and stored as described previously (175). Infections were performed using an MOI of 3 as described in Chapter II before removal of extracellular virus and replacement with complete media or media supplemented with IFNγ (Abcam, Cambridge, MA) to a final concentration of 500 U/mL.

Western Blotting: Samples prepared as described in Chapter II and were electroblotted and probed using the following antibodies and concentrations: mouse anti-LMP2 and mouse anti-LMP7, both 2 µg/mL (Biomol, Plymouth Meeting, PA); rabbit anti-L26, 1 µg/mL (Sigma, St. Louis, MO.); rabbit anti-20S core 1 µg/mL (CalBiochem, La Jolla, CA); mouse anti-PA28α 1 µg/mL (Biomol, Plymouth Meeting, PA); and rabbit anti-NC-1 (VP5), 1:10000 dilution of stock (provided by Drs. D.H. Cohen and R.J. Eisenberg). Detection of bound antibodies was done as described in Chapter II.

qRT-PCR: Total RNA was collected each hour for 8h using TRIzol (Invitrogen, Carlsbad, CA.), reverse transcribed using Superscript III RT (Invitrogen, Carlsbad, CA), and cDNAs subjected to real-time PCR using an ABI9300 (Applied Biosystems, Carlsbad, CA) using the following primer sets (IDT, Coralville, IA): LMP7 (FP– 5’ ggattctcggccctcagc 3’, RP– 5’ ctgccccatagagagggccc 3’); LMP2 (FP– 5’ gtgcactcttgctctcagc 3’, RP– 5’ gacctcctcacggtgctc 3’); and 18S rRNA (FP– 5’ ttgcaacgtcgtcctatatca 3’, RP– 5’ ggatgtagtgtagcgggttctca 3’), which was used to normalize transcript levels.
Proteasome Purification: Proteasomes were purified from infected and uninfected cells as previously described (32). Briefly, 2 x 10^7 HeLa cells were collected in 5 mL of 37°C 1x PBS and pelleted at 500 x g for 10 min at room temperature following treatment (as described in text) with IFNγ, HSV-1, and ΔICP0. The pellet was resuspended in 4 mL of buffer A (40 mM Tris-Cl, pH 7.5, 5 mM MgCl₂, 1 mM DTT, 1 mM PMSF, 2 mM ATP, 10 mM sodium pyrophosphate, and 40% (v/v) glycerol) and dounced 10x with a small homogenizer. Samples were then centrifuged at 115,000 x g for 150 min to remove cellular debris and ribosomes. The supernatant was spun twice at 243,000 x g for 240 min per spin and the resulting pellet resuspended in 200 µL of buffer B (50 mM HEPES, 5 mM MgCl₂, 100 mM NaCl, 1 mM DTT, and 40% (v/v) glycerol). These are crude proteasome preps and contain a mixture of 26S, 20S, and immuno- proteasomes. Proteasome preparation concentrations were measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE).

Proteasome activity assay: Proteasome activity was assessed using the fluorogenic proteasomal substrate Suc-LLVY-AMC (N-succinyl-Leu-Leu-Val-Tyr-AMC) (Enzo Life Sciences, Plymouth Meeting, PA). 10 µg of purified proteasomes were incubated in a solution containing 2 mM ATP and 2 µg of Suc-LLVY-AMC for 1 h at 37°C. The reactions were stopped by addition of SDS to a final concentration of 1%, and proteasome activity was assessed by monitoring of fluorescence in triplicate samples using a Chemidoc XRS Imager (BioRad,
Hercules, CA). Fluorescence intensity was normalized to that of buffer alone (2 mM ATP, 2 µg Suc-LLVY-AMC, 1% SDS).

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CHAPTER V

Identification and characterization of miRNAs produced
during lytic HSV-1 infection
Abstract

The influence of miRNAs on the host-pathogen environment is largely unknown and under intensive investigation. Whether produced by the pathogen or by the host cell, miRNAs have the ability to sculpt the intracellular landscape by directly regulating target proteins. Using high-throughput sequencing of Herpes Simplex Virus Type 1 (HSV-1) infected epithelial cells, we have identified 19 novel small RNAs produced during the early hours of infection. Six of the novel RNAs have predicted folds characteristic of known miRNAs. RT-PCR, Northern blot, and stem-loop PCR analyses were used to provide further evidence of the production and kinetics of the viral miRNA miR-92944. Direct sequencing was used to reveal the 5’ and 3’ ends of the mature miR-92944. Mutation of miR-92944 resulted in a 3-4 log decrease in viral titers in either a single or multi-step growth analysis and a 4 fold reduction in plaque size. This study identifies a novel HSV-1 miRNA produced with early kinetics that influences the production of a global regulator of HSV-1 replication in epithelial cells. Additionally, this study reveals potential new targets for antagonistic molecules that may curtail the establishment of lytic or latent virus infection.
**Introduction**

MicroRNAs (miRNAs) are ~22 nt regulatory RNAs that control gene expression via association with target mRNAs (8). Although a relatively new discovery, already thousands of miRNAs have been described for a host of organisms (79). Following the discovery of miRNAs in eukaryotic cells, it was quickly realized that DNA viruses had evolved the use of miRNAs to regulate both host and viral gene expression (155, 156). Although found in SV40 (188) and HIV-1 (149), the majority viral miRNAs described thus far are encoded by members of the *Herpesviridae* family (39, 187). Herpesviruses are able to undergo both lytic (productive) and latent (dormant) infections (172). Because of the suspected importance of miRNAs in controlling latency, the identification of miRNAs isolated from latently infected cells or late-stage lytically infected cells has been under intensive investigation, yielding considerable information on miRNA activity during latency (23, 38, 39, 76-78, 101, 187, 195). Several miRNAs have been identified from HSV-1 latently infected ganglia and end-stage lytic infection, but only a few have been functionally characterized (76-78, 101, 195). Most act to reduce the levels of lytic proteins in order to maintain the latent state or down-regulate the levels of cellular proteins involved in immune detection. Recent analysis of HSV-1 and HSV-2 has revealed an additional 8 miRNAs produced between 8 and 24 hours post-infection (hpi), some of which are conserved between the viruses (101). The miRNAs that are produced immediately after infection (0-6 hpi) have yet to be identified. In this report, we describe the identification, annotation, and characterization of unique small RNAs...
Results

Small RNA identification

In order to identify miRNAs produced by HSV-1 during early lytic infection, we applied massively parallel sequencing of small RNAs from HSV-1 infected HEK293 cells. Small RNA enriched samples were collected at 2 h intervals post infection up to 24 h, pooled, cloned, and sequenced, yielding $1.3 \times 10^7$ total sequence reads equating to $5.7 \times 10^5$ unique sequences (Fig. 5-1A). Bowtie (71, 112) was used to map the reads to the HSV-1 genome, resulting in 20,165 mapped reads (Fig. 5-1A). We recovered 11 of the 16 known HSV-1 viral miRNAs (Fig. 5-1B), which comprised the majority of the recovered sequences. Nineteen HSV-1-derived novel small RNA sequences were recovered at a frequency of 3+ reads (Fig. 5-1C), the accepted cutoff for miRNA identification (3). The 19 novel RNAs mapped throughout the viral genome and originated from both the top and bottom strand of the virus (Fig. 5-1C). Recovered sequences ranged in length from 18-24 nucleotides (Table 5-1). Some of the novel miRNAs likely represent sequences for star strand molecules. For example, given the proximity to the novel RNA originating from position 425 and lower read number, it is possible that the RNA from position 470 is the star strand
A. 13,609,783 raw sequence reads (571,042 unique sequences)
   → Map the reads to the HSV-1 genome using Bowtie (20,165 reads)
   → 11 known HSV-1 miRNAs and 19 novel HSV-1 small RNAs with 3+ reads

B. HSV-1 miRNAs sequenced

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</tr>
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<td>2/-</td>
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<tr>
<td>miR-H12</td>
<td>1</td>
</tr>
<tr>
<td>miR-H14</td>
<td>3p/5p</td>
</tr>
</tbody>
</table>

* For miR-H5, 6, and 14 the order is 3p/5p

C. Novel HSV-1 Derived Small RNAs

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<th>Genomic Position</th>
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<tr>
<td>132792</td>
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Bottom strand

| Top strand | 34208 | 5 | UL15 |
| Bottom strand | 40262 | 5 | UL19 |
| 47588 | 3 | no |
| 56054 | 3 | UL28 |
| 105617 | 5 | UL49 |
| 106947 | 4 | UL49A |

^ Indicates the first nucleotide in the small RNA with respect to the 5' -> 3' directionality of the strand

^b Arise from the repeat regions of the genome, only the most 5' location is listed
Figure 5-1 – Discovery of novel small RNAs produced by HSV-1 during lytic infection of epithelial cells.  

**A.** Sequence results for a small RNA library generated from HSV-1 infected epithelial cells. Cloned small RNAs were sequenced and aligned to the HSV-1 genome strain 17, resulting in the assignment of 19 novel small RNAs and the recovery of many known miRNAs.  

**B.** Identity and copy number of known HSV-1 miRNAs recovered from the sequence analysis.  

**C.** Identity and copy number of 19 novel RNA species that were sequenced 3+ times. The genome position of the first nucleotide in a novel RNA sequence is listed as well as whether the RNA is found in a coding sequence and/or the repeat regions of the genome.
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<tr>
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<td>4/-</td>
</tr>
<tr>
<td></td>
<td>3' CU U -- -- A</td>
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(a) Refers to the position of the first nucleotide of the predicted miRNA sequence
(b) Originates from the bottom strand
**Figure 5-2:** Mfold and RNAfold analysis of the 19 novel small RNAs yielded 6 folds typical of known miRNAs. Mfold and RNAfold analyses were performed on 150 nt regions of the HSV-1 genome with the novel small RNA sequences centered in the 150 nt region. Positive folds were defined by the novel RNA being present in the arm of the hairpin, proximal to the loop. The novel RNA sequence is shown in bold and possible star strands in bold italics. The number of reads and which arm of the hairpin the RNA is found in are also shown.
to the mature 425 (Fig. 5-1C, *). We were surprised to find that only four of the 19 novel RNAs originated from repeat regions of the genome, since these areas contain the majority of the immediate early genes, which are highly expressed during lytic infection. Furthermore, although most known miRNAs arise from intergenic regions, of the 19 novel RNAs, only seven were found there (Fig. 5-1C).

Characterization of putative miRNAs

Folding of novel HSV-1 small RNAs: We next subjected all 19 novel miRNAs to Mfold (212) and RNAfold (94) analysis to determine if the 150 nucleotide sequence, with the novel RNA at its center, could form a hairpin indicative of known miRNAs using parameters established for miRNA identification (3). Six of the 19 novel RNAs formed hairpin structures representative of known pre-miRNAs and included two novel RNAs with possible star strands (Fig. 5-2; star strands in bold italics). This included the previously mentioned 425/470 pair and RNAs originating from genome positions 107631/107653.

miRNA Expression and Determination of the mature miRNA sequence: We next sought to determine the timing of expression of the known and novel HSV-1 miRNAs using RT-PCR. Small RNA libraries from each time point were created and subjected to PCR using primers specific for the miRNA in
A. Total length ~ 70 nt

B. Hour Post Infection

M | 2  | 4  | 6  | 8  | 10 | 12 | 16 | 20 | 24  
---|----|----|----|----|----|----|----|----|-----
|    |    |    |    |    |    |    |    |    |     
| 160 bp | miR-92944 |
| 75 bp   |          |
| 150 bp  | hsv1-mir-H1 |
| 75 bp   |          |
| 160 bp  | let-7a   |
| 75 bp   |          |

C. miR-92944

TTCGCTTTGCTCGCTGAGGACGGCTTTAACCS
TTGCTTTTGGCCTGATGGAGGACGGCTTTAACCS
TTGCTTTTGGCCTGATGGAGGACGGCTTTAACCS
TTGCTTTTGGCCTGATGGAGGACGGCTTTAACCS
TTGCTTTTGGCCTGATGGAGGACGGCTTTAACCS
Figure 5-3 – RT-PCR analysis of miR-92944. A. Schematic diagram of the small RNA linking and amplification strategy. Small, enriched RNAs were ligated with 5’ and 3’ linkers, reverse transcribed and PCR performed using the designated primers to produce the indicated fragment size. B. PAGE gel images of RT-PCR reactions for putative and known miRNAs over the course of HSV-1 infection (2-24 hours). miR-92944 produced a band of expected size and contained little to no background in the mock lane. hsv-miR-H1 and let-7a and were used as viral and loading controls, respectively. Positions of nucleotide markers are given on the left. M — uninfected samples. C. Sequencing results for the 5’ and 3’ ends of miR-92944 from 10 independent isolates of cloned products from the miR-92944 RT-PCR (asterisk in B).
Figure 5-4: RT-PCR of 13 of the 16 known HSV-1 miRNAs using primers a and b as illustrated in Figure 5-3. Numerous HSV-1 miRNAs, most prominently H6 and H13 produce a signal pattern which increases along the course of infection. hsv1-miR-H1 is included on Figure 5-4.
Figure 5-5: RT-PCR of the remaining 18 novel HSV-1 small RNAs isolated from Figure 5-1, using primers a and b as illustrated in Figure 5-3. Numerous samples had large amounts of signal in the Mock lane, making signal in the infected lanes difficult to interpret.
question and the 5’ linker (Fig. 5-3A, primers a and b) added during library construction. We first analyzed 14 of the 16 known HSV-1 miRNAs, using hsv1-miR-H1 as a control, as its expression has been reported previously (38) (Fig. 5-3B, Fig. 5-4). Levels of the human miRNA let-7a were used as a cDNA loading control (Fig. 3B). hsv1-miR-H1 showed the expected temporal expression based on previous findings (38). Little to no signal was detected in the uninfected control sample and the signal from samples derived from infected cells was much higher. Time course analysis of miR-92944 produced a band at the expected size of 70 bp for a mature miRNA in this assay (Fig. 5-3B). RT-PCR analysis of the other 18 novel small RNAs did not produce any additional expression patterns of note (Fig. 5-5).

Mapping the ends of novel HSV-1 miRNA miR-92944 was performed by direct DNA sequence analysis of cloned fragments (* band in Fig. 5-3B). For each miRNA, 10 clones were sequenced for either the 5’ or 3’ end, with the recovered viral sequences shown boxed in grey, flanked by the linker sequences (Fig. 5-3C). For miR-92944, the mature miRNA appears to be 20 nt in length (Fig. 5-3C).

**Northern blot and stem loop PCR:** Since miR-92944 produced distinct temporal expression pattern in our RT-PCR experiment, we wanted to further confirm candidacy as a bona fide HSV-1 miRNA via Northern blotting. Levels of let-7a and tRNA were similar at all time points and served as loading controls (Fig. 5-6B, D). hsv1-miR-H1 was used as a positive control for virus infection,
A. miR-H1

B. let-7a

C. miR-H1

D.
Figure 5-6 – Northern blot analysis of miR-92944.  A. Northern blot analysis of miR-92944 over the course of HSV-1 infection (2-24 hours). The positions of the 70 and 45 nt products are shown (arrowheads). Positions of $^{32}$P-labeled Decade markers are given on the left. Northern blot for let-7a (B) and hsv-miR-H1 (C) are provided as an RNA load control and infection control, respectively. D. Image of an ethidium bromide stained PAGE gel of tRNA for each sample. M — uninfected samples. Positions of let-7a and hsv-miR-H1 are shown with arrowheads.
Figure 5-7 – Stem-loop quantitative PCR analysis of miR-92944.  

A. Relative levels of Hsv-miR-H1 in uninfected (M) or HSV-1-infected epithelial cells over time (2-24 hours).  

B. Relative levels of miR-92944 in uninfected (M) or HSV-1-infected epithelial cells over time (2-24 hours). All samples were normalized to the let-7a levels.
showing an increase in signal of an ~25 nt species from 8-24 h.p.i. (Fig. 5-6C). Signal for the novel HSV-1 miR-92944 was observed at ~70 nt and ~45 nt from 4-24 h.p.i. for both species (Fig 5-6A, black arrowheads). The expression pattern observed in the Northern blot was similar to that observed in the RT-PCR experiment (Fig. 5-3C). Although we did not recover any signal for a mature miRNA (20 nt) when probing for miR-92944, the signals observed at 45 nt and 70 nt may represent the pre- and pri-miRNAs species, respectively, whose sizes fit with the predicted secondary structures (Fig. 5-2).

In order to better visualize the kinetic expression of mature miR-92944 we utilized stem-loop PCR because of its increased sensitivity over Northern blotting. hsv-miR-H1 was used as the viral control and produced detectable signal as early as 2 hrs post infection with peak signal levels plateauing from 12-24 hrs post infection (Fig. 5-7A). miR-92944 levels were detectable at 2 hrs post infection and increased slightly at 4 and 6 hrs post infection before reaching peak signal intensities at 8 hrs post infection and remaining high throughout infection (Fig. 5-7B). These results are consistent with those seen in Fig. 5-3 for both hsv-miR-H1 and miR-92944, with the exception being that the increased sensitivity of the stem-loop PCR was able to detect the mature miRNAs at an earlier time point. We conclude from these analyses that miR-92944 is an early miRNA produced during lytic infection of HSV-1 in epithelial cells.

**Mutation of miR-92944 severely decreases viral growth**
Single overlap extension PCR and bacmid based recombination (Fig. 5-8) was used to mutate the miR-92944 sequence by swapping the paired nucleotides from the 5’ and 3’ arm (Fig. 5-9A, grey boxes on hairpin structure, miR-92944 sequence in bold italics). Mutations were made in this way to avoid affecting UL42 expression since it is unknown if there are structural motifs required for transcription or translation (Fig. 5-9A, hairpin location relative to UL42 transcript). Mfold and RNAfold were used to fold the mutated sequence which formed a hairpin similar in structure to the wild-type sequence (Fig. 5-9A, bottom hairpin, miR-92944 sequence in bold italics). Following Bacmid based recombination, we were able to isolate 3 separate viral clones which contained the mutation of interest and had no mutations in the intergenic region between UL41 and UL42, nor any mutations in the transcripts of these genes (data not shown). In order to verify the deletion of miR-92944, we infected 293 cells and collected RNA for stem-loop PCR 8 or 10 h post infection. Stem-loop PCR confirmed that miR-92944 was not produced (Fig. 5-9B). Unexpectedly, levels of our positive viral control, miR-H1, were at or near our levels of detection at both time points (Fig. 5-9B). This apparent growth defect was explored further by single and multi-step growth analyses of the ΔmiR-92944 clones. In both the single step (Fig. 5-9C) and multi-step (Fig. 5-9D) growth analyses, we observed a 3-4 log decrease in viral titers when compared to wild-type for all three viral clones. Furthermore, plaques from the mutant viruses were about 4 times smaller than wild-type plaques allowed to grow the same amount of time (Fig. 5-9E).
Figure 5-8: Recombination scheme to mutate miR-92944. The mutation was made using single overlap extension as described in the Materials and Methods section for this chapter. The 600 bp fragment that was produced was cloned into the shuttle vector pGS284, which has an inducible origin of transfer and the \textit{sacB} gene for sucrose sensitivity. Cross streak mating was performed to achieve the first recombinant event. Resolution of the recombination was screened for the absence of the \textit{sacB} gene which should not grow on plates containing sucrose. As there are two possible outcomes for this event, additional screening by PCR and confirmation of the sequences by Sanger sequencing was done.
Figure 5-9 – Analysis of ΔmiR-92944 viral mutants.  A. Single overlap extension PCR was used to exchange the nucleotides in the grey boxes thereby mutating the sequence of miR-92944 (bold italics) and preserving the hairpin structure.  B. Stem-loop PCR from virus infected 293 cells collected either 8 or 10 hours post infection for hsv1-miR-H1 and miR-92944.  Growth analyses of the wild-type (black line) and three ΔmiR-92944 viruses (grey lines) performed either in a single step (C) or multi-step (D) growth curve.  E. Quantification of the plaque sizes from infected Vero Cre cells.  Plaques were allowed to grow for 5 days before fixation and staining.  Images of at least 15 plaques were collected and measured.
Discussion

MicroRNAs have been established in a number of systems as a simple yet effective means of gene regulation. In addition to simplicity, miRNA regulation is rapid and requires highly conserved proteins for processing and function. DNA viruses have evolved ways to exploit the miRNA pathway by encoding miRNAs of their own which target both host and/or viral transcripts. HSV-1 has previously been shown to encode at least 16 miRNAs, only 2 of which have known function. These two miRNAs repress translation of the important transactivators ICP0 and ICP4, thereby enhancing latency. During the reactivation from latency, the gene expression pattern of HSV-1 is nearly identical to that of lytic infection. Therefore, miRNAs produced during lytic infection may be important during reactivation as well. Using high-throughput sequencing of small RNA libraries from HSV-1 infected cells, we were able to recover 19 novel viral RNA species, 6 of which formed hairpin structures reminiscent of known pre-miRNA folds. Of these 6, miR-92944 was validated using a RT-PCR based assay, Northern blotting and stem-loop PCR. Cloning and sequencing of the bands produced by RT-PCR was used to define the ends of the miR-92944. Northern blotting of miR-92944 showed detectable signal at ~45 and 70 nucleotides, sizes indicative of the pre- and pri-miRNA, respectively. Stem-loop PCR was used to determine the kinetics of mature miR-92944 expression, which increased from 2 hours post infection to a plateau level around 8 hours post infection. The position of miR-
92944 within the 5’ UTR of the UL42 transcript agrees with the observed expression kinetics as UL42 is an early gene whose transcription occurs between 2-4 hours post infection. Mutation of miR-92944 resulted in a 3-4 log decrease in both single and multi-step growth analyses and yielded plaques which were significantly smaller in size when compared to wild-type HSV-1. Studies are underway to determine the targets of this novel viral miRNA and to further characterize the ∆miR-92944 mutants. Although it is possible that miR-92944 fine-tunes the cellular landscape during lytic infection by targeting host mRNAs, we believe that miR-92944 targets a viral mRNA which affects replication/transcription on a global scale. Evidence for this comes from our observation that miR-H1 levels do not accumulate during infection, similar to results seen when acyclovir is used to inhibit DNA replication (38). Additionally, if our mutation affected UL42 transcription or translation, we would expect there to be little or no viral replication, as mutations to UL42 are not viable (25, 28). Instead, we observed a blunting of viral replication which implies miR-92944 may target a global regulator of replication, perhaps one of the immediate-early genes. Characterization of miR-92944 and its potential targets will likely reveal novel mechanisms of pathogenicity and/or gene regulation, as well as providing possible new targets for antiviral molecules that curtail the establishment of lytic or latent virus infection.

Materials and Methods
Cells and Virus. HEK 293 cells (ATCC #CRL-1573) and Vero Cre cells (Dr. David Leib, Dartmouth University) were maintained at 37°C and 5% CO₂ in D-MEM containing 10% fetal bovine serum. Vero Cre cells were treated every 3rd passage with 500 µg/mL Hygromycin B. HSV-1 strain KOS was maintained and infections performed as described previously (197). HSV-1 ΔmiR-92944 clones were plaque purified and titered on Vero Cre cells before being used to infect 293 cells at the indicated MOIs. Plaque sizes were determined by measuring the area of at least 15 separate plaques, which were defined by the area of clearance following crystal violet staining.

Small RNA isolation. 10 confluent 10 cm² dishes of HEK 293 cells were infected with HSV-1 strain KOS at an MOI of 10. At 2, 4, 6, 8, 10, 12, 16, 20, and 24 h post infection, small RNAs (≤200 nt) and total RNAs (depleted of small RNAs) were purified using the miRVANA small RNA isolation kit according to the manufacturer's protocol (Ambion, Austin, TX). An uninfected control was also collected in parallel. Small RNA prep purity was assessed by running 300 ng of material on a 15% PAGE gel containing 8 M Urea in order to observe the 5.8S and 5S rRNA bands, as well as the tRNA smear.

miRNA purification and cloning for high-throughput sequencing. Small RNAs were prepared and cloned following previously published methods (84). Briefly, 2 µg of each small RNA fraction was pooled and size fractionated on a 15% PAGE gel containing 8 M Urea. RNAs which migrated between 15 and 30
nt were excised and eluted in 2.5:1 (v/w) 0.4 M NaCl, phenol chloroform extracted, and ethanol precipitated before ligation of a 3’ miRNA linker (miRNA linker 3, IDT, Coralville, IA) using RNA ligase2 (NEB, Ipswitch, MA). The reaction was gel purified and eluted as before and a 5’ RNA linker (5’ rGrUrUrCrArGrArGrUrUrCrUrArCrArGrUrCrCrGrArCrGrArUrC 3’) was ligated using RNA ligase (Ambion, Austin, TX) and gel purified and eluted as before. Reverse transcription with Superscript RT III (Invitrogen, Carlsbad, CA) and PCR amplification with linker specific primers (FP – 5’ aatgatacggcaccaccgccaggttcagttctacagtccga 3’, RP – 5’ gctggaattgcgtttaaa 3’) yielded a final PCR pool of miRNA candidates that were gel purified using a 4% low-melt agarose gel. 50 ng of purified PCR products were sent to the genomics facility at Albert Einstein College for high-throughput sequencing on an Illumina Genome Analyzer Ile (Illumina, San Diego, CA).

**Bioinformatic analysis of high-throughput sequencing data.** Data from the sequencing run was uploaded to the Galaxy server (http://main.g2.bx.psu.edu) for bioinformatic manipulation (71). The 13,609,783 sequence reads were trimmed of any linker sequence and condensed based on sequence similarity to yield a total of 571,042 unique reads. The reads were aligned to the HSV-1 strain 17 genome using Bowtie (112), allowing for 2 nt mismatches to account for strain to strain variability between strain 17 and strain KOS. Reads were aligned to both strands of the genome. 20,165 total reads mapped to the HSV-1 genome, of which 8398 were known HSV-1 viral miRNA or miRNA star strands.
The remaining reads were assigned positions along the genome according to where the first nucleotide of the read was located. Any reads which were sequenced less than 3 times were discarded as background (3). The remaining reads were manually screened for aberrations such as mononucleotide runs which could align to the GC rich genome of HSV-1 with our parameters, leaving 19 unique (296 total) reads which were analyzed further for their ability to fold into hairpin precursor structures. M fold (212) and RNAfold (94) were used to test a 150 nt region with the candidate miRNA in the center of the sequence. Positive results were considered to be the lowest mean free energy structures where the candidate miRNA was present in an arm of a hairpin, proximal to the loop (3).

**miRNA RT-PCR and sequencing.** 300 ng of miRVANA isolated small RNAs were ligated and reverse transcribed as described in the miRNA purification section. miRNA specific PCR amplification was done using a primer which annealed to the 5’ end of the 5’ linker (5’ aatgatacgccagtac 3’) or to the 3’ end of the 3’ linker (gcctgcagtcgagct 3’) and one specific to the miRNA sequence in question (See Supplementary Table 1 for sequences). Amplification using the 3’ PCR primer was done using miRNA specific primers to the reverse complement of the sequences in Supplementary Table 1, column 3. PCR products were electrophoresed on a 15% polyacrylamide gel and visualized using SyberGold (Invitrogen, Carlsbad, CA). Bands from the 5’ (~70 bp) and 3’ (~45 bp) PCR reactions were excised, crushed, and the DNA eluted overnight in
**Supplementary Table 1:** Table of sequences of the novel HSV-1 small RNAs recovered during sequencing, the miRNA-specific RT-PCR primer used in conjunction with primer (a) from Figure 5-3, and the Northern Blot probe used for the indicated novel RNAs. Primers used for 3' mapping were the reverse complement to the 5' end of the candidate sequence and were used with a primer that annealed to the 3' linker shown in Figure 5-3.

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a 2.5:1 v/w solution of 0.4 M NaCl at 4°C. The eluate was phenol extracted and ethanol precipitated before being cloned in a standard TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced at the Wadsworth Center Applied Genomic Technologies Core Facility.

**Northern blot analysis.** 10 µg of miRVANA isolated small RNAs were size fractionated on a 15% PAGE gel with 8 M Urea along with $^{32}$P labeled Decade Markers (Ambion, Austin, TX). Samples were electroblotted to BrightStar-Plus membranes (Ambion, Austin, TX) and probed with $^{32}$P labeled oligos (50 pmol with specific activity ≥ 2.8 x $10^6$ cpm/pmol) specific to the miRNAs (Supplementary Table 1) in ULTRAhyb-Oligo hybridization buffer (Ambion, Austin, TX) overnight at 42°C. Blots were washed according to the manufacturer’s directions and exposed to a phosphoimager screen for 2 weeks before imaging on a Storm 860 (Molecular Dynamics, Piscataway, NJ).

**Stem-Loop qPCR.** Stem-loop PCR assay kits were obtained from Applied Biosystems for hsa-let-7a (assay ID - 000377), hsv-mir-H1 (assay ID – 464923_mat), and for miR-92944 (designed against target sequence – UGGCUCGGUGAGCGACGGUC). Assays were performed in triplicate following the manufacturer’s directions. Real-time PCR was performed using an ABI7500 (Applied Biosystems, Carlsbad, CA). ∆∆Ct values were determined and normalized to the let-7a values for each sample (210).
**HSV-1 miR-92944 mutagenesis:** Single overlap extension PCR was used to mutate miR-92944 by exchanging the paired nucleotides on opposite arms (grey boxed pairs in Fig. 5-9A) of the predicted hairpin structure. This was done in order to preserve the predicted hairpin structure, using the following mutagenesis primers (FP – 5’ cgcgcgctcggcttcattctgcgagccggcaggaggtgcttgcttggtggtc 3’, RP – 5’ cacgctccgtccggctcgcagaatgaagcgcgcgcgcggttagcctgatt 3’) and primers which annealed ~300 bp upstream (5’ gctctagacgggtaattgtactgcggta 3’) and downstream (5’ cgagctccgcggccgagaagcgcctg 3’) and included SacI and XbaI sites, respectively. The ~660 bp PCR product was cloned into the SacI/XbaI sites of the pGS284 shuttle plasmid (courtesy of G. A. Smith, Northwestern University) to create pGS284-ΔmiR-92944. Recombination of the mutated miR-92944 sequence into HSV-1 strain KOS was done using Bacmid based recombination techniques (69). Briefly, recA+ bacteria (GS500, courtesy G. A. Smith, Northwestern University) harboring the wild type HSV-1 strain KOS bacmid were mated with BL21pLysS bacteria containing pGS284-ΔmiR-92944 and plated on double selective media. 100 colonies were collected and grown overnight and serial dilutions plated on no salt, 10% sucrose LB agar for recombination screening. 75 colonies obtained following sucrose selection were screened by colony PCR and sequenced to determine if they contained the mutation. Positive clones were transfected into Vero-Cre cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and multiple plaques purified and sequenced to recover the HSV-1 ΔmiR-92944 mutant.
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CHAPTER VI

Discussion and Future Directions
Herpes simplex virus type 1 is a complex human pathogen with wide ranging importance in human health and scientific research. Insight gained into the processes of HSV-1 correlate to like processes in numerous other Herpesviruses. As a virus, HSV-1 encounters various barriers during the course of infection that must be overcome for successful replication and progeny release to occur. We explored the relationship of HSV-1 with two important cellular pathways, the proteasome and miRNA pathways, with the goal of learning more about HSV-1 biology and developing possible therapeutics.

The cellular proteasome is an important protein machine required for protein degradation, recycling, and immune presentation (11). It is curious that components of the proteasome are relocalized during HSV-1 infection to sites (VICE domains) adjacent to active replication compartments. Along with 20S proteasomal cores and several chaperones, Mss1 is also localized to VICE domains. Mss1 and the other AAA-ATPases have been shown to have numerous important functions when uncoupled from the proteasome, most importantly DNA repair and transcription initiation (66, 129, 186, 189). As HSV-1 has a gapped genome in places, DNA repair and the ability to restart stalled transcription complexes would seem to be of crucial importance to the virus. However, removal of Mss1 from permissive cells or supplementation of Mss1 to a naturally reduced cell line does not alter HSV-1 replication.

Interestingly, in a wild-type infection, only Mss1 is present at VICE domains while the other AAA-ATPases have perinuclear or cytoplasmic staining. Although Mss1 co-immunoprecipitates with ICP6, ICP6 is not required for Mss1
localization to VICE domains. Whether the other AAA-ATPases localize to VICE domains when Mss1 has been reduced using siRNAs was not explored and would be an interesting question to answer. Of the six AAA-ATPases that comprise the base of the 19S regulator, 3 have C-terminal anchors which mediate attachment to the 20S core particle, Rpt2, 3, and 5 (193). Thus, logic would dictate that Mss1 localization to VICE domains as a consequence of 20S core relocalization would be unlikely. However, this prospect cannot be disregarded without evidence to the contrary. Using FRET based techniques to determine if Mss1 is in fact bound to 20S cores may answer this question.

Recently a number of cellular chaperones have been shown to be required for 19S base assembly (7). Specifically, Hsm3 binds to Mss1 to facilitate assembly into the 19S base (115). As there are a number of other cellular chaperones at VICE domains, it would be interesting to revisit the mass spectrometry data from the Mss1 immunoprecipitates and see if Hsm3 is present. If so, it is possible that free Mss1 complexed with the chaperone Hsm3 is relocalized to VICE not for Mss1’s accessory function(s) but perhaps for Hsm3’s function as a chaperone.

Because no change was observed in HSV-1 replication in ECP19 cells supplemented with Mss1, another factor(s) must be playing a role in the inhibition of HSV-1 replication. Exploring this defect further, perhaps using immunofluorescence to observe the localization of markers of viral replication, viral nucleocapsid proteins, glycoproteins, and looking for aberrations in capsid trafficking to the nucleus might provide additional avenues leading to possible therapeutics and/or viral interacting proteins. Alterations in the cellular miRNA
landscape in undifferentiated versus differentiated ECP19 cells cannot be ignored (95, 96). Differentiated ECP19 cells support HSV-1 replication (13) and thus the altered miRNA profile of these cells would alter not only the miRNA landscape but also the proteome as well, possibly having effects on HSV-1 replication efficiency.

Proteasome function during immune signaling is well established, as is the primary mechanism by which HSV-1 blocks TAP mediated transport (67). In accordance, our research into immunoproteasome functionality during HSV-1 infection showed no inhibition of proteolysis of a preferred substrate. In fact, we observed an increase in proteolysis in proteasomes purified from HSV-1 infected and IFNγ-treated cells. Previous work in our lab has shown a dramatic increase in the amount of oxidized proteins occurs during HSV-1 infection (130, 179). While the reason for this increase is under investigation, the mechanism by which the damaged proteins are degraded has been defined. It has been demonstrated that inhibition of proteasome function leads to an inability of infected cells to clear oxidized proteins (81, 130). This may, in part, elucidate why an increase in proteasome mediated protein turnover is observed in our experiments. Nonetheless, why would protein turnover capacity increase in uninfected IFNγ treated cells? Part of the reason may be an effort by the cell of damage limitation during the IFN response. IFNγ triggers the production of numerous reactive oxygen and nitrogen producing enzymes to aid in the destruction of foreign proteins (73). As such, having a system in place to remove damaged proteins would serve to protect the cell and inhibit apoptosis induced
by the presence of too many oxidized proteins (14).

HSV-1 degrades host and viral mRNAs via the non-specific RNAse \textit{vhs} and encodes the US3 kinase which inhibits the activity of PKR (121). However, our results show a 1 log increase in LMP2 transcripts levels when IFNγ signaling occurred after HSV-1 infection. As \textit{vhs} is a tegument protein, it is active as soon as the capsid enters the cytosol, and even nascent LMP2 transcripts would be degraded at this point (consistent with our findings). Our data suggest that the coordinated efforts of \textit{vhs} and US3 kinase do not degrade mRNA transcripts (and hence abrogate transcription) entirely. Whilst this appears to hold true for LMP2, it would interesting to see if other IFNγ stimulated genes also experience "leakiness" during HSV-1 infection.

MicroRNAs at first appear to be a primitive mechanism for regulating RNA expression, as RNA processing and binding proteins are required but little else. However, more detailed investigations have uncovered a veritable multitude of small RNAs with the capability to regulate diverse cellular processes (26). Perhaps because of the basic requirement for only RNAse type enzymes and the relatively small footprint a miRNA gene requires, viruses have evolved the use of miRNAs to regulate expression of host and/or cellular mRNAs (39). For HSV-1, the research has predominantly focused on miRNA production during latency. Our studies chose instead to focus on miRNAs produced during lytic infection, as these would have importance both in lytic infection as well as during latent reactivation. Using high-throughput sequencing, we discovered 19 novel small RNAs which appeared 3 or more times during our analysis. While this is the
established cutoff for new miRNA identification (3), we recovered a number of known HSV-1 miRNAs at a frequency of 1 or 2 sequence reads. Although the possibility remains that this could be due to differences in cell type or assay design, the distinct possibility remains that discarded small RNA sequences are in fact bona fide viral miRNAs. Additionally, Mfold and RNAfold, although scientifically accepted as predictors of RNA folding, are just that, predictors. There still exists no way to accurately define how an RNA will fold in vivo, due to the presence of proteins, other RNAs, etc. which could influence the folding. Thus, although only a few of our novel viral small RNAs passed the folding test, any of the ones that failed may again be bona fide viral miRNAs. Stem-loop PCR is becoming more and more the standard for miRNA (mature and pri-/pre-miRNAs) detection as opposed to Northern blotting because of its higher sensitivity, reproducibility, and non-reliance on radioactive labeling. For these reasons, it would be interesting and important to design stem-loop PCR assays against the remaining 17 (or more) novel HSV-1 derived small RNAs.

From our experiments, miR-92944 looks to be a bona fide HSV-1 derived miRNA produced as early as 2-4 hours post infection and persisting throughout the course of our experimental infection. As our analysis was done in epithelial cells (HEK293), it would be interesting to compare miR-92944 abundance and/or production (especially temporality) between various cell types using the same virus strain. Production of miR-92944 across a number of different cell types would shed light on the potential function of the miRNA. Because of the variability in HSV-1 replication in different cells, ubiquitous expression of miR-
92944 would suggest that the target of the miRNA is viral and contributes to viral replication in some way. The lack of miR-H1 and the diminished growth of ΔmiR-92944 in either a single and multi-step growth analysis strengthen this argument as well. This however does not preclude the possibility that miR-92944 is targeting a cellular mRNA, since numerous cellular effectors of viral replication are provided externally by the immune system and thus would not be present in cultured cells (99). Furthermore, numerous important genes (i.e. cell cycle, apoptotic, etc.) are conserved/required across all cell types and play important roles in HSV-1 replication efficiency (173).

Determining the target of a given miRNA is challenging since a single miRNA can influence the translation of hundreds of mRNAs (6, 180). Although miRANDA and other programs can predict potential human genomic targets, issues arise when trying to extrapolate these programs to fit a viral miRNA targeting a viral genome. Additionally, since there are currently no microarray chips which monitor protein changes, with a given miRNA differential labeling of proteins must be used (6, 180). We have performed preliminary miRANDA screens using miR-92944 and produced some interesting genomic targets. Perhaps the most curious is the human miRNA miR-1181. miR-1181 is predicted to down regulate NeuroD2 levels, which in turn allows for REST levels to increase. REST is involved in chromatin silencing and thus increased REST expression would lead to increased chromatin silencing and a decrease in transcription. HSV-1 has already been reported to interact with the REST pathway (83), and allowing chromatin silencing machinery to function during
replication would negatively affect replication efficiency. Interestingly, miR-1181 levels increase in response to hepatitis C infection (125). Although HSV-1 and hepatitis C are very dissimilar viruses, perhaps inhibition of the common pathway of chromatin silencing is important for robust replication. It would thus be interesting to use antimirs to inhibit miR-1181 and then infect with HSV-1. If miR-92944 is in fact targeting miR-1181, then we would expect to see more viral replication in antimir-treated HSV-1 infected cells than in untreated infected cells.

Given that the currently annotated HSV-1 miRNAs function by targeting the HSV-1 genome, it seems likely that miR-92944 would follow suit. The kinetics of miR-92944 production suggests it is produced as an early gene product. Thus, it is possible that miR-92944 targets the transcript(s) of an immediate early or early protein. Although this may seem counterintuitive since vhs function should be degrading most transcripts, previous evidence in our immunoproteasome experiments suggest that some transcripts are not degraded by vhs and instead degrade naturally, even during robust HSV-1 infection. Therefore, miR-92944 may be playing an inhibitory role but also providing an easily removable switch, allowing for expression of the regulated gene (or genes) quickly if the need arises. Preliminary scans of the HSV-1 genome for miR-92944 seed region complementarity have revealed two locations within the transcript of ICP4. ICP4 is a known target of viral miRNAs during the regulation of latency (195) and is known to compete with ICP0 during lytic infection for the establishment of latency, with high levels of ICP4 leading to latency (125). Interestingly, our data support ICP4 as a potential target, since ablation of miR-
92944 would allow for ICP4 levels to increase, delaying or inhibiting lytic viral replication altogether. In order to test for this possibility, a number of approaches can be explored. Perhaps the most straightforward approach would be to look at ICP4 protein levels in ∆miR-92944 infected cells vs. wild-type infected cells at various time points post infection. Since ICP4 is an immediate-early gene product that is also a tegument protein, we would expect to see low levels of ICP4 in all samples upon initial infection. If ICP4 transcripts are in fact the target of miR-92944, then protein levels should remain low or possibly decrease in the mutant infected samples. In parallel, looking at ICP4 transcript levels using RT-PCR would shed light on whether transcripts are degraded in the presence or absence of miR-92944.

In order to conclusively determine that ICP4 is the target, complementation studies would need to be completed in order to restore miR-92944 functionality to ∆miR-92944 HSV-1 infections. Because miR-92944 is present in the 5' UTR of UL42, complementation of the entire transcribed region may not sufficiently answer the question of miR-92944 function. However, a number of different constructs could be used to not only complement the mutation, but also shed light on the location of the promoting elements for miR-92944. Using constructs containing the entire UL42 transcript, just the 5' UTR, only the translated region, and only the predicted pre-miRNA sequence to complement would define what elements are indeed required for miR-92944 production. Complementation could be analyzed in a similar fashion to how the mutation affected viral growth and miR-H1 production by using growth analyses.
and step-loop PCR. Additionally, monitoring ICP4 protein levels as compared to both wild-type infection and non-complemented ΔmiR-92944 infection would allow direct visualization of changes in ICP4.

Similar to the use of antimirs to inhibit miR-1181 to verify it as a target of miR-92944, antimirs could be used in a wild-type infection to target miR-92944. If miR-92944 antimir inhibition mirrors the growth results and stem-loop PCR results seen in the mutational analyses, it would be further validation of the importance of this HSV-2 miRNA. Conversely, either custom siRNAs, or shRNA constructs could be used to mimic miR-92944 function in ΔmiR-92944 infected cells and a restoration of function, as observed by wild-type like levels of ICP4, viral growth, and miR-H1 production could be monitored.

In conclusion, HSV-1 is a complex human DNA virus that provides a tractable genetic platform for manipulation and experimentation. HSV-1 interacts extensively with the proteasome, relocalizing its constituent proteins while requiring the proteolytic activity for replication. Although the uncoupled activity of Mss1 does not appear to be required for HSV-1 replication, the relocalization to VICE domains (while the other 5 AAA-ATPases do not) cannot be overlooked or dismissed. HSV-1 induces an increase in proteasome proteolytic activity, possibly due to the increase in oxidized or otherwise damaged proteins during the infectious course. The requirement of the proteasome during HSV-1 infection illustrates an intriguing symbiosis between virus and host in an otherwise parasitic relationship. Furthermore, HSV-1 produces at least one miRNA during lytic infection, with an as yet undetermined target. However, the advent of more
sensitive miRNA detection tools may well reveal that some of the other novel RNA species we detected are in fact bona fide HSV-1 miRNAs. Hopefully insight gained from these studies and those that come afterward will advance our knowledge of HSV-1 biology and Herpesviridae biology in general. Armed with this knowledge, perhaps Herpesviruses, which comprise a large number of serious human pathogens, will no longer be the burden to human health and well-being that they are today.
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