Characterization of Rat Cytomegalovirus Gene Expression During Persistence

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List of Abbreviations

α: alpha

aa: amino acid

Ad: adenovirus

AGO: argonaute protein

β: beta

BAC: bacterial artificial chromosome

bp: base pair

cDNA: complementary DNA

CMV: cytomegalovirus

CR: chronic rejection

DB: dense body

DC: dendritic cell

Dpi: days post-infection

E: refers to the β or early class of CMV expressed genes

EC: endothelial cell

ER: endoplasmic reticulum

ERGIC: endoplasmic reticulum-Golgi intermediate compartment

EBV: Epstein Barr Virus

FOS: foscarnet, foscavir, PFA

γ: gamma

GAG: glycosaminoglycan

GalK: galactose kinase

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GCV: ganciclovir

HCMV: human cytomegalovirus

HHV: human herpesvirus

hpi: hours post-infection

HSPG: heparan sulfate proteoglycan

HSV: Herpes Simplex Virus

IE: refers to the α or immediate early class of CMV expressed genes

IP: immunoprecipitation

kbp: kilobase pair

kDa: kilodalton

KSHV: Kaposi's Sarcoma-associated Herpesvirus (HHV-8)

L: refers to the γ or late class of CMV expressed genes

LAT: latency associated transcript

Mac: macrophage

MCMV: mouse cytomegalovirus

MCP: major capsid protein

mCP: minor capsid protein

mC-BP: minor capsid binding protein

MHV-68: murine gammaherpesvirus-68

MIEP: major immediate early promoter and enhancer region of cytomegaloviruses

miRNA: microRNA

miRISC: miRNA induced silencing complex

mRNA: messenger RNA

moi: multiplicity of infection

NIEP: noninfectious enveloped particle

nm: nanometer

nM: nanomolar

nt: nucleotide

ORF: open reading frame

oriLyt: HCMV origin of replication

P-body: processing body

PBMC: peripheral blood mononuclear cells

PEG: polyethylene glycol

pp: phospho-protein

RCMV: rat cytomegalovirus

RGD: arginine-glycine-aspartic acid

RISC: RNA induced silencing complex

RNAi: RNA interference

SCP: smallest capsid protein

siRNA: small-interfering RNA

SMC: smooth muscle cell

SMG: submandibular gland

SOT: solid organ transplantation

ssRNA: single-stranded RNA

TGN: trans-golgi network

TVS: transplant vascular sclerosis

UL: unique long

US: unique short

UTR: untranslated region

WT: Wild-type

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I lost a great friend this year; I met him the first day of my first rotation of graduate school. Dr. Nelson came by the lab one day and told him he was “the rock of the lab,” we later nicknamed him Drock. This statement was true in many ways, even some I had not realized at the time. He was always there for me everyday in the lab, a great comfort in the volatile world of science. I don’t think I ever had the chance to properly thank him for all the wisdom he gave freely from his many years experience with molecular biology and with life.

Abstract

Cytomegalovirus (CMV) is a ubiquitous species-specific β-herpesvirus that establishes lifelong persistence and latency following primary infection. CMV persistence involves the continual production of low levels of virus, whereas viral latency is associated with a lack of virus production but both states maintain limited viral gene expression. While much is known about viral gene transcription during lytic infections *in vitro*, little is known about the specific gene transcription profiles that are associated with *in vivo* persistence, latency and reactivation. Persistence is likely to play a role in the development of chronic disease associated with human CMV (HCMV) infection including vascular diseases and chronic inflammatory diseases. Our laboratory has developed a rat heart transplant chronic rejection (CR) model that exhibits the hallmarks of transplant vascular sclerosis (TVS) in humans. In this transplant model, rat CMV (RCMV) accelerates the development of chronic allograft rejection by enhancing TVS formation. Using microarrays to analyze viral gene expression, we have shown that RCMV gene expression is highly restricted in tissues from infected allograft recipients. However, RCMV gene expression is also highly dynamic, changing in the same tissue over time. For instance, in the salivary glands the viral transcription profile significantly changes between 7 and 10 days post infection. Initially, RCMV expresses genes involved in viral replication but that quickly changes to a profile primarily involved in persistence (i.e. immune evasion and host manipulation). Identification of CMV genes expressed and the level of expression in specific cell types, tissue types and during different stages of the

viral lifecycle is critical to our understanding of CMV persistence and pathogenesis. Our laboratory is actively determining the function of the viral genes expressed during persistence and identifying the control mechanisms involved in regulating viral gene expression *in vivo*.

In this dissertation, I document the identification of RCMV microRNAs (miRNAs), which are a class of small non-coding RNAs involved in post-transcriptional regulation. Using a direct cloning/sequencing approach we discovered RCMV encodes 24 miRNAs and similar to RCMV mRNA expression, RCMV miRNA expression is dynamic and tissue specific in infected rat heart allograft recipients. We hypothesize that RCMV miRNAs are involved in regulating viral gene expression *in vivo*. During our study of RCMV gene expression *in vivo* we found a viral open reading frame (ORF) R116 with an unknown function highly expressed in infected salivary glands, the site of viral persistence. I characterized the RCMV gene R116 and show R116 is important for the production of infectious virus. We hypothesize that R116 plays a significant role in an entry step of the virus lifecycle. The expression, regulation and function of CMV genes involved in the establishment and maintenance of a persistent infection within host cells is critical to our understanding of CMV pathogenesis.

Chapter 1: Introduction and Review of the Literature

I. Herpesviridae

A. Overview

The family Herpesviridae is a group of large (120-250 kb) linear double-stranded DNA viruses sharing similar virion architecture. There exist over a hundred animal Herpesviruses, eight of which are known to infect humans (Pellett and Roizman 2007). Herpesvirus virions range in size from 120 to 260 nanometers (nm) in diameter and are composed of three structural layers. The icosahedral capsid (n=16 symmetry) is approximately 125 nm in diameter and encloses the double-stranded DNA genome. A structured layer composed of viral and cellular proteins, as well as RNAs, termed the tegument surrounds the capsid. The tegument is enclosed in a lipid bilayer envelope, derived from modified cellular membranes and studded with viral glycoproteins. In addition to their structural features, herpesviruses share four biological properties: 1) herpesviruses encode enzymes involved in nucleic acid metabolism, DNA synthesis, and protein processing. 2) The nucleus is the site of viral DNA synthesis and assembly of capsids, but completion of the infectious virion occurs in the cytoplasm. 3) Production of infectious progeny causes irreversible cellular damage or death. 4) Herpesviruses enter a latent phase wherein the viral genomes become closed, circular molecules associated with chromatin. During latency only a limited subset of viral genes are expressed, which are generally not involved in viral replication and new progeny are not produced. Herpesvirus infections are life-long and virus is able to reactivate from latency over the lifetime of the host ensuring spread through the population. The immune response to herpesvirus

infections is typically sufficient to prevent or suppress disease, but virus is never cleared from the infected host.

B. Subfamilies

The family Herpesviridae is divided into three subfamilies *alphaherpesvirinae*, *betaherpesvirinae* and *gammaherpesvirinae* based on genome sequence and virus biology such as host range, growth kinetics, tissue tropism, and ability to transform cells (Roizman, Carmichael et al. 1981).

i. Alphaherpesvirinae

The α-herpesviruses share several characteristics including a variable host range, relatively short replication cycle, rapid and destructive spread in cell culture and these viruses primarily establish latency in neurons of the sensory ganglia (Pellet Fields 2007). α-herpesviruses infecting humans include Herpes simplex virus types 1 and 2 (HSV-1, HSV-2) and Varicella-zoster virus (VZV). Other notable α-herpesviruses include Pseudorabies virus (*Suid herpesvirus 1)* and Herpes B virus (Cercopithecine herpesvirus 1), the endemic simplexvirus of macaque monkeys. Typically humans zoonotically infected with herpes B virus develop fatal encephalomyelitis or severe neurologic impairment (Huff and Barry 2003; Whitley and Hilliard 2007). Symptoms associated with α-herpesvirus infections in humans range from asymptomatic to life threatening. HSV-1 infections are typically asymptomatic, however this virus is the causative agent of cold sores and viral reactivation can lead to lesions on mucosal surfaces

innervated by latently infected neurons of the trigeminal ganglia. HSV-2 shares significant sequence homology with HSV-1 and is the major cause of genital herpes infection with the reservoir of latent virus established in the sacral ganglia. Severe complications of HSV disease occur in neonates where infection can be fatal due to virus dissemination involving multiple organs and herpes simplex encephalitis is the most common cause of sporadic, fatal encephalitis in the US (Roizman, Knipe et al. 2007). The α-herpesvirus, VZV, is the causative agent of the common childhood infection chicken pox and is associated with fever, malaise, and rash (Cohen, Straus et al. 2007). After primary infection, the virus establishes latency in dorsal root ganglia and can reactivate as herpes zoster (shingles) later in life. Herpes zoster is most often observed in older adults and in immunocompromised patients of any age manifesting as a painful rash (Cohen, Straus et al. 2007).

ii. Betaherpesvirinae

The β-herpesviruses are characterized by a restricted host range, broad tissue tropism, a long replication cycle in cell culture, infected cells become enlarged (cytomegalic), and latency occurs in secretory glands, lymphoreticular cells and other tissues (Pellett and Roizman 2007). Members of the β-herpesvirus family that infect humans include human cytomegalovirus (HCMV, HHV-5), human herpes virus 6 (HHV-6), and human herpes virus 7 (HHV-7). Other notable members of the β-herpesvirus family include rat, mouse, rhesus and chimpanzee cytomegaloviruses. The pathogenesis of HCMV will be discussed in detail below.

HHV-6 and HHV-7 are T-lymphotropic viruses and are ubiquitous in the human population similar to HCMV (Ward 2005). HHV-6 and HHV-7 are the causative agents of exanthem subitum (roseola) (Yamanishi, Mori et al. 2007). Primary infections with HHV-6 and HHV-7 are typically asymptomatic with rash being the most common symptom. Several studies have proposed an association between HHV-6 and multiple sclerosis (MS) although without a proper animal model this association has been difficult to prove (Yamanishi, Mori et al. 2007). Viral DNA and proteins have been found in brain samples of MS patients, however other studies show an insignificant correlation, suggesting further experiments are still needed (Cermelli, Berti et al. 2003; Tuke, Hawke et al. 2004).

iii. Gammaherpesvirinae

γ-herpesvirus infections are limited to their natural host, display specificity for infecting T or B cells and establish latency in lymphoid tissues (Pellett and Roizman 2007). These viruses can cause lymphoproliferative disorders and are linked to tumor formation (Pellett and Roizman 2007). Members infecting humans include Epstein-Barr virus (EBV, HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8). Other members of the γ-herpesvirus family include rhesus rhadinovirus (RRV), which is a homolog to KSHV, herpesvirus saimiri (saimiriine herpesvirus 2) and murine gammaherpesvirus-68 (MHV-68, Murid herpesvirus 4). Among the γ-herpesviruses, EBV is a ubiquitous virus that is the etiologic agent of infectious mononucleosis. Symptoms range from mild fever to several weeks of general malaise, pharyngitis, and lymphodenopathy (Evans,

Niederman et al. 1968; Rickinson and Kieff 2007). EBV establishes latency in B cells and is associated with several lymphoproliferative disorders such as Burkitt's lymphoma, Hodgkin's disease and Nasopharyngeal carcinoma (Rickinson and Kieff 2007). KSHV also establishes latency in B cells and is implicated in lymphoproliferative disorders, such as Multicentric Castleman's disease (MCD), Primary Effusion Lymphoma (PEL) and Kaposi's sarcoma (KS) (Cesarman, Chang et al. 1995; Soulier, Grollet et al. 1995). KS is a multi cell type tumor whose frequency was significantly increased with the onset of AIDS in human immunodeficiency virus (HIV) infected hosts. Serologic studies indicate KSHV is found throughout the world, however the infection rate of KSHV in the United States and Western Europe is low (1-7%) compared to parts of Africa and the Amazon basin where KSHV prevalence is greater than 50% (Ganem 2007).

II. Cytomegalovirus

A. Brief History

HCMV infected cells are characterized by cell enlargement, increased cytoplasmic volume (cytomegaly), and intranuclear and cytoplasmic inclusions. In 1881, Dr. Ribbert first observed the presence of cytomegalic cells in the kidney from a stillborn child and presumed that the cells were infected with protozoa (Jesionek 1904; Ribbert 1904; Lòwenstein 1907). Due to the distinct tissue morphology observed in patients with fatal infections, this disease was named cytomegalic inclusion disease (CID) (Wyatt, Saxton et al. 1950). The agent responsible was named salivary gland virus due to pathological findings from

infants dying from a variety of diseases and studies done in guinea pigs, which showed cytomegalic cells within the salivary and parotid glands (Goodpasture and Talbot 1921; Cole and Kuttner 1926). In the mid-1950s, HCMV was isolated by multiple laboratories (Rowe, Hartley et al. 1956; Smith 1956; Craig, Macauley et al. 1957). The cytopathic effect of this agent was similar to the cells seen in patients with either CID or salivary gland virus infections; therefore, cytomegalovirus was named (Craig, Macauley et al. 1957). CMV was later classified as a member of Herpesviridae due to the characteristic virion morphology and large linear double-stranded DNA genome (Huang, Chen et al. 1973).

B. Epidemiology and Pathogenesis

HCMV is a ubiquitous human pathogen with an incidence of infection ranging from 30-100% depending on geographical location, age, and socioeconomic status of the host (Staras, Dollard et al. 2006; Britt 2008). HCMV is most often acquired within the perinatal period (0-2 years of life) usually via maternal transmission (*in utero* or breast milk) or later in life following puberty when the virus is transmitted via sexual contact. The virus establishes a life-long infection that is generally asymptomatic in healthy individuals. However, the virus is a major cause of morbidity and mortality in immunocompromised hosts (neonates, transplant, late-stage AIDS, and cancer patients) who are less able to control primary infection or reactivation of latent virus (Khoshnevis and Tyring 2002; Whitman, Bryan et al. 2005). Immunity to HCMV is generally protective against disease although immunity cannot prevent re-infection (Drew, Sweet et al. 1984;

Chandler, Holmes et al. 1985). HCMV has a broad cellular tropism *in vivo*, resulting in the infection of most host organs. The ability to infect multiple cell types enables the virus to shed in most human body fluids, increasing viral fitness and spread. Antiviral agents are able to limit viral replication and reduce the severity of disease associated with acute HCMV infections but do not appear to affect latent virus. Therefore, the current antiviral agents are unable to clear CMV from the infected host.

i. Immunocompetent Host

HCMV infection of immunocompetent hosts is generally asymptomatic although the acute infection phase can result in mononucleosis-like symptoms including fever, myalgia, headache, lymphadenopathy, and splenomegaly (Britt 2008). During acute infection the virus spreads throughout the body, infecting most host tissues and organs. Viremia is typically cleared after a period of weeks to months. Persistent CMV infection is usually established within 2 weeks in the salivary glands where virus can be detected years after the acute infection state has been cleared from the host (Britt 2008). Persistent HCMV infections are linked to the acceleration of vascular diseases such as atherosclerosis, restenosis following angioplasty and transplant vascular sclerosis (Melnick, Petrie et al. 1983; Speir, Modali et al. 1994; Melnick, Adam et al. 1998). HCMV has also been linked to the development of other chronic diseases including inflammatory bowel disease, periodontal disease, rheumatologic disorders, and some malignancies (Cobbs, Harkins et al. 2002; Britt 2008).

ii. Immunocompromised Host

HCMV infection is more problematic in individuals with an absent, weak or immature immune system. Sources of infection can either be primary infection, reactivation of latent virus, or re-infection with a different strain of CMV. Congenital HCMV infections are the most common cause of virus-induced birth defects. Acute infection of the fetus or newborn can lead to hepatitis, retinitis, and neurological disease while chronic infection can lead to hearing loss and neuronal developmental abnormalities (Britt 2008). HCMV is also an important infectious agent affecting organ transplant recipients and AIDS patients leading to fever, hepatitis, retinitis, gastrointestinal disease, pneumonia and bacterial superinfections (Britt 2008). Although the use of HAART (highly active anti-retroviral therapy) to treat HIV infections has significantly decreased the severity of HCMV disease in AIDS patients (Deayton, Mocroft et al. 1999; O'Sullivan, Drew et al. 1999). Chronic HCMV infection of transplant recipients is associated with increased severity of vascular disease and decreased time to allograft rejection (Britt 2008). Immunosuppressive therapies administered following solid organ and bone marrow transplantation render the recipient susceptible to HCMV disease due to either primary infection with the virus or secondary infection following reactivation of the virus from latency (Tanaka 2003).

iii. HCMV and Vascular Disease

Despite advances in medicine, cardiovascular disease remains a common cause of death in the United States. A majority of these deaths are due to vascular

disease linked to atherosclerosis (Colaco and Roser 1994) and other vasculopathies such as restenosis after coronary angioplasty and transplant vascular sclerosis (TVS), the hallmark lesion of chronic solid organ graft rejection (Heck, Shumway et al. 1989). Although the initial causes of these vascular disease processes have differences, many aspects of their pathogenesis and formation are similar, with the end result being vessel narrowing leading to diminished blood supply and end organ ischemia. There are various risk factors connected with vascular disease, including hypertension, chemicals/tobacco, hyperlipidemia, diabetes, immunological factors and infectious agents (bacteria and viruses).

Long-term HCMV infections are associated with chronic inflammatory disease leading to the acceleration of vascular diseases such as atherosclerosis, restenosis, and TVS (Rubin, Tolkoff-Rubin et al. 1985; Grattan, Moreno-Cabral et al. 1989; Speir, Modali et al. 1994; Melnick, Adam et al. 1998). Studies have shown a direct association between HCMV and accelerated TVS and restenosis following angioplasty both in humans and in rodent models (Streblow, Orloff et al. 2001). These diseases are usually the result of a mechanical or immune-mediated injury followed by inflammation and subsequent smooth muscle cell migration contributing to the plaque or neointimal formation that leads to occlusion of the vessel. In HCMV-positive individuals, epidemiological studies have indicated an increased percentage of vascular disease associated with a decrease in time to rejection (Sorlie, Nieto et al. 2000). HCMV antigens and DNA

have been detected in vascular lesions and diseased vessels (Melnick, Petrie et al. 1983; Hendrix, Dormans et al. 1989). Treatment with the antiviral drug ganciclovir, in human and animal transplant recipients, results in prolonged allograft survival, indicating that virus replication is necessary for the acceleration of disease (Merigan, Renlund et al. 1992; Valantine, Gao et al. 1999; Tikkanen, Kallio et al. 2001; Zeng, Waldman et al. 2005; Tu, Potena et al. 2006). Determining the mechanisms involved in the development of HCMV-associated TVS has been challenging due to the multifactorial nature of the underlying causes of vascular disease. Human studies are difficult to control and verification of mechanisms is impossible, therefore animal models provide an optimal tool to study the association between CMV and TVS.

C. Genome Organization

At 196 to 241 kbp, HCMV has the largest genome of all known herpesviruses. The genome contains unique long (UL), unique short (US) and internal and terminal repeat regions in the arrangement *ab*-UL-*b*'*a*'*c*'-US-*ca* (Figure 1.1). The genome arrangement designates it a class E genome producing four possible isomeric forms of the viral genome in progeny. In contrast, the animal CMVs are classified as class F viruses because their genomes are linear without internal repeat regions but contain repeated *a* sequence at the genome termini, facilitating packaging into capsids. The HCMV genome contains over 230 potential open reading frames (ORFs) (Murphy E, Yu D et al. 2003; Murphy, Rigoutsos et al. 2003; Murphy and Shenk 2008). HCMV encodes 40 core genes,

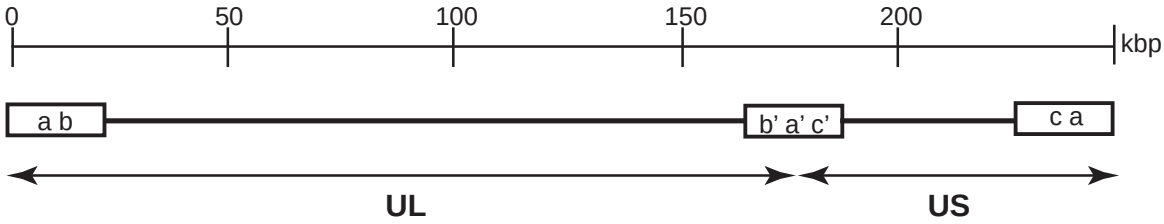

Figure 1.1 Schematic of the HCMV genome. Depicted is the HCMV genome with the internal and terminal repeat regions shown in boxes. The arrows denote the unique long (UL) and the unique short (US) regions that can isomerize to produce four unique progeny genomes. The ruler is in kilobases. Adapted from (Landolfo, Gariglio et al. 2003).

which are common to all herpesviruses and are located within the central region of the genome (UL domain). The core genes are involved in viral DNA replication, virion assembly, and other essential functions (Yu, Silva et al. 2003; Mocarski, Shenk et al. 2007). Within the US domain and the terminal regions of the HCMV genome are CMV specific genes, which are typically nonessential for virus replication in cell culture and often contain immune evasion activity (Yu, Silva et al. 2003; Mocarski, Shenk et al. 2007). Two laboratory strains, AD169 and Towne, and five clinical isolates, Toledo, Fix, Phoebe, Merlin, and TR have been sequenced, revealing a number of previously unrecognized ORFs within the clinical strains not present in laboratory strains (Chee, Bankier et al. 1990; Cha, Tom et al. 1996; Murphy E, Yu D et al. 2003; Dolan, Cunningham et al. 2004). The significant differences amongst the strains enable clinical isolates and not laboratory isolates to productively infect endothelial cells, epithelial cells and monocyte/macrophages (Hahn, Revello et al. 2004; Wang and Shenk 2005; Sinzger, Digel et al. 2008).

D. Virion Structure

i. The HCMV Particle

In tissue culture, HCMV produces three distinct particles including infectious mature virions, noninfectious enveloped particles (NIEPs), and dense bodies (DBs) (Mocarski and Courcelle 2001). NIEPs are defective particles composed of the same viral proteins as infectious virions but lack viral genomic DNA. NIEPs are distinguished from mature virions by a lack of an electron dense DNA core by

electron microscopy. DBs are unique to human and simian CMVs and are large enveloped structures containing several tegument proteins (primarily pp65 encoded by UL83) but lack an assembled nucleocapsid and viral DNA (Varnum, Streblow et al. 2004). The production and quantity of the different particles depends on the number of passages in cell culture as well as the multiplicity of infection and viral strain used to infect the cells (Streblow, Varnum et al. 2006).

ii. Capsid

The 125 nm diameter icosahedral nucleocapsid (Figure 1.2) is assembled in the nucleus and is composed of two main proteins, pUL86 and pUL85. pUL86 or major capsid protein (MCP) forms the pentamers and hexamers while pUL85 or minor capsid protein (mCP) is believed to anchor the viral DNA genome within the capsid (Irmiere and Gibson 1985; Chee, Rudolph et al. 1989). pUL48.5 or smallest capsid protein (SCP) and pUL46 or minor capsid-binding protein (mC-BP), are the other two capsid-associated proteins involved in capsid assembly and virion maturation (Irmiere and Gibson 1985; Welch, McGregor et al. 1991; Gibson, Clopper et al. 1996; Borst, Mathys et al. 2001). mCP and mC-BP associate to form triplexes in a two to one ratio that link together the capsomeres (Butcher, Aitken et al. 1998; Chen, Jiang et al. 1999). Six copies of SCP decorate the tips of each hexon (Yu, Shah et al. 2005). In addition, the portal protein pUL104 is in one of the pentamer positions through which viral DNA is loaded into the capsid and viral DNA is also thought to exit through the portal (Dittmer and Bogner 2005).

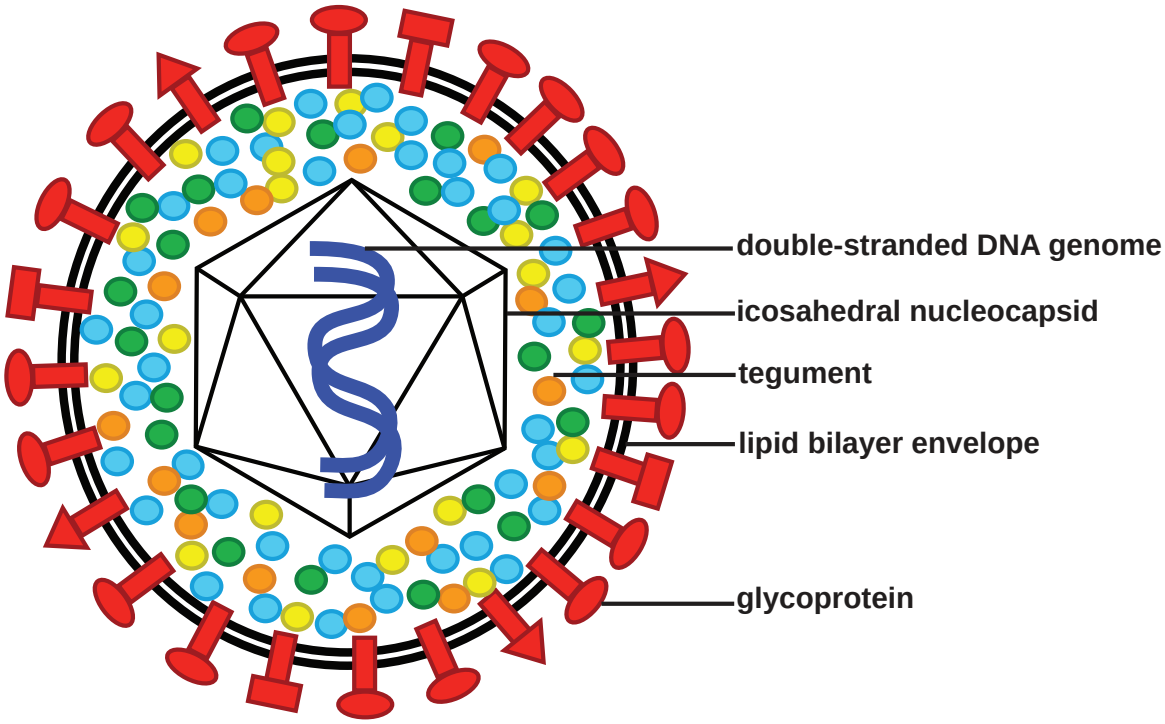

Figure 1.2 The HCMV virion. The double-stranded linear DNA genome is enclosed in the nucleocapsid that is surrounded by the tegument composed of viral and cellular proteins. The lipid bilayer envelope is acquired from modified cellular membranes and contains viral glycoproteins necessary for entry.

iii. Tegument

The tegument consists of multiple viral and cellular proteins obtained in both the nucleus and cytoplasm of the infected cell (Varnum, Streblow et al. 2004). The functions of the majority of the tegument proteins are unknown but they are hypothesized to be involved in regulation of cell signaling and initiation of viral gene transcription. The major tegument proteins are UL83 (pp65) and UL32 (pp150). The multi-functional phospho-protein pp65 is the most abundant tegument protein and is involved in inhibition of natural killer (NK) cell cytotoxicity (Arnon, Achdout et al. 2005), inhibition of interferon signaling (Browne and Shenk 2003; Abate, Watanabe et al. 2004), and mediating the down-regulation of HLA-DR in infected cells (Odeberg, Plachter et al. 2003). pp150 is important in directing capsids to the site of envelopment and is essential for tegument assembly (AuCoin, Smith et al. 2006). pp71 (UL82) and ppUL69 are minor tegument proteins. pp71 enhances expression from the major immediate early promoter and ppUL69 functions to block the infected cell from entry into the S phase of DNA synthesis (Liu and Stinski 1992; Lu and Shenk 1999; Hayashi, Blankenship et al. 2000).

iv. Envelope and Glycoproteins

The viral lipid envelope is derived from host cell endoplasmic reticulum (ER) and/or endoplasmic reticulum-Golgi intermediate compartment (ERGIC) membranes and contains at least 19 viral encoded glycoproteins that play various roles in virus entry, cell-to-cell spread, and virion maturation (Britt and

Mach 1996; Varnum, Streblow et al. 2004; Mocarski, Shenk et al. 2007). The essential envelope glycoproteins of HCMV are members of the herpesvirus family core set and include gB, gH, gL, gM and gN (Hobom, Brune et al. 2000; Mocarski, Shenk et al. 2007). The α- and γ- herpesviruses also encode subfamily specific envelope glycoproteins, in addition to the core set, that are also essential for entry (Mocarski, Shenk et al. 2007). The five core glycoproteins are required for CMV replication (Hobom, Brune et al. 2000; Dunn, Chou et al. 2003; Yu, Silva et al. 2003).

The most abundant glycoprotein of the HCMV virion envelope is gM (UL100), which can be complexed with gN (UL73) and acts as an attachment receptor binding to heparan sulfate proteoglycans (HSPGs) (Kari and Gehrz 1992; Compton, Nowlin et al. 1993; Mach, Kropff et al. 2000; Varnum, Streblow et al. 2004). Mass spectrometry analysis implies most of gM is uncomplexed because ten percent of the HCMV virion mass is credited to gM while gN is present at only one percent of gM levels (Varnum, Streblow et al. 2004). Both gM and gN are essential for CMV replication supporting a role in the initial interaction of the virion with the cell surface (Hobom, Brune et al. 2000; Dunn, Chou et al. 2003; Yu, Silva et al. 2003). gN is highly polymorphic and is proposed to play an auxiliary role in secondary envelopment of HCMV particles (Mach, Osinski et al. 2007). The significance of gN's polymorphism is thought to be important for evasion of the host neutralizing antibody response and to facilitate re-infection in previously CMV infected hosts (Burkhardt, Himmelein et al. 2009).

The second most abundant glycoprotein of the HCMV virion is gB (UL55), which exists as a disulfide-linked homodimer in the viral envelope and is highly conserved amongst the human herpesviruses (Varnum, Streblow et al. 2004; Mocarski, Shenk et al. 2007). gB functions both during attachment where it is critical for the initial virion tethering to HSPGs on the cell surface and also serves as a fusion receptor during virus entry (Kari and Gehrz 1992; Compton, Nowlin et al. 1993; Navarro, Paz et al. 1993; Bold, Ohlin et al. 1996). The dual roles for gB were confirmed in studies utilizing gB specific antibodies that were able to inhibit attachment and/or block fusion (Gicklhorn, Eickmann et al. 2003; Lantto, Fletcher et al. 2003). CMV receptor identification is controversial but studies have shown that gB interacts with epidermal growth factor receptor (Wang, Huong et al. 2003), toll-like receptor 2 (TLR 2) (Compton, Kurt-Jones et al. 2003), and β1 integrins (Feire, Koss et al. 2004). The presence of integrin binding sequences such as the amino acid motif Arg-Gly-Asp (RGD) among the core herpesvirus envelope glycoproteins suggests that integrins may play an important role during entry and signaling (Mocarski, Shenk et al. 2007). The binding of HCMV virions to cells results in the activation of cellular signaling pathways partially attributed to gB, which alone initiates cellular signaling similar to an interferon toll receptor or growth factor response (Boyle, Pietropaolo et al. 1999; Compton, Kurt-Jones et al. 2003; Wang, Huang et al. 2005; Boehme, Guerrero et al. 2006). Another function for gB in various herpesviruses is during virion assembly and egress where gB is involved in exit from the outer nuclear membrane into the cytoplasm

(Peeters, de Wind et al. 1992; Peeters, de Wind et al. 1992; Lee and Longnecker 1997; Krishnan, Sharma-Walia et al. 2005).

The glycoprotein complex of gH (UL75) and gL (UL115) is common to the herpesvirus family and required for replication, specifically for entry into cells (Forrester, Farrell et al. 1992; Roop, Hutchinson et al. 1993; Molesworth, Lake et al. 2000; Dunn, Chou et al. 2003). The gH:gL complex can be additionally modified by viral glycoproteins in both β- and γ- herpesviruses. gL acts as a chaperone required to properly localize gH within infected cells and in the virion envelope (Kaye, Gompels et al. 1992; Spaete, Perot et al. 1993). Studies using soluble gH or blocking antibodies to gH suggest a role for gH in fusion (Keay and Baldwin 1991; Huber and Compton 1997; Lopper and Compton 2004; Kinzler and Compton 2005). gH like gB, has been shown to interact with both αvβ3 integrins and TLR2 suggesting possible co-receptors for HCMV entry into cells (Wang, Huang et al. 2005; Boehme, Guerrero et al. 2006). In addition to the heterodimeric complex, gH:gL associated with gO (UL74) or with UL128:UL130:UL131 has been identified in HCMV virions (Hahn, Revello et al. 2004; Patrone, Secchi et al. 2005; Wang and Shenk 2005). Knockout of gO in HCMV revealed a modest role in the efficiency of viral replication in fibroblasts and gO was thought to act by enhancing the efficiency of gH:gL-mediated fusion (Hobom, Brune et al. 2000; Paterson, Dyer et al. 2002; Dunn, Chou et al. 2003; Jiang, Adler et al. 2008). Recently, the role of gO was analyzed in lab-adapted vs. clinical strains of HCMV and found to function as the chaperone to promote

incorporation of gH:gL into the virion envelope (Ryckman, Chase et al. 2010; Wille, Knoche et al. 2010). The other gH:gL complex incorporates UL128, UL130 and UL131 enabling HCMV to infect clinically relevant cell types such as epithelial, endothelial and dendritic cells (Hahn, Revello et al. 2004; Gerna, Percivalle et al. 2005; Wang and Shenk 2005; Wang and Shenk 2005).

E. HCMV Replication Cycle

i. Entry

The first step in HCMV entry is attachment of the virus to HSPGs present on the cell surface in a process mediated by gB and/or the gM:gN complex (Figure 1.3) (Compton, Nowlin et al. 1993). Attachment is followed by a stable binding step where gB and/or gH:gL interact with receptor/s on permissive cell types and mediate the fusion step (Wang, Huong et al. 2003; Feire, Koss et al. 2004; Wang, Huang et al. 2005; Boehme, Guerrero et al. 2006). The gH:gL complex is required for pH-independent fusion into fibroblasts (Compton, Nepomuceno et al. 1992; Ryckman, Jarvis et al. 2006). While the 5-member complex gH:gL:pUL128:pUL130:pUL131, which is found in clinical strains of HCMV, is required for pH-dependent entry via endocytosis into endothelial and epithelial cells (Ryckman, Jarvis et al. 2006; Ryckman, Rainish et al. 2008).

ii. Post-Entry

Fusion of the virus and cell membranes is followed by entry of the nucelocapsid and tegument proteins into the host cell cytoplasm. Some of the viral tegument

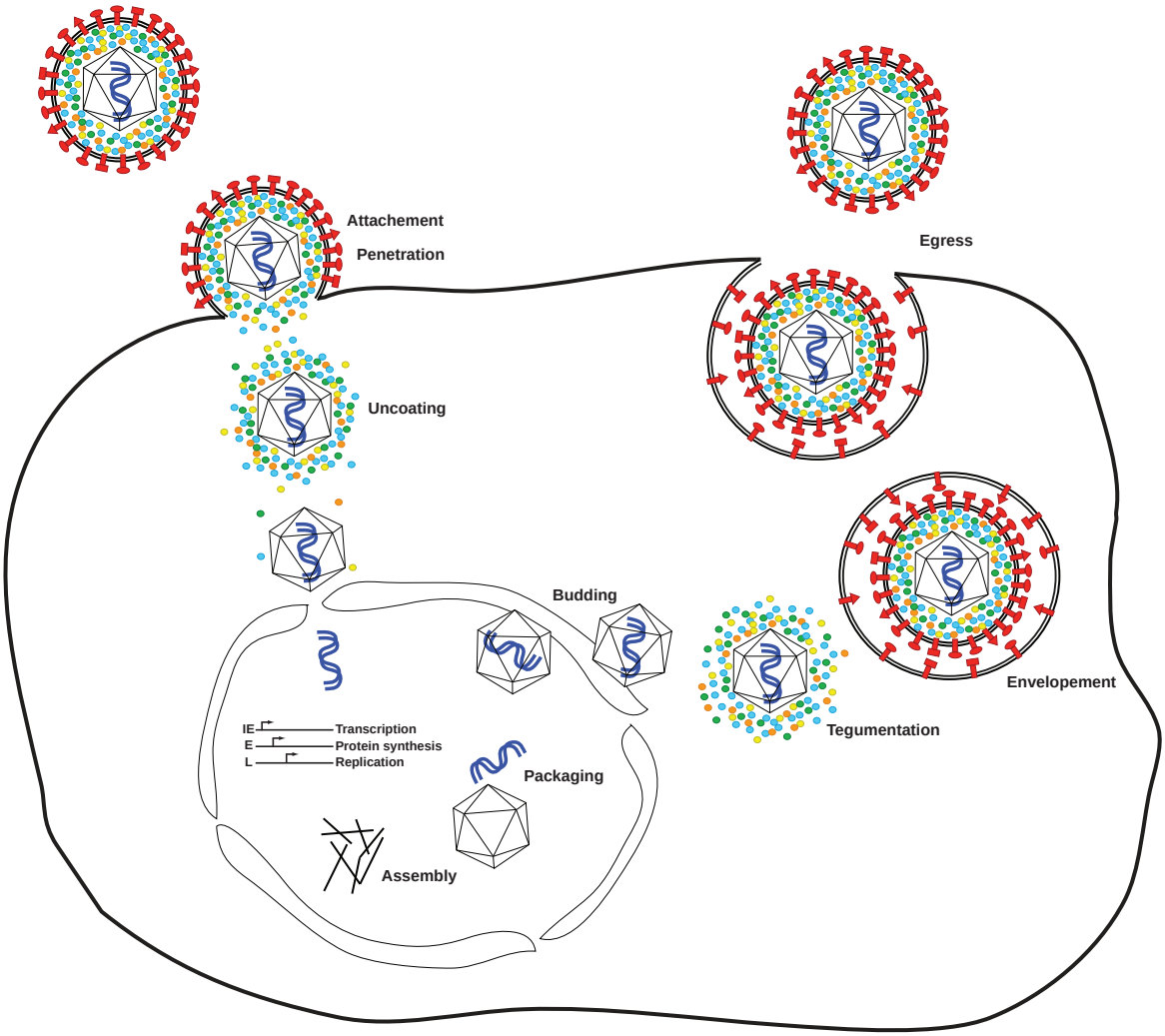

Figure 1.3 The HCMV lifecycle. Virus attachment to the host cell and penetration into the cytoplasm is mediated by the interaction between viral glycoproteins and cellular proteins. Once in the cytoplasm, the tegumented capsids uncoat and are delivered to the nucleus where the capsids dock with nuclear pores and inject the viral DNA into the nucleus. Gene expression occurs in three kinetic phases IE, E and L generating the necessary components for viral DNA replication and virion assembly. Once viral DNA replication has begun, capsid scaffold proteins assemble into nucleocapsids. The progeny DNA genomes are packaged into assembled nucleocapsids that acquire nuclear tegument proteins before budding through the inner and outer nuclear membranes into the cytoplasm. Tegumentation is completed in the cytoplasm prior to final envelopment, which occurs by budding into cytoplasmic vesicles. Mature virions exit via the secretory-exocytic pathway to the cell surface. Fusion with the plasma membrane releases progeny virions.

proteins (pp150, pUL47 and pUL48) remain associated with the viral nucleocapsids and mediate the rapid translocation of the capsid to the nucleus along the microtubule network (Schmolke, Drescher et al. 1995; Bechtel and Shenk 2002; Ogawa-Goto, Tanaka et al. 2003). Dynein, a cellular motor protein, facilitates this process in HSV capsid transport (Dohner, Wolfstein et al. 2002). Through unknown mechanisms, nucleocapsids dissociate from microtubules, dock at nuclear pore complexes and release their DNA into the nucleus. The nuclear viral DNA rapidly associates with histones and chromatin remodeling proteins such as Daxx, which act to suppress viral transcription (Tang and Maul 2006; Everett and Chelbi-Alix 2007). Other tegument proteins such as pp65 and pp71 are transported to the nucleus independently of the capsid. Once in the nucleus, pp71 acts to inactivate Daxx and promote viral gene transcription (Hofmann, Sindre et al. 2002).

iii. Viral Gene Expression

During productive infection, CMV gene transcription, like that of other herpesviruses, is controlled temporally in three major kinetic categories including immediate-early (IE or α), early (E or β), and late (L or γ) genes (Figure 1.4). Categorization of viral gene expression was originally determined by timing and sensitivity to drugs that target protein synthesis (i.e. cycloheximide) or the viral DNA polymerase (i.e. ganciclovir). The general pattern of herpesvirus gene expression has been obtained from studies where cultured cells are infected under selected and/or optimized conditions. In addition, these studies were

typically focused on only a subset of viral genes that were thought to be important. More recently virus-specific DNA microarray analysis has proven to be a valuable tool to measure global viral gene transcription of herpesviruses (Chambers, Angulo et al. 1999; Stingley, Ramirez et al. 2000; Goodrum, Jordan et al. 2002; Goodrum, Jordan et al. 2004; Inkinen, Lahesmaa et al. 2005; Tang, Murphy et al. 2006; Streblow, van Cleef et al. 2007; Chan, Bivins-Smith et al. 2008). The first microarray analysis of CMV gene expression examined 151 of the approximately 200 known HCMV ORFs and classified their temporal kinetic class in human fibroblasts (Chambers, Angulo et al. 1999). Interestingly, the study found the presence of potential regulatory motifs common to the promoters of a kinetic class, implying HCMV can regulate viral gene transcription using the cis-elements built into the viral genome. Stingley et al. a year later examined HSV-1 transcription using microarray analysis of infected HeLa cells and rabbit skin cells (Stingley, Ramirez et al. 2000). The study revealed that the cell type infected is significant to the relative proportion of viral transcripts detected at different times and conditions of infection. Cell type specific differences in CMV gene expression were also investigated in HCMV infected $CD34^+$ hematopoietic progenitor cells as compared to fibroblasts (Goodrum, Jordan et al. 2002). The microarray results showed that HCMV infected $CD34^+$ hematopoietic progenitor cells expressed a small subset of mRNAs, which were not expressed in the established temporal cascade typical of lytic infection of fibroblasts. Furthermore, the HCMV genes expressed were typically not essential for replication in fibroblasts. Reactivation from infected $CD34^+$ hematopoietic progenitor cells

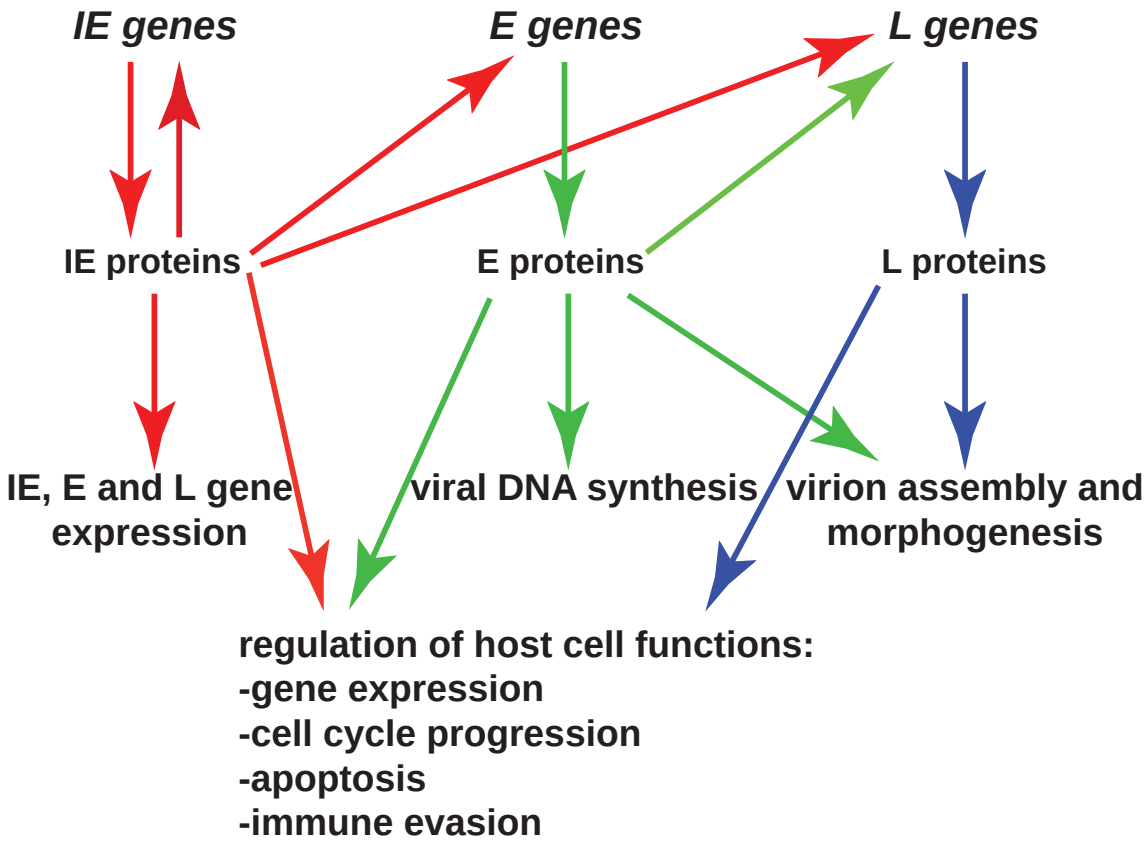

Figure 1.4 HCMV gene expression and function. The cascade of HCMV gene expression is temporally controlled. Immediate early (IE) proteins are necessary for early (E) and late (L) gene expression. E proteins are necessary for viral DNA synthesis while L genes are not expressed until viral DNA replication has occurred. IE, E and L proteins all regulate host cell functions. Adapted from (Landolfo, Gariglio et al. 2003).

suggested these cells were latently infected and the authors concluded that the profile of HCMV gene expression is distinct and may include genes that contribute to latency. Subsequently, the mouse cytomegalovirus (MCMV) transcriptome was examined in infected NIH 3T3 fibroblasts as well as the macrophage cell line IC21 (Tang, Murphy et al. 2006). Ninety percent of viral transcripts were detected in infected fibroblasts at 24 hours post-infection (hpi) with two ORFs specifically expressed in the macrophage cell line. The authors speculate that the viral transcripts not detected in MCMV infected fibroblasts are potentially expressed in specific cell types during the viral replicative lifecycle or during reactivation from latency. As discussed in Appendix 1 our study of rat CMV (RCMV) gene expression revealed that CMV gene expression is cell- and tissue- type specific (Streblow, van Cleef et al. 2007). Microarray analysis of different RCMV infected cells in culture and tissues from RCMV infected rats revealed that viral gene transcription *in vitro* does not reflect viral gene transcription *in vivo*. Lastly, as monocytes are believed to be important for HCMV latency, persistence and dissemination, microarray analysis demonstrated that HCMV changed the host transcriptional profile of infected human monocytes *ex vivo* (Chan, Bivins-Smith et al. 2008). The study showed that most of the induced genes in response to infection are associated with an activated macrophage phenotype potentially affecting subsequent viral transcription. Therefore, the question that remains is what are the potential gene regulation strategies used by CMV to allow for the differences in viral gene expression between tissues and during the different phases of the CMV lifecycle? The above studies illustrate

different potential aspects of regulation. Viral promoters and enhancers are important for controlling viral temporal gene regulation as many genes in a specific kinetic class contain common potential regulatory motifs within their promoter regions. A second approach is through the use of specific viral proteins, such as the IE proteins, that are known as powerful gene transactivators and at late times post infection can act as repressors of viral gene transcription. Third, the cellular environment is important. Depending upon the cell type infected, the host can contain specific transcription factors and the presence or absence of these factors can influence viral gene transcription. The activation and differentiation state of the infected cell are also significant to viral transcriptional profiles. Lastly, microRNAs regulate viral gene expression and are introduced below.

Numerous transcriptional control regions exist within the viral genome and are active at various times during the infection cycle. RNA polymerase II and its associated basal transcription machinery are used to transcribe the HCMV genome in the nucleus (Fortunato and Spector 1999). Productive HCMV infection requires efficient expression of IE genes, which is driven by the major immediate early promoter (MIEP) and adjacent enhancer region in a tissue- and cell-type-specific manner (Nelson, Gnann et al. 1990; Meier and Stinski 1996). The MIEP of HCMV is considered to be one of the strongest promoters and is commonly used in mammalian expression vectors. The MIEP enhancer is a large genomic region (>1 kilobase of DNA) that controls transcription of both the IE1 and the IE2

genes (Hermiston, Malone et al. 1987; Hermiston, Malone et al. 1990). The MIEP enhancer upstream region is only necessary for infection at a low MOI while a promoter proximal region is critical for MIEP expression under all conditions (Isomura and Stinski 2003; Isomura, Tsurumi et al. 2004). IE2 interacts with a cis-acting repressive sequence between the TATA box and the transcription initiation site to repress MIEP expression (Huang and Chen 2002). Within the MIEP enhancer of HCMV are encoded several binding sites for cellular transcription factors such as NF-κB, AP-1, Sp1, and CREB/ATF (Boshart, Weber et al. 1985; Stinski and Roehr 1985; Meier and Stinski 1996). The availability of transcription factors in specific cell types or during specific differentiation states contributes to the activity of the MIEP and plays an important role in determining the permissiveness of cells for HCMV IE expression (Kohwi-Shigematsu and Nelson 1988; Lubon, Ghazal et al. 1989; Shelbourn, Kothari et al. 1989; Ghazal, Lubon et al. 1990; Kothari, Baillie et al. 1991; Sinclair 2009). The MIEP plays an essential role in regulating viral gene expression immediately following entry and is thought to also play a transcriptional role in latency and reactivation (Reeves, MacAry et al. 2005; Wright, Bain et al. 2005).

iv. Immediate Early Viral Genes

IE transcription occurs in the absence of *de novo* protein synthesis by host transcription factors usually within 1 hour post-infection (Wathen, Thomsen et al. 1981). Therefore, the IE transcripts are produced during treatment with cycloheximide, an inhibitor of protein translation. In general, the IE proteins are

viral transcriptional transactivators that also regulate expression of a number of host genes. IE proteins also mediate the shut down of host cell defenses and activate the expression of the viral early genes. The major IE gene products, IE1 (IE72) and IE2 (IE86), are expressed as alternatively spliced transcripts from the UL122/123 locus (Spector 1996; Stenberg 1996). IE1, a nuclear phosphoprotein is not essential for viral growth in fibroblasts when infected at a high multiplicity of infection but is required for replication during multistep growth following infection at a low multiplicity (Mocarski, Kemble et al. 1996). IE1 functions to positively autoregulate expression of IE genes and to augment IE2 activation of E and L genes (Mocarski, Kemble et al. 1996; Fortunato and Spector 1999). IE2 is essential for the HCMV lifecycle and is the master regulator of HCMV transcription. IE2 promotes the transition from IE to E gene transcription and also regulates L gene transcription (Marchini, Liu et al. 2001; Heider, Bresnahan et al. 2002; White, Clark et al. 2004). Another important function of IE2 is the down-regulation of transcription from its own promoter, therefore, IE2 mediates autoregulation of its own expression and contributes to the reduction of IE gene expression in the late stages of infection (Pizzorno and Hayward 1990; Cherrington, Khoury et al. 1991; Liu, Hermiston et al. 1991; Macias and Stinski 1993). Both IE proteins are involved in reactivation of lytic virus from latency by promoting viral gene expression; however the mechanisms involved in this process are mostly unknown (Meier and Stinski 2006). In addition, both IE1 and IE2 transactivate cellular gene promoters (dihydrofolate reductase, DNA polymerase α, c-*fos*, c-*myc*, NF-κB p65 subunit, and thymidylate synthase

genes) (Hagemeier, Walker et al. 1992; Wade, Kowalik et al. 1992; Hayhurst, Bryant et al. 1995; Yurochko, Kowalik et al. 1995; Gribaudo, Riera et al. 2002) and interact with cellular transcription factors (histone acetyl-transferase, CREB, Sp1, c-Jun, and ATF-2) and cell cycle regulators (p53, pRb, and p21) to promote viral gene expression and replication (Hagemeier, Caswell et al. 1994; Sommer, Scully et al. 1994; Speir, Modali et al. 1994; Fortunato, Sommer et al. 1997). Other less abundant IE proteins, TRS1, IRS, US3 and UL36-38, have diverse roles in viral replication and these genes are, for the most part, dispensable for replication *in vitro*. TRS1 and IRS assist IE1 and IE2 transactivation of HCMV early gene expression. US3 is an immune evasion protein involved in the down-regulation of MHC class I (Stasiak and Mocarski 1992; Jones, Wiertz et al. 1996) while the UL36-38 region encodes anti-apoptotic proteins that inhibit the caspase cascade (Skaletskaya, Bartle et al. 2001; Goldmacher 2002).

v. Early Viral Genes

The E genes encode for a number of nonstructural proteins involved in viral DNA synthesis, proteins that act during capsid assembly, modify the cellular environment for replication or block host anti-viral responses (Mocarski, Shenk et al. 2007). E genes are distinguished by their dependence on the presence of functional IE proteins, thus their expression is sensitive to drugs that block protein expression (cycloheximide). Another distinguishing feature that differentiates early genes from late genes is their ability to be synthesized in the presence of inhibitors of viral DNA replication (Ganciclovir, Foscarnet) (Wahren

and Eriksson 1985; Wahren, Larsson et al. 1987; Fortunato and Spector 1999). E gene expression is divided into two subclasses; the β1 (early) genes are transcribed from 4-8 hpi and the β2 (early-late) genes are transcribed within 8-24 hpi. E genes induce the infected host cell to enter the cell cycle but block the cell from entering S phase thus allowing viral genome replication in the nucleus without competing with cellular genome replication (Kalejta and Shenk 2003). Early gene products such as UL54 (DNA polymerase), UL44 (polymerase processivity factor), UL57 (single-stranded DNA-binding protein), and the heterotrimeric helicase-primase consisting of UL70, UL102, and UL105 are required for viral replication at the origin of lytic replication (oriLyt) (Smith and Pari 1995; McMahon and Anders 2002; Mocarski, Shenk et al. 2007).

vi. Viral DNA Replication

Herpesviruses use two different strategies to initiate DNA replication. In HCMV and other β-herpesviruses, except the Roseolavirus family, initiation of DNA synthesis is controlled by IE transactivators and depends on transcriptional activation at the oriLyt, a replication strategy that is similar to the γ-herpesviruses (Mocarski, Shenk et al. 2007). α-herpesviruses and members of the Roseolavirus genus of β-herpesviruses encode origin-binding proteins (OBP) to control initiation at OBP sites within the origin of replication. OBPs nucleate the DNA replication machinery and play a role in initiating DNA replication (McGeoch, Dalrymple et al. 1988; Inoue, Dambaugh et al. 1994). OBP homologs or sites are not encoded in other β-herpesviruses or in γ-herpesviruses.

All β-herpesvirus genomes contain a positionally conserved single oriLyt in the middle of the UL region (Hamzeh, Lietman et al. 1990; Anders and Punturieri 1991; Anders, Kacica et al. 1992; Masse, Karlin et al. 1992). The large (~1500 bp) region of the oriLyt required to support CMV-dependent DNA replication is complex and the essential core region includes a pyrimidine-rich sequence, reiterated elements, direct and inverted repeats, transcription factor-binding sites, and sites that form RNA-DNA hybrid structures (Anders, Kacica et al. 1992; Masse, Karlin et al. 1992; Zhu, Huang et al. 1998; Borst and Messerle 2005). The significance of the RNA-DNA hybrid structure is not understood but it contains multiple G+C-rich repeat sequences that are able to form a stem loop arrangement and interact with UL84 (Prichard, Jairath et al. 1998; Colletti, Xu et al. 2005).

An essential element of the oriLyt also includes a promoter element involved in initiating and regulating lytic DNA synthesis (Huang and Chen 2002). The promoter element contains IE2 binding sites proposing viral transcription is involved in the activation of DNA synthesis (Xu, Cei et al. 2004). HCMV IE2 and UL84 binding to the oriLyt induce the start of viral DNA replication (Xu, Cei et al. 2004). UL84 is a unique protein that has no known homolog to any other viral or cellular protein. EBV and KSHV also contain promoter elements within lytic origins essential for viral replication (Hammerschmidt and Sugden 1988; Aiyar, Tyree et al. 1998; Wang, Li et al. 2004). HCMV DNA synthesis, inversion and

packaging occur later than 24 hpi in the nucleus of *in vitro* infected fibroblasts. HCMV does not encode enzymes necessary for dNTP synthesis and, therefore, relies on host cell metabolism, which is also induced by virus infection (Chee, Bankier et al. 1990; Lembo, Gribaudo et al. 1999; Gribaudo, Riera et al. 2002; Gribaudo, Riera et al. 2003). While HCMV stimulates proteins necessary for DNA replication the virus inhibits cellular DNA synthesis and infected cells do not divide during viral DNA replication (Bresnahan, Boldogh et al. 1996).

As with other herpesviruses, HCMV replication associates with nuclear domain 10 (ND10) sites where initiation of IE gene transcription also occurs (Everett 2006). pp71 interaction with Daxx causes derepression of viral IE gene expression and stimulates replication (Cantrell and Bresnahan 2005; Preston and Nicholl 2006). pp71 prevents recruitment of Daxx-dependent histone deacetylases (HDAC) to viral DNA (Saffert and Kalejta 2006). Regulation of HDAC is important in fully permissive cells as well as during latency in cells such as myeloid progenitors (Reeves, Lehner et al. 2005; Reeves, MacAry et al. 2005). Once replication is initiated HCMV uses the herpesvirus core set of six replication fork proteins to synthesize viral DNA, which includes UL44, UL54, UL57, UL102, UL105 and UL170 (Pari and Anders 1993; Pari, Kacica et al. 1993). The core six proteins along with four UL112-113 gene products and the transactivators form the replication compartment (Park MY 2006 JV, Penfold M 1997 Virology). The HCMV viral genome circularizes within 4 hpi following entry and associates with sites of viral transcription where DNA synthesis initiates

(Ishov and Maul 1996; Ishov, Stenberg et al. 1997; Ishov, Vladimirova et al. 2002). Initiation is followed by bidirectional theta DNA replication that switches to late-phase rolling-circle replication by 16 hpi, resulting in concatemeric viral DNA (McVoy and Adler 1994). Replication begins in one or two regions of the nucleus but gradually forms the hallmark nuclear inclusion of HCMV-infected cells (Penfold and Mocarski 1997). Viral DNA replication peaks between 60 and 80 hpi in HCMV infected fibroblasts.

vii. Late Viral Genes

Late protein synthesis occurs more than 24 hpi and late gene products are typically viral structural proteins involved in assembly and morphogenesis of the virion. The L class, similar to the E class, is further subdivided into two classes. The Leaky late or γ1 genes are transcribed at approximately 24-36 hpi and inhibitors of viral DNA replication may or may not modulate their expression. The true late or γ2 genes are transcribed at 24-48 hpi and their expression is strictly dependent on prior viral DNA replication (Leach and Mocarski 1989; Depto and Stenberg 1992; Adam, Jervey et al. 1995; Mocarski, Shenk et al. 2007).

viii. Virion Assembly and Egress

The assembly precursor (pAP, pUL80.5) and protease precursor (pPR, pUL80a) coordinate capsid assembly but are ultimately eliminated from the mature capsid (Gibson 1996). Procapsid formation begins in the cytoplasm where MCP interacts with pAP in a 1:1 stoichiometric ratio (Beaudet-Miller, Zhang et al. 1996;

Wood, Baxter et al. 1997). This complex translocates into the nucleus where oligomerization is catalyzed by pAP leading to the formation of hexons and pentons (Plafker and Gibson 1998). These larger assemblies interact with mCP and mC-BP to form the capsid precursor (Newcomb, Homa et al. 1999). The capsid precursor becomes decorated with SCP at the tips of the hexons in a process that completes the formation of capsids (Lai and Britt 2003; Yu, Shah et al. 2005). At one site on the capsid pUL104 forms a portal through which the viral DNA genome is loaded (Butcher, Aitken et al. 1998). Direct interaction of pUL56 and pUL89 (DNA terminase and cleavage/packaging enzyme), which bind to packaging (pac) sites at or near the ends of replicating, concatameric viral DNA, position the DNA for packaging into capsids at the portal protein (pUL104) (Thoma, Borst et al. 2006). Viral DNA is cleaved into unit lengths and packaged into capsids when terminase (pUL56) arrives at the next pac site (Bogner, Radsak et al. 1998; Scheffczik, Savva et al. 2002; White, Stow et al. 2003).

The current model of HCMV egress supports a two-stage envelopment process that begins in the nucleus and leads to virion release by exocytosis at the plasma membrane. This mechanism of envelopment/de-envelopment/re-envelopment has been controversial but is now widely accepted based on studies utilizing egress mutant viruses as well as electron microscopic analysis (Gibson 1996; Mettenleiter 2002; Leuzinger, Ziegler et al. 2005; Campadelli-Fiume and Roizman 2006). Nuclear egress begins when nucleocapsids bud into the inner nuclear membrane. These enveloped particles then bud out of the outer nuclear

membrane into the cytoplasm and are subsequently de-enveloped in a process that requires two core proteins UL50 and UL53 (Gibson 1996; Mettenleiter 2004). Tegumentation of nucleocapsids occurs in the nucleus, perinuclear space and the cytoplasm, supporting the envelopment/de-envelopment/re-envelopment theory of viral egress (Hensel, Meyer et al. 1995; Sanchez, Greis et al. 2000; Sanchez, Sztul et al. 2000). Strong evidence suggests that a stable perinuclear cytoplasmic compartment also called the virion assembly compartment exists wherein virion tegument and envelope glycoproteins accumulate during the late stage of the infectious cycle (Sanchez, Greis et al. 2000; Sanchez, Sztul et al. 2000; Homman-Loudiyi, Hultenby et al. 2003). Non-enveloped but completely tegumented capsids acquire their final envelope and viral glycoproteins by budding into cytoplasmic vesicles resulting in viral particles that also contain small amounts of cellular protein and RNA (Eickmann, Gicklhorn et al. 2006). Mature infectious virions within the vesicles exit the host cell via the secretory-exocytic pathway after the vesicles fuse to the plasma membrane at approximately 72 hours post-infection (Eickmann, Gicklhorn et al. 2006; Mocarski, Shenk et al. 2007).

F. Cellular Tropism

During natural infection HCMV productively infects nearly all organ systems in the human body. As such, the virus is capable of infecting multiple cell types. The most prominent *in vivo* target cells include epithelial cells, endothelial cells, smooth muscle cells and fibroblasts (Sinzger, Digel et al. 2008). HCMV can also

productively infect connective tissue cells and parenchymal cells in various organs as well as hematopoietic cell types including monocyte/macrophages (Sinzger, Digel et al. 2008). Lymphocytes and polymorphonuclear leukocytes are two cell types that are generally considered non-permissive for HCMV replication (Grefte, Harmsen et al. 1994). HCMV transmission primarily occurs through contact with virus shed in bodily fluids. Therefore, infection of epithelial cells of the salivary glands, mammary tissues, kidney, and intestinal mucosa is critical for viral transmission.

Infection of hematopoietic stem cells, monocytes and/or dendritic cells is thought to be critical for dissemination of viral infection. Dendritic cells (DCs) are capable of taking up virions at mucosal surfaces and distributing the virus to secondary lymphoid tissues (Hertel, Lacaille et al. 2003). Infection of DCs has been shown to alter their function as well as prevent their ability to mount an effective immune response (Grigoleit, Riegler et al. 2002; Moutaftsi, Mehl et al. 2002; Hertel, Lacaille et al. 2003). Studies have shown CMV infection of DCs down-regulates MHC class II as well as CCR1 and CCR5 cell surface expression leading to a reduction in stimulation of T cell proliferation and migration respectively (Varani, Frascaroli et al. 2005; Varani, Frascaroli et al. 2005). Hematopoietic stem cells and monocytes can be infected *in vitro*, either through direct infection or by co-culture with infected endothelial cells (Maciejewski, Bruening et al. 1992). In fact, infected monocytes are also capable of transendothelial migration, which induces their differentiation into tissue macrophages capable of lytic viral replication

(Ibanez, Schrier et al. 1991; Smith, Bentz et al. 2004). Infection of monocytes is potentially one of the most significant modes of virus dissemination to various tissues during primary infection or reactivation from latency in hematopoietic stem cells.

Infection of organ parenchymal cells such as hepatocytes is considered a major source of virus during acute viremia and may help to maintain viral persistence (Emery, Cope et al. 1999; Sinzger, Bissinger et al. 1999). Lytic infection of these cell types can lead to organ dysfunction by promoting chronic inflammation (Britt 2008). The human vasculature is an additional site of HCMV persistence (Streblow, Orloff et al. 2007). Viral DNA and proteins have been detected in vascular endothelial cells, smooth muscle cells, and macrophages at sites of turbulent flow and near sites of atherosclerosis (Pampou, Gnedoy et al. 2000). Infection of the vasculature is associated with the acceleration of vascular disease (Melnick, Adam et al. 1998; Sorlie, Nieto et al. 2000).

Clarifying the viral determinants of HCMV infection of clinically relevant cell types has been an important topic of recent research efforts. In general, the viral determinants of cellular tropism include genes with functions involved in entry (gH/gL interactions with gO vs. pUL128-131), inhibition of apoptosis, replication, virion morphogenesis and virion egress. Loss of these functions has been attributed to passage of clinical virus strains in culture, a process that generates a number of mutations, deletions, and rearrangements in the UL region. In fact,

the highly passaged tissue culture laboratory-adapted strain AD169 is missing 22 genes from the ULb' region, some of which are required for infection of epithelial and endothelial cells as well as the maintenance latency (UL138) (Cha, Tom et al. 1996; Hahn, Revello et al. 2004; Wang and Shenk 2005; Wang and Shenk 2005; Adler, Scrivano et al. 2006; Goodrum, Reeves et al. 2007; Ryckman, Rainish et al. 2008).

G. Persistence and Latency

HCMV infections are life-long, however, there is still controversy as to whether persistent HCMV infection is a chronic productive infection in sequestered sites, a true latent infection with periodic reactivation or a combination of both mechanisms. Following primary HCMV infection and a robust immune response, latency is established. The mechanisms of HCMV latency (character of the latent state, how latency is maintained, reactivation to the lytic state) are still relatively unknown when compared to the knowledge of CMV molecular biology and anti-CMV immunology. HCMV persistence involves the continual production of low levels of virus, whereas HCMV latency involves the maintenance of the viral genome without production of infectious virions, but retains the ability to reactivate under specific stimuli. The critical sites of viral persistence and latency remain unclear, however, proposed sites for persistence include ductal epithelial cells and mucosal epithelium (Kloover, Hillebrands et al. 2000; Chen and Hudnall 2006). Sites of latency may include bone marrow, monocyte/myeloid lineage, endothelial cells and smooth muscle cells (Lemstrom, Bruning et al. 1993;

Minton, Tysoe et al. 1994; Fish, Soderberg-Naucler et al. 1998). The idea that the myeloid lineage or undifferentiated monocytes contain latent HCMV infection is attractive as these cells would be able to distribute the virus throughout the body (Taylor-Wiedeman, Sissons et al. 1991; Fish, Britt et al. 1996; Sinclair and Sissons 1996; Sindre, Tjoonnfjord et al. 1996; Hahn, Jores et al. 1998; Prosch, Docke et al. 1999; Soderberg-Naucler, Streblow et al. 2001). Differentiation or activation of latently infected monocytes releases latent infection, which promotes lytic virus replication and virus production (Taylor-Wiedeman, Sissons et al. 1991; Soderberg-Naucler, Fish et al. 1997; Hahn, Jores et al. 1998; Prosch, Docke et al. 1999; Reeves, Lehner et al. 2005; Reeves, MacAry et al. 2005). Latency is tied to the expression of IE viral proteins. Repression of IE expression results in a loss of virus production and is thought to involve post-translational modification of histones associated with the major immediate early promoter (Murphy, Fischle et al. 2002; Reeves and Sinclair 2009). Interestingly, the UL138 ORF, part of the ULb' region deleted from laboratory strains of HCMV, acts to promote the development of latency in infected monocytes (Goodrum, Reeves et al. 2007). Virus lacking the UL138 ORF failed to establish/maintain a latent infection and replicated like laboratory strains in cultured $CD34^+$ cells implying that HCMV contains gene products that actively promote and maintain latency (Goodrum, Reeves et al. 2007). Further studies to dissect the role of UL138 in latency are highly warranted.

H. Reactivation

There are two conditions thought to be responsible for HCMV reactivation 1) HCMV reactivation occurs sporadically when certain inflammatory events present in the host or 2) more commonly when latently infected cells differentiate, for example monocytes differentiating into macrophages. Both scenarios can be efficiently controlled by the immune response of a healthy host. In contrast, during organ transplantation the resulting pro-inflammatory environment allows lytic virus replication and subsequent spread throughout the host. Thus, the cellular environment is probably the most critical factor in determining HCMV reactivation. HCMV reactivation *in vivo* has a strong dependence on the host immune system as well as the basic regulation of viral IE expression. Observations from both experimentally and naturally latent cells suggest that reactivation may be promoted by pro-inflammatory signals and is cellular differentiation-state dependent (Jarvis, Wang et al. 1999; Soderberg-Naucler, Streblow et al. 2001; Murphy, Fischle et al. 2002; Reeves, Lehner et al. 2005; Reeves, MacAry et al. 2005). Several *in vitro* models have been developed to examine the mechanisms of HCMV latency and reactivation in monocytes and progenitor cells. A system was developed to examine HCMV latency in granulocyte-macrophage progenitor (GMPs) cells (Kondo, Kaneshima et al. 1994). HCMV infection of GMPs *in vitro* caused a latent infection and reportedly did not affect cell growth or phenotype. However, treatment of these cells with cytokines, promoting myeloid differentiation, induced virus reactivation (Hahn, Jores et al. 1998). Notably, treatment with cytokines promoting differentiation

down the erythroid pathway failed to support latency or reactivation. A second model examines HCMV reactivation in naturally, latently infected $CD14^+$ monocytes. Allogeneic stimulation of peripheral blood mononuclear cells (PBMCs) differentiated monocytes into macrophages in which viral proteins were detected and infectious virus was isolated (Soderberg-Naucler, Fish et al. 1997; Soderberg-Naucler, Streblow et al. 2001). Finally, HCMV infection of cultured $CD34^+$ hematopoietic progenitors on an immortalized murine stromal cell line retained virus for up to 20 days and cells could reactivate virus at 10 days post-infection (Goodrum, Jordan et al. 2002).

I. Antivirals

Currently, five compounds are used clinically for the treatment of HCMV-related diseases (Michel and Mertens 2006). The antiviral drugs have produced clinical improvement by reducing or eliminating viral load in patients. On the other hand, the drugs are accompanied by problematic toxic side effects and their use has been attributed to the creation of drug-resistant HCMV strains rendering the compounds less effective. Ganciclovir (GCV) and Valganciclovir (ValGCV) (orally applicable valine ester of GCV) are guanosine analogs while Cidofovir (CDV) is a cytosine analog. In their tri-phosphorylated forms these drugs are nucleotide analogs that act as competitive inhibitors of the viral DNA polymerase, promoting the slowing of the viral DNA polymerase or termination of DNA chain elongation (Plotkin, Starr et al. 1982; Snoeck, Sakuma et al. 1988; Crumpacker 1996; Curran and Noble 2001). Foscarnet (PFA) is a pyrophosphate analog that binds

selectively to the pyrophosphate binding site of the viral DNA polymerase and works at concentrations that are low enough to not affect cellular DNA polymerases (Chrisp and Clissold 1991). Lastly, the drug Fomivirsen (ISIS2922) is an antisense oligonucleotide directed against HCMV IE2 mRNA and exerts its antiviral effect by inhibiting translation of IE2 mRNA (Azad, Driver et al. 1993; Anderson, Fox et al. 1996).

Emergence of resistant strains are mainly seen in patients undergoing prolonged maintenance therapy (AIDS patients, solid organ transplant recipients (Erice, Chou et al. 1989; Lurain, Spafford et al. 1994; Baldanti, Sarasini et al. 1995; Chou, Erice et al. 1995; Chou, Guentzel et al. 1995; Baldanti, Simoncini et al. 1998; Limaye, Corey et al. 2000; Limaye 2002). Mutations conferring HCMV drug resistance are typically found in two critical viral enzymes, the viral DNA polymerase (UL54) and the viral phosphotransferase (UL97). UL97 phosphorylates GCV and ACV to their monophosphate forms, followed by diphosphorylation to their active triphosphate forms by cellular kinases (Littler, Stuart et al. 1992; Sullivan, Talarico et al. 1992). Importantly, CDV and PFA do not require phosphorylation making them resistant to UL97 mutations (Cihlar, Fuller et al. 1998). PFA resistance is attributed to mutations in UL54 (Baldanti, Underwood et al. 1996). A Fomivirsen-resistant HCMV strain was isolated but the mechanism of resistance is uncertain as the mutant virus still contained an intact IE2 target sequence (Mulamba, Hu et al. 1998).

III. microRNAs

Interest in post-transcriptional regulation of viral and cellular mRNAs has increased recently with the discovery of a new family of noncoding RNAs – microRNAs (miRNAs). As HCMV is a common pathogen in humans and can cause serious morbidity and mortality in congenital infections and in the immunocompromised individual, it is important to understand the regulatory mechanisms controlling CMV gene expression and replication. CMV gene expression, like cellular gene expression, is subject to multiple levels of regulation, which can include initiation of transcription, RNA processing, nuclear transport, and mRNA stability. Infection with β-herpesviruses unlike α- and γ-herpesviruses does not result in a global shutoff of host cellular gene expression. Instead, HCMV infection has been shown to increase the abundance and alter the subcellular localization of cellular post-transcriptional processing factors to ensure maximal viral gene expression (Adair, Liebisch et al. 2003; Su, Adair et al. 2003; Adair, Liebisch et al. 2004; Adair, Liebisch et al. 2006). Post-transcriptional regulation mechanisms, such as miRNAs, provide an ideal mechanism to control viral and cellular protein expression, which would promote a favorable environment for the viral life cycle.

RNA interference (RNAi) is a ubiquitous and highly conserved mechanism found in fungi, plants and animals. Along with a role in the immune response to viruses and other pathogens, RNAi is also used in gene regulation where small noncoding RNAs are used to direct sequence-specific silencing of gene

expression. Classes of small RNAs include miRNAs, small-interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs), which all serve as guide RNAs in RNA silencing but differ in their biogenesis. siRNAs derive from exogenous transcripts or are chopped from endogenous double-stranded transcripts. In addition to their role in the RNAi pathway, siRNAs also act in RNAi-related pathways, such as antiviral mechanisms and shaping the chromatin structure of genomes (Hamilton and Baulcombe 1999; Elbashir, Harborth et al. 2001; Reinhart and Bartel 2002). To date no virus-derived siRNAs have been found in virus infected mammalian cells (Pfeffer, Sewer et al. 2005; Lin and Cullen 2007). piRNAs are expressed in mammalian cells, form RNA-protein complexes through interactions with Piwi proteins and are important in gametogenesis (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Grivna, Beyret et al. 2006; Lau, Seto et al. 2006; Watanabe, Takeda et al. 2006).

A. miRNA Defining Features and Identification

The current criteria that defines miRNAs include size, approximately 22 nucleotides for the mature miRNA and approximately 70 nucleotides for the precursor-miRNA detected by Northern blot. The precursor-miRNA forms a stem loop structure with the mature miRNA present in one arm of the stem loop. Usually both the mature and the precursor miRNAs are phylogenetically conserved. Another test of a true miRNA is when Dicer, an enzyme involved in miRNA biogenesis, function is disrupted then the precursor miRNA should still be observed but the mature miRNA should be absent.

Multiple methods are used to identify miRNAs. Computational methods for miRNA identification include computer algorithms such as Stem-loop Finder (SLF) (Combimatrix) and miRscan (Lim, Glasner et al. 2003; Lim, Lau et al. 2003). The programs predict potential RNA transcripts that can form stem loop secondary structures and determine whether the stem loops encode miRNAs based on structural aspects. Bioinformatics methods can generate false positives as well as imprecisely determine the 5' end of the mature miRNA, which is required for accurate target prediction and function. Cloning and sequencing of small RNA-derived cDNAs is also used to identify miRNAs. Some miRNAs are expressed in a temporal-, spatial- and tissue-specific manner, which make their detection rare. An alternative approach to the discovery of miRNAs that has proven robust and accurate is high-throughput or deep sequencing of small RNAs (Lu, Tej et al. 2005; Babiarz, Ruby et al. 2008; Kuchenbauer, Morin et al. 2008). This method can provide additional information such as quantitation of miRNA expression levels and the detection of rare transcripts and isoforms.

B. miRNA Biogenesis

miRNAs are transcribed by RNA Polymerase II in the nucleus creating the primary transcript called pri-miRNAs (Figure 1.5) (Lee, Jeon et al. 2002). The pri-miRNA stem loop structures are recognized by a nuclear RNase III endonuclease called Drosha in conjunction with its binding partner DGCR8 (DiGeorge syndrome critical region gene 8), which processes the pri-miRNA transcript into a 60 to 100-nucleotide stem loop structure called the precursor-

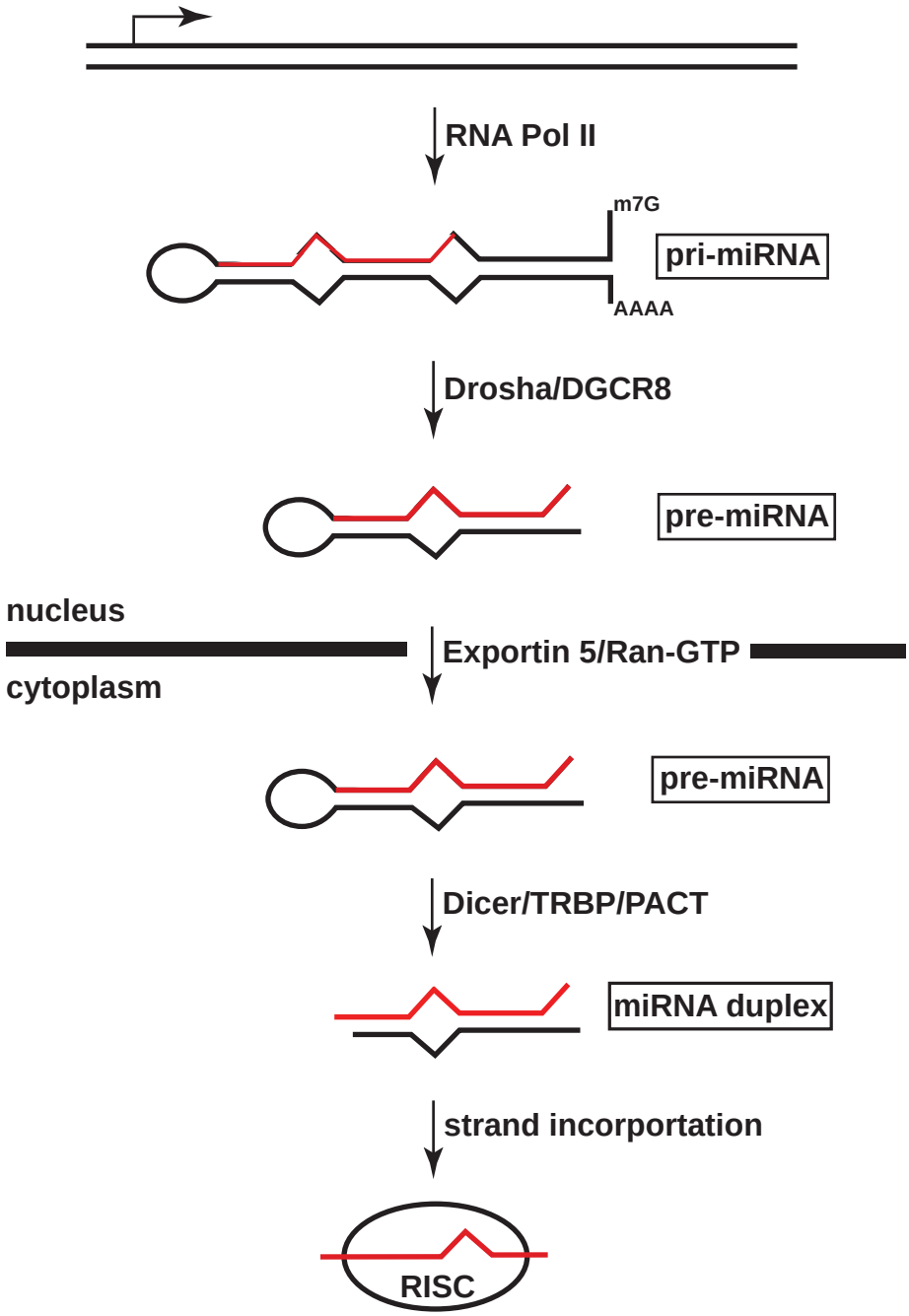

Figure 1.5 miRNA biogenesis. miRNAs are transcribed in the nucleus by RNA polymerase II generating pri-miRNAs that are capped and polyadenylated. Pri-miRNAs are processed by Drosha and DGCR8 producing pre-miRNA stem loops. Exportin-5 and Ran-GTP export pre-miRNAs into the cytoplasm. Dicer, TRBP, and PACT catalyze the final processing step to produce a mature miRNA duplex. One strand is incorporated into RISC. Adapted from (Cullen 2006; Rana 2007; Ganem and Ziegelbauer 2008).

miRNA (pre-miRNA) (Lee, Ahn et al. 2003; Zeng and Cullen 2005; Zeng, Yi et al. 2005; Han, Lee et al. 2006; Yeom, Lee et al. 2006). DGCR8 binds the ssRNA region that is flanking the terminal loop structure (Han, Lee et al. 2006). The Drosha cleavage site is ~11 base pairs from the stem-ssRNA junction (Han, Lee et al. 2006). The pre-miRNA interacts with the nuclear export machinery exportin-5 and Ran-GTP and is exported into the cytoplasm (Yi, Qin et al. 2003; Bohnsack, Czaplinski et al. 2004; Lund, Guttinger et al. 2004). The cofactor Ran binds GTP in the nucleus and upon entering the cytoplasm the GTP is hydrolyzed to GDP releasing the pre-miRNA from exportin-5 (Yi, Qin et al. 2003; Bohnsack, Czaplinski et al. 2004; Lund, Guttinger et al. 2004). In the cytoplasm, Dicer, a cytoplasmic RNase III endonuclease, which works in concert with TRBP (TAR RNA binding protein) and PACT (protein kinase R-activating protein) (Chendrimada, Gregory et al. 2005; Haase, Jaskiewicz et al. 2005; Lee, Hur et al. 2006), catalyzes the final processing step to produce a 21-25 nucleotide mature miRNA/miRNA* duplex containing a 2 nucleotide overhang that typically occurs at the 3'end of the base of the hairpin (Bernstein, Caudy et al. 2001; Grishok, Pasquinelli et al. 2001; Hutvagner, McLachlan et al. 2001; Ketting, Fischer et al. 2001). The miRNA from the corresponding arm of the stem loop (the "star strand") or the strand with the stronger base pairing at the 5' end is released and degraded, though either strand may actually be a functional miRNA (Khvorova, Reynolds et al. 2003; Lim, Lau et al. 2003; Schwartz, Decker et al. 2003). The mature miRNA is incorporated into a ribonucleocomplex called the RNA induced silencing complex (RISC), which uses the miRNA as a guide to

identify and bind target mRNAs (Hutvagner and Zamore 2002; Martinez, Patkaniowska et al. 2002).

C. miRNA Targeting

Target recognition and the rules governing target recognition are essential to determining miRNA specificity and function. The canonical miRNA seed sequence is positioned in the 5' region of the miRNA at nucleotides 2-7 with Watson-Crick base pairing (Lewis, Burge et al. 2005) (Figure 1.6). This 6mer-site needs to perfectly match the target mRNA sequence and enables target prediction above false background levels (Krek, Grun et al. 2005). Three other types of seed-matched sites are conserved including the 7mer-m8 site, 7mer-A1 site, and the 8mer site (Figure 1.6) (Lewis, Burge et al. 2005). The 7mer-m8 comprises the seed match with the addition of a Watson-Crick match to the miRNA at nucleotide 8. The 7mer-A1 site contains the seed match with the addition of an A across nucleotide 1 of the miRNA. Finally, the 8mer site consists of the seed match extended by both the nucleotide match at position 8 and the A at position 1. The hierarchy of site efficacy follows seed matches at the 8mer > 7mer-m8 > 7mer-A1 > 6mer sites with the same true for protein expression (Grimson, Farh et al. 2007; Nielsen, Shomron et al. 2007; Selbach, Schwanhausser et al. 2008). Depending on the search parameters, specificity vs. sensitivity, a search for a conserved 8 nt site yields greater prediction specificity whereas a search for a conserved 6 nt seed match yields greater sensitivity. Additional factors influence miRNA targeting including: 1) Watson-Crick base

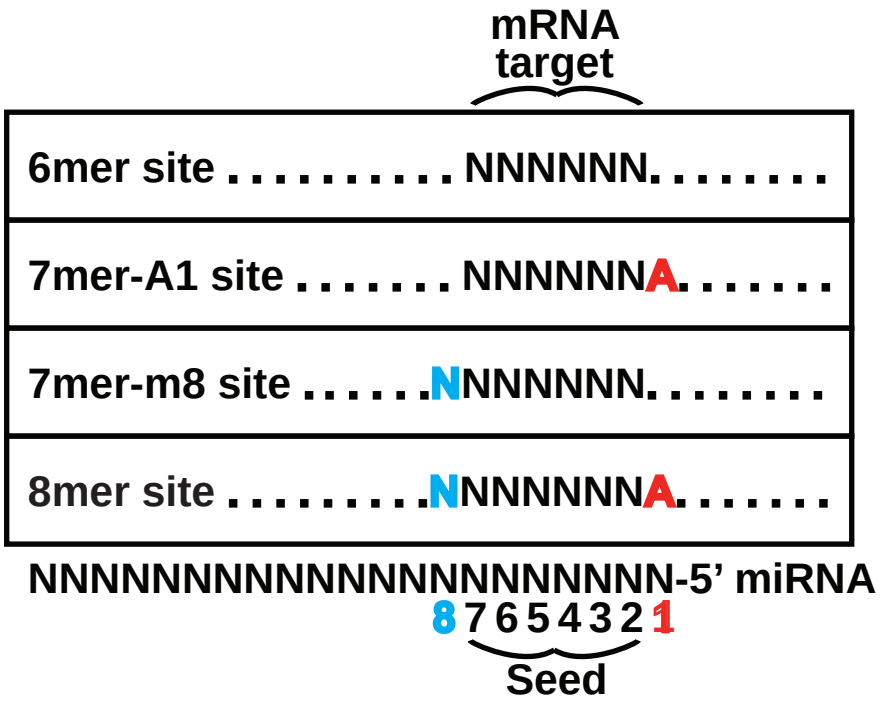

Figure 1.6 Canonical miRNA target sites. Canonical seed match sites include nucleotides 2-7 (6mer) of the miRNA with an additional A match at nucleotide 1 (7mer-A1) or a nucleotide match at position 8 (7mer-m8). A target search for an 8mer site including both the A1 and 8 nucleotide matches increases prediction specificity. Adapted from (Grimson, Farh et al. 2007; Baek, Villen et al. 2008).

pairing at nucleotides 12-17 of the miRNA. 2) miRNA seeds within a locally AU-rich sequence. 3) Target sites in the 3' UTR are at least 15 nucleotides from the stop codon. Lastly, target sites are away from the centers of long UTRs i.e. reside near either end. These additional restrictions possibly increase site accessibility (weaken mRNA secondary structure) and site affinity for the association and dissociation of RISC (Grimson, Farh et al. 2007).

To validate miRNA:mRNA interactions multiple methods are used including indirect and direct experimental techniques. Indirect methods based on high-throughput experiments including transcriptional microarray analysis and proteomics (i.e. stable isotope labeling with amino acids in cell culture (SILAC)) are able to identify overall changes in a large number of genes or proteins (Selbach, Schwanhausser et al. 2008). These methods give indirect information and may be complicated by secondary and nonspecific or off-target effects. Conversely, direct experimental methods to validate specific miRNA:mRNA interactions include the quantification of a reporter construct (luciferase, GFP) that contains the 3' UTR of a potential mRNA target transfected alongside the miRNA into cells (Kiriakidou, Nelson et al. 2004). After transfecting the miRNA of interest, quantitative RT-PCR can be used to report levels of the target mRNA. This method can potentially have a high false-negative rate by missing targets that are repressed at the translational level rather than degraded, so protein analysis also needs to be used. These *in vitro* assays must be followed up with

site directed mutagenesis of the miRNA or 3’ UTR to disrupt the miRNA:mRNA interaction to show specificity of the seed sequence for the target.

D. RISC and miRNA post-transcriptional regulation

RISC is hypothesized to identify its target by either scanning mRNAs for target sites similar to the way in which ribosomes locate translational start sites or through a diffusion-controlled mechanism where RISC binds nonspecifically until the matched target is found (Kozak 1999). The type of post-transcriptional regulation elicited by RISC depends upon the location of the target sequence and the level of base pairing between the miRNA and the target (Figure 1.7). For example, if the miRNA is a perfect or near-perfect match then RISC can mediate mRNA cleavage, which is often observed in plants (Elbashir, Harborth et al. 2001; Elbashir, Lendeckel et al. 2001; Selbach, Schwanhausser et al. 2008). If the miRNA sequence is less homologous to the mRNA or if the target sequence is located within the 3’ UTR of the mRNA, then translational repression occurs by blocking ribosomal initiation or elongation or mRNA destabilization occurs by poly-(A) shortening (Lee, Feinbaum et al. 1993; Zeng, Wagner et al. 2002; Lim, Lau et al. 2005). The latter mechanism is most often observed for mammalian and viral miRNA targets.

The miRNA duplex is inserted into the Argonaute (AGO) protein subunit of RISC and then the two strands are unwound and one strand dissociates from AGO (Kawamata, Seitz et al. 2009). Strand selection is not random but depends on

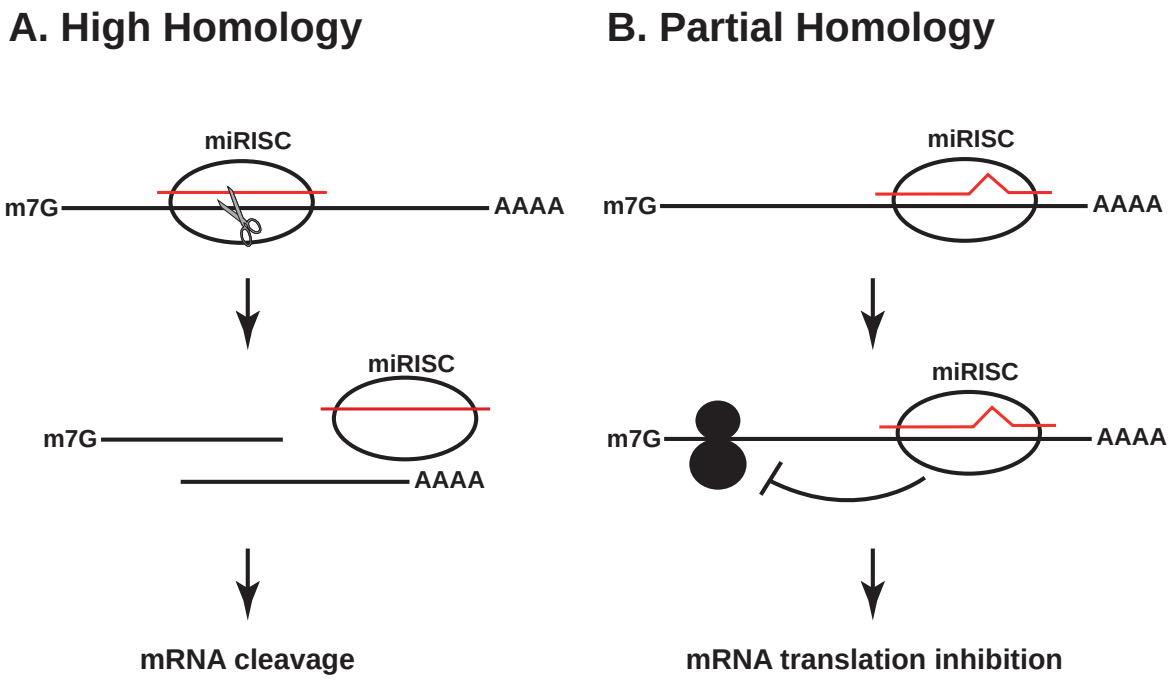

Figure 1.7 Mechanisms of miRNA post-transcriptional regulation.
A. Perfect or near perfect homology between the miRNA and the target mRNA leads to RISC mediated mRNA cleavage.
B. Low homology between the miRNA and the 3' UTR of the target mRNA leads to translational repression. Adapted from (Bartel 2004).

the relative thermodynamic stability of the first 1-4 bases at each end of the RNA duplex where the less stable strand serves as the guide strand and the other is discarded (Schwarz, Hutvagner et al. 2003; Iwasaki, Kawamata et al. 2009). We as well as others have shown that pre-miRNA dicing can produce distinct isoforms of miRNA:miRNA* duplex with 5' and 3' heterogeneity (Ruby, Stark et al. 2007; Wu, Ye et al. 2009; Chiang, Schoenfeld et al. 2010). Differential processing at the 5' end is able to alter the seed sequence (position 2-7), which is necessary for target recognition and can broaden the regulatory impact of the miRNA but can also switch the preference for AGO species (Azuma-Mukai, Oguri et al. 2008; Ghildiyal, Xu et al. 2009). There are four AGO proteins in mammals (AGO1-4) all of which can repress translation of their target mRNAs but it is only AGO2 that is capable of endonucleolytic cleavage with its ribonuclease H-like domain cutting the phosphodiester bond in the target mRNA opposite the 10^{th} and 11^{th} residue in the miRNA (Liu, Carmell et al. 2004; Meister, Landthaler et al. 2004). RISC is minimally composed of the mature miRNA and one of the Argonaute proteins.

The two main structural features of the Argonaute protein family include the PAZ domain and the PIWI domain (Figure 1.8). The PAZ domain contains a binding pocket that anchors the characteristic two-nucleotide 3' overhang of RNAs digested by Dicer (Lingel, Simon et al. 2003; Song, Liu et al. 2003; Yan, Yan et al. 2003; Lingel, Simon et al. 2004; Ma, Ye et al. 2004). The PIWI domain is an RNase H domain that is present in a large number of nucleic acid-binding

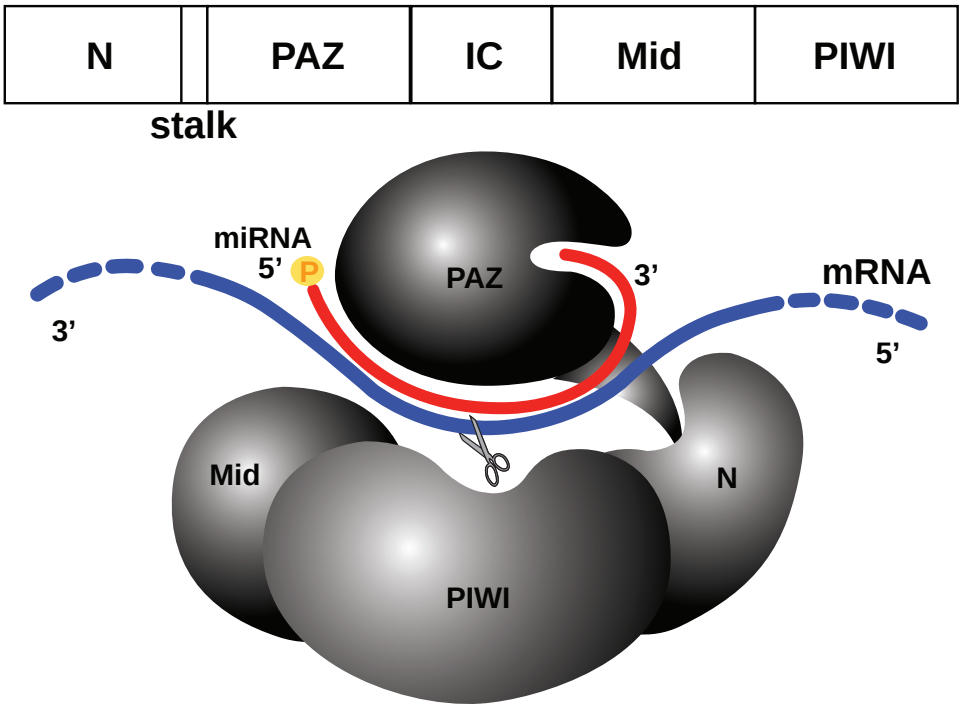

Figure 1.8 Human Argonaute protein 2 (AGO2). AGO2 is subdivided into four domains. The model depicts the miRNA and mRNA interaction with AGO. The PAZ cleft anchors the 3’ end of the miRNA. The PIWI domain contains a catalytic site (scissors) capable of cleaving the bound mRNA. The Middle domain (Mid) functions to bind the 5’ phosphate of the miRNA. N=N-terminal domain. IC=interdomain connector. Adapted from (Song, Smith et al. 2004).

proteins, especially those that bind and cleave RNA (Song, Smith et al. 2004). Another domain called the Mid domain is situated between the PAZ and PIWI domains and this domain anchors small RNAs into the AGO protein by binding the characteristic 5' phosphate (Ma, Yuan et al. 2005; Parker, Roe et al. 2005).

Processing bodies (P-bodies) are specific sites within the cytoplasm that contain proteins important for mRNA metabolism. Since AGO proteins are localized to the cytoplasm and are observed to be concentrated in P-bodies, AGO proteins are proposed to target mRNAs to P-bodies for degradation or translational repression (Figure 1.9) (Sen and Blau 2005). P-bodies are enriched with enzymes for RNA turnover (decapping, deadenylation, exonucleases), contain untranslated mRNA and mRNA decay intermediates and exclude translational machinery (Cougot, Babajko et al. 2004; Rehwinkel, Behm-Ansmant et al. 2005; Teixeira, Sheth et al. 2005; Behm-Ansmant, Rehwinkel et al. 2006; Eulalio, Behm-Ansmant et al. 2007). The current hypothesis proposes that destabilization of the target mRNA takes place in P bodies through deadenylation when imperfect base pairing between the miRNA and the mRNA leads to translational repression (Behm-Ansmant, Rehwinkel et al. 2006; Giraldez, Mishima et al. 2006; Wu, Fan et al. 2006). The translation repressor protein RCK/p54 (RNA helicase) is thought to be the effector molecule that shuttles target mRNAs to P-bodies for storage or processing by decapping enzymes and cap-binding proteins that trigger mRNA decay (Sheth and Parker 2003; Sen and Blau 2005; Chu and Rana 2006). P-bodies are not necessary for translational silencing by miRNAs and miRNA-mediated entry into

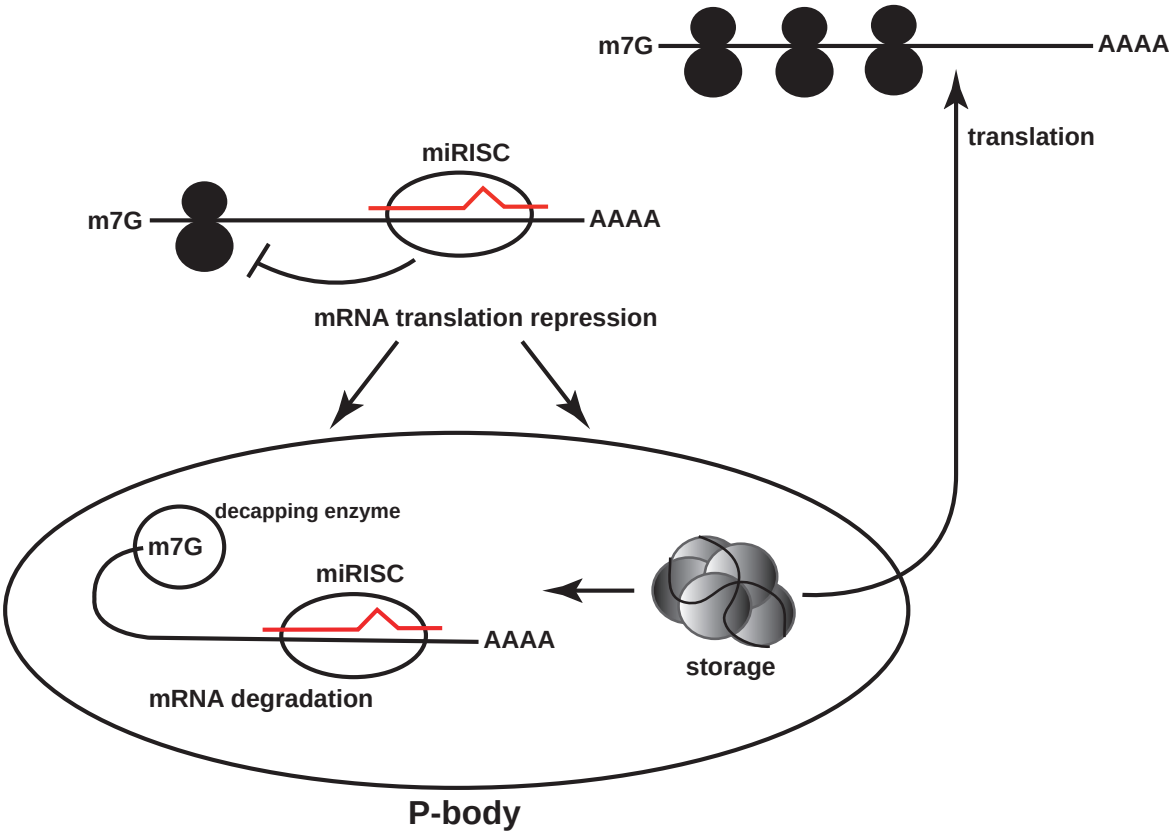

Figure 1.9 Mechanisms of miRNA translational repression.
AGO proteins of RISC target mRNAs to P-bodies for degradation or translational repression. mRNAs can exit P-bodies and resume translation. Adapted from (Chu and Rana 2006; Rana 2007; Skalsky and Cullen 2010).

P-bodies is reversible thus is not the absolute end for targeted mRNAs (Brengues, Teixeira et al. 2005; Bhattacharyya, Habermacher et al. 2006).

Genetic screens & biochemical assays have identified additional proteins thought to be required for miRNA expression and function with some of these factors being species, target, or miRNA specific. The miRNA-induced silencing complex (miRISC) is not completely understood but reportedly contains AGO as well as other cellular factors necessary for miRNA-mediated mRNA decay including: the P-body component GW182, the CCR4-CAF1-NOT deadenylase complex, the decapping enzyme DCP2, Ge-1, HPat, EDC3, and RCK/p54 (Rehwinkel, Behm-Ansmant et al. 2005; Chu and Rana 2006; Eulalio, Rehwinkel et al. 2007; Piao, Zhang et al. 2010). RCK/p54, a DEAD box helicase, interacts with AGO1 and AGO2 *in vivo*, and facilitates the formation of P-bodies (Chu and Rana 2006). Rck/p54 is also a general repressor of translation. The GW182 family of proteins has been shown to be associated with miRISC and to be required for miRNA-mediated gene silencing (Liu, Rivas et al. 2005; Rehwinkel, Behm-Ansmant et al. 2005). Recently, the LIM domain-containing proteins, LIMD1, Ajuba, and WTIP have been reported to bind AGO1/2, RCK, Dcp2 and eIF4E *in vivo* (James, Zhang et al. 2010).

E. miRNA Function and Expression

miRNAs regulate cellular processes such as development, differentiation, proliferation, hematopoiesis and apoptosis, though the vast majority of miRNAs

do not have validated targets or regulatory functions (as reviewed by (Bartel 2004)). Several miRNAs can regulate a single target, and a single miRNA can target multiple mRNAs, expanding the regulatory effects of miRNAs (Krek, Grun et al. 2005). To determine the phenotype of a miRNA:mRNA interaction can be challenging in the laboratory since disrupting one such interaction is potentially subtle. Possibly, the interactions of multiple miRNAs need to be mutated before one can detect changes in message levels. Also, some protein levels can be reduced or increased without measurable phenotypic changes. Many miRNAs potentially have redundant functions, which when mutated singly, would result in a subtle or immeasurable change in phenotype. Lastly, laboratory *in vitro* systems without the stresses and competition observed *in vivo* could potentially mask the phenotype associated with the disruption of miRNA regulation of a target.

Many tissues have been examined thus far and miRNAs have been expressed in all, including some found specifically in tissue or cell types, others only expressed during certain developmental stages and others expressed ubiquitously (Landgraf, Rusu et al. 2007). Comparing cellular miRNA expression from various tissues has revealed that approximately 50% of miRNAs are expressed in a tissue-specific manner (Sempere, Freemantle et al. 2004; Wienholds and Plasterk 2005; Lee, Baek et al. 2008). Ramkissoon and colleagues examined miRNA expression in 17 hematopoietic cell lines and compared it to purified human B cells, T cells, monocytes, and granulocytes. The

study identified considerable differences between the cell types, which is important to consider when studying model systems (Ramkissoon, Mainwaring et al. 2006). Differential miRNA expression is also documented in viruses. Our laboratory identified the viral miRNAs expressed in RCMV infected fibroblasts and salivary gland tissues. Our findings of tissue specific RCMV miRNA expression will be described in detail in Chapter 3.

F. Viral miRNAs

Bioinformatics, sequencing, and direct cloning approaches have led to the identification of over 200 viral miRNAs (as reviewed by (Skalsky and Cullen 2010)). The majority of these viral miRNAs have been identified in herpesviruses. All three families of herpesviruses (α, β, γ) encode miRNAs suggesting they have utilized the RNAi machinery throughout their evolution (Grey, Hook et al. 2008). Herpesvirus biology is conducive to the use of miRNAs for the following reasons. Herpesviruses are double-stranded-DNA viruses that replicate in the nucleus where they are able to utilize RNA polymerase II for transcription of their miRNAs as well as the host miRNA processing machinery (Lee, Kim et al. 2004). Second, viruses with longer life cycles and those capable of latent infections could profit from miRNA regulatory mechanism because miRNAs must be produced to sufficient quantities to exert inhibition of target protein production. Lastly, it is important to evade immune detection during latency, viral miRNAs are not recognized by the host immune system and therefore are able to attenuate immune responses by down-regulating key cellular genes. For example, MICB a

ligand of the natural killer (NK) cell activating receptor NKG2D critical for NK cell killing of virus infected cells and tumor cells, is down-regulated by HCMV miR-UL112-1 (Stern-Ginossar, Elefant et al. 2007). miRNAs, in contrast to viral proteins, are non-immunogenic, require less coding capacity, and can co-evolve with the host to target new or changing transcripts. Thus miRNAs are an ideal tool to alter gene expression in herpesvirus infected cells.

The first virally encoded miRNA was discovered in B cells latently infected with the γ-herpesvirus EBV (Pfeffer, Zavolan et al. 2004). Since then virally encoded miRNAs have been detected in all herpesviruses tested to date. In the α-herpesviruses, HSV type-1 encodes 8 (Cui, Griffiths et al. 2006; Umbach, Kramer et al. 2008; Jurak, Kramer et al. 2010), HSV type-2 encodes 6 (Tang, Bertke et al. 2008; Tang, Patel et al. 2009; Umbach, Wang et al. 2009; Jurak, Kramer et al. 2010), MDV type-1 encodes 14 (Burnside, Bernberg et al. 2006), and MDV type-2 encodes 18 (Yao, Zhao et al. 2007) known pre-miRNAs. In the β-herpesviruses, HCMV encodes 11 (Dunn, Trang et al. 2005; Grey, Antoniewicz et al. 2005; Pfeffer, Sewer et al. 2005) while MCMV encodes 18 (Buck, Santoyo-Lopez et al. 2007; Dolken, Perot et al. 2007) known pre-miRNAs and our laboratory detected 24 miRNAs in RCMV. Lastly in the γ-herpesviruses, EBV encodes 25 (Pfeffer, Zavolan et al. 2004; Cai, Schafer et al. 2006; Zhu, Pfuhl et al. 2009), rhesus lymphocryptovirus encodes 32 (Cai, Schafer et al. 2006), KSHV encodes 12 (Cai, Lu et al. 2005; Pfeffer, Sewer et al. 2005; Samols, Hu et al.

2005), rhesus rhadinovirus encodes 15 (Schafer, Cai et al. 2007) and MHV-68 encodes 9 (Pfeffer, Sewer et al. 2005) known pre-miRNAs.

Other dsDNA viruses including adenovirus (Aparicio, Razquin et al. 2006; Sano, Kato et al. 2006), simian, human and murine polyomaviruses (Cantalupo, Doering et al. 2005; Sullivan, Grundhoff et al. 2005; Seo, Fink et al. 2008; Seo, Chen et al. 2009; Sullivan, Sung et al. 2009) and the insect virus *Heliothis virescens* ascovirus (HvAV) (Hussain, Taft et al. 2008) also encode miRNAs. Several other viruses including human papilloma virus (HPV), hepatitis C virus (HCV), HIV type-1, yellow fever virus (YFV) and human lymphotropic virus-1 (HTLV-1) have been reported to lack virally encoded miRNAs using standard sequencing approaches (Pfeffer, Sewer et al. 2005; Cai, Li et al. 2006; Lin and Cullen 2007). Using deep sequencing techniques viral miRNAs were also not detected for HPV, HCV, cowpox virus, poliovirus, vesicular stomatitis virus (VSV), West Nile Virus, dengue virus or influenza virus (Lui, Pourmand et al. 2007; Parameswaran, Sklan et al. 2010). Many of these viruses replicate exclusively in the cytoplasm and as such they do not have contact with the nuclear enzyme Drosha for processing of the pri-miRNA. Additionally, some of these viruses have RNA genomes allowing the viral genomic RNA to be cleaved and destroyed if a miRNA were produced.

G. Viral miRNA Expression and Function

miRNA expression profiles have been reported to change following viral infection (Triboulet, Mari et al. 2007; Cameron, Fewell et al. 2008; Wang, Weber et al. 2008). These changes could be due to the cellular response to viral infection, for example, the induction of the innate immune response (Pedersen, Cheng et al. 2007). Conversely, the virus could specifically induce changes in cellular miRNA expression to increase virus replication. For example, Hepatitis C virus (HCV) binds cellular miRNA miR-122 leading to an increase in HCV RNA (Jopling, Yi et al. 2005; Jopling, Schutz et al. 2008) Similarly, human papilloma virus (HPV) down-regulates cellular p53 regulated miRNA miR-34a resulting in cell proliferation (Wang, Wang et al. 2009).

miRNA targeting of IE genes is a common strategy amongst herpesviruses. A leading hypothesis for the role of miRNAs in the establishment of latency is their ability to down-regulate IE protein expression during the later phases of the lytic replication cycle. Also, once latency is established, viral miRNAs targeting IE genes may maintain latency by inhibiting the initiation of the lytic cascade. Using a bioinformatics approach, a miRNA-target-predicting algorithm calculated that four herpesviruses encode miRNAs targeting the 3' UTRs of IE transactivators. HCMV miR-UL112-1 targets IE1, HSV-1 miR-LAT targets ICP0, EBV miR-BHRF-1 and miR-BART15 target BZLF1 and BRLF1 respectively, and KSHV miR-K10-6-3p targets Rta and Zta (Murphy, Vanicek et al. 2008). A common mechanism to maintain latency is thus possible as these viral transactivators are necessary

for efficient initiation of the lytic program. The expression of viral miRNAs provides herpesviruses a nonimmunogenic strategy to stably alter the cellular environment during latency.

The majority of identified targets of virally encoded miRNAs are from the α- and γ-herpesviruses. EBV was the first virus known to express miRNAs and they are encoded in two distinct clusters (BHFR1 and BART) that are differentially expressed in latent EBV-infected lymphoid and epithelial cells (Figure 1.10) (Pfeffer, Zavolan et al. 2004; Cai, Schafer et al. 2006). The expression of the BHFR1 miRNA cluster is only detected in Burkitt lymphoma (BL) cells and in lymphoblastoid cell lines (LCL) that use promoters characteristic of type III latency (Cai, Schafer et al. 2006), whereas the BART miRNAs are expressed in cells during all forms of latency but primarily in type II latency (Cai, Schafer et al. 2006; Grundhoff, Sullivan et al. 2006; Pratt, Kuzembayeva et al. 2009; Zhu, Pfuhl et al. 2009). In epithelial cells, BART miRNAs are expressed more abundantly than in B cells (Cai, Schafer et al. 2006). Also interestingly, it was discovered using quantitative stem loop real-time PCR that EBV miRNA expression differed approximately 50-fold among all tested cell lines and 25-fold among EBV-positive Burkitt lymphomas (Pratt, Kuzembayeva et al. 2009). EBV-associated primary tumors were analyzed and found to have high miR-BART expression (Kim do, Chae et al. 2007; Xia, O'Hara et al. 2008). Conversely, in AIDS diffuse large B cell lymphomas only BHRF miRNAs were detected (Xia, O'Hara et al. 2008).

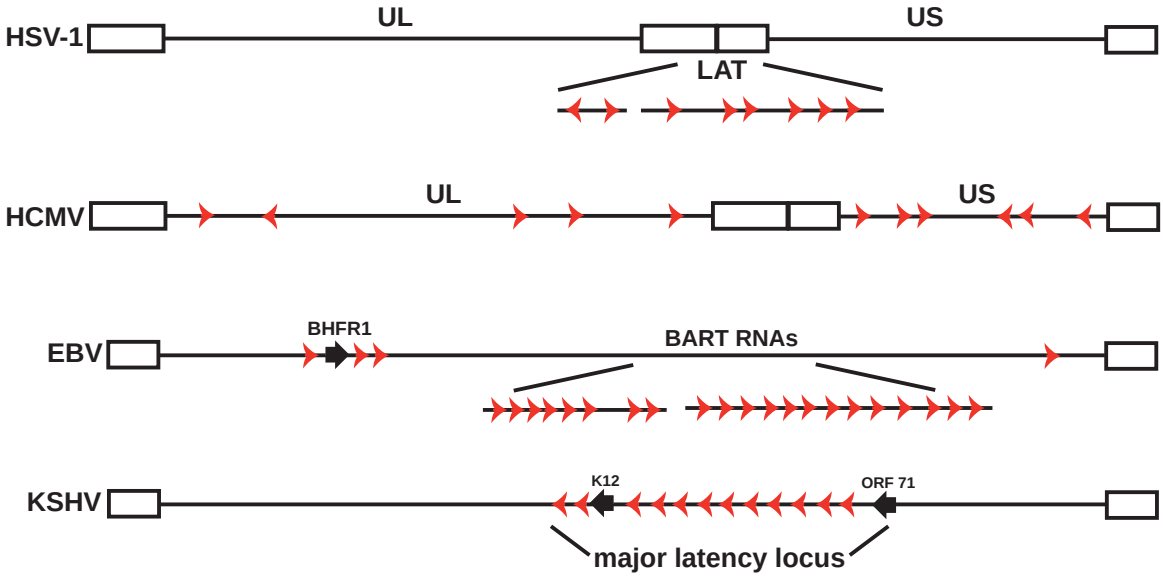

Figure 1.10 Location of viral pre-miRNAs within herpesvirus genomes. Schematic depicting the pre-miRNAs (red arrows) encoded by HSV-1, HCMV, EBV and KSHV. HSV-1 and KSHV miRNAs are clustered in latency-associated regions. miRNAs within the EBV genome are encoded in a cluster near the BHRF1 ORF and within the BART RNAs. HCMV miRNAs are encoded across the genome. ORFs are shown in black arrows. LAT = latency associated transcript. Adapted from (Boss, Plaisance et al. 2009; Skalsky and Cullen 2010).

miRNA profiling of nasopharyngeal carcinoma (NPC) tumors revealed BHRF2 miRNAs were not expressed (Cosmopoulos, Pegtel et al. 2009).

Another γ-herpesvirus, KSHV, also encodes miRNAs that are clustered and positioned within the latency-associated region (Figure 1.10) (Samols, Hu et al. 2005). KSHV miRNAs are highly expressed in latently infected primary effusion lymphoma cells and are believed to be regulated by one latent and two lytic RNA polymerase II promoters (Xu, Rodriguez-Huete et al. 2006). One study found that deletion of the 14-miRNA cluster from the KSHV genome significantly enhances viral lytic replication as a result of decreased NF-κB activity (Lei, Bai et al. 2010).

Similar to the γ-herpesviruses, the α-herpesvirus HSV-1 and HSV-2 miRNAs are predominately associated with latency (Figure 1.10). During latency HSV viral gene expression is restricted to the noncoding latency-associated transcripts (LATs). Deep sequencing of HSV-1 latently infected mouse and human trigeminal ganglia revealed six viral miRNAs encoded in the LAT (Umbach, Kramer et al. 2008; Umbach, Nagel et al. 2009; Umbach, Wang et al. 2009). Likewise in HSV-2, five LAT-associated miRNAs were identified from latently infected human sacral ganglia by deep sequencing (Umbach, Wang et al. 2009). Thus in both the α– and the γ-herpesviruses, miRNAs play a pivotal role in maintaining stable latent infections.

So far β-herpesvirus miRNAs have been isolated from fibroblasts undergoing acute lytic replication (Dunn, Trang et al. 2005; Grey, Antoniewicz et al. 2005; Pfeffer, Sewer et al. 2005; Buck, Santoyo-Lopez et al. 2007; Dolken, Perot et al. 2007). Both HCMV and MCMV miRNAs are found scattered across the viral genome with some encoded in small clusters (Figure 1.10), which is different from the α- and γ- herpesviruses as discussed previously, where miRNAs are found clustered and primarily in latency-associated areas (Pfeffer, Zavolan et al. 2004; Cai, Lu et al. 2005; Pfeffer, Sewer et al. 2005; Samols, Hu et al. 2005; Cai, Schafer et al. 2006; Grundhoff, Sullivan et al. 2006; Landgraf, Rusu et al. 2007; Schafer, Cai et al. 2007). CMV latency is difficult to study because currently there is a lack of an appropriate *in vitro* system. Despite this problem, it is hypothesized that viral miRNAs in CMVs are expressed early (IE and E expression kinetics) in infection to inhibit viral replication to establish and maintain persistence/latency. HCMV miR-UL112-1 targets MIC-B, a ligand for an activating receptor on natural killer (NK) cells, leading to a reduction in NK cell mediated killing of infected cells (Stern-Ginossar, Elefant et al. 2007). HCMV miR-UL112-1 targets the immediate early protein IE1 resulting in a significant decrease in viral genomic DNA levels (Grey, Meyers et al. 2007; Stern-Ginossar, Elefant et al. 2007). Lastly, HCMV miR-UL112-1 has also been reported to target UL114, uracil DNA glycosylase, which is involved in viral DNA replication (Stern-Ginossar, Saleh et al. 2009). The UL114 protein is required during the transition to late-phase viral DNA replication thus is an important checkpoint for the virus life cycle (Courcelle, Courcelle et al. 2001; Prichard, Britt et al. 2005). HCMV

miR-US25-1 and miR-US25-2 could also contribute to the establishment of latency because they reduce viral replication and DNA synthesis but this effect is not limited to HCMV since other DNA viruses (HSV-1 and adenovirus) but not RNA viruses (influenza virus) were also targeted (Stern-Ginossar, Saleh et al. 2009). Thus, the authors hypothesized that miR-US25-1 and miR-US25-2 target unknown cellular genes essential for virus growth. Recently, Grey and colleagues demonstrated that HCMV miR-US25-1 targets multiple cellular genes related to cell cycle control, specifically cyclin E2. Therefore, miR-US25-1 may contribute to levels of viral replication in infected cells by contributing to HCMV's ability to block cell cycle progression (Grey, Tirabassi et al. 2010). Clearly this data, along with the data demonstrating that α- and γ-herpesvirus miRNAs typically function during latency, leads to the hypothesis that, in general, herpesviruses encode miRNAs to inhibit viral replication to help establish and maintain latency, which would have the overarching affect of promoting persistence in the infected host.

Identification of miRNAs in small animal models such as MHV-68, a mouse model for the γ-herpesviruses EBV and KSHV, MCMV a mouse model for HCMV and RCMV a rat model for HCMV, are important to the study of miRNA function in herpesviral pathogenesis *in vivo* (Pfeffer, Sewer et al. 2005; Buck, Santoyo-Lopez et al. 2007; Dolken, Perot et al. 2007). The characterization of 18 miRNAs expressed during MCMV lytic replication in mouse fibroblasts was achieved through cloning, sequencing, Northern blot, and nuclease protection assays (Dolken JV 2007). Similar to HCMV, the MCMV miRNAs are found distributed

across the viral genome individually or clustered in small groups (Dolken, Perot et al. 2007). The MCMV miRNAs were expressed with IE or E kinetics and accumulated over time in fibroblasts *in vitro* (Dolken, Perot et al. 2007). Three different knockout MCMV viruses were constructed; deletion of 3 miRNAs clustered in the m01 locus, deletion of miR-M23-2/m21-1, and lastly deletion of miR-M44-1. All three mutant viruses showed the miRNAs to be dispensable for growth in fibroblasts *in vitro* (Dolken, Perot et al. 2007). MCMV miRNAs were also sequenced in liver, lung, and spleen at 3 and 5 days post-infection from BALB/c mice infected with wild type MCMV. miR-m01-4 was detected in all organs tested and miR-M44-1 was detected in the liver with miRNA accumulation paralleling infectious virus production by plaque assay (Dolken, Perot et al. 2007). Another report by Buck et al. also identified miRNAs in MCMV and found 17 miRNAs expressed in fibroblasts as well as a macrophage cell line by bioinformatics, sequencing, and RT-PCR (Buck, Santoyo-Lopez et al. 2007). They found all miRNAs were sensitive to cycloheximide treatment indicating the requirement of immediate-early proteins for their expression (Buck, Santoyo-Lopez et al. 2007). Interestingly, they identified three miRNAs within the 7.2-kb stable intron of MCMV (Buck, Santoyo-Lopez et al. 2007). Research has shown *in vivo* that mutagenesis of the 7.2 kb MCMV stable intron splice sites influences the progression from the acute phase of infection to the persistent phase of infection (Kulesza and Shenk 2006). The MCMV and the HCMV stable introns are orthologous (Plachter, Traupe et al. 1988; Kulesza and Shenk 2006). HSV-1 also encodes a miRNA within the stable intron in the latency-associated

transcript (Cui, Griffiths et al. 2006; Gupta, Gartner et al. 2006; Jurak, Kramer et al. 2010). Our lab has identified RCMV miRNAs encoded within the region where the RCMV stable intron is believed to be located. Though no miRNAs have been identified in the HCMV stable intron it is unclear whether miRNAs within the stable intron are common and what part they play in herpesvirus pathogenesis *in vivo* (Dunn, Trang et al. 2005; Grey, Antoniewicz et al. 2005; Pfeffer, Sewer et al. 2005). In conclusion, it is possible that herpesvirus miRNAs play particular roles during different stages of the viral lifecycle. miRNA expression levels *in vivo* vs. *in vitro* cell culture systems and in different cell types will be important to understand in order to elucidate miRNA function.

IV. Rat Cytomegalovirus: An Animal Model for HCMV Pathogenesis

A number of animal systems are used to model HCMV infection and disease of humans. Experimental infection of mouse, rat, guinea pig, and rhesus macaque by their species-specific CMVs show a similar pattern of dissemination and pathogenesis to HCMV infection of humans. Our laboratory uses RCMV infection of rats to investigate CMV pathogenesis. Two main RCMV strains are used in laboratory settings; Maastricht strain and English strain, which were both isolated from the salivary glands of wild brown rats, *Rattus Norvegicus*, in 1982 (Bruggeman, Meijer et al. 1982; Priscott 1982). A third RCMV strain, ALL-03, isolated from the placenta/uterus of a house rat (*Rattus rattus diardii*), is described as a possible model system to study congenital CMV infection (Loh, Mohd-Azmi et al. 2003).

Our laboratory primarily uses the Maastricht strain of RCMV, which has a linear double stranded DNA genome of 230 kb with greater than 170 potential ORFs. Approximately half of these ORFs are conserved with HCMV and two-thirds with MCMV (Vink, Beuken et al. 2000). The RCMV genome has an overall G+C content of 61% and consists of a single unique sequence flanked by 504 bp direct terminal repeats (Vink, Beuken et al. 2000). RCMV infection of rats exhibits a similar pattern of pathogenesis to HCMV infection of humans, and as such can be used as a model system (Stals, Bosman et al. 1990). The acute phase consists of widespread systemic infection followed by a chronic phase starting approximately two weeks post-infection where appreciable levels of virus can only be detected in the salivary glands and saliva (Bruggeman, Meijer et al. 1985). RCMV infections are life-long and detectable levels of virus are present in the salivary gland for more than a year post-infection where the virus persistently infects the ductal epithelial cells (Bruggeman, Debie et al. 1983; Bruggeman, Meijer et al. 1985; Kloover, Hillebrands et al. 2000). Similar to HCMV infection in humans, immunocompetent rats infected with RCMV show no overt disease but infection of immunocompromised (CSA treated, UV irradiated) rats leads to high levels of infection and high morbidity and mortality rates (Stals, Bosman et al. 1990). Infection of immunocompetent rats results in the same viral tropism for the salivary gland duct cells, but the amount of virus produced and the number of infected cells is reduced or under detection limits (Kloover, Hillebrands et al. 2000). When eight-week old male Brown Norway rats were immunosuppressed

by total body irradiation and then infected with RCMV, by 10 days post-infection 90% of the rats had died (Stals, Bosman et al. 1990). The authors found heavy infection in the spleen, liver and bone marrow and the pathology resembled lesions in HCMV infection (Stals, Bosman et al. 1990). RCMV infects the same cell types as HCMV including endothelial cells, epithelial cells, macrophages, smooth muscle cells and fibroblasts. When anti-RCMV hyperimmune serum was given it was effective against mortality (Stals, Bosman et al. 1990). Thus the RCMV-rat model is an appropriate tool to study pathogenesis and anti-viral therapies targeted against infections in the immunocompromised host.

A. RCMV Acceleration of Vascular Disease and Chronic Allograft Rejection

Our laboratory has provided evidence that in rat solid organ transplantation, RCMV infection of rats accelerates TVS, leading to graft failure (Orloff, Streblow et al. 2002; Streblow, Kreklywich et al. 2003; Soule, Streblow et al. 2006). The effect of RCMV on acceleration of TVS occurs in many types of solid organ transplants including heart, kidney, lung, and small bowel (Tikkanen, Kallio et al. 2001; Orloff, Streblow et al. 2002; Streblow, Kreklywich et al. 2003; Soule, Streblow et al. 2006). To study CMV in the development of TVS, our laboratory utilizes F344 rats for the donor tissue, which is transplanted into Lewis recipient rats (Ely, Greiner et al. 1983; Klempnauer and Marquarding 1989; Lubaroff, Rasmussen et al. 1989). This strain combination exhibits reduced allogenicity upon transplantation because the donors and recipients are identical for classic MHC but differ at minor MHC loci (Klempnauer and Marquarding 1989; Lubaroff,

Rasmussen et al. 1989). We have used this transplant scenario to study TVS and chronic rejection (CR) in transplanted heart, kidney, and small bowel grafts (Orloff, Streblow et al. 2002; Streblow, Kreklywich et al. 2003; Streblow, Kreklywich et al. 2005; Soule, Streblow et al. 2006). Acute rejection is prevented by a short regimen of cyclosporine A (immunosuppressant) resulting in long-term surviving allografts developing histological evidence of CR (Orloff, Streblow et al. 2002). Using this rat heart transplant model we have determined that RCMV infection accelerates the time to develop TVS and graft failure as well as increases the severity of TVS compared to uninfected recipients with an overall affect of decreasing the time to chronic rejection (Streblow, Kreklywich et al. 2003). Antiviral therapy can prevent or at least reduce the acceleration of rejection in the rat transplant model, indicating that similar to the human disease, active viral replication is necessary for this disease process.

V. Thesis Overview and Aims

In this dissertation, I examine two aspects of RCMV biology; the miRNAs encoded within the RCMV genome and the role of the RCMV glycoprotein R116 in virus infectivity. My work is focused on two vital aspects of CMV persistence, the regulation of viral gene expression *in vivo* and the functions of highly expressed viral genes. Therefore, one aim of my thesis was to determine whether the viral miRNAs encoded by RCMV modulate viral gene expression *in vivo*. The other aim of my thesis was to determine the function of R116, a RCMV gene that is highly expressed *in vivo* during both the acute phase of viral infection

and during persistence in the salivary glands. In previous studies we have determined that RCMV gene expression *in vivo* during persistence is tightly regulated and occurs in a tissue and cell specific manner. We hypothesize that RCMV miRNAs are important for determining viral gene expression *in vivo* and that viral miRNA expression is tissue specific. While there are multiple factors affecting CMV tropism, we have identified a RCMV glycoprotein, R116 that is important for the production of infectious virus. We hypothesize that R116 plays a significant role in an entry step of the virus lifecycle. The elucidation of viral gene function and the regulation of viral genes during the different phases of the CMV lifecycle will aid in the design of novel targeted therapeutics.

A. RCMV miRNAs

In Chapter 2, we identify the RCMV miRNAs expressed in fibroblasts and in salivary glands of infected rats. The long-term goal of this project is to determine the role of HCMV in the development and acceleration of vascular disease. Currently it is proposed that CMV typically restricts viral gene expression to reduce acute replication and minimize the potential for antigen presentation, while still maintaining the ability to reactivate at a later stage. However the process by which HCMV restricts its own gene expression during persistence and reactivation is not well understood. The discovery of RNAi and widespread expression of miRNAs uncovered a new layer of post-transcriptional gene regulation. Although the functions of most virally encoded miRNAs are unknown, their potential to regulate multiple transcripts both viral and cellular and the lack

of an immunogenic response due to their RNA nature make miRNAs an ideal candidate to promote and maintain a persistent or latent viral gene expression profile. The aim of the miRNA study was to identify the miRNAs encoded in the RCMV genome and to determine the miRNA expression levels not only in RCMV infected tissue culture cells but also in RCMV infected rats. This knowledge will then enable us to characterize the role of RCMV miRNAs during acute and persistent infections as well as during reactivation and subsequent dissemination in our RCMV infection rat heart transplant model. In addition to the data presented in Chapter 2, in the Appendix, we present our findings that begin to identify the targets of the RCMV miRNAs using a RISC IP approach combined with a bioinformatics approach.

B. RCMV ORF R116

In Chapter 3, we demonstrate that RCMV R116 is a viral glycoprotein important for virus entry into fibroblasts. HCMV encodes a complex assortment of glycoprotein genes. Varnum et al. identified 19 structural glycoproteins as constituents of HCMV extracellular virions using mass spectrometry (Varnum, Streblow et al. 2004). Sequencing of the MCMV genome identified nearly 30 unknown ORFs referred to as putative glycoproteins (Rawlinson, Farrell et al. 1996). The envelope of CMV is studded with glycoproteins functioning during the initial phase of infection, attachment and fusion as well as later stages of infection with roles in assembly of infectious virions to envelopment and cell to cell spread. Several CMV glycoproteins have multiple roles and/or are not

essential for entry but have augmenting functions such as gB and gO. gB is essential for CMV entry but is also important in the activation of cellular signaling pathways similar to an interferon toll receptor or growth factor response and leads to many of the transcriptional changes that are activated by HCMV virion attachment (Yurochko, Hwang et al. 1997; Boyle, Pietropaolo et al. 1999; Simmen, Singh et al. 2001). It was recently discovered that gO was not present in extracellular virus particles in the HCMV clinical strain TR, but was detected in extracellular particles from the lab-adapted strain AD169 (Huber and Compton 1998; Ryckman, Chase et al. 2010). Additional findings concluded that TR gO is a chaperone and promotes the ER export of the gH:gL complex, but dissociates prior to incorporation into the HCMV TR envelope (Wille, Knoche et al. 2010). Thus it is important to determine the role of unknown glycoproteins in CMV biology and lifecycle. The identification of glycoprotein functions in CMVs infecting small animal models is necessary for the *in vivo* investigation of viral protein function. In Chapter 3, we characterize the protein encoded by RCMV ORF R116, a predicted glycoprotein with an unknown function. We found that R116 is a virion-associated glycoprotein that is important for virus infectivity in rat fibroblasts.

Chapter 2: Cytomegalovirus miRNA Expression is Tissue Specific and Associated with Persistence

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Abstract

MicroRNAs (miRNAs) are a class of small noncoding RNAs involved in post-transcriptional regulation. miRNAs are utilized in organisms ranging from plants to higher mammals and data demonstrates that DNA viruses also use this method for host and viral gene regulation. Here, we report the sequencing of the small RNAs in RCMV infected fibroblasts and persistently infected salivary glands. We identified 24 unique miRNAs that mapped to stem loop structures found within the viral genome. While most miRNAs were detected in both samples, four were detected exclusively in the infected fibroblasts and two were specific for the infected salivary glands. The RCMV miRNAs are distributed across the viral genome on both the positive and negative strands, with clusters of miRNAs at a number of locations including near viral genes r1 and r111. The RCMV miRNAs share a similar genomic positional orientation with the miRNAs described for MCMV but they do not share any substantial sequence conservation. Similar to other reported miRNAs, the RCMV miRNAs had considerable variation at their 3' and 5' ends. Interestingly, we found a number of specific examples of differential isoform usage between the fibroblast and salivary gland samples. We determined by real-time PCR that expression of the RCMV miRNA miR-r111.1-2 is highly expressed in the salivary glands and that miR-R87-1 is expressed in most tissues during acute infection. Our study identified the miRNAs expressed by RCMV *in vitro* and *in vivo*, and demonstrates that expression is tissue specific and associated with a stage of viral infection.

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs involved in post-transcriptional regulation through binding to complementary sequences in target mRNAs, resulting in gene silencing (Bartel and Chen 2004). miRNAs are ubiquitous among multicellular eukaryotic organisms where they have diverse roles in many different biological processes including development, differentiation, proliferation, apoptosis and hematopoiesis (Lee, Feinbaum et al. 1993; Rana 2007). In addition to eukaryotic miRNAs, DNA viruses, mainly of the herpesvirus family, have been shown to encode miRNAs. Bioinformatics, sequencing, and direct cloning approaches have led to the identification of over 140 viral miRNAs (reviewed in (Skalsky and Cullen 2010)). The role of viral miRNAs is proposed to include: the targeting of cellular genes to induce a favorable replication environment or to evade the host immune system; and the targeting of their own viral genome to regulate viral gene expression during persistence or latency/reactivation (Grey, Hook et al. 2008; Skalsky and Cullen 2010).

Human cytomegalovirus (HCMV) is known to encode at least 14 miRNAs (Dunn, Trang et al. 2005; Grey, Antoniewicz et al. 2005; Pfeffer, Sewer et al. 2005). HCMV is a ubiquitous β-herpesvirus and primary infection results in life-long persistent/latent infection of the host. Infection of immunocompetent hosts is generally asymptomatic but infection of immunocompromised hosts can lead to high morbidity and mortality. HCMV has been linked to the development of atherosclerosis, arterial restenosis following angioplasty, and solid organ

transplant vascular sclerosis (TVS) (Melnick, Petrie et al. 1983; Speir, Modali et al. 1994; Melnick, Adam et al. 1998). HCMV infection nearly doubles the 5-year rate of cardiac graft failure due to accelerated TVS (Grattan, Moreno-Cabral et al. 1989) and doubles the rate of liver graft loss at 3 years (Deotero, Gavalda et al. 1998; Rubin 1999). Since the β-herpesviruses including HCMV are highly species specific, effective animal models have been established to investigate the role of CMV infection in chronic disease. We have utilized a rat cytomegalovirus (RCMV) infection system to model a number of human diseases associated with HCMV infections, including solid organ transplant rejection and restenosis following angioplasty (Handa, Hatanaka et al. 1993; Lemstrom, Aho et al. 1994; Lemstrom, Bruning et al. 1994; Lemstrom, Koskinen et al. 1994; Steinhoff, You et al. 1995; Koskinen, Lemstrom et al. 1996; Koskinen, Yilmaz et al. 1996; Persoons, Daemen et al. 1997; Martelius, Scholz et al. 1999; Orloff, Streblow et al. 2002; Streblow, Kreklywich et al. 2003; Hillebrands, van Dam et al. 2005; Kloppenburg, de Graaf et al. 2005; Streblow, Kreklywich et al. 2005; Soule, Streblow et al. 2006; Streblow, Kreklywich et al. 2008). Similar to humans, infection of immunocompetent rats leads to a limited subclinical infection that persists life-long in host bone marrow and columnar epithelial cells of the salivary glands (Kloover, Hillebrands et al. 2000). RCMV infection of immunocompromised rats (i.e. those rats undergoing immunosuppressive treatment for the prevention of allograft rejection) leads to infection of most host tissues and organs. We have shown that RCMV gene expression is highly restricted in tissues from allograft recipients following infection. In fact, the highly expressed genes in tissues from the infected

rats are hypothesized to be involved in host cell manipulation and/or immune evasion, which allows the virus to persist by turning over low amounts of infectious virus while remaining undetected by the immune system (Streblow, van Cleef et al. 2007). However, the mechanisms by which CMV controls viral gene expression *in vivo* are still unknown. However, recent studies suggest that miRNAs may play an important role in regulating CMV gene expression and latency (Grey, Meyers et al. 2007). The aim of the current study was to identify the miRNAs encoded in the RCMV genome and determine expression levels in tissue culture infected fibroblasts as well as in persistently infected tissues from RCMV-infected heart allograft recipients. Our elucidation of the RCMV miRNAs expressed during lytic and persistent *in vivo* infections is crucial for our understanding of their role in CMV persistence, pathogenesis and disease.

Materials and Methods

Rat cytomegalovirus. Salivary gland-derived stocks of the Maastricht strain of RCMV were titered using primary rat lung fibroblasts (RFL6s) (Bruggeman, Schellekens et al. 1983; Beisser, Vink et al. 1998). Plaque assays were performed in confluent 24-well plates by infection with 10-fold serial virus dilutions in triplicate in 0.2ml of media. Virus was incubated at 37°C for four hours and then overlaid with 1ml Dulbecco's modified Eagle's Medium (DMEM) supplemented with 5% FBS, penicillin-streptomycin, 20mM L-Glutamine and 10% carboxyl methylcellulose. After 7 days, the cells were fixed in 3.7% formaldehyde in PBS

and stained with 0.05% aqueous methylene blue. The plaques were visualized and counted by light microscopy.

RCMV infection of tissue culture cells. RFL6s were maintained in DMEM supplemented with 10% FBS and pen/strep/glutamine. For the cloning and sequencing of the RCMV miRNAs from *in vitro* infected cells, RFL6 cells were plated onto 150mm dishes (Costar). These cells were infected with RCMV upon 75% confluence at a multiplicity of infection equal to 1.0 (moi=1.0). After 3 hours, the cells were washed three times with PBS. The infected cells were harvested at 72 hours post infection (hpi) by first washing once with PBS, then adding 3mls of Trizol reagent. The reagent was allowed to lyse the cells for 5 minutes at room temperature. Subsequently, the samples were stored frozen at –80°C. For Real Time-PCR analysis of the RCMV miRNAs miR-R87-1 and miR-r111.2-6 in infected tissue culture cells; RFL6s were plated into 6-well plates. The cells were infected with RCMV moi= 1.0 and harvested by addition of Trizol, as described above, at 6, 24 and 48hpi.

Preparation of rat tissues from heart allograft recipients. To identify the RCMV miRNAs expressed *in vivo*, we sequenced the small RNAs from total RNA samples isolated from the salivary glands of RCMV infected rat allograft recipients at 21 days post infection (dpi) (Streblow, Kreklywich et al. 2003; Streblow, van Cleef et al. 2007). Rat heart transplant surgeries were performed as previously described (Orloff, Streblow et al. 2002; Streblow, Kreklywich et al. 2003; Streblow,

Kreklywich et al. 2005; Streblow, van Cleef et al. 2007; Streblow, Kreklywich et al. 2008). Various tissues (native and graft heart, salivary glands, spleen, liver, kidney, and lung) were collected from infected heart allograft recipients at 7 and 28 dpi (Streblow, Kreklywich et al. 2003; Streblow, van Cleef et al. 2007; Streblow, Dumortier et al. 2008) for Real Time-PCR analysis of the RCMV miRNAs miR-R87-1 and miR-r111.1-2. For each analysis, total RNA was prepared from approximately 0.25g of rat tissue using the Trizol method (Invitrogen). All animals were housed in the Portland VA Medical Center animal facilities in a specific-pathogen-free room. This facility is AAALAC accredited and complies with the requirements for animal care as stipulated by USDA and HHS.

miRNA Deep Sequencing. Deep sequencing was performed by LC Sciences (Houston, TX). Accordingly, for each sample, about 10µg of total RNA was size fractionated on a 15% tris-borate-EDTA (TBE) urea polyacrylamide gel and a 15-50 base pair fraction was excised. The small RNA fraction was eluted from the gel slice in 500µl of 0.3M NaCl and precipitated by the addition of ethanol. According to Illumina/Solexa's instructions, both a 5' and 3' RNA adaptor were added using T4 RNA ligase and the ligated RNA was again size fractionated on a 15% TBE polyacrylamide gel. The fraction containing the 65-100 bp region was excised from the gel and precipitated. The recovered RNA was converted to single-stranded cDNA using M-MLV reverse transcriptase (RT) and Illumina/Solexa's RT primer. The cDNA was amplified with pfx DNA polymerase for 20 cycles by PCR using Illumina/Solexa's small RNA primers set. PCR products were purified on a

12% polyacrylamide gel and the 80-120 bp fraction was excised from the gel and eluted and precipitated as above. The samples were resuspended and sequenced using the Illumina/Solexa G1 sequencer. Raw sequencing data was filtered for composition, the presence of adapter dimmers, length, sequence repetition and copy number. The filtered data was then mapped to current miR databases, the *rattus norvegicus* genome, and the RCMV genome (AF232689).

Northern Blot Analysis. Total RNA was isolated from infected (72 hpi) or uninfected rat fibroblasts using the Trizol (Invitrogen) method. To determine the kinetics of RCMV miRNA gene expression, another set of fibroblasts were harvested at 0, 8 hpi, 48 hpi and 48 hpi in the presence of Foscarnet (1:250 125mM). An equal volume of formamide and loading dye were added to each 20µg sample of RNA. The samples were boiled for 5 minutes and cooled on ice prior to loading onto a 15% urea/acrylamide gel. The gel was transferred to Gene Screen plus (PerkinElmer) membrane in 1X TBE. Probes were produced by end-labeling oligos using polynucleotide kinase (Fermentas) with gamma P^{32} dATP. The total labeled probe was hybridized to the blot overnight at 38°C in PerfectHyb Plus (Sigma Aldrich). The blot was washed 2 times with a low stringency buffer (2X SSC, 0.05% SDS) and 1 time with a high stringency buffer (0.1X SSC, 0.1% SDS) at 38°C. The blot was exposed to autoradiographic film (Kodak Biomax MS) overnight at -80°C.

Quantitative RT-PCR detection of RCMV miRNAs. Real-time RT-PCR was used to quantify the RCMV miRNA expression in RCMV infected fibroblasts and tissues from RCMV infected heart allograft recipients. miRNA cDNA was generated from total RNA using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems) in 15µl reactions containing 1.5pmoles of the miRNA-specific RT primer and 100ng of total RNA. The sequences of the miR-R87.1 and miR-r111.1-2 RT primers are: GTC GTC TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG AGTT G and GTC GTC TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CGC CG, respectively. Samples were incubated in an Applied Biosystems 9700 thermocycler at 16°C for 30 minutes, 42°C for 30 minutes, and then at 85°C for 5 minutes. Real time PCR (TaqMan) was used to quantify miRNA levels from 1.5µl of the cDNA reaction run in a ABI StepOnePlus Real Time PCR machine using 40 cycles: 95°C for 15 sec and 60°C for 1 minute (Streblow, Kreklywich et al. 2005; Streblow, van Cleef et al. 2007; Streblow, Kreklywich et al. 2008). Primer and probe sets included: miR-R87-1 Forward: GCT CGA AGA ACG GGT GC, Reverse: GTG CAG GGT CCG AGG T, Probe: TGG ATA CGA CGA GTT G; and miR-r111.1-2 Forward: GCT CGA AAC AAC GTG GA, Reverse: GTG CAG GGT CCG AGG T, Probe: TGG ATA CGA CAC GCC G. Oligonucleotides with the miRNA sequences for miR-R87-1 and miR-r111.1-2 were used as quantification standards. Data are presented as relative copy number per 10ng of total input RNA.

Results

Identification of RCMV miRNAs

To identify the miRNAs encoded by RCMV, we deep sequenced the small RNAs expressed in RCMV-infected fibroblasts and in persistently infected salivary glands from a rat infected for 21 days. We have previously demonstrated that salivary glands from 21 day infected rats have highly restricted viral gene expression profiles, limited to a small number of genes involved in immune evasion and persistence (Streblow, van Cleef et al. 2007). For sequencing experiments, total RNA was extracted and the small RNAs (15-25 bp) were isolated by polyacrylamide gel electrophoresis. The RNAs were ligated to 5' and 3' primers and deep sequenced using Illumina/Solexa G1 sequencer. The total reads were filtered for composition, length and junk.

A total of 6,665,287 reads were made in the RCMV infected fibroblast sample and nearly 41% of the sequences were mapped to either the cellular miR database or the rat genome (Table 2.1). A total of 7,230,718 reads were made in the RCMV infected salivary gland sample and nearly 33% of the sequences were mapped to the miR database and/or the rat genome. Approximately 87% of the sequenced small RNAs from the infected fibroblast sample and 85% from the infected salivary glands were between 19-23 bp. A list of the cellular miRNAs detected in the infected fibroblasts and salivary glands is available in the Supplemental Table 2.1. Of the 976,611 unmapped sequences from the RCMV infected fibroblasts 305,522 (31%) of these mapped to stem loop structures within the RCMV

Table 2.1 Distribution of small RNAs in RCMV infected fibroblasts and salivary glands

RCMV-Infected Fibroblasts	**seq#**	**seq%**	**family#**	**family%**
Raw	6665287	100.00%	414447	100.00%
mappable	3731474	55.98%	27168	6.56%
mapped (total)	2754863	41.33%	13288	3.21%
unmapped (total)	976611	14.65%	13880	3.35%
mapped to RCMV genome	305522	4.60%	1611	0.39%
RCMV Infected Salivary Glands				
Raw	7230718	100.00%	377359	100.00%
mappable	2909115	40.23%	20029	5.31%
mapped (total)	2369736	32.77%	11139	2.95%
unmapped (total)	539379	7.46%	8890	2.36%
mapped to RCMV genome	4159	0.06%	145	0.03%

Supplemental Table 2.1 Abundant cellular miRNAs detected in rat fibroblasts and rat salivary glands.

Fibroblasts				Salivary Glands			
miR name	miR seq	miR len	copy# all isoforms	miR name	miR seq	miR len	copy# all isoforms
rno-let-7a-1-5p-1	TGAGGTAGTAGGTTGTATGGTT	22	1766918	rno-let-7a-1-5p-1	TGAGGTAGTAGGTTGTATGGTT	22	1513030
rno-let-7d-5p-11	AGAGGTAGTAGGTTGCATAGTT	22	106917	rno-let-7d-5p-24	AGAGGTAGTAGGTTGCATAGTT	22	35790
rno-let-7e-5p-3	TGAGGTAGTAGGTTGTATAGTT	22	428546	rno-let-7e-5p-6	TGAGGTAGTAGGTTGTATAGTT	22	162540
rno-let-7i-5p-45	TGAGGTAGTAGTTTGTGCTGTT	22	34850	rno-let-7i-5p-50	TGAGGTAGTAGTTTGTGCTGTTT	23	26102
rno-mir-1-3p-1679	TGGAATGTAAAGAAGTATGTAT	22	166	rno-mir-1-3p-131	TGGAATGTAAAGAAGTATGTAT	22	2068
rno-mir-7a-1-5p-5641	TGGAAGACTAGTGATTTTGTTGTTT	25	43	rno-mir-7a-1-5p-4816	TGGAAGACTAGTGATTTTGTTGTTT	25	30
rno-mir-7a-2-5p-10195	TGGAAGACTAGTGATTTTGTTGT	23	21	rno-mir-7a-2-5p-7925	TGGAAGACTAGTGATTTTGTTGT	23	12
rno-mir-7b-5p-5641	TGGAAGACTAGTGATTTTGTTGTT	24	43	rno-mir-7b-5p-4816	TGGAAGACTAGTGATTTTGTTGTT	24	30
rno-mir-15b-5p-2518	TAGCAGCACATCATGGTTTAC	21	129	rno-mir-15b-5p-6272	TAGCAGCACATCATGGTTTA	20	36
rno-mir-16-5p-188	TAGCAGCACGTAAATATTGGCG	22	2797	rno-mir-16-5p-325	TAGCAGCACGTAAATATTGGCG	22	882
rno-mir-17-1-5p-11672	CAAAGTGCTTACAGTGCAGGTAG	23	42	rno-mir-17-1-5p-4121	CAAAGTGCTTACAGTGCAGGTAG	23	47
rno-mir-17-1-3p-10121	ACTGCAGTGAAGGCACTTGTGG	22	18	rno-mir-17-1-3p-4325	ACTGCAGTGAAGGCACTTGTGG	22	32
rno-mir-18a-5p-6223	TAAGGTGCATCTAGTGCAGAT	21	19	rno-mir-18a-5p-19504	TAAGGTGCATCTAGTGCAGATAG	23	3
rno-mir-19a-3p-2848	TGTGCAAATCCATGCAAAACTGA	23	110	rno-mir-19a-3p-1015	TGTGCAAATCCATGCAAAACTGA	23	194
rno-mir-20a-5p-10413	TAAAGTGCTTATAGTGCAGGTA	22	23	rno-mir-20a-5p-5706	TAAAGTGCTTATAGTGCAGGTAG	23	19
rno-mir-21-5p-24	TAGCTTATCAGACTGATGTTGA	22	63810	rno-mir-21-5p-10	TAGCTTATCAGACTGATGTTGAC	23	72889
rno-mir-21-3p-3972	ACAGCAGTCGATGGGCTGTCT	21	71	rno-mir-21-3p-3097	CAACAGCAGTCGATGGGCTGT	21	123
rno-mir-22-5p-2288	AGTTCTTCAGTGGCAAGCTTT	21	87	rno-mir-22-5p-4346	AGTTCTTCAGTGGCAAGCTTT	21	35
rno-mir-22-3p-217	AAGCTGCCAGTTGAAGAACTGT	22	1849	rno-mir-22-3p-689	AAGCTGCCAGTTGAAGAACTGT	22	255
rno-mir-23a-5p-12529	GGGGTTCCTGGGGATGGGATTT	22	11	rno-mir-23a-5p-20214	GGGGTTCCTGGGGATGGGATTT	22	3
rno-mir-23a-3p-310	ATCACATTGCCAGGGATTTCC	21	3454	rno-mir-23a-3p-498	ATCACATTGCCAGGGATTTC	20	2037
rno-mir-23b-5p-5642	GGGTTCCTGGCATGCTGATTT	21	32	rno-mir-23b-5p-4452	GGGTTCCTGGCATGCTGATTT	21	30
rno-mir-23b-3p-310	ATCACATTGCCAGGGATTTCC	21	2157	rno-mir-23b-3p-516	ATCACATTGCCAGGGATTT	19	1934
rno-mir-24-1-3p-100	TGGCTCAGTTCAGCAGGAACAGT	23	9382	rno-mir-24-1-3p-106	TGGCTCAGTTCAGCAGGAACAGT	23	5716
rno-mir-24-2-5p-10356	GTGCCTACTGAGCTGAAACAGT	22	24	rno-mir-24-2-5p-22736	GTGCCTACTGAGCTGAAACAG	21	3
rno-mir-25-5p-659	AGGCGGAGACACGGGCAATTGCT	23	484	rno-mir-25-5p-2343	AGGCGGAGACACGGGCAATTGCT	23	53
rno-mir-25-3p-90	CATTGCACTTGTCTCGGTCTGA	22	6940	rno-mir-25-3p-249	CATTGCACTTGTCTCGGTCTGA	22	1129
rno-mir-26a-5p-49	TTCAAGTAATCCAGGATAGGCT	22	18576	rno-mir-26a-5p-65	TTCAAGTAATCCAGGATAGGCT	22	8958
rno-mir-26b-5p-164	TTCAAGTAATTCAGGATAGGTT	22	5906	rno-mir-26b-5p-152	TTCAAGTAATTCAGGATAGGTT	22	3692
rno-mir-27a-3p-486	TTCACAGTGGCTAAGTTCCG	20	1546	rno-mir-27a-3p-395	TTCACAGTGGCTAAGTTCCG	20	1548
rno-mir-27b-5p-22688	AGAGCTTAGCTGATTGGTGAACA	23	7	rno-mir-27b-5p-4434	AGAGCTTAGCTGATTGGTGAACA	23	38
rno-mir-27b-3p-486	TTCACAGTGGCTAAGTTCCG	20	1468	rno-mir-27b-3p-395	TTCACAGTGGCTAAGTTCCG	20	1878
rno-mir-28-5p-2071	AAGGAGCTCACAGTCTATTGA	21	161	rno-mir-28-5p-2871	AAGGAGCTCACAGTCTATTGA	21	67
rno-mir-28-3p-5414	CACTAGATTGTGAGCTCCTGGA	22	46	rno-mir-28-3p-6968	CACTAGATTGTGAGCTCCTGGA	22	23
rno-mir-29a-3p-28	TAGCACCATCTGAAATCGGTTA	22	32648	rno-mir-29a-3p-46	TAGCACCATCTGAAATCGGTTA	22	12816
rno-mir-29b-1-3p-2145	TAGCACCATTTGAAATCAGTGTT	23	159	rno-mir-29b-1-3p-2194	TAGCACCATTTGAAATCAGTGTT	23	148
rno-mir-29b-2-3p-2145	TAGCACCATTTGAAATCAGTGTT	23	159	rno-mir-29b-2-3p-2194	TAGCACCATTTGAAATCAGTGTT	23	148
rno-mir-29c-3p-28	TAGCACCATCTGAAATCGGTTA	22	30947	rno-mir-29c-3p-46	TAGCACCATCTGAAATCGGTTA	22	12007
rno-mir-30a-5p-292	TGTAAACATCCCCGACTGGAAGC	23	5325	rno-mir-30a-5p-438	TGTAAACATCCCCGACTGGAAGCT	24	3055
rno-mir-30a-3p-3567	CTTTCAGTCGGATGTTTGCAG	21	175	rno-mir-30a-3p-2859	CTTTCAGTCGGATGTTTGCAG	21	162
rno-mir-30b-5p-4865	TGTAAACATCCTACACTC	18	65	rno-mir-30b-5p-3021	TGTAAACATCCTACACTC	18	48
rno-mir-30c-1-5p-4929	TGTAAACATCCTACACTCTCAGC	23	83	rno-mir-30c-1-5p-3021	TGTAAACATCCTACACTCTCAGCT	24	75
rno-mir-30c-2-5p-4929	TGTAAACATCCTACACTCTCAGC	23	83	rno-mir-30c-2-5p-3021	TGTAAACATCCTACACTCTCAGCT	24	75
rno-mir-30c-2-3p-4525	CTGGGAGAAGGCTGTTTACTCT	22	67	rno-mir-30c-2-3p-4800	CTGGGAGAAGGCTGTTTACT	20	49
rno-mir-30d-5p-292	TGTAAACATCCCCGACTGGAAGC	23	5210	rno-mir-30d-5p-438	TGTAAACATCCCCGACTGGAAGCT	24	2867
rno-mir-30e-5p-341	TGTAAACATCCTCGACTGGAAGC	23	2197	rno-mir-30e-5p-568	TGTAAACATCCTCGACTGGAAGC	23	1376
rno-mir-30e-3p-3567	CTTTCAGTCGGATGTTTGCAG	21	150	rno-mir-30e-3p-2859	CTTTCAGTCGGATGTTTGCAG	21	147
rno-mir-31-5p-74	AGGCAAGATGCTGGCATAGCTGT	23	18556	rno-mir-31-5p-1825	AGGCAAGATGCTGGCATAGCTGT	23	157
rno-mir-31-3p-15033	TGCTATGCCAACATATTGCCATC	23	14				
rno-mir-33-5p-1749	GTGCATTGTAGTTGCATTGCA	21	206	rno-mir-33-5p-745	GTGCATTGTAGTTGCATTGCA	21	358
rno-mir-34a-5p-763	TGGCAGTGTCTTAGCTGGTTGT	22	520	rno-mir-34a-5p-2593	TGGCAGTGTCTTAGCTGGTTGT	22	78
rno-mir-34a-3p-9560	AATCAGCAAGTATACTGCCCTA	22	11	rno-mir-34a-3p-9800	AATCAGCAAGTATACTGCCCTA	22	7
rno-mir-34b-5p-153	AGGCAGTGTAGTTAGCTGATTG	22	7653	rno-mir-34b-5p-2276	AGGCAGTGTAGTTAGCTGATTG	22	154
rno-mir-34b-3p-10227	AATCACTAACTCCACTGCCATC	22	31				
rno-mir-34c-5p-33	AGGCAGTGTAGTTAGCTGATTGC	23	26107	rno-mir-34c-5p-638	AGGCAGTGTAGTTAGCTGATTGC	23	379
rno-mir-92a-1-5p-12234	AGGTTGGGATTTGTCGCAATGCT	23	11	rno-mir-92a-1-5p-14972	AGGTTGGGATTTGTCGCAATGCT	23	4
rno-mir-92a-1-3p-2369	TATTGCACTTGTCCCGGCCTGT	22	105	rno-mir-92a-1-3p-1816	TATTGCACTTGTCCCGGCCTGT	22	109
rno-mir-92a-2-3p-2369	TATTGCACTTGTCCCGGCCTGT	22	105	rno-mir-92a-2-3p-1816	TATTGCACTTGTCCCGGCCTGT	22	97
rno-mir-92b-3p-10213	TATTGCACTTGTCCCGGCCT	20	28	rno-mir-92b-3p-8746	TATTGCACTTGTCCCGGCCT	20	13
rno-mir-93-5p-411	CAAAGTGCTGTTCGTGCAGGTAG	23	1445	rno-mir-93-5p-681	CAAAGTGCTGTTCGTGCAGGTAG	23	323
				rno-mir-96-5p-4617	TTTGGCACTAGCACATTTTTGCT	23	38
rno-mir-98-5p-330	TGAGGTAGTAGGTTGTAT	18	3558	rno-mir-98-5p-220	TGAGGTAGTAGGTTGTAT	18	3616
rno-mir-99a-5p-785	AACCCGTAGATCCGATCTTGT	21	789	rno-mir-99a-5p-322	AACCCGTAGATCCGATCTTGTG	22	1371
rno-mir-99b-5p-522	CACCCGTAGAACCGACCTTGCG	22	1336	rno-mir-99b-5p-1637	CACCCGTAGAACCGACCTTGCG	22	192
rno-mir-100-5p-785	AACCCGTAGATCCGATCTTGT	21	645	rno-mir-100-5p-322	AACCCGTAGATCCGATCTTGTG	22	1121
rno-mir-101a-3p-201	GTACAGTACTGTGATAACTGA	21	6484	rno-mir-101a-3p-196	TACAGTACTGTGATAACTGA	20	4473
rno-mir-101b-3p-201	GTACAGTACTGTGATAACTGA	21	6592	rno-mir-101b-3p-196	TACAGTACTGTGATAACTGA	20	4117
rno-mir-103-1-3p-19	AGCAGCATTGTACAGGGCTAT	21	63534	rno-mir-103-1-3p-28	AGCAGCATTGTACAGGGCTAT	21	28952
rno-mir-106b-5p-449	TAAAGTGCTGACAGTGCAGAT	21	964	rno-mir-106b-5p-1726	TAAAGTGCTGACAGTGCAGAT	21	167
rno-mir-106b-3p-2637	CCGCACTGTGGGTACTTGCTGC	22	117				
rno-mir-107-3p-19	AGCAGCATTGTACAGGGCTAT	21	55294	rno-mir-107-3p-28	AGCAGCATTGTACAGGGCTAT	21	24949
rno-mir-10a-5p-1061	TACCCTGTAGATCCGAATTTGT	22	245	rno-mir-10a-5p-4270	TACCCTGTAGATCCGAATTTGT	22	37
rno-mir-10b-5p-1061	TACCCTGTAGATCCGAATTTGT	22	245	rno-mir-10b-5p-4270	TACCCTGTAGATCCGAATTTGT	22	37
rno-mir-122-5p-4497	TGGAGTGTGACAATGGTGTTT	21	65	rno-mir-122-5p-3055	TGGAGTGTGACAATGGTGTTTG	22	91
rno-mir-1224-5p-6679	GTGAGGACTGGGGAGGTGGAG	21	21				
rno-mir-124-1-3p-7629	TAAGGCACGCGGTGAATGCCAA	22	15	rno-mir-124-1-3p-7580	TAAGGCACGCGGTGAATGCCAA	22	10
rno-mir-125a-5p-1728	TCCCTGAGACCCTTTAACCTGT	22	179	rno-mir-125a-5p-4728	TCCCTGAGACCCTTTAACCTGT	22	26
rno-mir-125a-3p-6119	ACAGGTGAGGTTCTTGGGAGC	21	29	rno-mir-125a-3p-11903	ACAGGTGAGGTTCTTGGGAG	20	9
rno-mir-125b-1-5p-215	TCCCTGAGACCCTAACTTGTGA	22	2734	rno-mir-125b-1-5p-312	TCCCTGAGACCCTAACTTGTGA	22	857
rno-mir-125b-1-3p-4566	ACGGGTTAGGCTCTTGGGAGT	21	46				
rno-mir-125b-2-5p-215	TCCCTGAGACCCTAACTTGTGA	22	2734	rno-mir-125b-2-5p-312	TCCCTGAGACCCTAACTTGTGA	22	857
rno-mir-125b-2-3p-22648	ACAAGTCAGGCTCTTGGGA	19	13	rno-mir-125b-2-3p-3344	ACAAGTCAGGCTCTTGGGAC	20	31
rno-mir-126-5p-25277	CATTATTACTTTTGGTACGCGT	22	3	rno-mir-126-5p-3874	CATTATTACTTTTGGTACGCG	21	51
rno-mir-126-3p-26974	TCGTACCGTGAGTAATAATGCG	22	3	rno-mir-126-3p-2611	TCGTACCGTGAGTAATAATGCG	22	93
				rno-mir-127-3p-6235	TCGGATCCGTCTGAGCTTGGC	21	28
rno-mir-128-1-3p-352	TCACAGTGAACCGGTCTCTTT	21	1285	rno-mir-128-1-3p-1334	TCACAGTGAACCGGTCTCTTT	21	175
rno-mir-130a-3p-129	CAGTGCAATGTTAAAAGGGCAT	22	4631	rno-mir-130a-3p-544	CAGTGCAATGTTAAAAGGGCAT	22	452
rno-mir-132-3p-2452	TAACAGTCTACAGCCATGGTCG	22	101	rno-mir-132-3p-2796	TAACAGTCTACAGCCATGGTCG	22	44
				rno-mir-134-5p-5402	TGTGACTGGTTGACCAGAGGG	21	68
				rno-mir-135a-5p-2824	TATGGCTTTTTATTCCTATGT	21	56
				rno-mir-135a-3p-5411	TGTAGGGATGGAAGCCATGAA	21	68
rno-mir-138-1-5p-4246	AGCTGGTGTTGTGAATCAGGCCG	23	89	rno-mir-138-1-5p-3523	AGCTGGTGTTGTGAATCAGGCCG	23	55
rno-mir-140-5p-4711	CAGTGGTTTTACCCTATGGTAG	22	27	rno-mir-140-5p-10935	CAGTGGTTTTACCCTATGGTAG	22	6
rno-mir-140-3p-91	ACCACAGGGTAGAACCACGGA	21	19740	rno-mir-140-3p-182	TACCACAGGGTAGAACCACGGA	22	4698
				rno-mir-141-3p-989	TAACACTGTCTGGTAAAGATGG	22	862
rno-mir-142-5p-31259	CCCATAAAGTAGAAAGCACTAC	22	3	rno-mir-142-5p-721	CCCATAAAGTAGAAAGCACTA	21	834
				rno-mir-142-3p-1929	GTAGTGTTTCCTACTTTATGGA	22	143
rno-mir-143-5p-1662	GGTGCAGTGCTGCATCTCTGGT	22	309	rno-mir-143-5p-3198	GGTGCAGTGCTGCATCTCTGGT	22	91
rno-mir-143-3p-64	TGAGATGAAGCACTGTAGCTC	21	12253	rno-mir-143-3p-83	TGAGATGAAGCACTGTAGCTC	21	5377
rno-mir-145-5p-2827	GTCCAGTTTTCCCAGGAATCC	21	261	rno-mir-145-5p-4723	GTCCAGTTTTCCCAGGAAT	19	62
rno-mir-145-3p-7097	ATTCCTGGAAATACTGTTCT	20	29	rno-mir-145-3p-18962	ATTCCTGGAAATACTGTTCT	20	6

rno-mir-146a-5p-9904	TGAGAACTGAATTCCATAGG	20	25
rno-mir-148b-3p-2481	TCAGTGCATCACAGAACTTTGT	22	121
rno-mir-151-5p-1108	TCGAGGAGCTCACAGTCTAGT	21	265
rno-mir-151-3p-3875	CTAGACTGAGGCTCCTTGAGGA	22	173
rno-mir-152-3p-103	TCAGTGCATGACAGAACTTGGT	22	9814
rno-mir-181a-1-5p-451	AACATTCAACGCTGTCGGTGAGT	23	2454
rno-mir-181a-1-3p-9615	ACCATCGACCGTTGATTGTAC	21	21
rno-mir-183-5p-17527	TATGGCACTGGTAGAATTCACT	22	5
rno-mir-185-5p-212	TGGAGAGAAAGGCAGTTCCTGA	22	2816
rno-mir-186-5p-2337	CAAAGAATTCTCCTTTTGGGCT	22	165
rno-mir-187-3p-6066	TCGTGTCTTGTGTTGCAGCCGG	22	27
rno-mir-191-5p-305	CAACGGAATCCCAAAAGCAGCTG	23	4779
rno-mir-192-5p-283	CTGACCTATGAATTGACAGCC	21	2674
rno-mir-193-5p-8401	TGGGTCTTTGCGGGCAAGATGA	22	22
rno-mir-193-3p-1366	AACTGGCCTACAAAGTCCCAGT	22	227
rno-mir-194-1-5p-4333	TGTAACAGCAACTCCATGTGGAA	23	69
rno-mir-194-2-5p-4333	TGTAACAGCAACTCCATGTGGAA	23	69
rno-mir-195-5p-27411	TAGCAGCACAGAAATATTGGCAT	23	3
rno-mir-196a-5p-2177	TAGGTAGTTTCCTGTTGTTGGG	22	151
rno-mir-199a-5p-384	CCCAGTGTTCAGACTACCTGTT	22	1111
rno-mir-199a-3p-15	ACAGTAGTCTGCACATTGGTT	21	76647
rno-mir-200a-3p-10642	TAACACTGTCTGGTAACGATGT	22	13
rno-mir-204-5p-7433	TTCCCTTTGTCATCCTATGCCT	22	15
rno-mir-204-3p-9093	GCTGGGAAGGCAAAGGGACGT	21	22
rno-mir-206-3p-7527	TGGAATGTAAGGAAGTGTGTGA	22	21
rno-mir-210-3p-123	CTGTGCGTGTGACAGCGGCTGA	22	5146
rno-mir-211-5p-7433	TTCCCTTTGTCATCCTATGCCT	22	15
rno-mir-214-3p-4278	ACAGCAGGCACAGACAGGCAT	21	253
rno-mir-219-1-3p-10084	AGAGTTGCGTCTGGACGTCCCG	22	18
rno-mir-221-5p-4540	ACCTGGCATACAATGTAGATTT	22	49
rno-mir-221-3p-179	AGCTACATTGTCTGCTGGGTTT	22	4366
rno-mir-222-3p-1096	AGCTACATCTGGCTACTGGGTCT	23	579
rno-mir-298-5p-2995	GGCAGAGGAGGGCTGTTCTTC	21	302
rno-mir-301a-3p-12327	CAGTGCAATAGTATTGTCAAAG	22	21
rno-mir-301b-3p-12327	CAGTGCAATAGTATTGTCAAAG	22	15
rno-mir-320-3p-132	AAAAGCTGGGTTGAGAGGGCGA	22	8123
rno-mir-322-5p-3473	CAGCAGCAATTCATGTTTTGGA	22	133
rno-mir-322-3p-376	AAACATGAAGCGCTGCAACAC	21	1351
rno-mir-324-5p-1049	CGCATCCCCTAGGGCATTGGTGT	23	283
rno-mir-324-3p-8679	ACTGCCCCAGGTGCTGCTGGT	21	13
rno-mir-330-3p-2800	GCAAAGCACAGGGCCTGCAGAGA	23	172
rno-mir-331-3p-13493	GCCCCTGGGCCTATCCTAGAA	21	14
rno-mir-335-5p-13354	TCAAGAGCAATAACGAAAAATG	22	10
rno-mir-339-5p-13540	TCCCTGTCCTCCAGGAGCTCA	21	21
rno-mir-339-3p-8129	TGAGCGCCTCGACGACAGAGCCA	23	48
rno-mir-340-5p-670	TTATAAAGCAATGAGACTGATT	22	480
rno-mir-342-5p-17482	AGGGGTGCTATCTGTGATTGAGG	23	16
rno-mir-342-3p-2818	TCTCACACAGAAATCGCACCCGT	23	91
rno-mir-345-5p-10372	TGCTGACCCCTAGTCCAGTGCT	22	22
rno-mir-345-3p-8600	CCCTGAACTAGGGGTCTGGAGT	22	54
rno-mir-350-3p-13362	TTCACAAAGCCCATACACTTTC	22	11
rno-mir-351-5p-15214	TCCCTGAGGAGCCCTTTGAGC	21	18
rno-mir-361-5p-7388	TTATCAGAATCTCCAGGGGTAT	22	51
rno-mir-362-5p-6811	AATCCTTGGAACCTAGGTGTGA	22	34
rno-mir-362-3p-5140	AACACACCTGTTCAAGGATTC	21	36
rno-mir-365-5p-10778	GAGGGACTTTCAGGGGCAGCTGT	23	10
rno-mir-365-3p-6512	TAATGCCCCTAAAAATCCTTAT	22	25
rno-mir-374-5p-1928	ATATAATACAACCTGCTAAGTG	22	145
rno-mir-375-3p-10302	TTTGTTCGTTCGGCTCGCGTGA	22	10
rno-mir-378-3p-442	ACTGGACTTGGAGTCAGAAGGC	22	1606
rno-mir-423-5p-244	TGAGGGGCAGAGAGCGAGACTTT	23	2389
rno-mir-423-3p-3496	AGCTCGGTCTGAGGCCCCTCAGT	23	76
rno-mir-425-5p-13489	AATGACACGATCACTCCCGTTG	22	17
rno-mir-429-3p-29091	TAATACTGTCTGGTAATGCCGT	22	3
rno-mir-449a-5p-3626	TGGCAGTGTATTGTTAGCTGGT	22	56
rno-mir-450a-5p-6682	TTTTGCGATGTGTTCCTAAT	20	40
rno-mir-455-3p-19425	GCAGTCCACGGGCATATACACT	22	11
rno-mir-466c-5p-3232	TGTGATGTGTGTATGTACATGT	22	99
rno-mir-497-5p-23372	CAGCAGCACACTGTGGTTTGT	21	4
rno-mir-499-5p-651	TTAAGACTTGCAGTGATGTTT	21	482
rno-mir-500-3p-9469	AATGCACCTGGGCAAGGGTTC	21	22
rno-mir-503-5p-776	TAGCAGCGGGAACAGTACTGCAG	23	398
rno-mir-505-5p-9919	GGGAGCCAGGAAGTATTGATGT	22	22
rno-mir-532-5p-1115	CATGCCTTGAGTGTAGGACTGT	22	236
rno-mir-532-3p-12974	CCTCCCACACCCAAGGCTTGCA	22	14
rno-mir-542-5p-6848	CTCGGGGATCATCATGTCACGA	22	17
rno-mir-542-3p-3886	TGTGACAGATTGATAACTGAA	21	70
rno-mir-592-5p-10268	TTGTGTCAATATGCGATGATGT	22	10
rno-mir-598-3p-1685	TACGTCATCGTCGTCATCGTT	21	305
rno-mir-652-3p-2843	AATGGCGCCACTAGGGTTGTGT	22	86
rno-mir-664-2-5p-24237	CTGGCTGGGGAAAATGATTGGATA	24	4
rno-mir-672-5p-456	TGAGGTTGGTGTACTGTGTGTGA	23	1018
rno-mir-674-5p-1244	GCACTGAGATGGGAGTGGTGT	21	322
rno-mir-872-5p-1464	AAGGTTACTTGTTAGTTCAGG	21	383
rno-mir-872-3p-2617	TGAACTATTGCAGTAGCCTCCT	22	100
rno-mir-877-5p-3054	TAGAGGAGATGGCGCAGGGGAC	22	215

rno-mir-146a-5p-2002	TGAGAACTGAATTCCATGGGTT	22	218
rno-mir-148b-3p-4308	TCAGTGCATCACAGAACTTTGT	22	34
rno-mir-151-5p-2604	TCGAGGAGCTCACAGTCTAGT	21	73
rno-mir-151-3p-6688	CTAGACTGAGGCTCCTTGAGGA	22	57
rno-mir-152-3p-263	TCAGTGCATGACAGAACTTGGT	22	1936
rno-mir-154-5p-4156	TAGGTTATCCGTGTTGCCTTCG	22	25
rno-mir-154-3p-4611	AATCATACACGGTTGACCTATT	22	25
rno-mir-181a-1-5p-1466	AACATTCAACGCTGTCGGTGAGT	23	350
rno-mir-181a-1-3p-8461	ACCATCGACCGTTGATTGTACC	22	24
rno-mir-182-5p-4136	TTTGGCAATGGTAGAACTCACA	22	57
rno-mir-183-5p-4209	TATGGCACTGGTAGAATTCACT	22	65
rno-mir-183-3p-8604	TGAATTACCGAAGGGCCATAA	21	12
rno-mir-185-5p-145	TGGAGAGAAAGGCAGTTCCTGA	22	2423
rno-mir-186-5p-3584	CAAAGAATTCTCCTTTTGGGCT	22	62
rno-mir-187-3p-13690	TCGTGTCTTGTGTTGCAGCCGG	22	5
rno-mir-191-5p-190	CAACGGAATCCCAAAAGCAGCTG	23	4203
rno-mir-192-5p-608	CTGACCTATGAATTGACAGCC	21	635
rno-mir-193-5p-5173	TGGGTCTTTGCGGGCAAGATGA	22	16
rno-mir-193-3p-1318	AACTGGCCTACAAAGTCCCAGT	22	149
rno-mir-194-1-5p-6701	TGTAACAGCAACTCCATGTGGAA	23	24
rno-mir-194-2-5p-6701	TGTAACAGCAACTCCATGTGGAA	23	24
rno-mir-195-5p-1638	TAGCAGCACAGAAATATTGGCA	22	143
rno-mir-199a-5p-3699	CCCAGTGTTCAGACTACCTGTT	22	74
rno-mir-199a-3p-125	ACAGTAGTCTGCACATTGGTT	21	4826
rno-mir-200a-5p-13069	CATCTTACCGGACAGTGCTG	20	12
rno-mir-200a-3p-229	TAACACTGTCTGGTAACGATGTT	23	2669
rno-mir-200c-3p-109	TAATACTGCCGGGTAATGATGGA	23	6111
rno-mir-203-3p-3087	GTGAAATGTTTAGGACCACTAG	22	65
rno-mir-204-5p-14802	TTCCCTTTGTCATCCTATGCCT	22	4
rno-mir-204-3p-6552	GCTGGGAAGGCAAAGGGACGT	21	15
rno-mir-205-5p-2715	TCCTTCATTCCACCGGAGTCT	21	113
rno-mir-206-3p-15401	TGGAATGTAAGGAAGTGTGTGA	22	4
rno-mir-210-3p-800	CTGTGCGTGTGACAGCGGCTGA	22	309
rno-mir-211-5p-14802	TTCCCTTTGTCATCCTATGCCT	22	4
rno-mir-221-5p-2041	ACCTGGCATACAATGTAGATTT	22	94
rno-mir-221-3p-121	AGCTACATTGTCTGCTGGGTTT	22	4220
rno-mir-222-3p-429	AGCTACATCTGGCTACTGGGTCT	23	1009
rno-mir-223-5p-6340	CGTGTATTTGACAAGCTGAGTTGGACAGT	29	15
rno-mir-224-5p-6764	CAAGTCACTAGTGGTTCCGTTT	22	20
rno-mir-298-5p-15956	GGCAGAGGAGGGCTGTTCTT	20	4
rno-mir-301a-3p-15174	CAGTGCAATAGTATTGTCAAAG	22	4
rno-mir-301b-3p-15174	CAGTGCAATAGTATTGTCAAAG	22	4
rno-mir-320-3p-135	AAAAGCTGGGTTGAGAGGGCGA	22	4035
rno-mir-322-5p-5017	CAGCAGCAATTCATGTTTTGGA	22	45
rno-mir-322-3p-1772	AAACATGAAGCGCTGCAACAC	21	191
rno-mir-324-5p-5605	CGCATCCCCTAGGGCATTGGTGT	23	26
rno-mir-330-3p-6169	GCAAAGCACAGGGCCTGCAGAGA	23	38
rno-mir-337-5p-4518	CGGCGTCATGCAGGAGTTGATT	22	29
rno-mir-339-5p-15063	TCCCTGTCCTCCAGGAGCTC	20	8
rno-mir-339-3p-11830	TGAGCGCCTCGACGACAGAGCCA	23	24
rno-mir-340-5p-688	TTATAAAGCAATGAGACTGATT	22	363
rno-mir-341-3p-7771	TCGGTCGATCGGTCGGTCGGT	21	28
rno-mir-342-3p-7805	TCTCACACAGAAATCGCACCCGT	23	15
rno-mir-345-5p-19591	TGCTGACCCCTAGTCCAGTGCT	22	9
rno-mir-345-3p-9057	CCCTGAACTAGGGGTCTGGAGA	22	18
rno-mir-361-5p-9832	TATCAGAATCTCCAGGGGTAC	21	15
rno-mir-362-5p-8109	AATCCTTGGAACCTAGGTGTGA	22	12
rno-mir-362-3p-4660	AACACACCTGTTCAAGGATTC	21	28
rno-mir-363-3p-4692	AATTGCACGGTATCCATCTGTA	22	43
rno-mir-365-5p-14696	AGGGACTTTCAGGGGCAGCTGTG	23	4
rno-mir-369-3p-10514	AATAATACATGGTTGATCTTT	21	13
rno-mir-374-5p-3472	ATATAATACAACCTGCTAAGTG	22	33
rno-mir-375-3p-136	TTTGTTCGTTCGGCTCGCGTGA	22	2597
rno-mir-376a-3p-4168	ATCGTAGAGGAAAATCCACGT	21	46
rno-mir-376b-3p-16928	ATCATAGAGGAACATCCACTTT	22	10
rno-mir-378-3p-59	ACTGGACTTGGAGTCAGAAGGC	22	14703
rno-mir-379-5p-4892	TGGTAGACTATGGAACGTAGG	21	40
rno-mir-382-5p-4234	GAAGTTGTTCGTGGTGGATTCG	22	43
rno-mir-411-5p-7442	ATAGTAGACCGTATAGCGTAC	21	25
rno-mir-423-5p-387	TGAGGGGCAGAGAGCGAGACTTT	23	856
rno-mir-423-3p-6006	AGCTCGGTCTGAGGCCCCTCAGT	23	31
rno-mir-425-5p-18495	AATGACACGATCACTCCCGTTG	22	3
rno-mir-429-3p-470	TAATACTGTCTGGTAATGCCGT	22	497
rno-mir-449a-5p-8477	TGGCAGTGTATTGTTAGCTGGT	22	9
rno-mir-450a-5p-14227	TTTTGCGATGTGTTCCTAAT	20	4
rno-mir-451-5p-7486	AAACCGTTACCATTACTGAGTTT	23	10
rno-mir-455-3p-13907	GCAGTCCACGGGCATATACACT	22	5
rno-mir-466c-5p-18474	TGTGATGTGTGCATGTACATGT	22	3
rno-mir-495-3p-8645	AACAAACATGGTGCACTTCTTT	22	11
rno-mir-497-5p-1099	CAGCAGCACACTGTGGTTTGT	21	189
rno-mir-499-5p-933	TTAAGACTTGCAGTGATGTTT	21	237
rno-mir-500-3p-5432	AATGCACCTGGGCAAGGGTTCA	22	21
rno-mir-503-5p-1244	TAGCAGCGGGAACAGTACTGCAG	23	148
rno-mir-511-3p-3202	AATGTGTAGCAAAAGACAGGA	21	37
rno-mir-532-5p-1326	CATGCCTTGAGTGTAGGACTGT	22	110
rno-mir-541-5p-5314	AAGGGATTCTGATGTTGGTCA	21	59
rno-mir-542-3p-13186	TGTGACAGATTGATAACTGAAA	22	5
rno-mir-543-3p-5554	AAACATTCGCGGTGCACTTCTTT	23	30
rno-mir-598-3p-5865	TACGTCATCGTCGTCATCGTTAT	23	26
rno-mir-652-3p-8213	AATGGCGCCACTAGGGTTGTGT	22	13
rno-mir-664-2-5p-10714	CTGGCTGGGGAAAATGATTGGAA	23	13
rno-mir-672-5p-366	TGAGGTTGGTGTACTGTGTGTGA	23	744
rno-mir-674-5p-1100	GCACTGAGATGGGAGTGGTGT	21	236
rno-mir-872-5p-2639	AAGGTTACTTGTTAGTTCAG	20	109
rno-mir-872-3p-5787	TGAACTATTGCAGTAGCCTCCT	22	21

*miRNA isoforms identified in fibroblasts and salivary glands are highlighted in yellow (3' end differences) or green (5' end differences).

genome. However, the number of sequences mapping to the RCMV genome from the infected salivary gland sample was only 0.7% of the total reads (4159 total counts). The remainder of the unmapped sequences most likely represents RNA degradation products, non-coding RNAs, or yet unrealized cellular miRNAs.

We identified 24 unique miRNAs in the RCMV infected samples (fibroblasts and salivary gland combined) that mapped to stem loop structures found within the viral genome (Table 2.2 and Supplemental Table 2.2). Of the 24 RCMV-miRNAs, 22 were expressed in the fibroblast sample with 4 (miR-r1-4, miR-r43.1-2, miR-R90-1, and miR-r111.2-1) being uniquely detected in this sample and not in the salivary gland tissues. Twenty viral miRNAs were detected in the salivary glands; 18 of these were also detected in the fibroblasts and two RCMV encoded miRNAs (miR-r95.1-1 and miR-r170-1) were only present in the salivary gland tissues. The viral miRNAs ranged from 16 to 23 nucleotides (nt) in length; however most were between 19 to 22 nt. As expected, the overall viral miRNA copy number in the salivary glands was lower than in the infected fibroblasts, which is most likely due to the lower infection rate found *in vivo*. Expression profiles (types of miRNAs and relative levels) of the RCMV miRNAs are, for the most part, consistent under both *in vitro* and *in vivo* conditions. Accordingly, the top most highly expressed of the viral miRNAs, miR-R87-1, miR-r111.1-2, miR-r111.2-5, and miR-r1-1 are expressed under both conditions (Table 2.2). A small number of RCMV miRNAs are expressed at low levels and it is possible that their discovery alone might be due to the high sensitivity of the deep sequencing

Table 2.2 RCMV miRNAs (miR) detected in infected fibroblasts and salivary glands.

Sequenced from RCMV infected Fibroblasts (72hpi)

RCMV-miR name	miR sequence[b]	miR length	Copy Number of the isoform	Copy Number all isoforms	strand	RCMV Genomic Position[a] miR start	miR end
miR-r6-1	TCGACCTCAAGCCGTTCGGGAC	22	131	996	+	8671	8692
miR-OriLyt-1	GACGGGGTCTCGGGCTCCTGA	21	178	583	+	77402	77422
miR-OriLyt-2	TGGCTCGCGTCGCCATGGAGAC	22	30	97	+	78953	78974
miR-R87-1	TCGAAGAACGGGTGCAACTC	20	68629	166117	+	113930	113949
miR-R91-1	GGACTCGGAGTCGTCGGACGCT	22	1706	6491	+	121165	121186
miR-r111.1-1	CGCACCGGCGTCGAGCACGTAC	22	753	2857	+	152214	152235
miR-r111.1-2	TCGAAACAACGTGGAACGGCGT	22	11212	30084	+	152728	152749
miR-r111.1-3	TCGGGGGCGGTCGGAAGGTCC	21	78	427	+	152891	152911
miR-r1-1	GTAAGATGGAATCACCGGAGG	21	5265	26402	-	1024	1044
miR-r1-2	TTTCTCTCGTGCTCCGTGTCGC	22	610	1030	-	1187	1208
miR-r1-3	TGATGCGGGGTAGGGGAGTGAG	22	183	473	-	1154	1175
miR-r1-4	CCAGGTGGAGGAGTCCGGTC	20	85	156	-	1281	1300
miR-r37-1	TTGTCGTGGGTTTCGT	16	72	77	-	34740	34755
miR-r43.1-1	TTATCAGCCGGCAAGCACCCAGG	23	1261	5577	-	40748	40770
miR-r43.1-2	AGGGGTTCGCCGGCCGATATCG	22	40	58	-	40795	40816
miR-R90-1	GACCGGGGATCGTCGAACGAC	21	66	192	-	121129	121149
miR-r111.2-1	TACGTGCTCGACGCCGGTGCGG	22	13	47	-	152213	152234
miR-r111.2-2	CTTCGAGTGCGTGTCCGATAGC	22	101	347	-	152238	152259
miR-r111.2-3	AATCGGACACCCGCTCGCGAAGG	23	164	1072	-	152292	152314
miR-r111.2-4	CCCGAAACTCCGTCGAACGCG	21	418	1075	-	152762	152782
miR-r111.2-5	TTCCACGTTGTTTCGAGGCCT	21	1637	3770	-	152723	152743
miR-r111.2-6	TTCGCGGACGATCGAGGAG	19	14331	57461	-	152854	152872

Sequenced from Salivary glands (21dpi)

RCMV-miR name	miR sequence[b]	miR length	Copy Number of the isoform	Copy Number all isoforms	strand	RCMV Genomic Position[a] miR start	miR end
miR-r6-1	TCGACCTCAAGCCGTTCGGGAC	22	9	74	+	8671	8692
miR-OriLyt-1	GACGGGGTCTCGGGCTCCTGA	21	22	42	+	77402	77422
miR-OriLyt-2	TGGCTCGCGTCGCCATGGAGACA	23	5	8	+	78953	78975
miR-R87-1	TCGAAGAACGGGTGCAACTC	20	402	926	+	113930	113949
miR-R91-1	GGACTCGGAGTCGTCGGACGCT	22	12	54	+	121165	121186
miR-r111.1-1	CGCACCGGCGTCGAGCACGTAC	22	28	113	+	152214	152235
miR-r111.1-2	TCGAAACAACGTGGAACGGCGT	22	646	1942	+	152728	152749
miR-r111.1-3	TCGGGGGCGGTCGGAAGGT	19	7	19	+	152891	152909
miR-r170-1	ACCGACTGAGCGGACGG	17	6	6	+	224343	224359
miR-r1-1	GTAAGATGGAATCACCGGAGGC	22	51	198	-	1023	1044
miR-r1-2	TTTCTCTCGTGCTCCGTGTCGC	22	6	6	-	1187	1208
miR-r1-3	TGATGCGGGGTAGGGGAGTGAGA	23	3	3	-	1153	1175
miR-r37-1	TTGTCGTGGGTTTCGT	16	143	147	-	34740	34755
miR-r43.1-1	TTATCAGCCGGCAAGCACCCA	21	15	36	-	40750	40770
miR-r95.1-1	GACGGAGAGCGAACGGT	17	4	4	-	128079	128095
miR-r111.2-2	CTTCGAGTGCGTGTCCGATAG	21	10	20	-	152239	152259
miR-r111.2-3	ATCGGACACCCGCTCGCGAAGGA	23	12	31	-	152291	152313
miR-r111.2-4	CCCGAAACTCCGTCGAACGCG	21	17	30	-	152762	152782
miR-r111.2-5	TTCCACGTTGTTTCGAGGCCT	21	45	101	-	152723	152743
miR-r111.2-6	TTCGCGGACGATCGAGGAGGCC	22	87	399	-	152851	152872

[a] positions are given relative to the published genomic sequence of the Maastricht Strain of rat cytomegalovirus (GenBank Accession Number NC_002521)

[b] shown is the predominate isoform sequence

Supplemental Table 2.2 RCMV pre-miRNA (mir) sequences.

		RCMV Genomic Position[a]		
RCMV-miR name	strand	mir start	mir end	mir sequence
miR-r6-1	+	8653	8751	TCCGCCGCGGCCGGCCGATCGACCTCAAGCCGTTCGGGACCTCTCTCCGTACGTACTCGGGTCCCGTCCACTCCGAGGTCGGTCGGCCGCGCGCGCCCG
miR-OriLyt-1	+	77355	77427	CCCGGCCCCGGAGCTCGAAACCCGGTTCGCGTGTCGTTTCGCGAGGCGACGGGGTCTCGGGCTCCTGACCCGG
miR-OriLyt-2	+	78934	79034	ACGGGCCGCCGCCTCGAGGTGGCTCGCGTCGCCATGGAGACCGAGACCCGTCAATCAGCGGTCACCATGGCGACGCGAGCCCGCCTCCGAGTCCGCGGCCG
miR-R87-1	+	13882	113959	GGCGTGCACAGCGATTGCACTCGTCCTTCCGCTGTGTGATGTGCGAAGTCGAAGAACGGGTGCAACTCTGTGTACAGC
miR-R91-1	+	121114	121200	CGAGAGGGCGTACGGGTCGTTCGACGATCCCCGGTCCTTCGTGACGGTCGAGGACTCGGAGTCGTCGGACGCTCCGTACACCTCGCA
miR-r111.1-1	+	152159	152251	GAATGTCCGATGACGATTATGTGCTCGTCACCGGAGGGTCTCTCGAATAGTCTGCCGCACCGGCGTCGAGCACGTACTCGCTATCGGACACGC
miR-r111.1-2	+	152707	152802	GGCGAACCGTATTTAGAGGCCTCGAAACAACGTGGAACGGCGTTTCGATCGATAGCGCGTTCGACGGAGTTTCGGGGCTTCGAAAGTACGTTCTCC
miR-r111.1-3	+	152844	152917	GCAGGGGGGCCTCCTCGATCGTCCGCGAAGAATCGATTTATTAAGCTTCGGGGGCGGTCGGAAGGTCCCCCGAC
miR-r170-1	+	224298	224361	CGACGCCGCCGAACGGGGCGGCGGCGGTTTTGTAACAAACGCCGAACCGTCTGAGCGGACGGGA
miR-r1-1	-	1017	1091	AGACCGGCCGCCGGTGCTGCCGTCTTATAGTCCGTGCGATGAAGAGGGTAAGATGGAATCACCGGAGGCCGGGCG
miR-r1-2	-	1126	1232	CGGGGCCGGGGCCGCCTTCCTCTCTTTCTCTCGTGCTCCGTGTCGCGGGGGAAGACGTGATGCGGGGTAGGGGAGTGAGAGAGGGAGGGCGGAGGAGGCGACCGAGA
miR-r1-3	-	1126	1232	CGGGGCCGGGGCCGCCTTCCTCTCTTTCTCTCGTGCTCCGTGTCGCGGGGGAAGACGTGATGCGGGGTAGGGGAGTGAGAGAGGGAGGGCGGAGGAGGCGACCGAGA
miR-r1-4	-	1268	1358	GAGGCGGACGGCAGCCGGACGCTTCCTTACCGCGGTCGTGTCGGCGGCAGGCGCCGGACCAGGTGGAGGAGTCCGGTCGTCGTCCGTCGAC
miR-r37-1	-	34697	34756	CTTGTCGTGGGTTTCGTGTTCTGTCGAGATCGTGTCCGATGTGTCGTCGCCCGCGATGTC
miR-r43.1-1	-	40737	40825	ACGCGCGATAGGGGTTCGCCGGCCGATATCGCGTCTGTGTCTCGATCGTGTCGCGTTATCAGCCGGCAAGCACCCAGGTCGCCGCGGGC
miR-r43.1-2	-	40748	40818	ATAGGGGTTCGCCGGCCGATATCGCGTCTGTGTCTCGATCGTGTCGCGTTATCAGCCGGCAAGCACCCAGG
miR-R90-1	-	121100	121208	GCGAGGCGTGCGAGGTGTACGGAGCGTCCGACGACTCCGAGTCCTCGACCGTCACGAAGGACCGGGGATCGTCGAACGACCCGTACGCCCTCTCGAGCGTCTTCGTCCT
miR-r95.1-1	-	128035	128102	GGGGACCGACGGAGAGCGAACGGGGCCTCGGGTACCCTACCGATCCGCCCTCGCCTCCCACGGTCGCG
miR-r111.2-1	-	152158	152252	TGCGTGTCCGATAGCGAGTACGTGCTCGACGCCGGTGCGGCAGACTATTCGAGAGACCCTCCGGTGACGAGCACATAATCGTCATCGGACATTCG
miR-r111.2-2	-	152237	152315	CAATCGGACACCCGCTCGCGAAGGATCCCCCAGACTCCGGGTCGGGCGGACGGTTCCTTCGAGTGCGTGTCCGATAGCG
miR-r111.2-3	-	152233	152323	CCCGCTCGCAATCGGACACCCGCTCGCGAAGGATCCCCCAGACTCCGGGTCGGGCGGACGGTTCCTTCGAGTGCGTGTCCGATAGCGAGTA
miR-r111.2-4	-	152707	152800	AGAACGTACTTTCGAAGCCCCGAAACTCCGTCGAACGCGCTATCGATCGAAACGCCGTTCCACGTTGTTTCGAGGCCTCTAAATACGGTTCGCC
miR-r111.2-5	-	152707	152800	AGAACGTACTTTCGAAGCCCCGAAACTCCGTCGAACGCGCTATCGATCGAAACGCCGTTCCACGTTGTTTCGAGGCCTCTAAATACGGTTCGCC
miR-r111.2-6	-	152844	152917	GTCGGGGGACCTTCCGACCGCCCCCGAAGCTTAATAAATCGATTCTTCGCGGACGATCGAGGAGGCCCCCCTGC

[a] positions are given relative to the published genomic sequence of the Maastricht Strain of rat cytomegalovirus (GenBank Accession Number NC_002521)

methods employed for this study. To validate the expression of the RCMV miRNAs, we performed Northern blot analysis of RNA extracted from RCMV infected RFL6 fibroblasts at 72hpi. Figure 2.1 shows the results obtained from 16 out of 24 RCMV miRNAs we were able to detect by Northern blot compared to the control cellular miRNA miR-16. We observed the mature form (~22nt) for all of the miRNAs as well as some of the pre-miRNA forms.

Identification of RCMV miRNA Isoforms

Similar to what has been observed in other studies of viral and cellular miRNAs, a number of the RCMV miRNAs contained considerable variation at their 3' and 5' ends. As shown in Table 2.3, the variation, for the most part, was conserved between the miRNAs detected in the infected fibroblasts and those in the rat salivary gland tissues. However, there were specific examples where the percentage of the particular miRNA isoform differed between the two samples. For example, RCMV miR-r6-1 has four major isoforms but only three of these were dominant in the fibroblasts. One of the isoforms for miR-r6-1 (TCGACCTCAAGCCGTTCGGGGACA) was the most highly expressed isoform detected in the salivary glands but this isoform was expressed to very low levels in fibroblasts. Similarly, one of the isoforms of the RCMV miR-OriLyt-2 was only detected in the *in vivo* sample (60% of sequenced sequences for this miRNA) but not in infected fibroblasts. We also detected the miRNA* (passenger strand) for 13 of the viral miRNAs and some were detected as major isoforms including RCMV miR-r6-1, miR-OriLyt-1, miR-R91-1, and miR-r111.1-1 (Table 2.3).

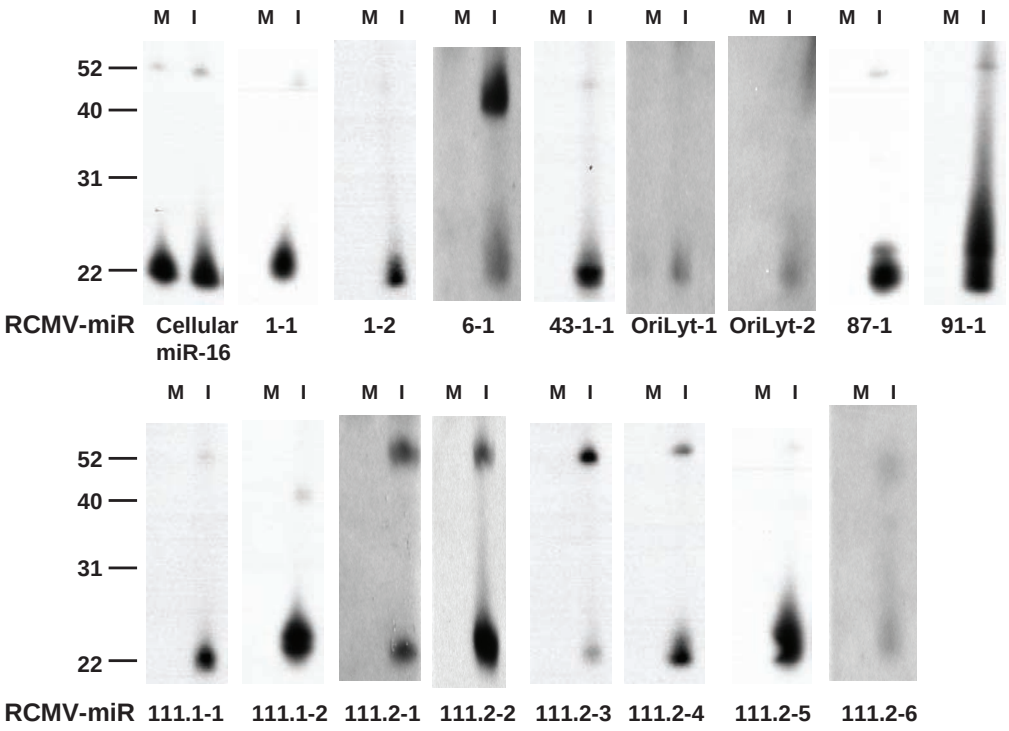

Figure 2.1 Northern blot analysis of RCMV miRNAs. Rat RFL6 fibroblast cells were infected for 72 hrs at a multiplicity of 1 PFU per cell and subjected to Northern blot analysis using probes specific for the predicted RCMV miRNA sequences. Lane M, mock infected; lane I, infected. The cellular miRNA, miR-16, was used as a control.

Table 2.3 RCMV miRNA sequence isoforms detected in fibroblasts and salivary glands.

			Fibroblasts		SMG	
miR	**RCMV miR Sequence**	**Length**	**Frequency**	**%**	**Frequency**	**%**
miR-r6-1	TCGACCTCAAGCCGTTCGGGAC	22	131	13%	9	12%
	TCCCGTCCACTCCGAGGTCGGT	22	127	13%	8	11%
	TCGACCTCAAGCCGTTCGGGA	21	97	10%	6	8%
	TCGACCTCAAGCCGTTCGGGACA	23	25	3%	17	23%
miR-OriLyt-1	GACGGGGTCTCGGGCTCCTGA	21	178	31%	22	52%
	GACGGGGTCTCGGGCTCCTGAC	22	110	19%	11	26%
	CCCGGAGCTCGAAACCCGGTTCG*	23	27	5%	6	14%
miR-OriLyt-2	TGGCTCGCGTCGCCATGGAGAC	22	30	31%		nd
	GCTCGCGTCGCCATGGAGACA	21	13	13%	3	38%
	TGGCTCGCGTCGCCATGGAGACA	23	0	nd	5	62%
miR-R87-1	TCGAAGAACGGGTGCAACTC	20	68629	41%	402	43%
	TCGAAGAACGGGTGCAACTCT	21	33774	20%	170	18%
	GAAGAACGGGTGCAACTC	18	14028	8%	99	11%
miR-R91-1	GGACTCGGAGTCGTCGGACGCT	22	1706	26%	12	22%
	GGACTCGGAGTCGTCGGACGCTT	23	972	15%	12	22%
	GACTCGGAGTCGTCGGACGCT	21	467	7%	0	nd
	CGTTCGACGATCCCCGGTCCTT*	22	191	3%	7	13%
miR-r111.1-1	CGCACCGGCGTCGAGCACGTAC	22	753	26%	28	25%
	CGCACCGGCGTCGAGCACGTACT	23	427	15%	24	21%
	CGCACCGGCGTCGAGCACGT	20	291	10%	18	16%
	TATGTGCTCGTCACCGGAGGGT*	22	333	12%	13	12%
miR-r111.1-2	TCGAAACAACGTGGAACGGCGT	22	11212	37%	646	33%
	TCGAAACAACGTGGAACGGCG	21	4115	14%	161	8%
	TCGAAACAACGTGGAACGGCGTT	23	3707	12%	488	25%
miR-r1-1	GTAAGATGGAATCACCGGAGG	21	5265	20%	27	14%
	GTAAGATGGAATCACCGGAGGC	22	5234	20%	51	26%
	GTAAGATGGAATCACCGGAG	20	4523	17%	42	21%
	GTAAGATGGAATCACCGGAGGCA	23	3869	15%	34	17%
	GTAAGATGGAATCACCGGAGGCT	23	2748	10%	26	13%
miR-r1-2	TTTCTCTCGTGCTCCGTGTCGC	22	610	60%	6	100%
	TTTCTCTCGTGCTCCGTGTCG	21	94	9%	0	nd
miR-r43.1-1	TTATCAGCCGGCAAGCACCCAGG	23	1261	23%	9	25%
	TTATCAGCCGGCAAGCACCCA	21	1183	21%	15	42%
	TTATCAGCCGGCAAGCACCCAG	22	905	16%	12	33%
miR-r111.2-1	TACGTGCTCGACGCCGGTGCGG	22	13	28%	0	nd
	TACGTGCTCGACGCCGGTGCGGA	23	11	23%	0	nd
miR-r111.2-2	CTTCGAGTGCGTGTCCGATAGC	22	101	29%	5	25%
	CTTCGAGTGCGTGTCCGATAG	21	95	27%	10	50%
	CTTCGAGTGCGTGTCCGATAGT	22	58	17%	5	25%
miR-r111.2-3	AATCGGACACCCGCTCGCGAAGG	23	164	15%	6	20%
	AATCGGACACCCGCTCGCGAAG	22	155	14%	3	10%
	ATCGGACACCCGCTCGCGAAGGA	23	114	11%	12	39%
	GGACACCCGCTCGCGAAGGA	20	76	7%	4	13%
miR-r111.2-4	CCCGAAACTCCGTCGAACGCG	21	418	39%	17	57%
	CCCGAAACTCCGTCGAACGCGC	22	346	32%	13	43%
miR-r111.2-5	TTCCACGTTGTTTCGAGGCCT	21	1637	43%	45	45%
	CACGTTGTTTCGAGGCCT	18	739	20%	22	22%
miR-r111.2-6	TTCGCGGACGATCGAGGAG	19	14331	25%	77	19%
	TTCGCGGACGATCGAGGAGGCC	22	7799	14%	87	22%
	TTCGCGGACGATCGAGGAGGC	21	6162	11%	31	8%

nd=not detected

* denotes miRNA* or passenger strand sequences

RCMV miRNA Genomic Organization

The genomic positions of the RCMV miRNAs are depicted in Figure 2.2. The RCMV miRNAs are generally distributed across the viral genome. However, similar to what was observed for other herpesviruses, a number of the RCMV miRNAs are encoded in clusters. The largest cluster of nine viral miRNAs exists in a 700bp region encoding miR-r111.1-1, miR-r111.1-2, miR-r111.1-3, miR-r111.1-4, miR-r111.2-1, miR-r111.2-2, miR-r111.2-3, miR-r111.2-4, miR-r111.2-5, and miR-r111.2-6. Of these, only two pairs (miR-r111.2-2/miR-r111.2-3 and miR-r111.2-4/miR-r111.2-5) are encoded within the same stem loop structure (Figure 2.3), indicating that within this region there are at least 7 individual stem loop structures. A cluster of 4 viral miRNAs also exists in and near the coding region for the RCMV gene r1, and two of these (miR-r1-2/miR-r1-3) also share a common stem loop. Six of the RCMV encoded miRNAs could target RCMV mRNAs because they are directly complementary to RCMV genes including: miR-R87-1 (R86), miR-R91-1 (R90), miR-r170-1 (r164) as well as miR-r111.2-1, miR-r111.2-2 and miR-r111.2-3, which target r111.1. One additional finding that may have implications in viral persistence is the fact that we identified two viral miRNAs expressed from the viral origin of replication (OriLyt).

We compared the miRNA sequences for MCMV and RCMV using the phylogenetic analysis program Clustal 3.1. There was minimal sequence conservation between the miRNAs from these two related viruses. However, when we compared the genomic positions of the miRNAs for MCMV and RCMV

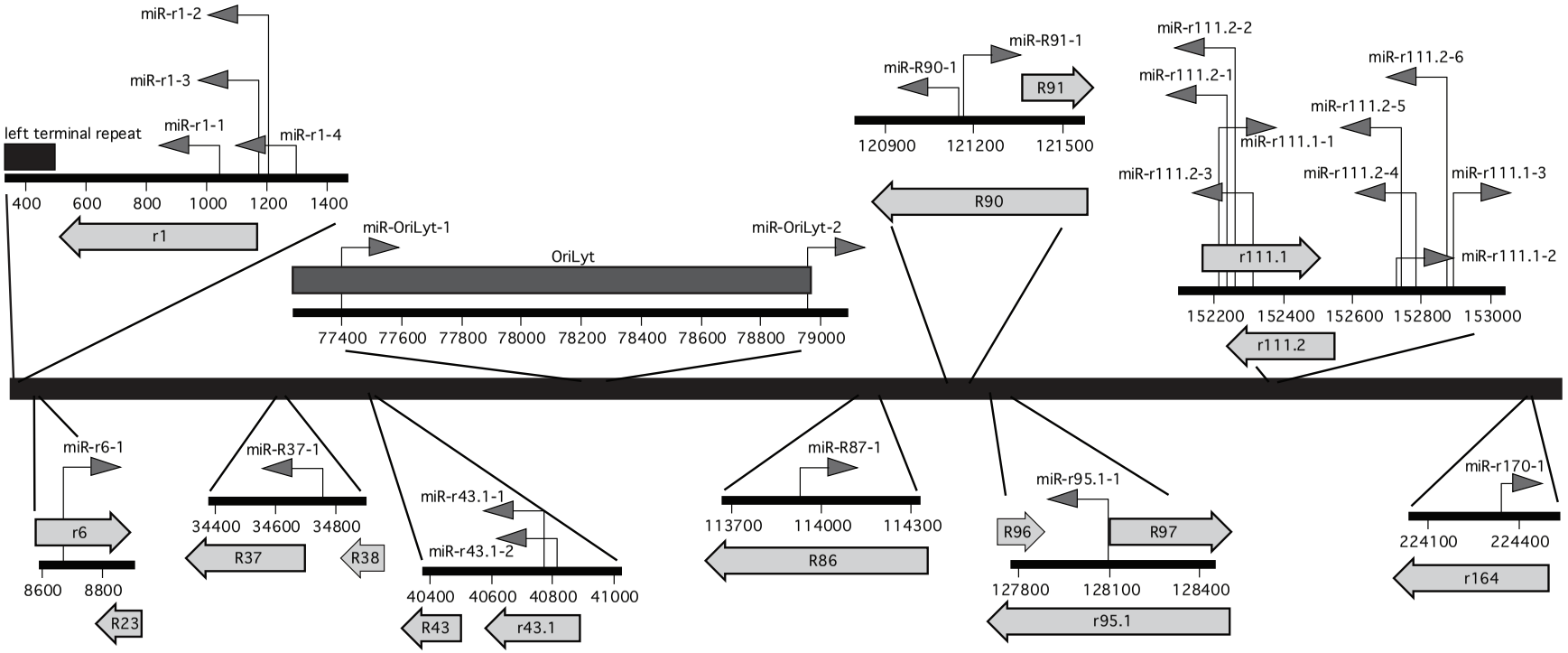

Figure 2.2 Genomic organization of RCMV miRNAs. Diagram depicting the regions of the RCMV genome that contain RCMV miRNAs. The dark gray arrows indicate the position and direction of the RCMV miRNAs where right-pointing arrows indicate RCMV miRNAs on the sense strand and left-pointing arrows indicate miRNAs on the complement strand. Open reading frames that contain or are near viral miRNAs are shown in light gray block arrows. The RCMV origin of lytic replication is depicted in a dark grey box.

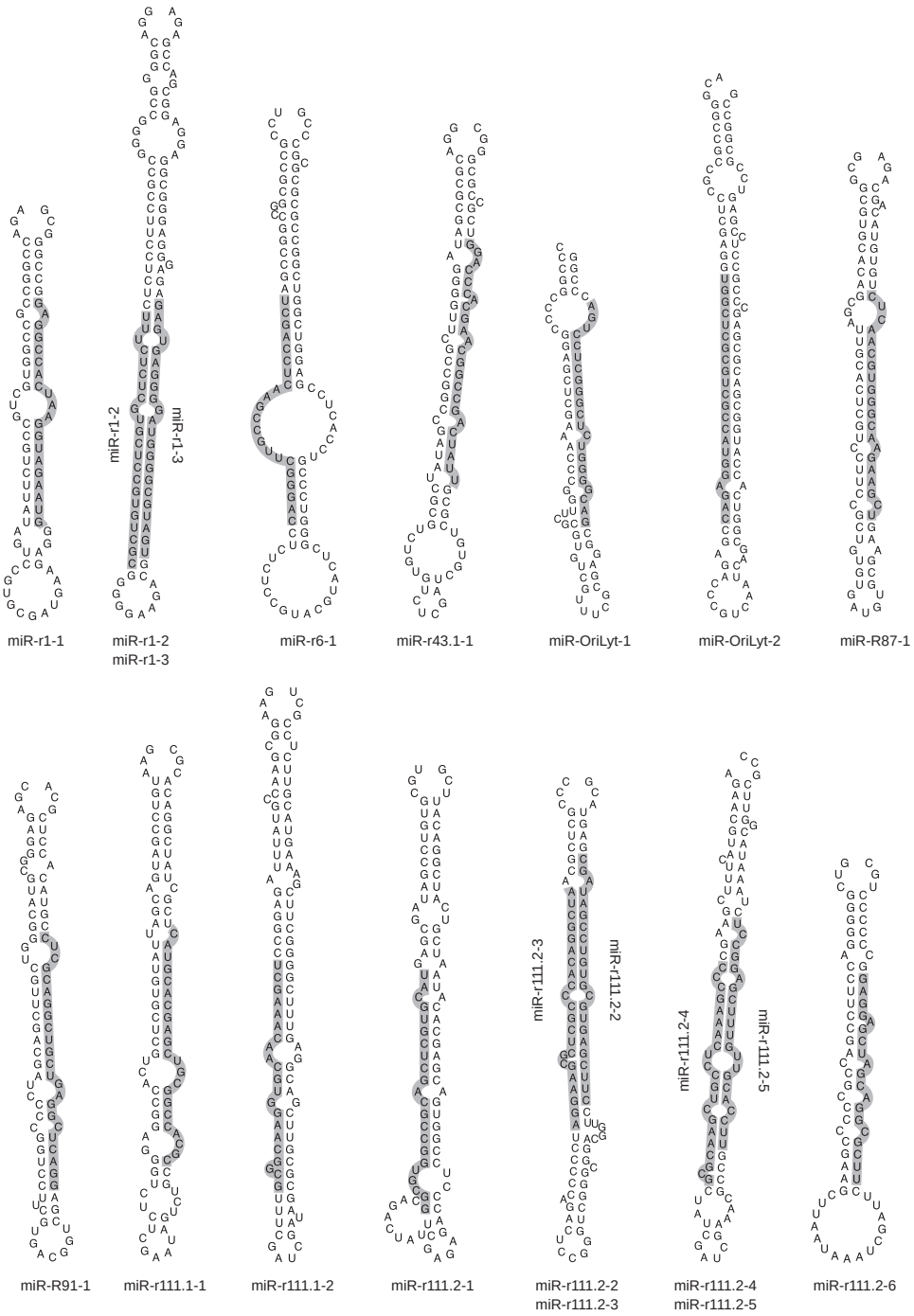

Figure 2.3 Secondary structures of RCMV precursor miRNAs. The predicted stem loop secondary structures of the RCMV pre-miRNAs from the mfold program (Zuker 2003) are depicted with the mature miRNAs shaded in gray. When two miRNAs are transcribed from one stem loop the position of each miRNA is indicated on the 5’ or 3’ arm of the stem loop.

we observed that most of the miRNAs had similar orientation in the viral genomes (Figure 2.4). For example, both RCMV and MCMV contain clusters of miRNAs at the extreme 5' end of the genome. MCMV encodes 6 miRNAs and RCMV encodes 4 miRNAs in this region. RCMV lacks the MCMV genes m7-m22 however; RCMV miR-r6-1 is positioned near R23, which is a similar location to the cluster of MCMV miRNAs encoded near m21, m22, and M23. Both viral genomes contain miRNAs in or near the OriLyt. While the original description of miR-m59-1, 2, and 3 did not suggest this orientation, further review by our group puts the miRNAs near the origin of lytic replication. Interestingly, both viruses also contain clusters of miRNAs on complementary strands of the viral genomes in a central region near the RCMV and MCMV 112 open reading frames. The 112 region is close to the large stable intron in MCMV and most likely RCMV, although the stable intron has not yet been specifically mapped for RCMV. Similar to other cytomegaloviruses, the location of the RCMV miRNAs share a genome wide distribution profile, which is different than the observed clustering of the α- and γ-herpesvirus miRNAs to latency associated regions.

Characterization of RCMV miRNA expression

CMV gene expression *in vitro* can be divided into three kinetic classes: Immediate Early, Early and Late, based on the requirements of protein synthesis and viral DNA replication. Therefore, we next sought to determine whether the cluster of nine RCMV miRNAs found within the 700bp region near RCMV r111 are all expressed with the same kinetics or differentially regulated. We performed

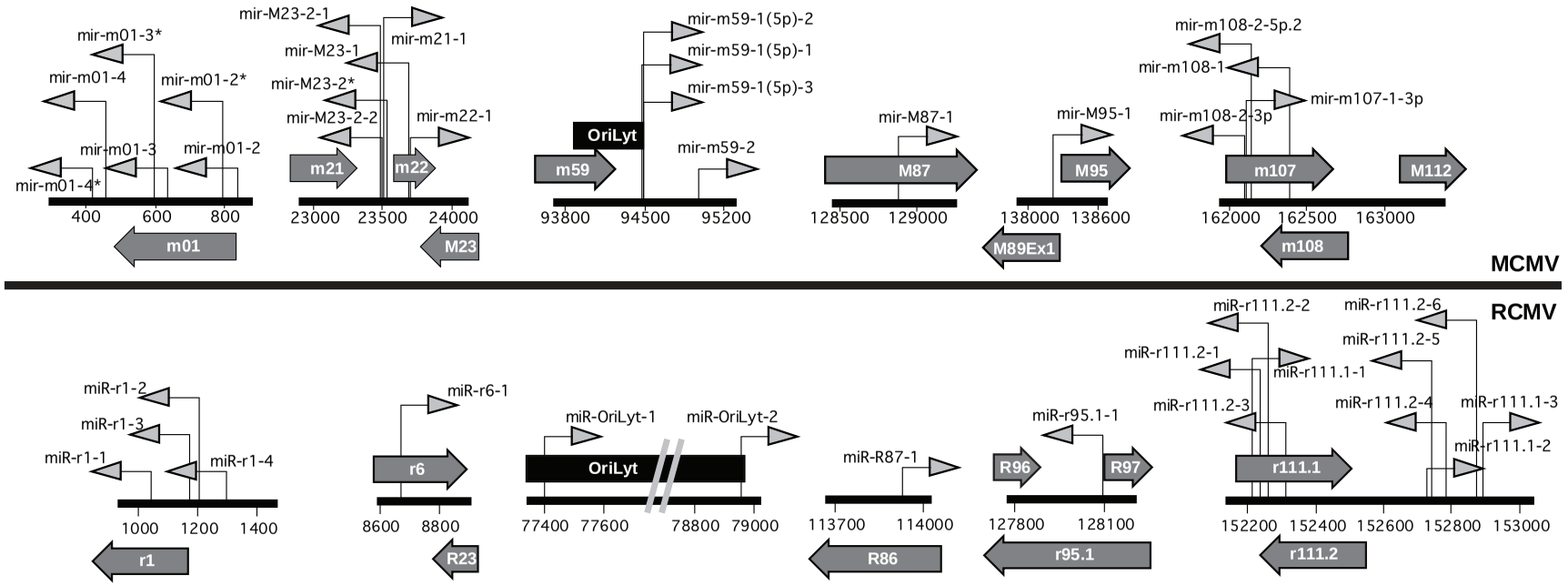

Figure 2.4 Comparison of the genomic positions of RCMV and MCMV miRNAs. Diagram depicting the genomic positioning of the miRNAs expressed by RCMV (lower) and MCMV (upper). The dark gray arrows indicate the position and direction of the CMV miRNAs where right-pointing arrows indicate miRNAs on the sense strands and left-pointing arrows indicate miRNAs on the complement strands. Open reading frames that contain or are near viral miRNAs are shown in light gray block arrows. The MCMV and RCMV origins of lytic replication are depicted with a black box.

Northern blot analysis on RNA isolated from RFL6 fibroblasts infected with RCMV for 8, 24 and 48 hours, as well as 48 hours in the presence of Foscarnet, which differentiates early from late viral gene expression. Figure 2.5 demonstrates that miR-r111.1-1 and miR-r111.2-2 and 3 are expressed with early kinetics, as their expression was not inhibited by Foscarnet treatment. In contrast, miR-r111.1-2 and 3, miR-r111.2-1 and 4 were expressed with late kinetics since their expression was blocked at late times in the presence of the viral DNA inhibitor.

Since we found that a portion of the RCMV miRNAs were differentially expressed in fibroblasts compared to salivary glands, we hypothesized that, similar to viral gene expression in tissues (Streblow, van Cleef et al. 2007), viral miRNA expression is also tissue specific. Therefore, we performed real-time PCR analysis on the two most highly expressed miRNAs of RCMV miR-R87-1 and miR-r111.1-2 from the tissues of RCMV infected rat heart allograft recipients harvested at 7 and 28 days post-transplantation. In infected RFL6 fibroblasts, RCMV miR-R87-1 is expressed at higher levels than miR-r111.1-2 (Figure 2.6A), which confirmed the sequencing data described above. Similarly, RCMV miR-R87-1 was more highly expressed (by at least 10-fold), compared to miR-r111.1-2, in tissues from infected rat heart allograft recipients (Figure 2.6B & C). Interestingly, miR-R87-1 was expressed to high levels in all tissues except the native heart at 7 days post-transplantation, which is the acute phase of infection. In fact, miR-R87-1 was most highly expressed in the allograft heart at day 7.

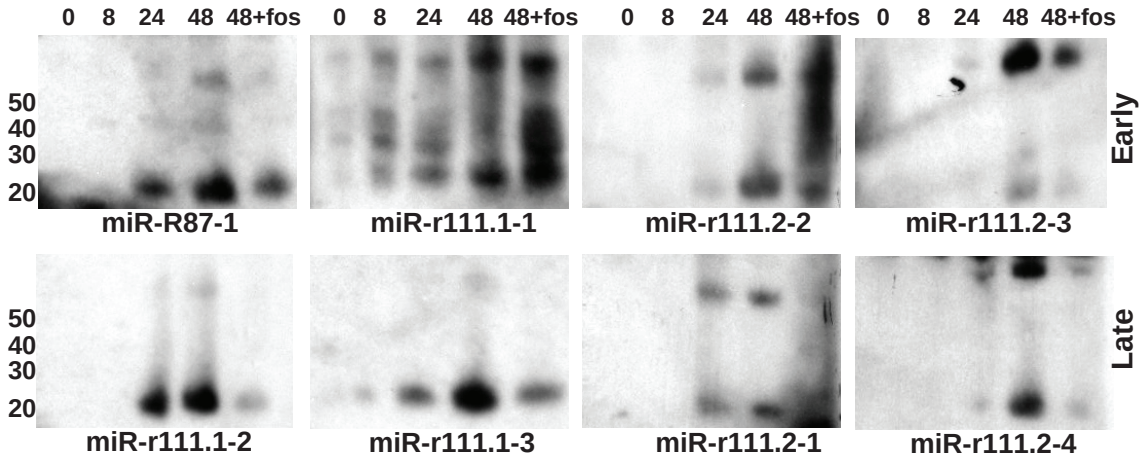

Figure 2.5 Kinetic analysis of miRNA expression by Northern blot. Total RNA was harvested with Trizol from RCMV-infected RFL6 rat fibroblasts at 0, 8, 24 and 48hpi. Cells were infected at a multiplicity of infection equal to 1.0. An additional sample harvested at 48hpi was treated with Foscavir (125mM) to prevent late gene expression. RNA was subjected to Northern blot analysis using probes specific for the predicted RCMV miRNA sequences. Lane 1 (0hpi) was mock infected.

However, miR-R87-1 expression was dramatically reduced to undetectable levels at 28 days in allograft heart, spleen, liver, kidney and lung tissues. The only tissue that expressed miR-R87-1 at 28 days post-transplantation was the salivary gland albeit at levels that were 7-fold lower compared to day 7. RCMV miR-r111.1-2 is almost exclusively expressed in salivary gland tissues and the level of expression increased from day 7 to day 28. Importantly, we have previously shown that viral gene expression profiles are tissue specific and the profiles do not correlate with viral DNA load or viral mRNA levels (Streblow, van Cleef et al. 2007).

Discussion

In the current study, we utilized a robust cloning/sequencing approach (Babiarz, Ruby et al. 2008; Kuchenbauer, Morin et al. 2008) to identify the miRNAs expressed by the Maastricht strain of RCMV in cultured fibroblasts as well as salivary glands from infected rats during the persistent phase of infection. Our study differs from previous studies of CMV miRNA identification in that we sequenced the RCMV miRNAs from both acutely infected cells as well as from *in vivo* persistently infected tissues (Dunn, Trang et al. 2005; Grey, Antoniewicz et al. 2005; Pfeffer, Sewer et al. 2005; Buck, Santoyo-Lopez et al. 2007; Dolken, Perot et al. 2007). We identified 24 small RNAs expressed from the RCMV genome that map to regions predicted to fold into stem loop structures. In fact, many of the RCMV pre-miRNAs were detected by Northern blot analysis. In addition, we cloned and sequenced the corresponding miRNA* or passenger

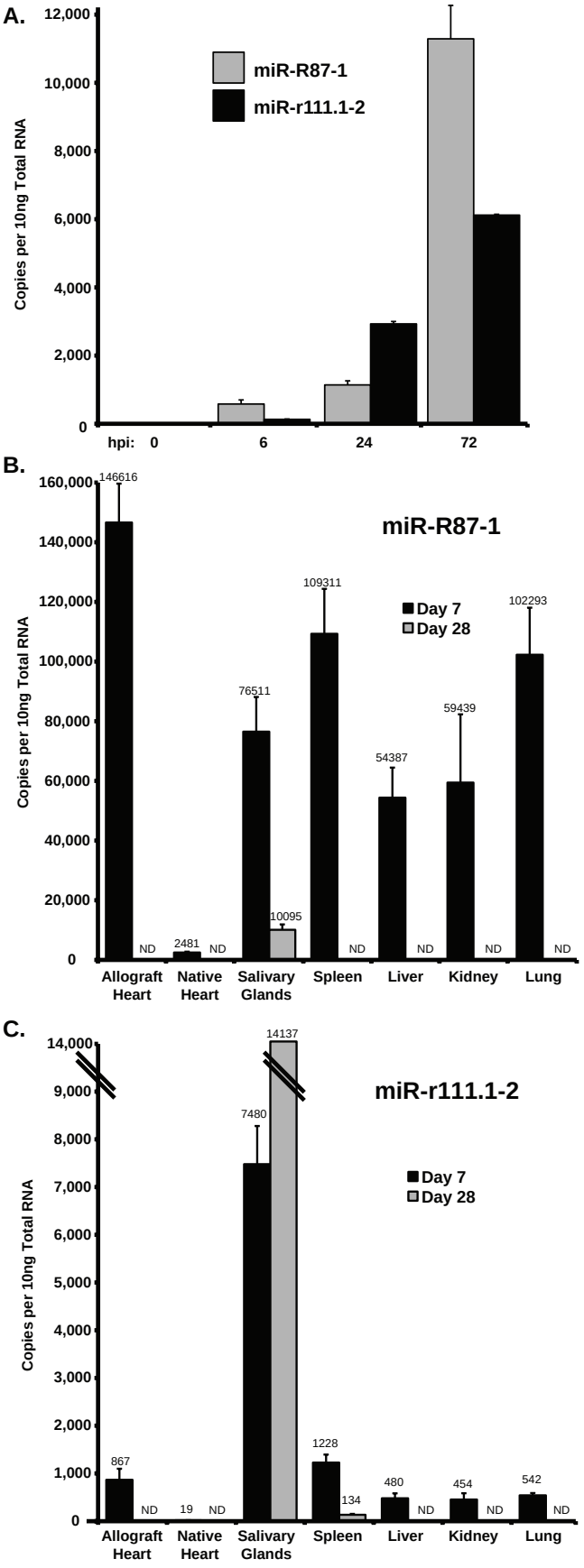

Figure 2.6 RCMV miRNA expression in tissues from RCMV infected allograft recipients. The levels of RCMV miRNA expression were determined by RT-PCR analysis of total RNA samples. Shown are the viral miRNA copy numbers as determined using dilutions of an oligo standard with the specific miRNA sequences for miR-R87-1 and miR-r111.1-2. A) Quantification of RCMV miRNA miR87-1 and miR-r111.1-2 expression in RCMV-infected fibroblasts at 0, 6, 24 and 72 hpi. Uninfected cells (Time=0) were included as a negative control. Both viral miRNAs accumulate with increasing time. B) RT-PCR results for RCMV miR-87-1 from tissues harvested from RCMV-infected heart allograft recipients (n=3) at 7 and 28 days post-transplantation. RCMV miR-R87-1 was most highly expressed in the allograft heart, spleen and lung tissues at 7 days post-transplantation. C) RT-PCR results for RCMV miR-111.1-2 from tissues harvested from RCMV-infected heart allograft recipients (n=3). miR-r111.1-2 was most highly expressed in salivary glands at 7 and 28 days post-transplantation.

strand for a number of the miRNAs discovered in our study. Similar to both HCMV and MCMV, the RCMV miRNAs are found distributed individually or in clusters across the viral genome, which differs substantially from the α- and γ-herpesviruses (Pfeffer, Zavolan et al. 2004; Samols, Hu et al. 2005; Cai and Cullen 2006; Grundhoff, Sullivan et al. 2006; Landgraf, Rusu et al. 2007; Schafer, Cai et al. 2007). Of the 24 identified RCMV miRNAs, four were uniquely detected in the infected fibroblast sample and two were unique to the persistently infected salivary gland tissue, which is most likely attributed to the natural variation observed between *in vivo* and *in vitro* infection scenarios (Streblow, van Cleef et al. 2007). The fact that RCMV miR-r111.1-2 was exclusively expressed in salivary glands and increased during persistence whereas miR-R87-1 was down regulated during persistence suggests that viral miRNA expression is dynamic and regulated *in vivo*. The conclusion is consistent with our results from a previous study documenting RCMV viral gene expression in different tissues from RCMV infected cardiac allograft recipients (Streblow, van Cleef et al. 2007). Our data demonstrates that the profiles, as well as amplitude, of RCMV viral gene and miRNA expression are tissue specific.

Viral miRNA target discovery and determination of the relevance of this targeting to virus infection is intrinsically difficult. This problem is compounded for HCMV due to the species specificity of CMVs and the lack of an appropriate *in vivo* animal model for HCMV. However, various *in vitro* and bioinformatics studies have identified both cellular and viral targets of the HCMV miRNAs. For example,

HCMV miR-UL112-1 targets the viral transactivator IE1 and UL114 the virally encoded uracil DNA glycosylase, which is important for the transition to late-phase viral DNA replication (Courcelle, Courcelle et al. 2001; Grey, Meyers et al. 2007; Stern-Ginossar, Saleh et al. 2009). A leading hypothesis for the role of miRNAs is in the establishment of latency due to their ability to down-regulate IE protein expression especially during the late phase of lytic replication when the viral miRNAs accumulate to high levels. Subsequently, once latency is established, viral miRNA targeting of the IE genes may help to maintain latency by inhibiting initiation of the lytic cascade. In fact, a common feature among herpesvirus miRNAs is the targeting of expression of viral transactivators including: HSV-1 miR-LAT targets ICP0, EBV miR-BHRF-1 and miR-BART15 target BZLF1 and BRLF1 respectively, and KSHV miR-K10-6-3p targets Rta and Zta (Murphy, Vanicek et al. 2008). In addition, HCMV miR-US25-1 and miR-US25-2 miRNAs may contribute to the establishment of latency since they were recently shown to reduce viral replication and DNA synthesis of not only HCMV but other DNA viruses as well (HSV-1 and adenovirus) (Stern-Ginossar, Saleh et al. 2009). Thus it was hypothesized that miR-US25-1 and miR-US25-2 target cellular genes essential for virus growth. Recently, Grey et al. demonstrated that HCMV miR-US25-1 targets multiple cellular genes related to cell cycle control, specifically cyclin E2 (Grey, Tirabassi et al.). Multiple EBV and KSHV miRNAs also target cellular pathways involved in apoptosis and cell cycle regulation (Lagana, Forte et al. ; Choy, Siu et al. 2008; Ziegelbauer, Sullivan et al. 2009). HCMV, KSHV and EBV encode a miRNA that targets the NK cell activating

ligand MICB and its down-regulation leads to decreased viral infected cell killing (Stern-Ginossar, Elefant et al. 2007). Thus it appears that expression of viral miRNAs provides herpesviruses a non-immunogenic strategy to stably alter the cellular environment during persistence.

Importantly, the types of genes and pathways targeted by viral miRNAs are most likely cell-type specific and this fact must be taken into consideration when identifying targets of the viral miRNAs. To date, most tissues examined for miRNA expression show that not only is cellular miRNA expression cell type specific but also dependent upon differentiation of that cell type (Bartel 2004; Landgraf, Rusu et al. 2007). Examination of miRNA expression in 17 hematopoietic cell lines was compared to purified human B-cells, T-cells, monocytes and granulocytes and was found to have considerable differences based upon cell-type and differentiation status (Ramkissoon, Mainwaring et al. 2006). Similarly, differential miRNA expression is documented for viruses. We identified two RCMV miRNAs that were expressed in salivary glands but not in acutely infected fibroblasts; and four that were not expressed during persistence in salivary gland (Table 2.2). In addition, the two most highly expressed miRNAs miR-R87-1 and miR-r111.1-2 differ in their tissue expression and timing following infection (Figure 2.6). Different virus latency programs control EBV BART and BHFR miRNAs and they are differentially expressed in lymphoid and epithelial cells (Cai, Schafer et al. 2006; Pratt, Kuzembayeva et al. 2009). EBV-associated primary tumors have high levels of miR-BART (Xia, O'Hara et al. 2008).

Conversely, in AIDS diffuse large B cell lymphomas, only BHRF miRNAs were detected but BHRF2 miRNAs were not detected in nasopharyngeal carcinoma tumors (Cosmopoulos, Pegtel et al. 2009). An additional level of control of viral miRNA expression and targeting specificity exists due to the heterogeneous processing of the 5' and 3' ends of the miRNA. We and others have observed that pre-miRNA processing can produce distinct isoforms of miRNA:miRNA* duplexes with 5' and 3' heterogeneity (Table 2.3 and (Chiang, Schoenfeld et al. 2010)). The mechanisms of how miRNA isoforms are derived and the extent of their differential function are still unknown. However, recent studies provide evidence to suggest that miRNA length isoforms are relatively common and that a single pre-miRNA hairpin can be differentially processed to give rise to several functional miRNAs with varying biological properties (Ebhardt, Tsang et al. 2009; Ameres, Horwich et al. 2010; Ebhardt, Fedynak et al. 2010; Ghildiyal, Xu et al. 2010). Interestingly, the differences in biological function may be attributed to the fact that the length isoforms can utilize unique Argonaute-RISC protein complexes (Ebhardt, Fedynak et al. 2010). Alternative processing at the 5' end would switch the seed sequence of the miRNA and consequently alter target recognition and act to broaden the regulatory impact of the miRNA (Ghildiyal, Xu et al. 2009). Interestingly, we also observed differential isoform usage between the *in vitro* fibroblast samples and the *in vivo* salivary gland samples. A specific isoform of miR-OriLyt-2 was only detected in the salivary gland sample and not in the fibroblast sample. This finding highlights the importance of determining the role of miRNA heterogeneity *in vivo*. Thus, herpesvirus miRNAs possibly play

particular roles during infection of differentiated cell types, making it critical to understand cell- and tissue-type specific miRNA expression in order to elucidate miRNA function.

An important characteristic of the miRNAs encoded by MCMV and RCMV is the finding that there are at least two miRNAs for each virus encoded in or directly adjacent to the origin of lytic replication. The function of OriLyt miRNAs is still unknown. However, they may play an important role in viral DNA replication. A small RNA stem loop structure encoded by EBV mediates the recruitment of the origin recognition complex to the OriP by binding ORC1 and the RGG motifs of EBNA-1 (Norseen, Thomae et al. 2008). This promotes the assembly of the replication complex at the OriP but occurs in the absence of RNA:DNA binding. Thus, another plausible role for viral miRNAs encoded in the OriLyt might be in DNA replication by directly binding DNA at the origin. Possibly, the viral miRNA might act as the RNA primer, normally synthesized by the host primase, that base pairs with the DNA at the origin of replication to initiate DNA synthesis. The role of a miRNA primer would be especially important for the initiation of viral DNA synthesis in non-dividing cells during reactivation from latency. Further experimentation to characterize the role of OriLyt miRNAs in viral replication is warranted and may lead to the development of novel therapeutics that target and exploit this interesting relationship. The aim of this study was to identify the small RNAs encoded in the RCMV genome and determine the *in vivo* viral miRNA expression in RCMV infected rats. This knowledge will enable us to characterize

the *in vivo* roles and relevance of CMV-encoded miRNAs during acute and persistent infections, latency/reactivation and during pathogenesis.

Acknowledgements

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Chapter 3: Rat Cytomegalovirus R116 is a Virion Envelope Glycoprotein Important for Virus Infectivity in Fibroblasts

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Abstract

There are multiple glycoproteins expressed by cytomegalovirus (CMV) with known functions in virus attachment, fusion, envelopment and egress, but there are still a number of putative glycoproteins with unknown function. Here, we characterize the open reading frame (ORF) R116 from rat cytomegalovirus (RCMV). Previously, we determined that R116 is highly expressed in most tissues during acute RCMV infection and also in the salivary glands during persistence. R116 is expressed as multiple mRNA species, which undergo a splicing event. R116 exists as four protein species but only the higher molecular weight species are incorporated into virions and produced in infected rat salivary glands. R116 protein localizes with a marker of the trans-Golgi network TGN-38 and the viral glycoprotein gB to a perinuclear site reminiscent of the virion assembly compartment. R116 is essential for RCMV replication in fibroblasts since mutation prevents reconstitution of virus and knockdown by siRNA reduces RCMV infectivity by approximately 100-fold. R116 knockdown failed to alter viral genome replication or affect the release of genome containing virus particles suggesting that the role of R116 is during entry or a post-entry event. The use of polyethylene glycol (PEG) to promote fusion between virion and cellular membranes restored the ability of virus lacking R116 to infect fibroblasts. The data we present here indicates that RCMV R116 is a virion glycoprotein required for an early step in the RCMV lifecycle.

Introduction

Human cytomegalovirus (HCMV) is a species specific β-herpesvirus that encodes over 200 unique open reading frames (ORFs) within its 230-kbp genome. HCMV is ubiquitous in the human population and primary infection results in a life-long persistent/latent infection of the host. Infection of immunocompetent hosts is generally asymptomatic but infection of immunocompromised hosts can cause high morbidity and mortality. CMV infections are life-long, and as such, HCMV has been associated with a number of chronic diseases. Rat cytomegalovirus (RCMV) infection of rats has been used to model a number of long-term human diseases associated with HCMV infections, including graft rejection, restenosis following angioplasty and fetal transmission (Handa, Hatanaka et al. 1993; Lemstrom, Bruning et al. 1994; Lemstrom, Koskinen et al. 1994; Steinhoff, You et al. 1995; Koskinen, Yilmaz et al. 1996; Lemstrom, Kallio et al. 1996; Martelius, Salmi et al. 2000; Orloff, Streblow et al. 2002; Loh, Mohd-Azmi et al. 2003; Streblow, Kreklywich et al. 2003; Hillebrands, van Dam et al. 2005; Streblow, Kreklywich et al. 2005; Soule, Streblow et al. 2006; Streblow, Kreklywich et al. 2008). Using RCMV-specific microarrays, we determined RCMV gene expression in infected cultured cells and in infected rat tissues (Streblow, van Cleef et al. 2007). One gene that was highly expressed in many different rat tissues was RCMV ORF R116. The protein encoded by R116 is a predicted glycoprotein with unknown function and shares 16% identity with MCMV M116 and 18% identity with HCMV UL116 (Vink, Beuken et al. 2000). M116 protein was found incorporated in the MCMV virion

but UL116 was not identified in the HCMV virion by mass spectrometry analysis (Varnum, Streblow et al. 2004). R116 is predicted to be a 1.2 kb type-1 glycoprotein with a N-terminal signal sequence, one predicted transmembrane domain and two predicted glycosylation sites.

CMVs encode a complex assortment of glycoprotein genes. Varnum et al. identified 19 HCMV structural glycoproteins as constituents of extracellular virions using mass spectrometry (Varnum, Streblow et al. 2004). The HCMV envelope is studded with glycoproteins with functions during the initial phase of infection including attachment to the target cell and fusion of the virion to host membranes (Spear and Longnecker 2003; Heldwein and Krummenacher 2008). CMV glycoproteins also have roles in later stages of infection during the assembly of infectious virions, envelopment of the capsid/tegument and cell-to-cell spread (Mettenleiter 2002). Five HCMV glycoproteins are essential for replication in fibroblasts; gB, gH, gL, gM and gN (Hobom, Brune et al. 2000; Mach, Kropff et al. 2000). The HCMV entry process begins with virion attachment to cell surface heparan sulfate proteoglycans (HSPGs), which is mediated by gB and/or the gM:gN complex (Compton, Nowlin et al. 1993). The initial tethering event is followed by a stable binding step of gB with the epidermal growth factor receptor (EGFR) or integrins present on many HCMV permissive cell types, or with a yet unknown receptor on other cell types (Wang, Huong et al. 2003; Feire, Koss et al. 2004). The gH:gL glycoproteins form a disulfide-linked complex that is essential for viral entry and fusion (Huber and Compton 1997; Li, Nelson et al.

1997; Huber and Compton 1998; Huber and Compton 1999). The gH:gL complex is required for pH-independent fusion into fibroblasts but the gH:gL:pUL128:pUL130:pUL131 complex is required for pH-dependent endocytosis into endothelial and epithelial cells (Compton, Nepomuceno et al. 1992; Ryckman, Jarvis et al. 2006; Ryckman, Rainish et al. 2008). What has become clear is that herpesviruses use an assortment of glycoproteins, sometimes redundantly, to adsorb onto cells and then bind more specifically to cellular receptors to mediate fusion. Because CMV is able to infect a number of cell types *in vivo* including epithelial cells, endothelial cells, fibroblasts, monocyte-macrophages, smooth muscle cells, dendritic cells, hepatocytes, neurons and glial cells, the complexity of the entry pathway increases as CMV protein functions are defined (Sinzger, Digel et al. 2008). Non-conserved structural glycoproteins between different herpesviruses may provide additional ligands that contribute to cell and tissue tropism. The RCMV/rat model is an important tool to study and dissect CMV pathogenesis *in vivo* and in the current manuscript, we characterize the protein encoded by RCMV ORF R116. We found that R116 is a virion-associated glycoprotein and the lack of R116 renders the virus less infectious. We conclude from our studies that R116 is a viral envelope glycoprotein important for RCMV infectivity of fibroblasts.

Materials and Methods

Antibodies. Rabbit polyclonal antibodies were generated to ORF R116 by immunizing rabbits with a HIS tag R116 (19-222aa) fusion protein. Rabbit anti-

RCMV-IE polyclonal antibody was previously described (Streblow, Kreklywich et al. 2008). A rat anti-RCMV gB monoclonal antibody was produced by Dan Cawley at the OHSU-VGTI Monoclonal Antibody Facility from splenocytes derived from RCMV-infected rats. Mouse monoclonal antibodies directed against GAPDH, KDEL, LAMP-1, and TGN-38 were purchased from AbCAM. Secondary anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated antibodies (NA934V and NA931V) were purchased from Amersham and rabbit anti-rat HRP from Southern Biotech. Secondary anti-mouse, anti-rabbit and anti-rat fluorescently tagged antibodies were purchased from BioSource International.

RCMV Virion Preparation and Analysis. The Maastricht strain of RCMV used for these studies was obtained from Drs. C. Bruggeman and C. Vink (The Netherlands). Construction of a recombinant RCMV constitutively expressing the green fluorescence protein (GFP) (RCMV-GFP) driven by the EF1α promoter was previously described (Baca Jones, Kreklywich et al. 2009). RCMV was grown and titered on rat lung fibroblasts (RFL6, ATCC). Plaque assays were performed in confluent 24-well plates by infection with 10-fold serial virus dilutions in triplicate in 0.2 ml of medium. Virus was incubated at 37°C for 4 hours and then overlaid with 1 ml of Dulbecco's modified Eagle's Medium (DMEM) supplemented with 5% FBS, penicillin-streptomycin, and 20mM L-glutamine and 10% carboxyl methylcellulose. After 7 days, the cells were fixed in 3.7% formaldehyde in PBS and stained with 0.05% aqueous methylene blue. The plaques were visualized and counted by light microscopy.

RCMV was purified by ultracentrifugation in order to determine whether R116 is incoporated into virions. At the time of maximum cytopathic effect, culture medium from infected RFL6 fibroblasts was first clarified by centrifugation at 12,000 x *g* for 15 min. Virus was pelleted through a 10% sorbitol cushion by centrifugation of the pre-cleared supernatants (SW28, 28,000 rpm for 2 hours). The virus pellet was resuspended in PBS and layered over a discontinuous 10-50% Histodenz step-gradient (Sigma). The gradient was centrifuged at 27,000 RPM for 2 hours at 4°C in a Beckman SW40.1 rotor. The collected virus band was resuspended in PBS and then pelleted through a 10% sorbitol cushion at 22,000 RPM for 1 hour. The protein composition of the pelleted virus was analyzed by SDS-PAGE and Western blot as described below. To determine whether RCMV R116 was an envelope protein, the resuspended viral pellet was split into 2 samples. One sample was treated with 2% NP40 in PBS added in a 1:1 ratio and the second half of the original sample was treated with an equivalent volume of PBS. The samples were rotated for 30 minutes at 4°C and centrifuged in a mini-ultracentrifuge at 100,000 x g for 30 minutes to acquire both the pellet and supernatant fractions. Samples were analyzed by SDS-PAGE and Western blot.

To determine whether R116 was on the virion surface an additional preparation of gradient banded virus particles was incubated with increasing concentrations of trypsin and incubated at 37°C for 15 minutes. Laemmli's sample buffer was

added to the virus/protease mixture to stop the reaction and the samples were analyzed by SDS-PAGE in combination with either Coomassie brilliant blue staining or Western blotting for R116.

To determine whether virion-associated R116 was glycosylated, a RCMV virus preparation from infected fibroblast cell lysates were treated with PNGaseF (NEB) or EndoH (Roche). The treated virus samples were analyzed by SDS PAGE and Western blot for R116 and gB.

Identification of RCMV R116 transcripts. A RCMV cDNA library was constructed from rat RFL6 fibroblasts infected with RCMV (multiplicity of infection equal to 1; moi=1.0) for 24 and 48 hours post infection (hpi) using the Superscript Plasmid System with Gateway Technology for cDNA synthesis and cloning (Invitrogen). The cDNA was ligated into the plasmid pSPORT and screened by Southern blotting using a R116 DNA probe (described below). The R116-positive clones were sequenced using oligonucleotides corresponding to Sp6 and T7 binding sites present in the plasmid flanking the cDNA insert. The predicted splicing of R116 was confirmed by RT-PCR using flanking primers. The products were analyzed by gel electrophoresis and the products were sequenced.

Southern blot. The RCMV cDNA libraries were screened using the NEN Life Sciences Colony/Plaque Screen protocol on GeneScreen Plus nylon membranes. R116 probes were generated from 500bp *Bam*HI fragments of

plasmids containing R116 using Roche Random Prime Labeling kit. Hybridization was performed with 1-2 x 10^6 CPM/ml in Express Hybe (Clonetech). The blots were washed first with a low stringency buffer (2XSSC 0.05% SDS) followed by a high stringency buffer (0.1XSSc 0.1% SDS). Detection was performed via exposure to autoradiography film (Kodak Biomax MS) using intensifying screens at -80C.

Northern blot. RFL6 fibroblasts plated on 10 cm dishes were infected with RCMV (moi=1.0). At 8, 24 and 48 hpi the cells were washed with PBS and lysed with Trizol (Invitrogen) for 5 min at room temperature. Subsequently, the samples were scraped and stored frozen at –80°C. RNA was isolated per the manufacturers instructions and electrophoresed through a 1% agarose/formaldehyde gel and transferred to GeneScreen Plus nylon membranes (Dupont/NEN). The blots were hybridized with probes specific for R116 and GAPDH generated from 500 bp *Bam*HI fragments of plasmids containing R116 or GAPDH using Roche Random Prime Labeling kit. Alternatively single stranded probes were made by end labeling DNA oligonucleotides complementary for R116 or GAPDH sequences using T4 ploynucleotide kinase (NEB). The Northern blots were hybridized in Express Hybe (Clontech) and washed with low stringency buffer (2xSSC with 0.05% SDS) followed by high stringency buffer (0.1xSSC with 0.1% SDS). The blots were exposed to autoradiography film (Kodak Biomax MS) using intensifying screens at -80°C, developed and visualized.

Western blot analysis. Samples of RFL6 fibroblasts infected with RCMV (moi=1.0), salivary gland tissue homogenates (described below), or virus preparations (described above) were lysed in Laemmlli's 2X sample buffer (4% sodium dodecylsulfate, 20% glycerol, 20% dithiothreitol, 0.004% bromphenol blue and 0.125M Tris HCl). Lysates were separated by 10% SDS-PAGE and the gels were transferred to Immobilon-P membranes (Millipore). Primary antibodies were diluted in 5% milk powder dissolved in TBST (10mM Tris, pH 7.2, 100mM sodium chloride, 0.1% Tween-20) and incubated for 1 hour. The blots were washed and then the appropriate secondary HRP-conjugated secondary antibodies were incubated for 30 minutes at room temperature. The blots were washed and developed using a chemiluminescent detection system (ECL Amersham Pharmacia Biotech) and visualized by autoradiography.

Immunofluorescence (IF) microscopy. Fibroblasts grown in 4-well chamber slides were infected with RCMV (moi=0.5) for 48hrs and treated with cycloheximide (100 µg/ml) for an additional 0, 1, 2, 4 and 6hrs to block new protein synthesis. At the indicated time points, cells were fixed in 2% paraformaldehyde in PBS, permeablized with Saponin buffer (0.2% saponin, 1% BSA, PBS) and blocked with normal goat serum. Localization of viral and cellular proteins was determined by IF microscopy utilizing primary antibodies directed against R116, gB, KDEL, LAMP-1, and TGN-38 followed by the appropriate fluorescently labeled species-specific secondary Ab and the DNA stain DAPI. Frozen thin-sections of rat salivary glands from rats infected with RCMV-GFP for

21 days were fixed and stained with antibodies directed against R116 using the same protocols described above. Deconvolution microscopy was used to visualize the stained cells in the 4-well chamber slides and tissue sections (mag=60X).

Salivary gland protein isolation. R116 *in vivo* protein expression was performed on approximately 100 µg of protein extracted from flash frozen salivary gland tissue from uninfected control rats and rats infected with RCMV for 28 days (n=3). Tissues were homogenized in RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 1mM PMSF and protease inhibitor cocktail) using a Precellys tissue homogenizer. Lysates were analyzed by SDS-PAGE and Western blotted for R116 and GAPDH as described above.

siRNA transfection and infection. Two R116-specific siRNAs (positions 308-326 nt and 520-538 nt) and a control siRNA were purchased from Dharmacon. The sequences of the R116 siRNA are: R116-308 CTA CAT TAC CCT CGC AAA T and siRNA R116-520 GCG ACG AGG CGA TAC GTT T. RFL6 fibroblasts seeded in 24-well plates were transfected twice with each siRNA using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturers protocol. At 24 hours after the initial transfection step, the cells were infected with RCMV (moi=1.0). At three days post-infection the cells and supernatants were collected and titered as previously described. The triplicate samples of cells were lysed in either DNAzol (viral DNA analysis, Invitrogen), Trizol (mRNA analysis,

Invitrogen) or Laemmli's buffer (viral protein analysis). Host cell production of the viral mRNAs R114, R115 and R116 was quantified by RT-PCR TaqMan and viral genomic DNA was analyzed by Real Time PCR for R54 (viral DNA polymerase) as described below. R116 protein levels were determined by Western blotting. Viral infectivity was determined by serial dilution using standard plaque assay.

The fusogenic substance polyethylene glycol (PEG) 6000 (44% in PBS, Calbiochem) was used to treat cells infected with virus prepared from the siRNA treated cells (Ryckman, Chase et al. 2008). After an initial 1 hour virus binding step RFL6 fibroblasts were treated with PEG for 30 seconds at 37°C and washed two times with PBS and once with media. The effects on infectivity were analyzed by standard plaque assay.

Quantitative PCR detection of RCMV genomic DNA and viral gene expression. For the quantification of RCMV DNA in virus particles and cell lysates of infected cells treated with siRNA, total genomic DNA was extracted from cells and supernatants using DNAzol (Invitrogen). A total of 0.5 µg of DNA was analyzed using a TaqMan probe/primer set recognizing RCMV R54 DNA polymerase sequence as previously described (Streblow, Kreklywich et al. 2003; Streblow, van Cleef et al. 2007). PCR reactions were set up using the TaqMan Universal PCR Master Mix (Applied Biosystems). Following thermal activation of AmpliTaq Gold (10 min. at 95°C), a total of 40 cycles were performed (15 sec. at

95°C and 1 min. at 58°C) using an ABI StepOnePlus Real Time PCR machine. The sensitivity of detection for this assay was <100 copies.

Real-time reverse transcriptase (RT)-PCR was used to quantify expression levels of RCMV R114, R115 and R116 from infected cells collected following treatment with siRNA (Streblow, van Cleef et al. 2007). cDNA was generated using Superscript III RT (Invitrogen) and analyzed by real-time PCR techniques using primer sets recognizing RCMV gene sequences. RT-PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems) as previously described using an ABI StepOnePlus Real Time PCR machine (Streblow, van Cleef et al. 2007; Streblow, Kreklywich et al. 2008). Plasmid clones containing each gene fragment were used as positive controls and quantification standards. The sensitivity of detection of this assay was <100 plasmid copies for all of the tested RCMV genes. Quantitative PCR data were analyzed by ANOVA and student's t-test.

Results

Since RCMV R116 transcripts were detected in nearly all of the tissues from RCMV infected rats (Streblow, van Cleef et al. 2007), we sought to characterize RNA and protein expression of this viral gene. The RCMV open reading frame encoding R116 is found on the complement strand of the RCMV genome from the genomic position 158983 to 157772 base pairs (bp) (Vink, Beuken et al. 2000). To determine the kinetics of R116 transcription, we performed Northern

blot analysis using a double stranded DNA probe to identify all of the transcripts sharing R116 sequence (Figure 3.1). We detected two unique transcripts containing R116 sequence, one is approximately 3 kb in length and the other approximately 1 kb. Both R116 transcripts were detectable by 24 hours post-infection (hpi) but expression was sensitive to Foscarnet treatment indicating that R116 is expressed with late viral gene expression kinetics. We confirmed that the two R116 transcripts are on the sense strand by Northern blot analysis using a R116 antisense oligonucleotide probe (data not shown). We also performed 5' primer extension, which confirmed the predicted ATG start site (data not shown). To determine the sequence of the two R116 transcripts, we constructed a cDNA library from RCMV infected rat fibroblasts harvested at 48 hpi. Sequence analysis of R116 positive clones revealed that the 3kb transcript contained R116, R115 and R114 and the 1kb transcript contained only R116 sequences (data not shown). A second cDNA library was constructed from rat fibroblasts infected for 24 hpi and a third transcript was discovered that contained a truncated form of R116 (880 bp) (data not shown). Analysis of all of the R116 transcripts revealed a common splice event such that 84 base pairs are removed from 353 to 437 bp of the gene. The spliced region is downstream from the predicted signal sequence cleavage site and upstream from the predicted transmembrane domain. We independently verified the presence of the splice site by RT-PCR (data not shown).

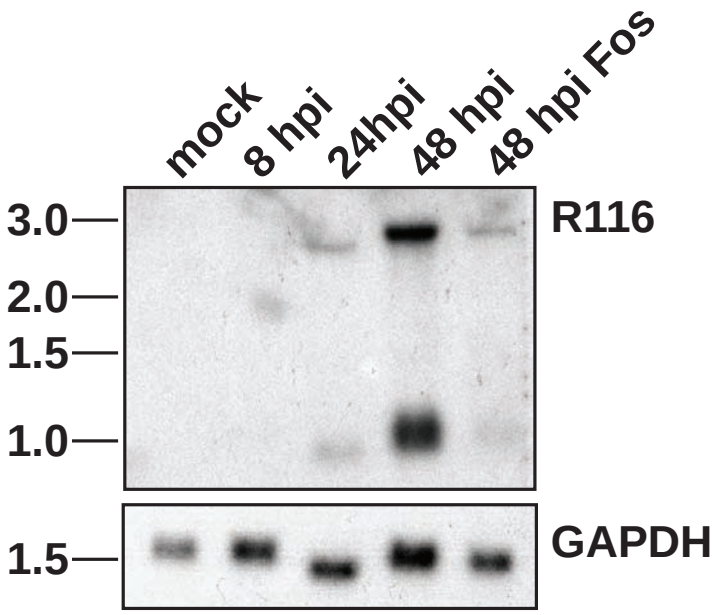


Figure 3.1 R116 is a spliced message expressed on two viral transcripts with late viral gene expression kinetics. Rat RFL6 fibroblasts were mock infected or infected with RCMV (MOI=1) and harvested at 8, 24, and 48 hpi. One infected cell sample was treated with Foscavir (100 µg/ml) and harvested at 48 hpi. RNA was separated by electrophoresis utilizing a 1% formaldehyde agarose gel. RNA was transferred to nitrocellulose membranes and probed for R116. The blot was then stripped and re-probed for GAPDH. The Northern blots were visualized by autoradiography.

In order to examine R116 protein expression, we generated a rabbit polyclonal antiserum to the predicted R116 amino acids 19-222. As shown in Figure 3.2, the antiserum was used to probe for R116 protein expression by Western blot using lysates from uninfected and RCMV infected fibroblasts (8, 24, and 48 hpi). Antibodies directed against the cellular protein GAPDH were used to equalize protein loading levels. In addition, we stained for RCMV IE proteins, which were detectable by 8 hpi. Interestingly, while the predicted molecular weight of R116 is 42 kDa, we detected four different molecular weight species under denaturing conditions that specifically reacted with R116. The lower molecular weight band is expressed with early kinetics since this form of the protein is detected by 24 hpi and expression is not sensitive to Foscarnet treatment. However, the higher molecular weight R116 protein species is expressed with late viral expression kinetics since the protein species is first detectable at 48 hpi and expression is sensitive to Foscarnet.

To identify the cellular localization of R116, we performed immunofluorescence microscopic analysis by co-staining RCMV infected RFL6 fibroblasts (48hpi) for R116 and either the endoplasmic reticulum (ER) marker KDEL, the trans-Golgi network (TGN) marker TGN-38, or the lysosomal marker LAMP-1. R116 protein localizes to the TGN but not within the ER or lysosomes in infected rat fibroblasts (Figure 3.3A). In order to chase R116 to its final or steady-state compartment we treated infected cells with cycloheximide, to block new protein synthesis, and compared R116 localization to that of the virion associated glycoprotein gB. We

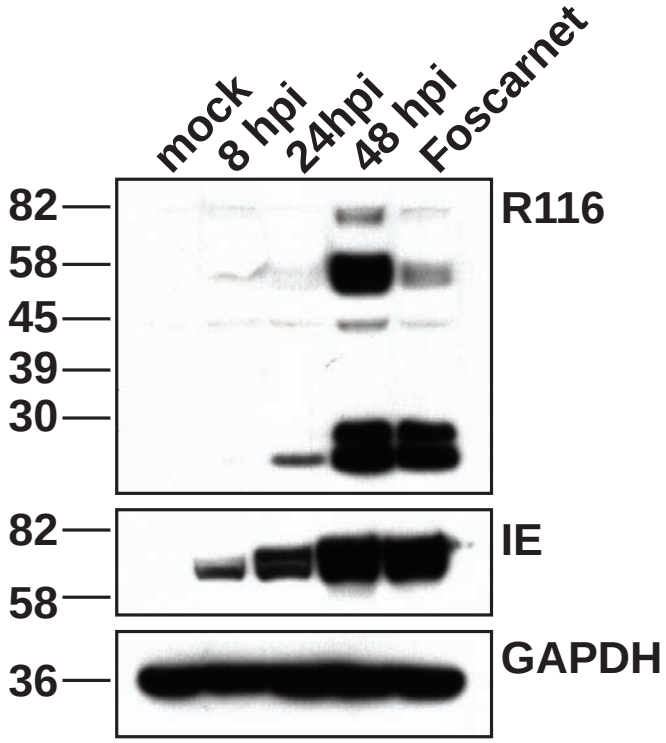


Figure 3.2 R116 protein is expressed with early and late viral kinetics *in vitro*. Rat RFL6 fibroblasts were mock infected or infected with RCMV (MOI=1) and harvested at 8, 16, 24, and 48 hpi in Laemmli's sample buffer. An additional infected cell sample was treated with Foscarnet (100 mg/ml) upon infection and harvested at 48 hpi. The cellular lysates were analyzed by SDS-PAGE and transferred to Immobilon-P membranes. The blot was probed with antibodies directed against the RCMV proteins R116 and IE1&2 as well as GAPDH.

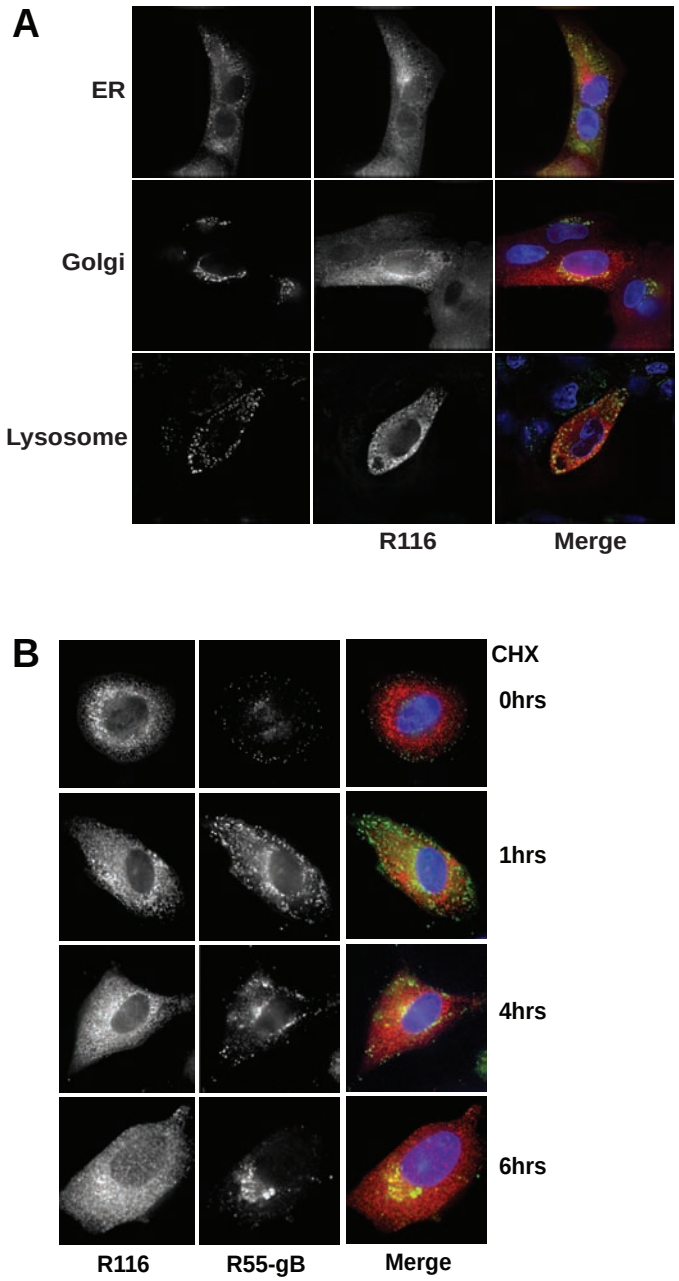


Figure 3.3 R116 localizes with the trans-Golgi network marker TGN-38 and the viral glycoprotein gB. Rat RFL6 fibroblasts were infected with RCMV (MOI=1). A) At 48 hpi the cells were fixed with 2% PFA. The fixed cells were stained for R116 (red) and antibodies to the cellular ER, TGN or lysosomal compartments using antibodies directed against KDEL, LAMP-1 or TGN-38 (green), and DAPI (blue). B) To confirm the cellular localization of R116 (red) in relationship to the viral glycoprotein gB (green), RCMV infected fibroblasts were fixed in 2% PFA at 0, 1, 2, 4, and 6 hrs post-treatment with cycloheximide (100 ug/ml) that was initiated at 48 hpi. Deconvolution microscopy was used to visualize the stained cells. Mag=60x.

show in Figure 3.3B that gB translocates from the surface into the assembly compartment within 4-6 hours. While R116 does not relocate to the same extent as gB, by 6 hours post-cycloheximide treatment a portion of the cellular R116 protein localizes with gB to a perinuclear site, reminiscent of the virion assembly compartment.

We next performed Western blot analysis on solubilized salivary gland tissue from mock infected and RCMV infected rats from 28 days post-infection to determine whether R116 protein was expressed *in vivo*. Interestingly, we only detected the higher molecular weight species of R116 in the lysates from salivary glands of infected rats (Figure 3.4A). The presence of R116 in the salivary glands was confirmed by immunofluorescence staining of frozen sections from rats infected with RCMV-GFP (21dpi). Only the GFP expressing salivary gland cells stained positive for R116 protein (Figure 3.4B).

RCMV R116 Is A Virion Associated Glycoprotein

Since R116 localized to the virus assembly compartment, we sought to determine whether R116 was incorporated into RCMV particles. Interestingly, Western blot analysis of gradient purified virions indicated that the high molecular weight species of R116 are preferentially expressed in RCMV virions, whereas the lower molecular weight species are excluded from the virions (Figure 3.5A). As a control, we also stained for RCMV IE and GAPDH and found that RCMV IE and GAPDH are not present in virions but are in the infected cell lysates. To

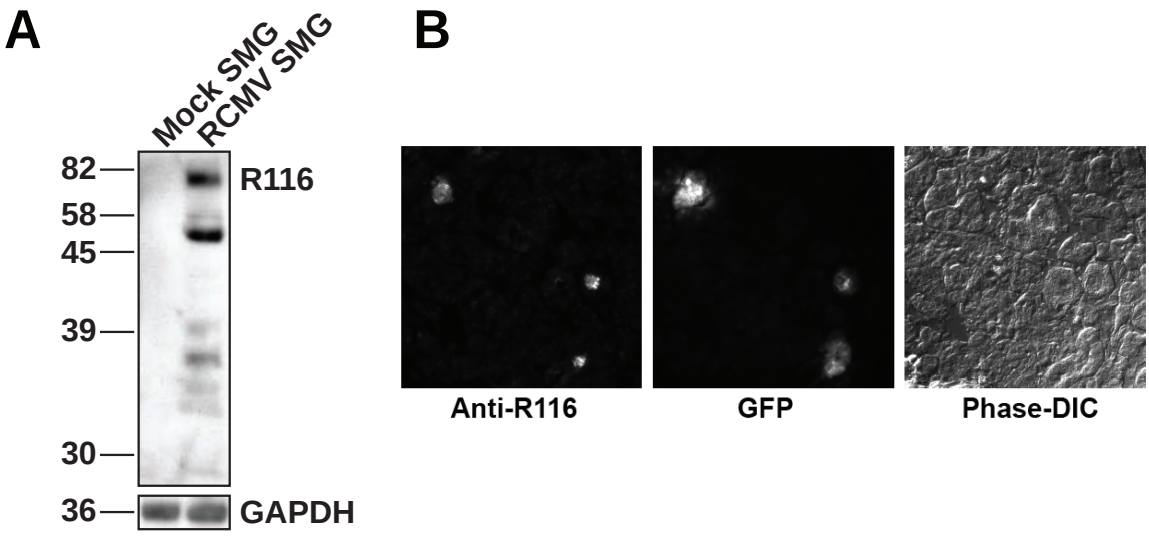


Figure 3.4 R116 is expressed in the salivary gland of RCMV infected rats. A) Salivary glands were harvested from RCMV infected rats at 28 dpi. Salivary glands from uninfected rats served as a control. The salivary glands were homogenized in RIPA buffer, analyzed by SDS-PAGE and probed using the anti-R116 polyclonal antibody. Equal loading was confirmed by staining for the cellular protein GAPDH. Western blots were visualized by autoradiography. B) Salivary glands were harvested from rats infected with RCMV-GFP RCMV at 21 dpi. Embedded frozen tissues were cut and sections were fixed with 2% PFA, washed and stained with antibodies directed against R116. Infected cells were detected by GFP expression. Deconvolution microscopy was used to visualize the stained cells. Mag=60X.

characterize the location (envelope vs. capsid/tegument) of the virion incorporated R116, we treated two different portions of a gradient purified RCMV sample with either 1% NP40 or PBS alone and centrifuged the treated samples to separate the virion envelope from the capsid/tegument fraction (Figure 3.5B). The different pellet and supernatant fractions were analyzed by Western blot for R116 and gB proteins. The pellet fraction in the control PBS treated sample represents intact virus particles and the supernatant fraction should contain any proteins that became soluble during the experimental procedures. In contrast, the pellet from the NP-40 treated preparation represents the capsid/tegument fraction whereas the supernatant fraction is the soluble detergent-stripped envelope. Similar to the viral glycoprotein gB, R116 protein is present in the pellet fraction of the PBS treated virus preparation but was only present in the supernatant fraction (envelope) when treated with detergent (Figure 3.5B). To confirm that R116 is a viral envelope protein and to determine whether R116 is on the virion surface, we treated gradient purified virions with increasing concentrations of trypsin for 15 minutes and analyzed total virion protein by Coomassie staining and R116 protein levels by Western blot. R116 was degraded by trypsin in the absence of detergent suggesting that R116 is present on the surface of the virion (Figure 3.5C).

Since R116 is a virion surface protein we next determined whether R116 is also glycosylated. We tested the sensitivity of virion associated R116 and gB to endoglycosidase H (Endo H) and Peptide N Glycosidase F (PNGaseF). Endo H

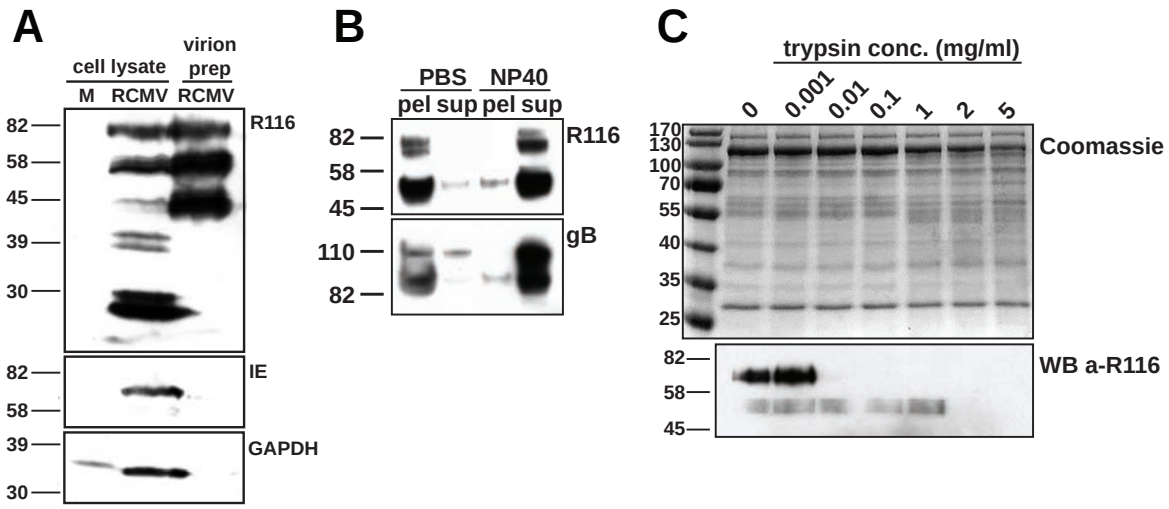


Figure 3.5 R116 is a virion surface envelope protein. RCMV virus particles were purified by layering over a discontinuous 10-to-50% Histodenz gradient and centrifuged at 110,000xg for 2 hours at 4°C. Virus banded at 20-30% Nycodenz was brought up in PBS and repelleted at 22,000 RPM for 1 hr. The virus pellet was resuspended in a minimal volume of PBS. A) The RCMV virion preparation, infected fibroblast lysate, and lysate from mock-infected fibroblasts were analyzed by SDS-PAGE for R116, IE1&2 and GAPDH by Western blot. B) RCMV virion preparation was split into two samples. One sample was treated with 1% NP40 and the control sample with an equal volume of PBS. Both samples were pelleted at 100,000xg for 30 minutes. The pellet and supernatant fractions were analyzed by SDS-PAGE and probed for RCMV R116 and gB. C) RCMV virion preparation was subjected to increasing concentrations of trypsin (0-5µg) for 15 minutes at 37°C. The samples were analyzed on two separate SDS-PAGE gels; one was stained with Coomassie Brilliant Blue and the other was probed for R116.

removes high mannose and some hybrid types of asparagine-linked (N-linked) carbohydrates, whereas PNGaseF cleaves all N-linked carbohydrates without regard to type. Similar to RCMV gB, a known virion envelope glycoprotein, both enzymes reduced the molecular weight of R116 suggesting that R116 is N-linked glycosylated (Figure 3.6). Examination of the R116 amino acid sequence indicates that the protein contains two predicted N-glycosylation sites.

R116 is important for virus entry into fibroblasts

Since R116 is a virion envelope glycoprotein, we investigated whether R116 is necessary for RCMV infectivity. We constructed a bacterial artificial chromosome (BAC) containing the RCMV genome and we mutated R116. However, exhaustive transfection experiments of the recombinant RCMV BAC mutant, RCMV-116GalK-BAC (containing a GalK selection marker inserted between nucleotides 1079 and 1080 of R116) in rat fibroblasts failed to produce infectious virus (data not shown). The transfected fibroblasts produced IE viral proteins and the BAC marker protein GFP indicating successful transfection of the BAC DNA and viral gene expression (data not shown). Importantly, a revertant virus constructed from RCMV-116GalK-BAC that replaced the GalK marker with wild-type (WT) R116 sequence produced infectious virus suggesting that R116 is necessary for RCMV replication in fibroblasts (data not shown). To confirm this finding, we performed R116 knockdown experiments in WT RCMV infected fibroblasts using two different siRNAs specific for R116. Western blot analysis of cell lysates from this experiment revealed that both R116 specific siRNAs, but

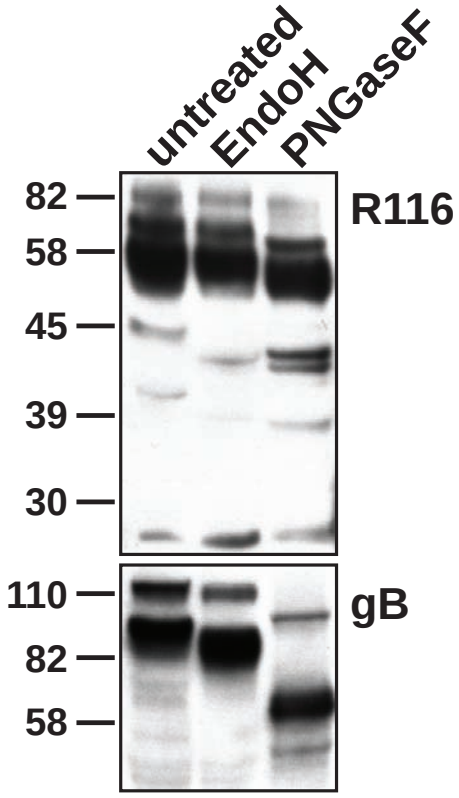
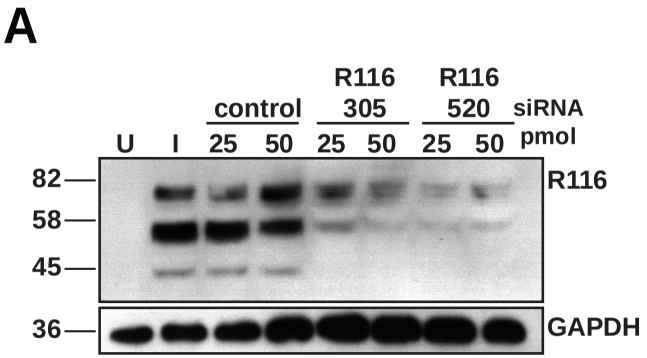
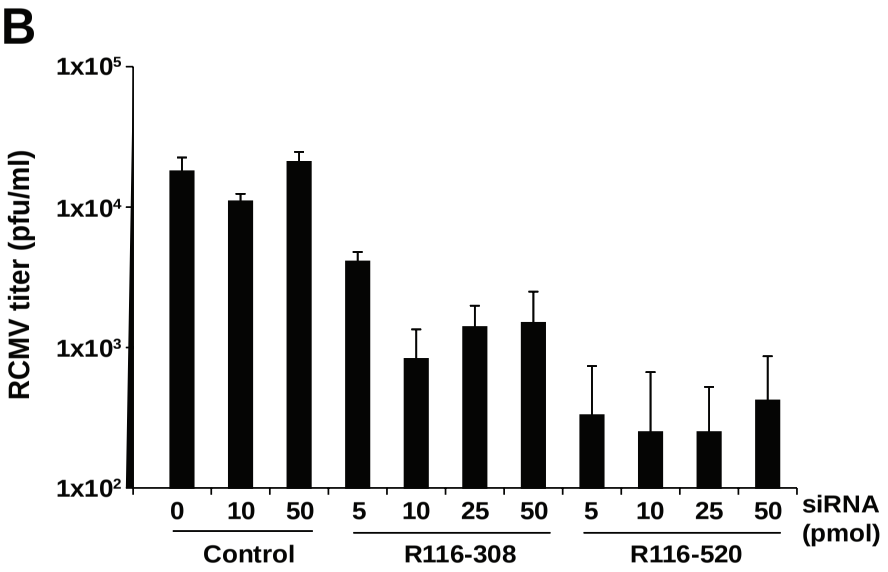


Figure 3.6 Virion associated R116 is glycosylated. RCMV particles was treated with either EndoH or PNGaseF and analyzed by SDS-PAGE. The Western blot was stained for RCMV R116 and gB and visualized by autoradiography.

not a control siRNA, reduced R116 protein production (Figure 3.7A). Titration of supernatant virus from this experiment revealed that knockdown of R116 reduces RCMV infectivity of fibroblasts by nearly 100-fold when compared to the negative control siRNA or untransfected controls (Figure 3.7B). To determine whether knockdown of R116 affected viral genome replication or the release of genome containing particles, we quantified viral DNA levels in supernatants and cell pellets from siRNA treated cells. Knockdown of R116 does not decrease viral genomic DNA levels in infected cells indicating that the effect of virus lacking R116 on decreasing infectious virus production was not at the level of DNA replication (Figure 3.7C). Similarly, by RT-PCR TaqMan, R116 knockdown did not affect transcription of either R114 or R115 (data no shown). Knockdown of R116 did not decrease the release of viral genome containing particles suggesting that R116 deficient viruses display a reduced infectivity per particle ratio (Figure 3.7C).

To examine whether depletion of R116 affects a step in the entry process or affects a post-entry step we utilized polyethylene glycol (PEG), a chemical fusogen, to attempt to reverse the defect (Lentz and Lee 1999). For this experiment, 10-fold dilutions of supernatants from siRNA treated cells infected with RCMV were allowed to bind for 1 hour and then treated with 44% PEG in PBS for 30 seconds at 37°C using a protocol designed by Ryckman et al. for the study of HCMV entry (Ryckman, Rainish et al. 2008). Following PEG treatment, the cells were washed, overlayed and incubated for 5 days to allow for virus





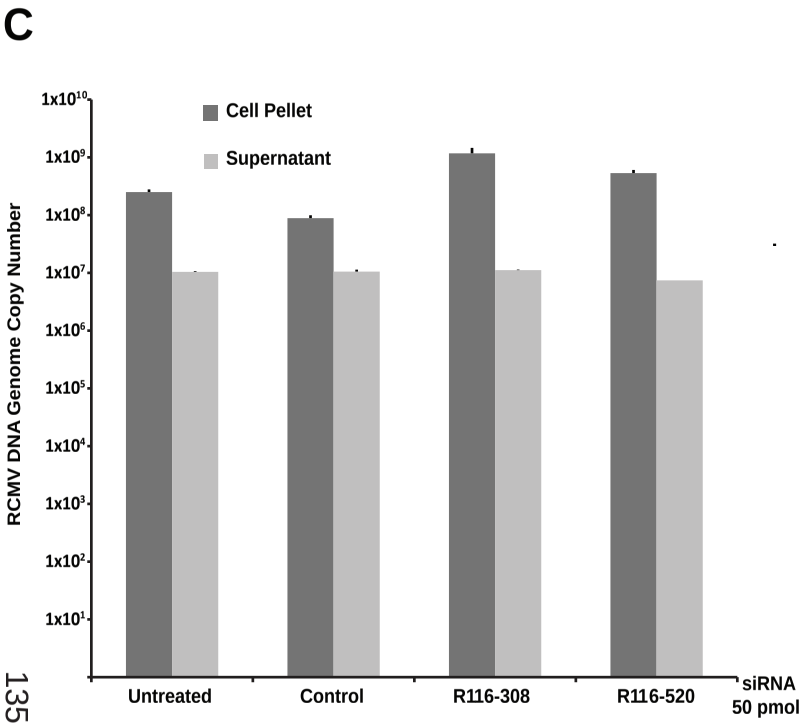


Figure 3.7 siRNA knockdown of R116 protein levels reduces RCMV infectivity but does not alter viral genome replication or release of genome containing particles. In triplicate wells fibroblasts were transfected with a control siRNA or R116 specific siRNAs. At 24 hours post-transfection, the cells were infected with RCMV (MOI=1.0). At 48 hpi, supernatants and cell pellets were collected and analyzed by: A) Western blotting for R116 and GAPDH; B) plaque assay for the presence of infectious virus [control vs. R116 siRNA at 10 and 50pmol $p<0.001$, n=3]; and C) quantitative PCR for the presence of viral DNA genomes [control vs. siRNA R116-308 cell pellet $p=0.02$ and supernatant $p=0.33$, n=3; control vs. siRNA R116-520 cell pellet and supernatant $p=0.01$, n=3].

plaquing. PEG restored infectivity of the R116-lacking virus to levels observed for virus produced by the control siRNA transfected cells that were similarly treated with PEG (Table 3.1). PEG treatment increased the infectivity of virus from control and R116 siRNA treated cells. However, the effect of PEG was greatest in cells infected with the R116-lacking virus wherein PEG treatment increases infectivity by 10-12 fold. The PEG result indicates there were similar numbers of RCMV virions present in the R116 knockdown supernatants compared to controls and confirms our findings of equal viral genome levels in the R116-lacking virions as shown in Figure 3.7C. Thus, R116-deficient virus particles have a reduced infectivity per particle ratio and are blocked at an early entry step.

Discussion

In this report, we demonstrate that RCMV ORF R116 is a virion surface envelope glycoprotein required for an early step of virus entry. R116 protein localizes to the TGN in a compartment that co-stains with RCMV glycoprotein gB. From this data and the fact that R116 is present within virions, we predict that this compartment is the virion assembly compartment. Transfection with a RCMV BAC disrupted in the R116 gene was not able to spread in fibroblasts *in vitro*. Similarly, siRNA knockdown of R116 protein decreased RCMV infectivity of fibroblasts by approximately 10-100-fold. Importantly, the knockdown of R116 did not significantly alter viral genome replication or release of viral genome containing particles. Polyethylene glycol treatment promotes the fusion of membranes and as such restored the infectivity of virus lacking R116 protein indicating that the

Table 3.1 PEG treatment restores infectivity of RCMV lacking R116.

	# plaque		fold increase with PEG[a]
	no treatment	PEG	
Control siRNA	725 +/- 199	2417 +/- 736	3.3
R116-308	158 +/- 143[b]	1950 +/- 421[c]	12.3
R116-520	208 +/- 174[d]	2183 +/- 640[e]	10.5

a=PEG treatment increases infectivity for all siRNA ($p<0.005$)
b, d=siRNA to R116 decreases infectivity vs. control ($p<0.0003$, $p<0.0008$ respectively)
c, e=PEG restores infectivity of RCMV lacking R116 to control levels ($p=0.21$, $p=0.57$ respectively)

R116-deficient particles are otherwise intact. This finding strongly implicates R116 functioning in an entry step in the RCMV lifecycle. Taken together the data presented in this manuscript demonstrate that R116 is a virion envelope glycoprotein important for virus entry.

Members of all three herpesvirus families can infect cells utilizing different routes or modes of entry. This property confers the ability of the virus to infect multiple host cell types through the use of different viral envelope glycoproteins. For example, HCMV is capable of infecting many types of human cells including epithelial cells, endothelial cells, macrophages, fibroblasts and neuronal cells. HCMV typically binds to surface receptors and fuses with the plasma membrane of fibroblasts (Compton, Nepomuceno et al. 1992). However, the virus is endocytosed in endothelial cells in a process that requires low pH (Compton, Nepomuceno et al. 1992; Ryckman, Jarvis et al. 2006; Ryckman, Rainish et al. 2008). Infection of these two different cell types is mediated by modification to the gH:gL complex. Entry into fibroblasts is mediated by the glycoprotein complex of gH:gL, whereas entry into endothelial cells and epithelial cells requires the addition of the UL128:UL130:UL131 complex. Epstein-Barr virus (EBV) also modifies its gH:gL complex in order to specifically infect different cell types. The EBV gH:gL complex mediates entry in epithelial cells but requires the gH:gL:gp42 complex for entry into B cells (Li, Turk et al. 1995; Wang, Kenyon et al. 1998). While the precise mechanisms of entry are currently under investigation, the different glycoprotein usage may enhance fusion efficiency, be

involved in determining specific entry pathways, and/or alter cell tropism at the level of receptor usage. A more detailed knowledge of the CMV glycoproteins and their functions during entry and tropism is necessary in order to understand the effects of glycoprotein usage on CMV disease.

We have used a number of protein sequence and motif analysis programs in order to identify specific domains within R116 that might shed light on its function during entry. A signal peptide within R116 is highly probable at amino acid position 1-18 with a cleavage site most likely between position 18 and 19. The TMpred program strongly preferred a model for R116 with the N-terminus of the protein outside, as a type-1 membrane protein, with the transmembrane domain at amino acids 253-272. We determined that R116 is present on the surface of virions and that it is glycosylated. Using the program NetNGlyc 1.0 there are two N-glycosylation sites predicted at positions 98 and 308, and multiple (>36) O-glycosylation sites. Further investigation into the sequence and domain attributes of R116 revealed an RGD motif located near the N-terminus of the protein. RGD motifs are found in many proteins associated with the extracellular matrix and the motif is recognized by integrins (Ruoslahti and Pierschbacher 1987). Integrins are potential CMV receptors on some cell types and act in a post-attachment step during entry. Interestingly, HCMV gB contains an ADAM disintegrin domain that confers RGD-independent binding to many types of integrin molecules, which highlights the importance of CMV virion interactions with integrins. According to our data, R116 may be acting at a post-attachment step, which

would be consistent with the involvement of integrins. Analysis of the R116 protein sequence also revealed the presence of a glycosaminoglycan (GAG) attachment site within the N-terminal extracellular domain. Both integrins and GAGs play a role in CMV attachment and entry. HSPGs are used by HCMV gB and and/or gM:gN, to tether the virion to the cell surface (Compton, Nowlin et al. 1993; Feire, Koss et al. 2004). Worthy of note, analysis of R116 using Propsearch, which finds putative protein families, showed R116 contained some similarity to rat CD44. CD44 is a cell surface glycoprotein involved in multiple cellular activities such as cell adhesion, migration, and cell-cell interactions. There are functionally distinct isoforms of CD44 achieved by alternative splicing (Goodison, Urquidi et al. 1999). Only the spliced form of R116 and not the full-length ORF displays homology to rat CD44. CD44 is a receptor for hyaluronic acid, a member of the GAG family that is expressed on connective, epithelial, and neural tissues. Thus R116 could function in attachment via binding to GAGs and/or integrins on the surface of cells though further studies are needed to test this hypothesis. R116 possibly functions as an accessory protein involved in the entry process or promotes efficient entry into specific cell types. Future studies will uncover the role of R116 in RCMV entry.

Acknowledgements

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Chapter 4: Discussion and Future Directions

Unquestionably, one of the most important characteristics of CMV's association with disease is the ability of the virus to persist for the lifetime of the infected host. Persistence allows the virus to be vertically transmitted to the fetus or newborn where the virus can lead to deafness, mental retardation and even death. The ability of the virus to persist also allows the virus to reactivate during times of stress in the host, which can lead to a number of diseases in AIDS patients and those undergoing immunosuppressive therapies following transplantation or for the treatment of inflammatory diseases. Chronic diseases associated with CMV infection such as atherosclerosis or cancers are probably a direct reflection of the ability of CMV to persist in the infected host undergoing multiple sporadic reactivations. Typically, formation of these diseases is accompanied by a chronic inflammatory response, which could be triggered or enhanced by the persistent CMV infection. Therefore it is absolutely critical that we discern the mechanisms by which CMV persists in order to develop proper therapies to abolish persistence or prevent reactivation, reducing the effects of the virus on disease.

A possible reason why CMV carries as many genes as it does is that different gene expression programs could be required in different cell and tissue types to modulate the host response to infection and maximize virus survival increasing the likelihood of transmission to a new host. A means for the establishment of CMV persistence within a host is to remain relatively undetected, this can be accomplished by regulating viral gene expression through the overexpression of

immune evasion genes and the repression of viral replication genes. Our *in vivo* viral gene expression analysis indicates that the expression of immune modulators occurs at higher levels while the expression of viral replication genes occurs at lower levels compared to *in vitro* viral gene expression (Streblow, van Cleef et al. 2007). The differential level of *in vivo* viral gene expression between immune evasion and viral replication genes could allow the virus to persist by producing few infectious virions while remaining relatively hidden from the host immune system. An interesting question related to our findings is how the virus is able to specifically up-regulate certain viral genes and not others in different tissue types. For example, in our study the RCMV encoded Fc receptor r138 was highly expressed in the spleen in contrast, r138 was expressed 30-fold lower in the salivary glands. The data suggests that CMV selectively regulates gene expression *in vivo* and does not produce the same degree of immune modulator genes in each infected cell type. Two important questions remain about CMV persistence: how is viral gene expression regulated *in vivo* and what are the functions of highly expressed viral genes? Therefore, one focus of my work was to determine whether the viral miRNAs encoded by RCMV modulate viral gene expression *in vivo*. The other focus of my work was to determine the function of R116, a RCMV gene that is highly expressed *in vivo*. In this dissertation I describe the identification of the miRNAs encoded by RCMV and the characterization of their expression in infected fibroblasts and in infected tissues from a rat heart transplant model. Chapter 2 is the first description of the miRNAs encoded by RCMV. However, the most important contribution to the field is that

RCMV miRNA expression is dynamic *in vivo* and occurs in a tissue specific manner. The information gained through the experiments described in this thesis contributes to our understanding of CMV post-transcriptional control mechanisms and persistence. Chapter 3 describes the characterization of the RCMV glycoprotein R116, a viral gene that is highly expressed in acutely infected rat tissues and in persistently infected rat salivary glands and I define a role for the viral glycoprotein in RCMV entry. My findings contribute to the identification of a function for an otherwise unknown viral ORF and extend our knowledge about CMV entry.

RCMV encoded miRNAs

The complex lifecycles of the herpesviruses requires an assortment of both positive and negative gene regulation mechanisms. Viral encoded miRNAs provide a means to alter viral gene expression to establish and maintain persistence/latency. However, the viral miRNAs have a huge advantage in that they require only a very limited amount of coding capacity and provide for a non-immunogenic means of controlling gene expression. In Chapter 2, I detailed our studies identifying the miRNAs encoded by RCMV expressed in infected fibroblasts *in vitro* and in salivary glands from infected rats. We identified 24 miRNAs expressed by RCMV. Similar to MCMV and HCMV, the RCMV miRNAs were distributed throughout the viral genome although some were clustered, with the largest cluster containing 9 miRNAs within a 700bp region of the genome near ORF r111. Other herpesvirus miRNAs are encoded in regions associated

with latency and reactivation. It is possible that the 700bp region is involved in RCMV persistence since at least one RCMV miRNA of this region, miR-R111.1-2, was detected in persistently infected salivary glands. miR-R111.1-2 was also detected in fibroblasts *in vitro* but similarly to RCMV gene expression, RCMV miRNA expression we believe will greatly differ *in vivo* vs. *in vitro*. Determining whether the r111 miRNA cluster is involved in RCMV latency and reactivation needs to be determined in future studies through mutation analysis utilizing our RCMV BAC and our *in vitro* and *in vivo* infection models.

We detected significant variation in the 3' and 5' processing of some of the RCMV miRNAs and there were differences in isoform usage between the *in vitro* and *in vivo* samples. Isoforms of miRNAs can have a significant impact by targeting different mRNAs because addition or subtraction of nucleotides at the 5' end of the miRNA can alter the seed sequence. The significance of the ability to process miRNAs with different 5' and 3' ends is yet to be fully established. An interesting study of miRNA isoforms in flies revealed that a single pre-miRNA stem loop gave rise to several functional miRNAs with different biological activities (Ameres, Horwich et al. 2010; Ghildiyal, Xu et al. 2010). The research was also able to show that the miRNA isoforms had different seed sequences, which associated with different AGO proteins. Thus a single compact genomic locus can produce multiple small RNAs, a different miRNA from each side of the pre-miRNA stem loop and each miRNA can produce different seed isoforms resulting in multiple miRNAs with distinct biological activity and target repertoire.

In addition, in flies the miRNA and miRNA* are differentially loaded between AGO1 and AGO2 depending on the 5' terminal nucleotide. Within mammalian cells, we do not yet know if a similar sorting pattern exists. However, our data supports the hypothesis that a single compact genomic locus can produce multiple small RNAs. RCMV miR-111.2-2 and miR-111.2-3 are encoded on the same pre-miRNA stem loop with three and four isoforms produced, respectively (Figure 2.3 and Table 2.3). While all the isoforms of miR-111.2-2 and miR-111.2-3 are expressed in both fibroblasts and salivary gland tissue they are expressed with different relative frequencies (Table 2.2). Differences in miRNA isoforms is of great interest, especially to determine whether the miRNAs contain different seed sequences, are able to target different viral or cellular mRNAs, and subsequently regulate different biological pathways. Using *in vitro* target RNA cleavage assays one can determine experimentally if a miRNA bound to AGO2 containing an extension of one or two nucleotides at the 5' end can produce two or more cleaved products that differ in size. Since AGO2 cleaves the bound mRNA target across from nucleotides 10 and 11, measured from the 5' end of the miRNA, 5' processing is significant. A study by Azuma-Mukai and colleagues found that miRNA isoforms with a 5' extension exist in mammalian cells *in vivo* (Azuma-Mukai, Oguri et al. 2008). Human AGO proteins were tested in RNA cleavage assays and the findings revealed that miRNAs with variants could exhibit different target specificities. Thus, there are assays able to determine the role of the 5' isoforms of RCMV miRNAs in virus replication and pathogenesis.

Persistent RCMV infections are established in the salivary gland specifically within the ductal epithelial cells (Kloover, Hillebrands et al. 2000). Different viral miRNA expression levels could facilitate exploitation of different host-cell types. Our lab was one of first to characterize the RCMV transcriptome of viral gene expression in various tissues *in vivo* (Streblow, van Cleef et al. 2007). We found that the genes highly expressed in persistently infected salivary glands, for example, are genes thought to be involved in immune evasion and host cell manipulation. In general, the genes that were highly detected *in vivo* are regarded as dispensable for virus replication *in vitro*. In infected tissues, the RCMV genes expressed at low levels or undetectable by microarray were involved in virus replication, which differs from the transcription profiles detected *in vitro* wherein approximately 90% of the viral genes were detected. Another feature of the RCMV transcriptome is that the pattern of RCMV gene expression *in vivo* is tissue specific, for example 10 of the genes detected were specific for the salivary glands. The ability of the virus to control gene expression in a tissue-specific manner may be regulated by specific motifs within viral promoters that are active in some tissues and not in others but may also be regulated by viral miRNA expression. EBV infection of B cells results in different transcriptional programs, depending on the activation and differentiation state of the host cell (Thorley-Lawson and Gross 2004). Additionally, HSV-1 expresses the viral LAT during all stages of replication, but LAT is exclusively expressed during latent infection of neurons (Rajcani, Andrea et al. 2004). As discussed in Chapter 2, EBV miRNAs are expressed during different viral transcription programs and are

differentially expressed depending on the cell type infected. RCMV miR-r111.1-2 is almost exclusively expressed in the salivary glands but of particular interest is the fact that expression of this miRNA increases from 7 dpi to 28 dpi. In our RCMV infected rat transplant model 21 dpi is the time when RCMV has established persistence within the host.

In the Appendix 2, I describe recent studies performed by our lab that are aimed at identifying the targets of the RCMV miRNAs. Using RISC immunoprecipitation techniques we have identified the RCMV mRNAs targeted in infected fibroblasts. Interestingly, most of the genes enriched in the RISC IPs are involved in virus replication such as R32, R45, R48, R88, R98, R51, r103, and r128 (Table A.1). Using a bioinformatics approach we identified the RCMV miRNA target sites within the 3' UTRs of all the RCMV ORFs. The computational analysis also showed that many of the target sites for the RCMV miRNAs are within genes involved in replication such as, ribonucleotide reductase (R45), DNase (R98), protein kinase (R97), and helicase-primase (R70). All the RCMV ORFs targeted in the RISC IP contained RCMV miRNA target sites found in the bioinformatics analysis. The use of miRNAs as a method of self-regulation has the potential to be a successful mechanism for the establishment of persistence and latency within an infected host and is described for many herpesviruses to date. We have yet to look for RCMV miRNAs targeting cellular genes such as immune modulators (MICB), proapoptotic proteins (PUMA) and cell cycle regulators (cyclin E2) all found targeted by other herpesvirus miRNAs, although these types

of studies will be performed in the future (Stern-Ginossar, Elefant et al. 2007; Choy, Siu et al. 2008; Grey, Tirabassi et al. 2010).

Clearly many, if not all, of the herpesviruses regulate their gene expression during persistence and latency. CMV expression in the salivary gland, a major site of viral persistence, is tightly regulated and highly skewed even in the presence of high levels of viral genome. To gain insight into persistence we can now isolate the ductal epithelial cells of the salivary gland and perform transcriptional analysis on the viral and cellular genes. We utilized microarray analysis in combination with laser capture microscopy to specifically capture cells from rat salivary glands infected with a recombinant RCMV expressing GFP in order to determine the viral transcriptome associated with the development and maintenance of persistence. We have observed that the viral transcription profile changes between 7 and 10 dpi from one associated with the expression of genes involved in viral replication, which shifts to a transcription program that excludes expression of replication genes for those primarily involved in persistence (Streblow unpublished data). Our data is the first demonstration of a switch in viral gene expression associated with viral persistence.

To investigate the miRNAs expressed during persistence we have begun constructing a recombinant RCMV expressing a myc-tagged version of AGO2 under the EF1α promoter into a non-essential region of the RCMV genome using our RCMV BAC. Using our recombinant RCMV BAC, we will perform RISC IP

analysis to identify the targets of the RCMV miRNAs during *in vivo* RCMV infection. With the above approach, we will also be able to provide a more complete list of the mRNAs targeted during allograft rejection.

The long-term goal of the Streblow laboratory is to determine the role of HCMV in the development of vascular diseases and chronic allograft rejection. In the future, we will characterize the role of CMV miRNAs during acute and persistent infections as well as during reactivation and subsequent dissemination in our latent RCMV infection rat model. We will first determine the RCMV miRNAs expressed in various clinically relevant cell types and tissues from allograft recipient rats. Cellular miRNAs have been implicated in heart disease and are potential biomarkers for predicting the disease process (Zhang 2010). Therefore, we will also characterize the changes in cellular miRNA expression profiles following infection and during transplantation to identify crucial changes associated with the development of TVS and CR using Illumina Rat Genome microarrays. We will then determine which RCMV miRNAs are important for viral replication and pathogenesis. Last, we will characterize the RCMV miRNA gene targets, viral and cellular, that are essential for persistence and reactivation in our rat transplant model.

To implement our aims we will first use over expression studies *in vitro* to determine the effect of RCMV miRNAs on virus replication, we will transfect fibroblasts with synthetic miRNAs or a negative control miRNA and at 24 hrs

post-transfection the cells will be infected with RCMV. At 8, 24 and 72 hpi the cells and supernatants will be collected. The production of infectious virus will be monitored by plaque assays from the supernatants. We will also examine protein expression using Western blot analysis using antibodies directed against the targeted gene(s). In the case of ORFs that do not have freely available antibodies, we will generate rabbit polyclonal antibodies to the proteins of interest. We have gained extensive experience with making antibodies as we have generated antibodies to RCMV R55-gB, R78, R115-gL, R116, r119.1, IE1/IE2, r129, r131, and r152.4.

We will then use recombinant RCMV viruses containing mutated or deleted viral miRNAs to characterize their role in viral replication. The disruption of the viral miRNAs can be complicated due to their genome localization. To circumvent this problem, we will introduce silent mutations to disrupt the secondary structure of the viral miRNA, which is an important feature in the processing of the miRNA. First, we will use the online secondary structure prediction tool mfold to predict silent mutations that disrupt pre-miRNA secondary structure. We will identify mutant miRNAs that fail to process and introduce them into the RCMV genome using BAC recombination. We use a modified two-step linear recombination protocol that utilizes galactokinase (*galK*) as the selectable marker (Warming, Costantino et al. 2005). Recombinant BACs and reconstituted viruses will be characterized using restriction enzyme analysis and Southern blotting. Direct sequence analysis will be used to confirm presence of the desired mutation.

Viruses will be analyzed for gene expression, as well as viral growth using high and low multiplicity infections of fibroblasts and other clinically relevant cell types. To ensure that other adjacent viral genes are not disrupted, we will analyze mRNA and protein expression of the genes adjacent to the site of mutation. We will also generate revertants of RCMV mutants to attribute the phenotype with the mutation introduced into the virus. We will use these recombinant RCMV viruses to characterize the role of the viral miRNAs *in vivo* during both acute and latent/reactivation infection scenarios as well as transplantation. A detailed investigation of the mechanisms of RCMV miRNA regulation during transplantation and the identification and validation of targets will enable us to determine important regulatory aspects of CMV gene expression during TVS/CR acceleration.

RCMV ORF R116

Only a relatively small percentage of the CMV virion-associated proteins have known functions. To this end, in Chapter 3 of my dissertation I describe the characterization of the virion-associated glycoprotein encoded by the RCMV open reading frame R116. RCMV R116 is encoded on the complementary strand of the genome and is expressed as three different transcripts, which differ in their expression kinetics (Figure 4.1). Similarly, a number of different R116 protein species are detected, but interestingly, only the higher molecular weight species are expressed *in vivo* and incorporated into virions. While RCMV R116 and MCMV M116 are the positional homologs of HCMV UL116, only RCMV R116

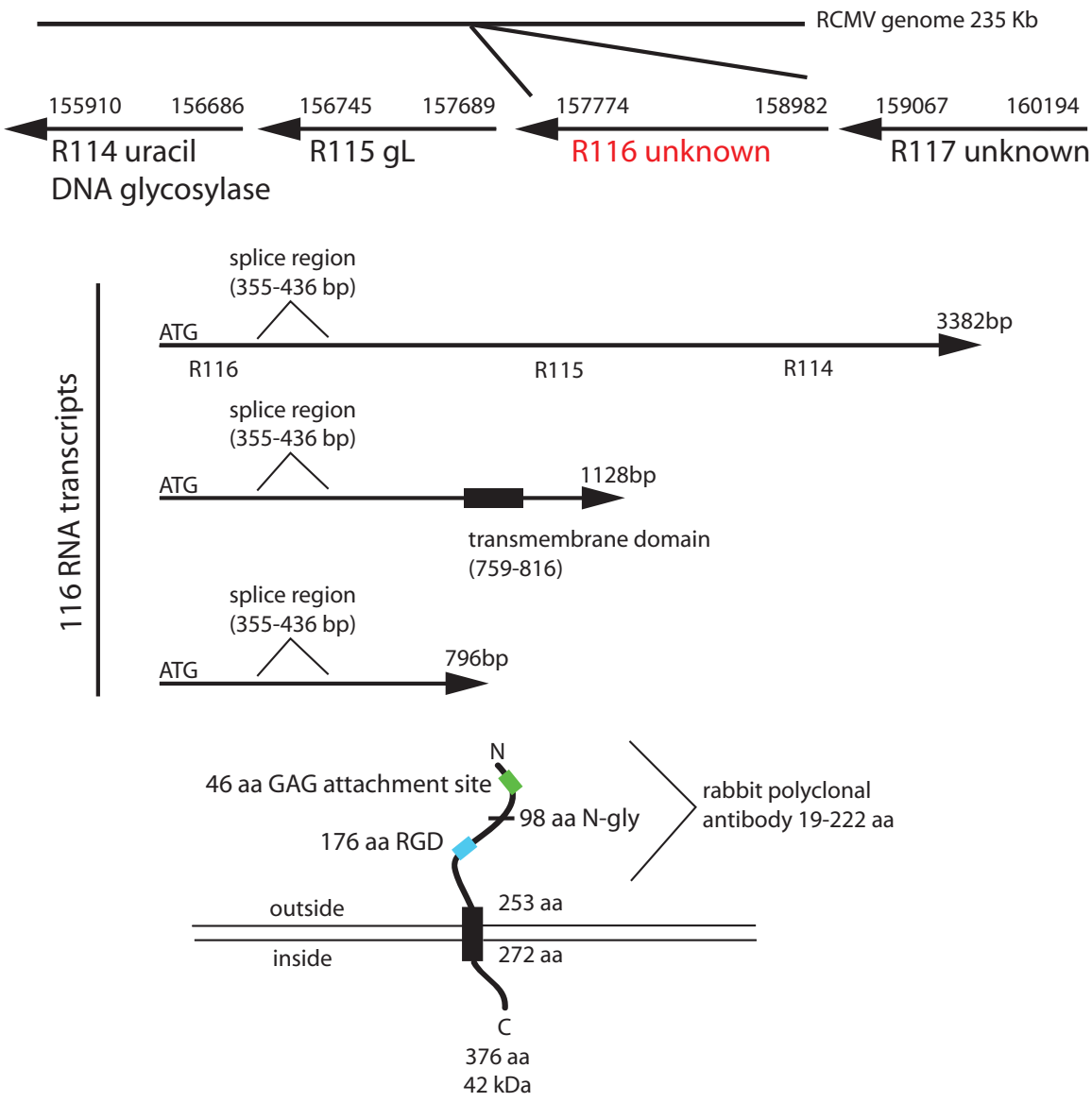

Figure 4.1 Schematic of R116 transcripts and predicted protein structure. R116 is encoded on the complement strand of the RCMV genome. Sequence analysis of a cDNA library from RCMV infected rat fibroblasts harvested at 48 hpi found two transcripts one containing R116, R115 (gL) and R114 (uracil DNA glycosylase) and the other containing only R116. A second cDNA library from rat fibroblast infected for 24hpi found a third transcript containing a truncated form of R116. All of the R116 transcripts contain a spliced region removing 84 base pairs downstream from the predicted signal sequence cleavage site (between 18 and 19 aa) and upstream from the predicted transmembrane domain (253-272 aa). The predicted protein from the 1128bp transcript is a type-1 membrane glycoprotein that is 42 kDa. The N-terminal protein contains a potential N-glycosylation site, GAG attachment site (green), and RGD motif (blue). Our rabbit polyclonal antibody was generated to a GST-fusion protein containing the N-terminal portion of R116 (19-222 aa).

and MCMV M116 are present in virions. Possibly HCMV UL116 was missed in the proteomics study (Varnum, Streblow et al. 2004). However, UL116 potentially differs from R116 since CMV is a species-specific virus and herpesviruses typically evolve with their host species. RCMV as well as MCMV are model systems to study HCMV and have potentially developed diverse viral glycoproteins utilized in the viral lifecycle.

One of the most important findings presented in Chapter 3 is that R116 is required for RCMV entry. I have shown that the RCMV ORF R116 is a virion surface envelope glycoprotein. Transfection of a RCMV BAC containing an insertional mutation of the R116 ORF did not spread in fibroblasts *in vitro*, which suggests that R116 was required for virus replication. Importantly, a repair RCMV BAC constructed from the mutant virus displayed wild type growth kinetics. However, at this point whether R116 was required for virus egress or entry was not clear because the assay did not control for these specific aspects of the virus lifecycle. Thus, to confirm the phenotype I performed a R116 siRNA knockdown experiment. Depletion of R116 decreased RCMV infectivity of fibroblasts by two logs but the treatment did not alter viral genome replication or release of viral genome containing particles nor disrupt the transcript levels of the downstream genes gL (R115) and uracil DNA glycosylase (R114). This suggested to us that the defect must be in virus entry. Therefore, I performed an experiment to determine whether virus entry was affected by R116 depletion. Polyethylene glycol is a chemical that causes membranes in close proximity to fuse together;

Drs. Johnson and Ryckman use this methodology to study HCMV entry. So after an initial binding step I treated the virus bound cells with PEG and found that PEG restored infectivity to control levels. The PEG experiment was extremely important because the data indicated that R116-lacking virions are otherwise normal and that the effect on infectivity is specific for R116. The data collectively implicate R116 as having a role in an entry step. Subsequent experiments to determine the precise role of R116 in the RCMV lifecycle are necessary.

Since I was unable to produce a recombinant RCMV R116 mutant virus from normal fibroblasts additional reagents will be necessary to study R116 function. Multiple complementing strategies exist that could be performed to allow the production of a RCMV-ΔR116 virus including transient transfection of an expression plasmid containing R116, producing a stable R116-expressing cell line or constructing a RCMV R116 conditional mutant. The latter could be accomplished by constructing a tetracycline-regulated mutant as was previously demonstrated for the essential MCMV gene M50 (Rupp, Ruzsics et al. 2005). To date I have attempted to express the full-length R116 ORF in expression vectors and non-replicating adenovirus (Ad) vectors but these attempts were unsuccessful. The multiple R116 transcripts including the spliced region could lend difficulty to protein processing in trans and the gene should be optimized for protein expression. With the elucidation of the R116 transcripts, we can now construct the R116 transcript with the splice region already removed in order to complement our R116 mutant virus. After complementation in trans by

adenovirus expression vector, transfection of the R116 expression plasmid and/or construction of a stable cell line we will be able to perform complementation experiments using our R116 mutant virus. CMV virions are thought to spread directly through cellular contacts or across cellular junctions during plaque formation (Johnson and Huber 2002). To determine if R116 is important for cell-to-cell spread of RCMV, we could perform an experiment using R116 complementing or non-complementing cells infected with the R116 mutant virus, overlayed with agarose and plaque size will be visualized one week post-infection. These experiments would enhance our understanding of the role of R116 in entry.

I performed sequence analysis aimed at identifying cellular or viral homologs to R116 and key protein motifs of R116. Interestingly, I identified an RGD motif and a GAG attachment site in the R116 extracellular domain. The spliced version of R116 also has minor domain similarity to rat CD44. RGD motifs mediate protein interactions with integrins and integrins are potentially important for CMV entry. GAG binding domains are important for herpesvirus attachment. Finding both domains in R116 points to the hypothesis that R116 could be functioning in attachment and/or entry of the virion into the host cell. To test whether R116 functions during RCMV entry, we can perform virion attachment and internalization experiments in rat fibroblasts. To facilitate our studies we will need to develop additional reagents such as a neutralizing antibody to R116 and/or use peptides encompassing the R116 RGD or GAG sites that could be used to

inhibit virus entry by binding to the cellular targets of R116. In addition, using BAC technology we could construct a recombinant RCMV containing a mutation in the R116 RGD motif and/or the GAG attachment site. HCMV gB contains a disintegrin-like domain (DLD), another known integrin recognition motif, that when blocked by peptides inhibited HCMV entry (Feire, Koss et al. 2004). Neutralizing antibodies have been used to study HCMV gB where their presence inhibited cell-to-cell spread shown by plaque assay (Britt 1984; Navarro, Paz et al. 1993; Bold, Ohlin et al. 1996). As discussed previously both integrins and GAGs play a role in CMV attachment and entry. The RGD motif is found in many proteins of the extracellular matrix and is recognized by different members of the integrin family (Ruoslahti and Pierschbacher 1987). Heparan sulfate proteoglycans, used by HCMV gB and and/or gM:gN, to tether the virion to the cell surface is a member of the GAG family (Compton, Nowlin et al. 1993). For RCMV binding assays, the R116 mutant virus is bound at 4°C to allow stable virus binding while restricting fusion and internalization. The cells are then washed and analyzed for bound virus compared to wild type. To assess virus binding rat fibroblasts are treated with a R116 neutralizing antibody or blocking peptides for 60 min at 4°C and infected with RCMV for 60 min at 4°C. Unbound virus is removed, and cells are fixed with paraformaldehyde. Bound virus is detected with anti-RCMV polyclonal antibody by Western blot. These experiments will determine whether R116 is involved in attachment.

However, our PEG experiments suggest that the R116 defect occurs in a post-attachment step of entry since PEG restored infectivity. There are a few methods that we could employ to differentiate between endocytosis and fusion entry pathways. Inhibitors of ATP synthesis can block endocytosis because it is an energy-dependent process and can be applied by pre-incubating cells with energy depletion medium (Mellman 1996). Many viruses that are internalized by endocytosis require a low-pH environment to trigger the viral glycoproteins that execute fusion. NH_4Cl and chloroquine are lysosomotropic agents that interfere with endosome acidification by buffering endosomal pH. The drug Bafilomycin prevents endosome acidification by interfering with the endosomal ATPase proton pump (Bowman, Siebers et al. 1988). The use of lysosomotropic agents has been experimentally proven to inhibit infection by viruses that require an acidic environment and the role of R116 could be analyzed in the presence of such agents. Electron microscopic (EM) analysis could be incorporated with the above experiment in order to identify blocks in the entry pathway in the presence of lysosomotropic agents.

In tissue culture, RCMV efficiently spreads in a cell-to-cell manner. Thus, R116 conceivably may also be involved in this process. Cell-to-cell fusion assays have been extensively used to understand how herpesvirus membrane proteins function in virus entry. Nonreplicating adenovirus vectors have been used to efficiently express HCMV glycoproteins in cells. Fusion assays can be performed with Ad vectors expressing different viral glycoproteins used for entry. To test

whether cells expressing R116 glycoproteins can fuse to form multinucleated syncytia we would use this approach. In the assay, cell-to-cell fusion is determined by monitoring syncytia formation by staining the nuclei with propidium iodide and plasma membranes with an antibody to β-catenin. This assay would identify whether R116 is involved in a fusion event.

Many of the viral glycoproteins encoded in herpesvirus genomes likely have redundant, cell type-specific and strain-specific functions necessary for virus spread. Transposon mutagenesis in AD169 and ORF deletion in Towne, both HCMV lab-adapted strains, classified gO as a nonessential but replication-enhancing ORF (Dunn, Chou et al. 2003; Yu, Silva et al. 2003). In contrast, gO in the clinical strain TR is required for entry into fibroblasts, epithelial and endothelial cells (Wille, Knoche et al. 2010). Others have stated that the mode of viral transmission changes with regard to clinical isolates of HCMV, which appear to spread by a cell-to-cell route whereas cell culture adapted HCMV predominantly releases enveloped progeny virions (Sinzger, Schmidt et al. 1999). Therefore, R116 possibly plays an accessory role to the already known and required glycoproteins of RCMV and future studies will elucidate the role of R116 in virus entry.

Effort Statement

In Chapter 2, C. Meyer carried out all the experiments except C. Kreklywich performed the quantitative RT-PCR on infected rat tissues and LC Sciences performed the deep sequencing. In Chapter 3, C. Meyer carried out all the experiments except C. Kreklywich constructed the RCMV cDNA libraries, J. Powel generated the rabbit polyclonal antibody to R116, P. Smith performed the immunofluorescence on the salivary gland frozen section, J. Vomaske performed the microscopy, and M. Denton aided in BAC construction and mutagenesis. In the Appendix, B. Poling wrote the seedseq and grayscale programs and R. Tirabiasi contributed Figure A.4.

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Appendix 1: RCMV *in vitro* and *in vivo* Gene Expression

Both animal and tissue culture models are used to study CMV infection and determine the function of viral ORFs in the development of disease. Therefore, our laboratory was interested in comparing the gene expression profiles in RCMV infected cultured cells to the gene expression profiles in tissues from RCMV infected rats. We hypothesized that CMV gene expression in tissue culture might not accurately represent gene expression *in vivo*. To determine viral gene expression we developed RCMV-specific DNA microarray chips containing two unique, nonoverlapping 70-mer antisense oligonucleotides for each of the 167 predicted viral ORFs and an additional 2,925 rat cellular genes were used to normalize the data (Vink, Beuken et al. 2000).

We determined RCMV gene expression in cultured rat embryonic fibroblasts, aortic smooth muscle cells, epithelial cells and endothelial cells as well as an alveolar macrophage cell line and bone marrow-derived dendritic cells (Streblow, van Cleef et al. 2007). RNA was purified from cells harvested at 4, 8, 16, 24, 36, and 48 hours post-infection. Only two genes, IE1 and r128, were highly expressed in all cell types at 4 hpi and by 16 hpi nearly 80% of RCMV ORFs were expressed at detectable levels. By 36 to 48 hpi greater than 90% of the RCMV genome was transcriptionally active. Sixty percent (96 viral genes) of the predicted viral ORFs were expressed with early kinetics in RCMV-infected fibroblasts determined by treatment with a viral DNA polymerase inhibitor. Interestingly, according to our microarray results the profile of viral genes and the

level of gene expression vary among the cell types. Fibroblasts are the most prolific cell type expressing nearly all of the viral genes by 24 hpi (Figure A.1). Macrophages and epithelial cells show the most limited gene expression profiles with other cell types displaying intermediate phenotypes (Figure A.1). A limited number of genes are expressed exclusively in one cell type or another. Our findings demonstrate that RCMV gene expression is cell-type specific. This phenomenon has interesting implications for viral infections *in vivo*.

To identify the viral genes expressed *in vivo* we harvested tissues (salivary gland, lung, kidney, spleen, PBMCs and native and allograft hearts) from rat heart transplant recipients at 7, 14, 21, and 28 days post-transplantation that were infected with RCMV at post-operative day 1. Each tissue examined displayed a unique RCMV transcription profile (Figure A.2). Compared to the 87% transcriptionally active genes observed in RCMV infected fibroblasts in culture, the number of transcripts detected *in vivo* were quite low. Importantly, this difference was not due to lower signal intensity but our data suggests that the number of viral genes expressed is specific to a given tissue and is not dependent upon viral load or viral mRNA levels. The average viral load in the allograft heart, spleen, lung, liver, salivary gland and kidney ranged from 5×10^4 to 2×10^5 RCMV genomic copies per 1 μg of total DNA. In the salivary gland tissue from post-transplantation day 7, only two RCMV genes, R116 and r152.4, were expressed to at least twice the background level. By post-transplantation day 14, the number of detectable RCMV genes increased to 11% and further

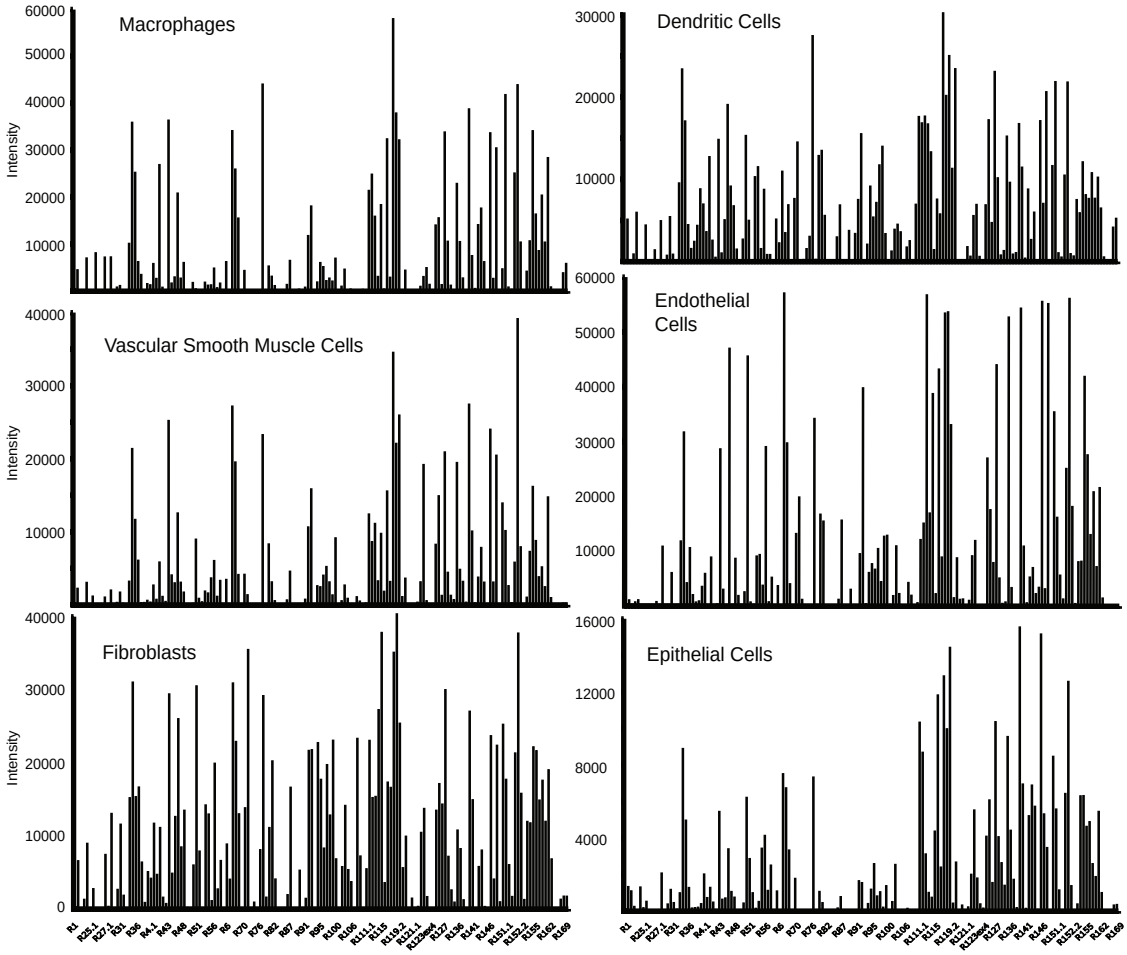

Figure A.1 Microarray analysis of RCMV gene expression *in vitro*. Total RNA was extracted from RCMV infected Macs, DCs, ECs, Epithelial cells, SMCs, and fibroblasts at 24hpi. The RNA was analyzed using RCMV microarray chips. The mean intensity is plotted on the y-axis and RCMV gene numbers are plotted on the x-axis.

increased to 25% by day 21. Due to normal viral clearance, none of the tissues except the salivary gland at post-transplantation days 14, 21, or 28 contained sufficient viral mRNA for microarray analysis. While viral gene expression levels were consistently the highest in the salivary gland, there were a greater number of RCMV genes expressed above background in the graft heart, spleen and liver than in the salivary gland. In all tissues examined only 9 RCMV ORFs were common: R78 (vGPCR), R116, r119.2, r119.3, r133, r138 (Fc receptor homolog), r142 (m142 homolog macrophage tropism determinant), r149, and r152.4. The results we obtained show that the transcriptional profiles differed dramatically between infected cells *in vitro* and RCMV infected rat tissues. This analysis also showed that RCMV gene expression is tissue specific indicating that the virus adapts to its cellular environment or viral gene expression is affected by the host's transcriptional control.

Transcriptional profiles from infected tissues revealed that the highly transcriptionally active genes are not those involved in virus replication, but are genes thought to be involved in immune evasion, cell cycle manipulation, anti-apoptosis, and/or cell survival. For example, r151 was highly expressed in the salivary gland of infected rats, which is a homolog of the MCMV m152 gene. The m152 gene blocks cell surface expression of MHC class I in infected cells an important action for virus immune evasion. The altered level of gene expression could allow the virus to persist by turning over small amounts of infectious virus while remaining undetected by the immune system. Most studies aimed at

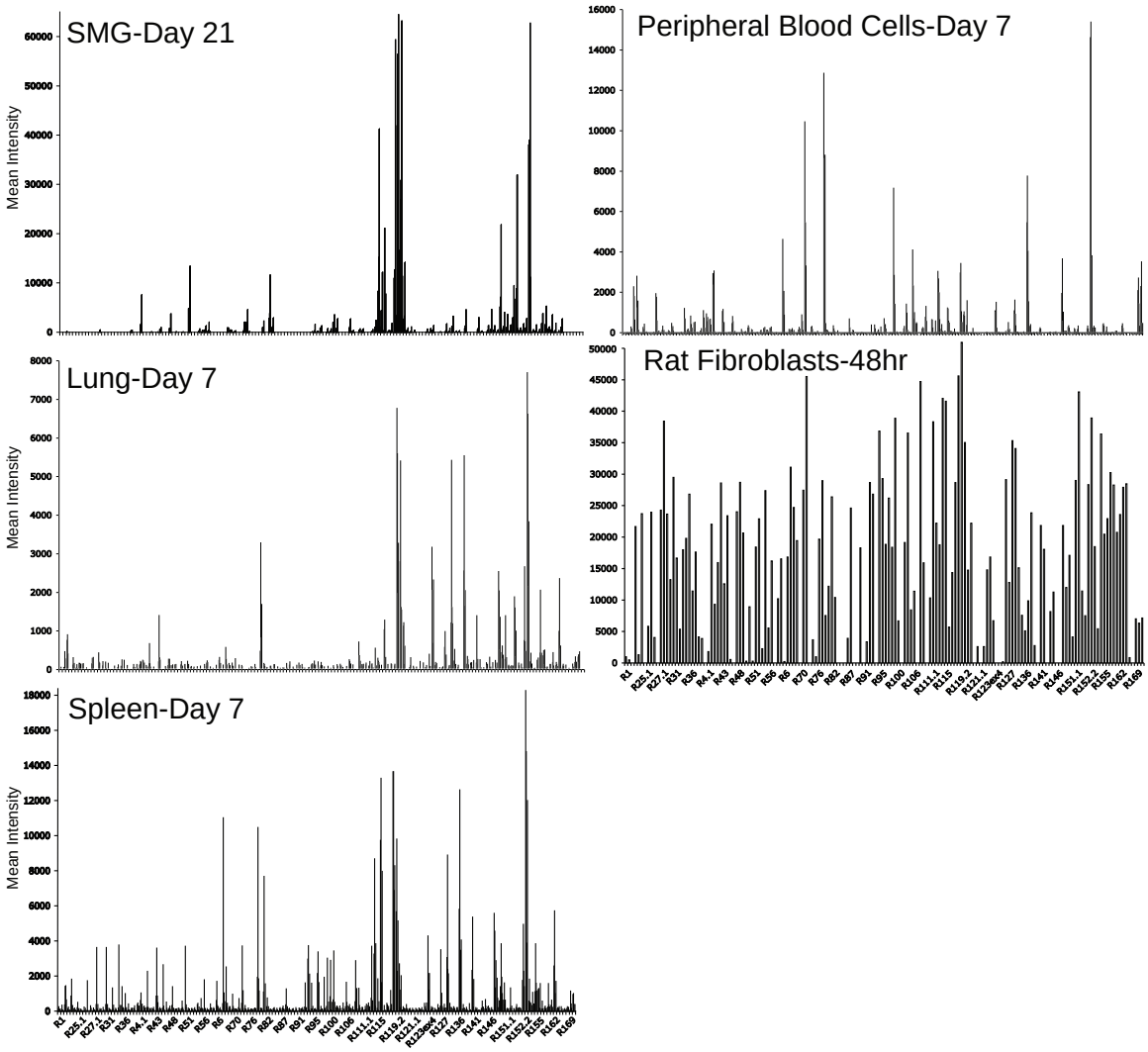

Figure A.2 Microarray analysis of RCMV gene expression *in vivo*. Total RNA was extracted from RCMV infected spleen, lung, peripheral blood at 7dpi, salivary gland at 21dpi, and cultured rat fibroblasts at 48 hpi. The RNA was analyzed using RCMV microarray chips (n=4). The mean intensity is plotted on the y-axis and RCMV gene number is plotted on the x-axis.

identifying viral gene expression *in vivo* have focused on the immediate early genes, which we show are expressed at very low levels compared to genes such as immune modulators. We used quantitative RT-PCR to confirm the microarray results. The levels of IE1 and other essential genes, such as gH were more than 100-fold lower in tissues compared to genes classified as non-essential for virus replication in fibroblasts in culture. This finding differs from that observed in cultured fibroblasts where IE1 expression was equal to, or significantly higher, than the non-essential genes highly expressed *in vivo*. The ability to control viral gene expression as a method of persistence in the infected host is critically important whether during latency or during persistent infection states. Our findings emphasize the importance of *in vivo* analysis using animal models in translational research.

Appendix 2: RCMV miRNA Target Identification within the RCMV Genome

Identification of target transcripts is still a major obstacle in determining the function of cellular and viral miRNAs. Bioinformatics approaches have yielded useful information but the method is affected by high false-positive rates. Other recently developed methodologies that use a biochemical approach have been shown to reduce the false discovery rate. Currently, combinations of biochemical and bioinformatics approaches are used to successfully identify important biological miRNA targets. Since we have identified the miRNAs expressed by RCMV, the next step is to determine their function by identifying specific targets. Our laboratory has used both indirect and direct methods to identify targets of RCMV miRNAs. We used a bioinformatics approach to identify RCMV miRNA targets within the RCMV genome by analyzing seed/target sequence matches within the 3' untranslated regions (UTRs) of the predicted RCMV ORFs. We combined bioinformatics with the RISC immunoprecipitation (IP) method. For the RISC IP assay, we infected cells with RCMV and then performed IP utilizing a specific antibody to AGO2, which pulls out the RISC complex containing transcripts targeted by miRNAs. We then performed microarray analysis to identify RCMV genes as well as rat cellular genes that were incorporated into the RISC complex in the infected cells compared to uninfected cells (Keene, Komisarow et al. 2006; Beitzinger, Peters et al. 2007; Easow, Teleman et al. 2007; Karginov, Conaco et al. 2007; Baroni, Chittur et al. 2008; Hendrickson, Hogan et al. 2008). Once targets are identified using these two methods we will

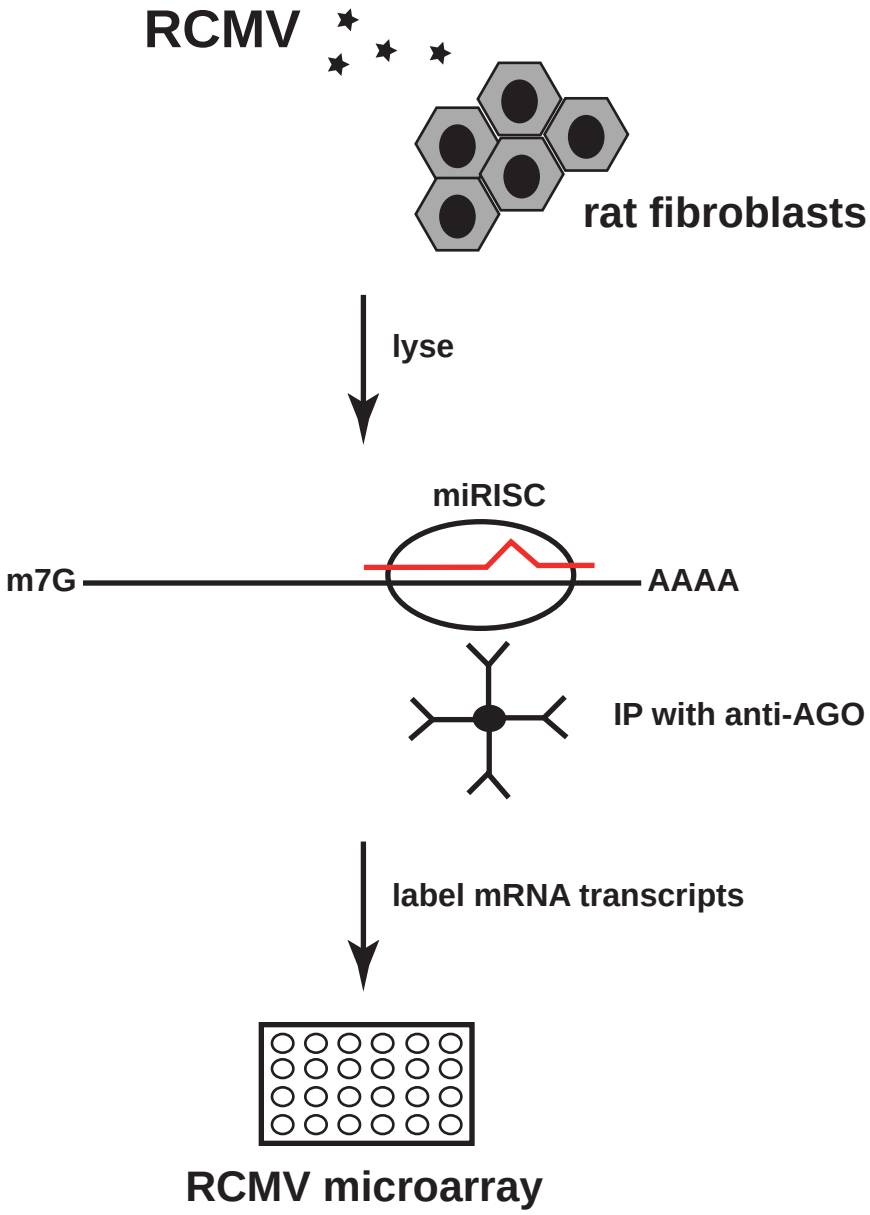

Figure A.3 Schematic of the RISC IP assay. Mock infected cells or RCMV-infected cells are lysed at 72 hpi in a standard NP-40 (Ipegal) lysis buffer containing RNase inhibitor. RISC complexes are pulled down using our polyclonal antibody to AGO2/4. Following IP RNA is extracted with Trizol, and converted to cDNA, labeled and hybridized to the RCMV genome microarray chips. A portion of the lysate is saved for microarray analysis (total RNA sample) to which the RISC-IP samples are compared for fold enrichment calculations.

use direct methods to specifically detect interactions between the miRNA and its target mRNA.

The method for our biochemical RISC IP approach to identify the targets of the RCMV miRNAs is depicted in Figure A.3. The use of the RISC IP has recently proved ideal for identifying miRNA targets (Grey, Tirabassi et al. 2010). In collaboration with Dr. Jay Nelson's laboratory we have generated an antibody to a C-terminal peptide sequence found in both human and rat Argonaute (AGO), which is a component of the RISC complex (Figure A.4A). The AGO antibody reacts with both rat AGO2 and AGO4 because the peptide sequence used for immunization is conserved in both proteins. AGO2 is larger than AGO4 making them easily differentiated by Western blot analysis. The AGO peptide used to make the antibody was able to compete for binding, demonstrating the specificity of the antibody (Figure A.4B). Immunoblot analysis of cell lysates from rat fibroblasts, smooth muscle cells (SMC), endothelial cells (EC), macrophages (Mac), as well as spleen and peripheral blood mononuclear cells (PBMC) indicates that expression levels of AGO2 and AGO4 vary amongst these cell types (Figure A.4C). We find this observation highly provocative and it may have important implications in CMV miRNA targeting in different cell types. Therefore, we plan to perform RISC IP analysis in different biologically relevant cell types including endothelial cells, macrophages, dendritic cells, smooth muscle cells and epithelial cells.

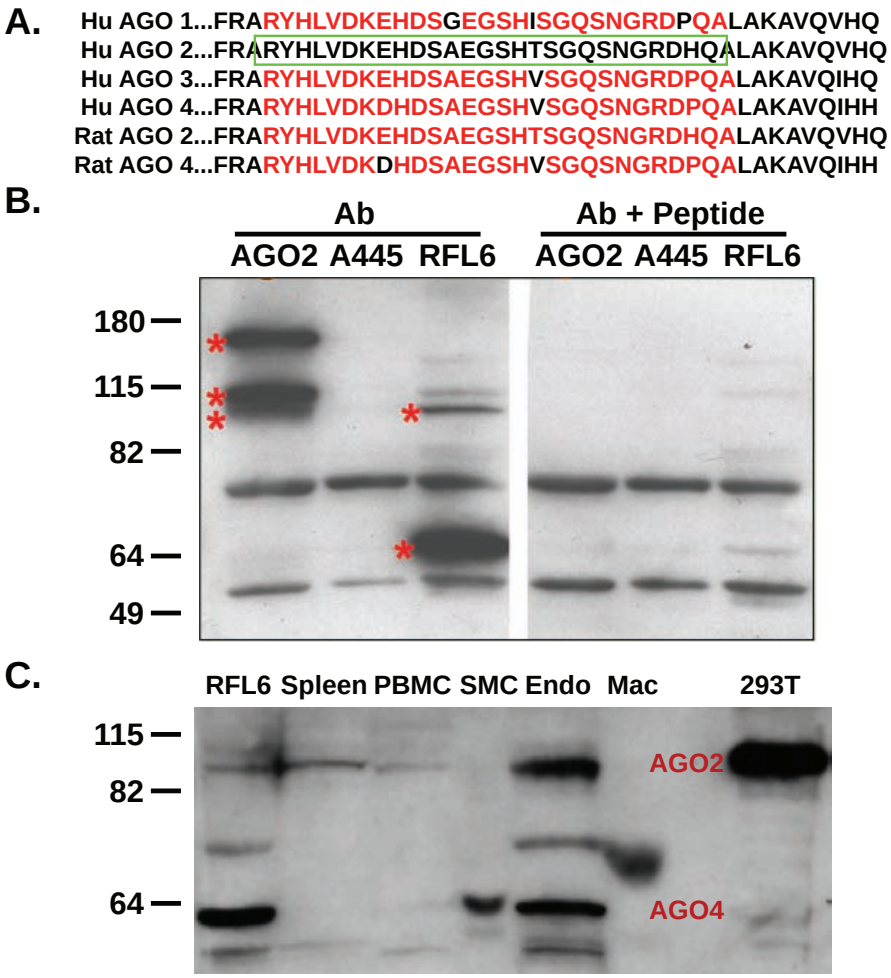

Figure A.4 Generation of the AGO antibody for the RISC IP. The antibody to the C-terminal peptide sequence found in rat Argonaute, which is a component of the RISC complex, reacts with AGO2 and 4 because the protein sequences are well conserved at that site. A) Ago peptide sequence, shown in green box is the peptide that the antiserum was made against (Hu=human). B) Western blot for AGO (AGO2=overexpression of AGO2 in 293T cells, A445=human fibroblast cell line, RFL6=rat fibroblast cell line). The red stars indicate AGO specific bands. In the right blot the antiserum was bound to peptide prior to probing the blot. C) Western blot for AGO expression in rat fibroblasts, smooth muscle cells (SMC), endothelial cells (EC), and macrophages (Mac) as well as lysates from rat spleen and peripheral blood mononuclear cells (PBMC).

In a preliminary study, fibroblasts were infected with RCMV and lysed at 72 hpi in a standard NP-40 (Ipegal) lysis buffer containing RNase inhibitor. A portion of the lysate was saved for microarray analysis (total RNA sample) to which the RISC IP samples are compared to for fold enrichment calculations. RISC complexes were immunoprecipitated with the anti-AGO antibody and RNA was extracted with Trizol. The RNA was converted to cDNA, labeled and hybridized to RCMV microarray chips, which contain all known RCMV ORFs and approximately 700 cellular genes. We identified 30 RCMV genes associated with the RISC complex that were enriched $\geq$ two-fold above the total RNA levels for each gene (Table A.1). Listed in the table are the RCMV gene functions or the homologs in HCMV or MCMV otherwise the ORF function is unknown. Interestingly, many of the viral genes that were brought down in the fibroblast RISC IP assay are involved in viral replication, such as R51, which functions in viral DNA packaging. Our RISC IP data is congruent with the hypothesis that miRNAs target their own viral replication genes in order to dampen replication and establish persistence.

While the RISC IP is useful for identifying targeted mRNAs, this technique does not discern which cellular or viral miRNAs are targeting the specific RCMV ORFs. To identify specific RCMV miRNA:mRNA target pairs, we used a bioinformatics approach to predict target sequences in the 3' UTRs of the identified genes in the fibroblast RISC IP experiment. We designated the 3' UTRs of the Maastricht RCMV genome as the sequence between the ORF stop codon and the polyadenylation signal (AATAAA). The sequences were placed into a text

Table A.1 RISC IP results from RCMV infected fibroblasts.

RCMV Gene	Fold Enrichment	Gene Function
R45	11.5	UL45 ribonucleotide reductase
R27	6.5	
r128	5.0	homolog MCMV IE 2 exon 3
R39	4.7	
r25.1	4.4	US22 family homolog
r44.3	3.6	
R98	3.3	UL98 DNase/exonuclease
R109	3.0	
r152.2	2.8	member m145 gene family
r125	2.8	
r44.1	2.8	
R97	2.7	UL97 phosphotransferase or protein kinase (PK)
R103	2.7	involved in virion morphogenesis
R51	2.7	involved in DNA packaging
r3	2.6	
r151.1	2.6	
R90	2.5	
r118	2.5	
r70.4	2.5	member m145 gene family
r38.5	2.4	
r70.2	2.3	member m145 gene family
R32	2.2	UL32 pp150 major tegument phosphoprotein
R31	2.2	
R88	2.1	tegument protein
R34	2.1	involved in gene regulation
R43	2.1	US22 family homolog, tegument protein
R48	2.0	UL48 large tegument protein, involved in capsid transport
r70.3	2.0	member m145 gene family
r95.1	2.0	
R42	2.0	
R70	1.6	UL70 helicase-primase (HP) complex component

document and analyzed using the program “seedseq” written in the programming language Python 2.6. The seedseq program outputs the number of times a particular target sequence, which is complementary to the seed sequence, is found within each RCMV 3’ UTR. The number of occurrences was calculated for 6mer, 7mer-m8, and 8mer seed sequences. The count numbers were tabulated using the program “grayscale,” also written in Python 2.6, and a heat map was generated as a visual representation of the data. Figure A.5 shows the heat map depicting the RCMV miRNA 7mer-m8 seed matches to the 3’ UTR target sites in the RCMV genome. Interestingly, the vast majority of target sites found in the RCMV 3’ UTRs are in genes involved in viral replication. There is far less targeting of genes at the 3’ end of the RCMV genome, which contains the genes involved in immune evasion and host cell manipulation. This result taken together with our RISC IP data indicates that RCMV miRNA targeting is aimed at reducing viral replication proteins. Figure A.6 shows an example of the target sites predicted in R45, the RCMV homolog of HCMV ribonucleotide reductase (UL45), which was the targeted viral transcript most highly enriched in our RISC IP experiment (Table A.1). R45 has 5 predicted RCMV miRNA target sites including miR-r1-4, miR-R87-1, miR-r111.2-2 and two sties for miR-r111.1-3. Members of Dr. Jay Nelson’s lab have shown that the HCMV miRNAs can work cooperatively to down-regulate protein expression of a single target (unpublished data). R45 is important for virus replication thus lends data to our hypothesis that RCMV miRNAs regulate viral replication in order to establish and maintain persistence.

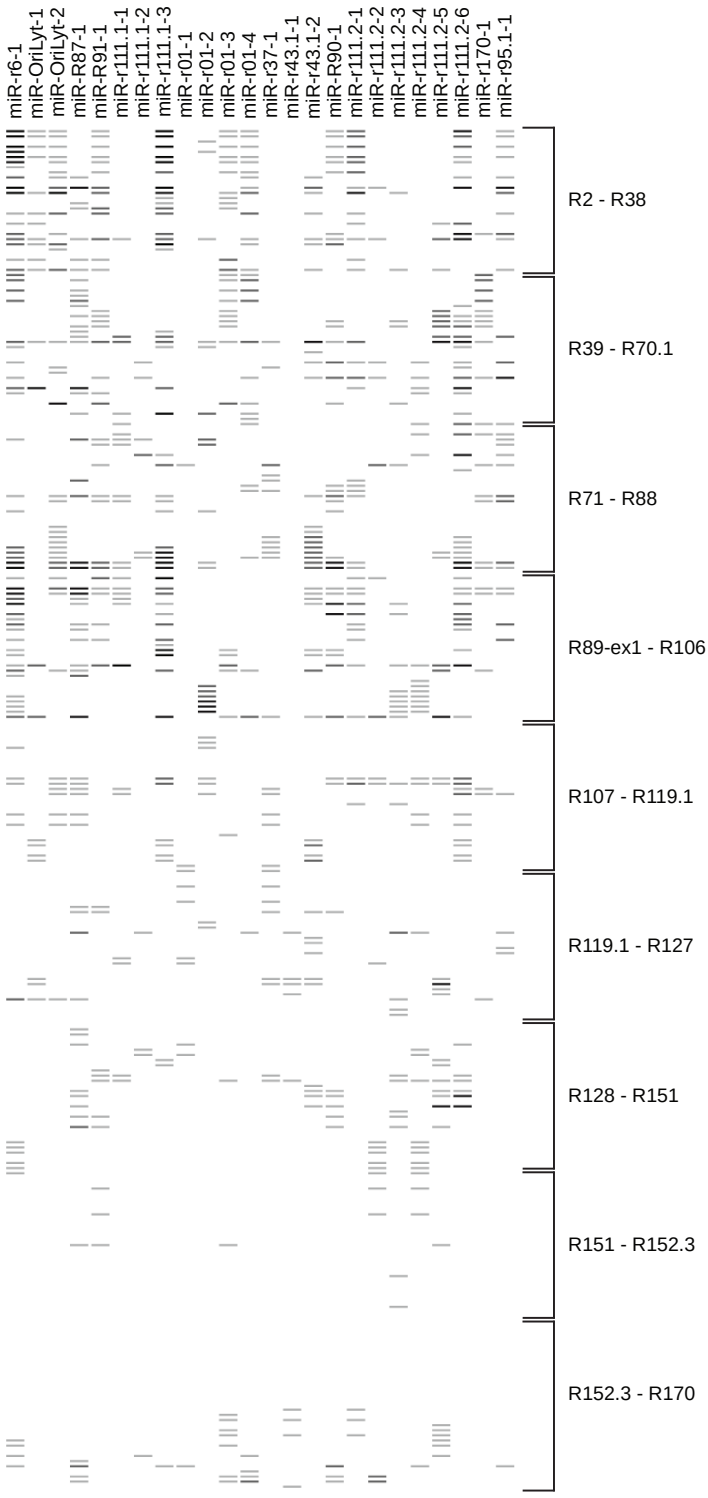

Figure A.5 Heat map of RCMV miRNAs targeting the RCMV genome. The heat map shows the number of RCMV miRNA 7mer-m8 seed matches to the 3' UTRs of the RCMV genome. The darker color represents multiple target sites. The RCMV miRNAs are labeled across the top and the RCMV ORFs are labeled in groups down the right.

R45-3'UTR

TGGCGGACGTCGCCTCGAAGTGCCCCCGGGCTTCGGGTCCGCCCCGCCG
CTCGCCTCGCGGCGCCCGCCCGTGTCACCTGTTTGTCCTGCTCCGCGCCC
CACGTCCGACAACGCGATTGGTTTTCGCGACGTCCCCCCGCGCTATAAAAG
CGAGAGTGTCTGTCCGCTGCCGCTTCATTCTTCGCCGAGGAGGCGGCCCG
TGTGAGTCCGCTCTGTAGTTTTCGTCGTCCGCCGAGAGAGAGGAAAAAATA
AA

RCMV miRNA	Type of seed match
miR-r01-4	6mer
miR-R87-1	7mer-m8
miR-r111.1-3	6mer and 7mer-m8
miR-r111.2-2	6mer

Figure A.6 RCMV miRNAs targeting the 3' UTR of R45. The sequence shown is the predicted 3' UTR of R45 from the stop codon to the poly(A) signal. The RCMV miRNA target sites are highlighted. The table lists the RCMV miRNA and the type of seed match found.

We also found target sites in other RCMV proteins important for replication including RCMV DNase (R98), protein kinase (R97), and helicase-primase (R70).

In the future, these miRNA:mRNA target pairs will need to be verified. Typically, the target 3' UTR is engineered into a reporter construct such as luciferase or GFP. The reporter construct and miRNA are co-transfected into cells and the ability of the miRNA to down-regulate protein expression is monitored. The miRNA and/or the target sequences are mutated to show specificity of the interaction. Next the mutations should be incorporated into the RCMV genome to dissect their function during *in vitro* replication and *in vivo* pathogenesis. The disruption of the viral miRNAs can be complicated due to their genome localization. To circumvent the problem, silent mutations can be introduced to disrupt the secondary structure of the viral miRNA, which is an important feature in miRNA processing. The target sites can also be mutated to confirm miRNA specificity for that particular target in the phenotype. In addition, antagomirs, which are RNA molecules antisense to the viral miRNA, can be used to interfere with the viral miRNA targeting in transfection studies. The antagomirs can be modified to work *in vivo* as well. In the future, we will use the combined biochemical and bioinformatics approach to identify transcripts associated with RISC in the context of viral infections in different cell types, and importantly, to determine whether miRNA targeting occurs in a cell type or tissue specific manner.