Summer 2001

Interactions Among Murine Cytomegalovirus US22 Family Gene Products That Influence Viral Pathogenesis

Zaruhi Karabekian
Old Dominion University

INTERACTIONS AMONG MURINE CYTOMEGALOVIRUS US22 FAMILY GENE PRODUCTS THAT INFLUENCE VIRAL PATHOGENESIS

by

ZARUHI KARABEKIAN
B.S. July 1987, Yerevan State University, Armenia
M.S. May 1989, Yerevan State University, Armenia

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

EASTERN VIRGINIA MEDICAL SCHOOL
OLD DOMINION UNIVERSITY
June 2001

Approved by:

Ann E. Campbell (Director)

Julie A. Kerry (Member)

Mark P. Birkenbach (Member)

Timothy J. Bos (Member)

ABSTRACT

INTERACTIONS AMONG MURINE CYTOMEGALOVIRUS US22 FAMILY GENE PRODUCTS THAT INFLUENCE VIRAL PATHOGENESIS

Zaruhi Karabekian
Eastern Virginia Medical School and Old Dominion University, 2001
Director: Dr. Ann E. Campbell

Cytomegalovirus (CMV) is a complex, ubiquitous herpesvirus that is characterized by acute, chronic, and latent infections. Monocytes-macrophages are the key target cell type involved in pathogenesis, which is most effectively studied using the murine model of CMV infection. Previously three murine CMV (MCMV) genes (M139, M140, and M141) were identified to regulate viral expression in cultured macrophages and in mice. These genes are members of the US22 gene family with respect to HCMV homology. There is no function assigned to the proteins encoded by these genes. However, deletion of M139, M140, and M141 significantly curtails growth of MCMV in macrophages *in vitro* and in macrophage-dense target organs *in vivo* (Hanson et al. 1999, J.Virol. 73(7): 5970-80). Therefore, M139, M140, and/or M141 gene products likely affect tissue specific viral infectivity.

The purpose of this study was to characterize these proteins (pM139, pM140, and pM141) and interaction among them. The M139, gene encodes two protein of 75 and 61 kD; M140 encodes a single protein of 56 kD, and M141 encodes a 52 kD protein. Most interestingly, when infected cell lysates were immunoprecipitated with anti-M139 antibody under non-denaturing (but not denaturing) conditions, five bands of 98-, 75-, 61-, 56-, and 52-kD proteins were co-precipitated. Likewise, anti-M140 antisera co-

precipitated two bands of 56- and 52-kD, and anti-M141 antibody precipitated a less abundant 56- and an abundant 52-kD band. The co-precipitating bands were identified as products of M139, M140, and M141 genes in experiments employing mutant viruses deleted of each gene. Complex formation between the M140 and M141 proteins (pM140 and pM141) was confirmed by sequential immunoprecipitations and combined immunoprecipitation and western blotting. These two proteins also formed a complex in the absence of other viral proteins. At least one function of the pM140/pM141 complex is to stabilize expression of pM141, which is unstable in the absence of pM140.

Given the complexity of viral pathogenesis and the fact that pM139, pM140, and pM141 proteins are dispensable for viral replication in tissue culture, it is possible that each single protein as well as the complex(s) they form may have a distinct function which influences tissue specific infectivity.

This thesis is dedicated to my parents,

my father, Hovannes Karabekian, and my mother, Inna Melkumova.

ACKNOWLEDGMENTS

I would like to thank many people that have helped me to make this day possible. First and foremost Dr. Ann E. Campbell. During all these years she has constantly supported, guided, and encouraged me, both scientifically and emotionally. I will always be grateful for the trust she has placed in me, which has given me the strength to persevere. Dr. Campbell was not only a model scientific mentor but also a friend, whom I needed so much being away from home. I will cherish our relationship for the rest of my life.

I would like to thank the current and former members of my committee, Dr. Kerry, Dr. Birkenbach, Dr. Bos, Dr. Stenberg, and Dr. Ratzlaff for their guidance and advice. My special thanks to the members of Dr. Campbell's laboratory, Jackie Slater, Laura Hanson, Victoria Cavanaugh, and Bridget Dalton, for their patience, help, support, and friendship, and to everybody in the Microbiology and Molecular Cell Biology Department, who have been there for me in the bad times and in the good times.

Last but not least, I would like to thank my family. To my daughter Alice, who has filled my life with joy and has given me the meaning for my existence. To my husband, for his high expectations of me, which have always forced me to be the best that I can, and ready to concur any challenge in life. To my parents, who have made everything possible in my life; have given me self-confidence, unconditional love, and enormous support from the day I was born.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Cytomegaloviruses

The cytomegaloviruses (CMV) are ubiquitous double-stranded DNA viruses that belong to the *betaherpesvirus* subfamily of the *Herpesviridae* family. Cytomegaloviruses infect a wide variety of rodents, domestic animals and primates. Human cytomegalovirus (HCMV) is the prototype for the *betaherpesvirus* subfamily. Viruses within this subfamily are distinguished by strict species specificity, a relatively long replication cycle, ordered and sequential gene expression, and slowly developing cytopathology depicted as an enlargement of the infected cell, or cytomegaly. Human cytomegalovirus is characterized by complex pathogenicity, which involves acute, chronic, and latent infections (reviewed by Mocarski, in press, 2001).

Human cytomegalovirus diseases

Epidemiology of primary infections

Between 50-80% of the adult population is seropositive for HCMV. Primary acute infection occurs early in life as a result of direct contact with infectious secretions (predominantly saliva) from an infected individual, usually a child or toddler. Typically primary infection starts with viral replication in the mucosal epithelium followed by a systemic phase in which viremia develops and virus disseminates within the host. CMV viremia is accompanied by constant viral shedding in the urine, saliva, breast milk, and genital secretions (reviewed by Mocarski, in press, 2001). HCMV is transferred by these

---

The model journal for this dissertation is *Virology*

bodily fluids, as well as by bone marrow and solid organ transplants. Although CMV can be detected in the blood of acutely infected individuals for several months, primary infection is normally inapparent in healthy individuals (Zanghellini et al., 1999). Rarely, HCMV infection of normal hosts results in the development of a mononucleosis syndrome, characterized by persistent fever and myalgia (reviewed by Britt and Alford, 1996). Moreover, unnoticed asymptomatic primary infection in a pregnant woman can result in severe defects in the newborn child such as blindness, mental retardation and others, because CMV can cross placenta and infect the immunologically immature fetus (reviewed by Britt and Alford, 1996).

Diseases associated with reactivated human cytomegalovirus

In healthy individuals, an acute infection is normally cleared from all target organs in conjunction with a slowly developing cell-mediated specific immune response. However, following primary infection, CMV persists in the host for life. It remains controversial whether the virus exists as a chronic infection, or as a true viral latency, because the virus has an ability to live in equilibrium with the host's immune system. Chronic infection is characterized by low-level viral replication, and results in continuous viral production and minimal release of infectious virus. During true latency the virus remains in a quiescent non-replicative state and infectious virus is undetectable. Latent virus reactivates upon differentiation of the infected host cell, or following immunosuppression or immunodeficiency (Mocarski et al., 1990). It is also possible that the virus could simultaneously persist within a host as a chronic infection in particular tissues and be latent in others (Campbell, 1999).

A series of diseases is associated with reactivation of latent HCMV in immunocompromised individuals, when the antiviral immune defenses fail to control a chronic or reactivated infection. Specifically, HCMV is the most frequent viral pathogen causing clinical complications in organ transplant recipients because of accompanying immunosuppressive therapies, and the influx of monocytes/macrophages into the graft tissues. This was illustrated in studies with the rat CMV model where CMV infection of the recipient led to histological damage of MHC class I-mismatched allografts, due to an increased perivascular influx of monocytes/macrophages early after transplantation (Li et al., 1998). Interestingly, reactivation of CMV following solid organ transplantation predominantly results in CMV pneumonia and host-versus-graft disease (Nguyen et al., 1999; Ljungman et al., 1998), whereas in bone marrow allografts recipients CMV reactivation augments graft-versus-host disease (Griffiths et al., 2000).

The immunosuppressed state in acquired immunodeficiency syndrome (AIDS) patients also creates ideal conditions for latent HCMV reactivation. Interestingly, these individuals predominantly develop CMV retinitis and colitis (reviewed by Britt and Alford, 1996).

Organs and cells infected by human cytomegalovirus

The diversity of HCMV associated diseases is explained by the wide variety of organs infected by HCMV *in vivo*. These organs include the bone marrow, liver, kidney, lung, spleen and salivary glands (reviewed by Campbell, 1999). Infection of the salivary glands is a hallmark of CMV infection. The virus exhibits a well-established tropism for this organ. The salivary gland remains chronically infected for long periods of time, and as a result represents a primary source of high-titer virus shedding into the saliva, the

principle route by which the virus spreads in a population. Within these organs HCMV infects epithelial cells, endothelial cells, monocytes/macrophages, hematopoietic progenitor cells, and immature dendritic cells (Jahn et al., 1999). Infected cells are characterized by cytomegaly, nuclear swelling, a reduction of cytoplasm, migration of chromatin, and formation of intranuclear and intracytoplasmic inclusions (Staczek, 1990). Prior isolation of the causative agent, these kinds of changes seen in tissues of patients led to designation of "cytomegalic inclusion disease" diagnosis. Peripheral blood mononuclear cells (monocyte/macrophages in particular) are thought to be the principle cell types responsible for disseminating CMV throughout the host (Michelson, 1997).

Understanding of the molecular mechanisms of HCMV pathogenesis is limited at this point due to many reasons. Several cell types, susceptible to HCMV infection *in vivo,* are mostly not susceptible in tissue culture. Conversely, tissue-culture propagated HCMV exhibit limited infectivity in terms of its ability to replicate to high titers in cultured cells, or the cell types it is able to infect *in vitro* (Waldman et al., 1991). In addition, strict species specificity of CMV makes it difficult to investigate HCMV pathogenesis and immunology in the natural host. Therefore, many animal CMV models were developed to overcome these obstacles.

Murine cytomegalovirus as a model for human cytomegalovirus disease

Murine cytomegalovirus (MCMV) serves as an appropriate model for HCMV disease because of the striking similarities in pathogenesis, overall structure, genome organization, regulation of gene expression, and the functions of known gene products (Campbell, 1999). This gives a great advantage to study the functions of viral proteins

within genetically altered recombinant virus in the environment of a natural host. In order to illustrate the suitability of the MCMV model, we will draw parallels between the structures, replicative cycles and pathogenesis of the two viruses.

Genome organization

All herpesviruses contain a double-stranded linear DNA genome. The genomes of both HCMV and MCMV have been completely sequenced (Chee et al., 1990; Rawlinson et al., 1996). Both genomes are approximately 230 kilobase pairs (kb) in length, and are estimated to encode over 200 genes.

The HCMV genes are arranged as an assemblage of two sequences, the unique long region (UL), surrounded by internal and terminal repeats (IRL and TRL), and the unique short region (US) flanked by shorter internal and terminal repeats (IRS and TRS) (Chee et al., 1990). The DNA sequence within the repeat elements is organized in a direct or inverted orientation with respect to each other. The presence of these sequences promotes genome inversion, in which the U_L and U_S regions can invert relative to each other during HCMV replication, giving rise to four genomic isomers. None of the other animal cytomegaloviruses, including MCMV, contain these repeat sequences within the genome, and therefore do not isomerize (reviewed by Mocarski, in press, 2001). The fact that MCMV does not have isomers should be taken into consideration when one is looking for genes homologous to HCMV. This may affect their position within the genome, placing these genes on the opposite end of either the U_L or the U_S fragment. For example, members of the US22 gene family (see section below) in MCMV are found on both ends of its genome.

Organization of the genome defines the nomenclature of the genes of HCMV as a number following either the UL or US prefix. The MCMV nomenclature follows similar rules with the exception of the prefix. A MCMV gene is designated by the "M" prefix if it is extensively homologous to the HCMV gene, or as "m" if there is no apparent homology found. Analysis of the MCMV genome showed that genes residing in the central part of the genome are essentially co-linear with those of HCMV. There is also a very similar distribution of G+C content across the two genomes (Rawlinson et al., 1996). Many HCMV, MCMV and other betaherpesvirus genes are classified into families based on the: 1) homology of DNA coding sequence, 2) presence of characteristic motifs, and 3) structure and function of expressed proteins.

Structure of the virion

HCMV and MCMV virion structure is prototypical for the *Herpesviridae* family, and consists of an inner core surrounded by a tegument, which together is enclosed by a lipid bilayer envelope embedded with numerous viral glycoproteins. The inner core is composed of a densely packed double-stranded DNA genome (Bhella et al., 2000) complexed with proteins, covered by a capsid shell. Capsids of all herpesviruses are characterized by icosahedral architecture and are classified into three types. Type A capsids do not contain DNA and accumulate within the infected cell because of a failure to package the viral genome; type B capsids also lack viral DNA, but contain the assembly protein and are found predominantly in the nucleus as precursors of mature capsids; and lastly, type C capsids are fully mature nucleocapsids (reviewed by Mocarski, in press, 2001).

A protein rich matrix surrounding the nucleocapsid in both HCMV and MCMV, called the tegument, contains many proteins, some of which are phosphorylated. In CMV virions, the tegument is icosahedrally ordered and interacts with the underlying capsid (Chen et al., 1999). Although some proteins localized to the HCMV and MCMV tegument are transcriptional transactivators (ppUL82 [pM82], ppUL69 [pM69], pTRS1, pIRS1), the function of most tegument proteins remains unclear. For example, the UL83 gene product is a phosphorylated protein, designated pp65 that is a major tegument protein of unknown function. The UL99 gene encodes another tegument phosphoprotein, pp28, which localizes to the endoplasmic reticulum-Golgi-intermediate compartment (ERGIC) around the infected cell nucleus (Sanchez et al., 2000). The MCMV homologs of both pp65 (M83 and M84) and pp28 (M99) have been identified (Morello et al., 2000; Morello et al., 1999; Cranmer et al., 1994). The UL25 gene product, pUL25, was recently characterized as a novel late tegument protein of HCMV. pUL25 colocalizes with pUL99 in the typical condensed structures in the perinuclear region, suggesting its possible function in the process of envelopment (Battista et al., 1999). Tegument proteins are highly conserved among the *betaherpesviruses*, but much less homologous among other herpesviruses (reviewed by Mocarski, in press, 2001).

The envelope of herpesviruses contains viral proteins, which are embedded in the lipid bilayer and are derived from membrane structures of an infected cell. There are three most prevalent herpesvirus–conserved glycoprotein complexes (gC-I, gC-II, and gC-III) present in the human cytomegalovirus envelope (Gretch et al., 1988).

The first most abundant complex, gC-I, is formed by glycoprotein B (gB) encoded by the UL55 gene (Kari et al., 1993). The full-length gB precursor is proteolytically

cleaved into two products, which form a functional heterodimer. This major heparan sulfate proteoglycan-binding glycoprotein is a type I integral membrane protein that is responsible for the first step in anchoring a virus particle to the cell membrane (Compton et al., 1993). Treatment of cells with heparinase, or the addition of exogenouse heparin, can prevent viral infection, because the interaction between the gB heterodimer (gC-I) and cell surface heparan sulfate is necessary for the initial attachment of the virion to the target cell (Compton, 1993). This interaction is characterized by low affinity and low specificity, but ensures the ability of the virus to interact with a broad variety of cells within the host as potential target cells (Britt and Mack, 1996). In MCMV, the M55 gene is homologous to UL55 and encodes a protein with similar functions (Rawlinson et al., 1996)

The gB-heparan sulfate interaction is quickly followed by receptor-mediated, but heparin-independent, fusion of the viral envelope to the cellular membrane and subsequent penetration. Two other major virion complexes, gM/gN (gC-II) and gH/gL/gO (gC-III), are thought to be the viral receptors involved in these secondary, high-affinity interactions (Mach et al., 2000; Kaye et al., 1992).

Two glycoproteins, gM and gN, constitute the second characteristic complex of the HCMV and MCMV envelope, gC-II (Kari et al, 1994). These proteins are encoded by UL100 (M100) and UL73 (M73), respectively, and exhibit homology with other human herpesviruses. The UL100 gene product (gM) is a type III membrane protein containing multiple hydrophobic sequences, which forms a disulfide-linked complex with the UL73 gene product in HCMV virions (Mach et al, 2000; Rawlinson et al., 1996).

The third prominent complex of the CMV envelope, gC-III, is composed of three proteins, gH/gL/gO, which are encoded by the UL75 (M75), UL115 (M115), and UL74 (m74) genes, respectively. This heterotrimeric complex is involved in viral penetration, since anti-gH antibody can prevent cell-to-cell transmission of the virus (reviewed by Mocarski, in press, 2001; Rawlinson et al., 1996).

Each one of these complexes is essential for viral growth in tissue culture (Britt and Mack, 1996). The components of these described complexes are highly antigenic and induce both humoral and cellular immune responses within the infected host, with gB being the most immunodominant (Gonczol et al., 1991).

Several other viral glycoproteins have been characterized and are likely to be minor envelope proteins, but none of them are apparently associated with these major complexes or are known to form additional, previously undetected complexes (reviewed by Mocarski, in press, 2001). It is possible that additional, and as yet undefined, viral glycoproteins determine the full repertoire of cell/tissue specific CMV infectivity. Despite extensive studies, the cellular counterparts for these viral receptors are poorly defined, although they are widely distributed, considering the diversity of the cells naturally susceptible to CMV infection.

In addition, in order to create the circumstances needed for the earliest events in the virus replication cycle, an attachment step (specifically gB binding) initiates a signaling cascade. Signaling results in up-regulation of immediate early gene transcription and, subsequently, expression of other viral genes during replication within a target cell (Boldogh et. al., 1991; Boyle et. al., 1999; Yurochko et. al., 1995; Zhu et. al., 1997).

Viral replication

A productive CMV infection occurs in permissive cells and results in the generation of infectious viral progeny. The replicative cycle of cytomegaloviruses consists of several sequential steps. Initial attachment and subsequent fusion of the virus particle to the target cell is ensured by the abundant envelope glycoproteins, which interact with cellular receptors. This is followed by the penetration of the viral genomic material into the cell nucleus, where the viral DNA undergoes replication. Then newly synthesized viral DNA is integrated with packaging proteins and is combined with the structural components of the virion, which result in new progeny that egress from the cell. If any of these steps is disrupted, the infection will be abortive. An abortive infection can occur due to following reasons. One is when an infected cell is nonpermissive and only a limited number of viral genes are expressed, and the other is when the cell is infected with a defective viral particle, which lacks genes essential for viral replication (reviewed by Roizman, 1996).

Viral DNA localizes to the nucleus soon after penetration. CMV gene expression occurs in an ordered and sequential manner, which is typical for all herpesviruses. Viral genes are classified into immediate-early (IE), early (E), and late (L) categories based on their expression kinetics during the replication cycle. Betaherpesviruses are characterized by a relatively slow replication cycle. Although the general replicative cycles of HCMV and MCMV are essentially identical, the kinetics of IE, E, and L genes expression are slightly different (see Table 1)

TABLE 1

Kinetics of HCMV and MCMV gene expression

	HCMV	MCMV
Immediate Early	0-12 h	0-3 h
Early	12-24 h	3-12 h
Late	24-72 h	12-48 h

Regulation and expression of the immediate-early genes

The immediate early genes are expressed instantly following penetration of the viral DNA into the target cell nucleus, and do not require any prior *de novo* viral protein synthesis. Genes expressed at IE times are found dispersed throughout the HCMV and MCMV genomes. Currently, four immediate-early gene regions within the HCMV genome have been identified. These regions are UL36-38, UL122-123 (ie1/ie2), TRS1-IRS1, and US3 (Colberg-Poley, 1996). *Cis*-acting DNA elements, composed of promoter-enhancer sequences localized upstream of the IE genes, enable the binding of cellular transcription factors and viral regulatory proteins, introduced into the cell as a part of the tegument, for the initiation of IE transcription.

The best characterized regulatory element in the HCMV genome is the regulatory DNA sequence upstream of the gene locus encoding for the major immediate early (MIE) proteins. This region, composed of promoter and enhancer sequences, is one of the strongest transcriptional enhancers identified in mammalian biology (Meier and Stinski, 1996). There are two repressor elements within this MIE regulatory sequence. The modulator (or silencer) is located upstream of the enhancer, and the *cis*-repression sequence (*crs*) is immediately downstream of the *ie1/ie2* start site. A functionally

analogous enhancer has also been identified upstream of the major immediate-early gene promoter in both the murine and simian cytomegaloviruses (Dorsch-Hasler et al., 1985; Chang et al., 1993). Moreover, chimeric murine cytomegaloviruses, containing the human CMV enhancer in place of the MCMV enhancer, demonstrated kinetics of infection similar to that of wild type MCMV (Angulo et al., 1998). These findings further proved functional homologies between HCMV and MCMV major immediate-early enhancers. The MIE promoter-enhancer is characterized by the presence of repetitive consensus binding sites for host transcription factors, such as CREB/ATF, NF-κB/REL, Sp1, AP1, NF-1, ELK-1, C/EBP, p53, Gfi-1, retinoic acid receptor, and serum response factor (reviewed by Mocarski, 2001). Some of these transcription factors are influenced by virion transactivators, which consequently contribute to the up-regulation of MIE protein expression. For example, ppUL82 (the upper matrix protein) transactivates promoters containing upstream ATF or AP-1 binding sites, and possibly regulate of other viral genes in a more general way (Liu and Stinski, 1992). The UL69 gene encodes a minor tegument phosphoprotein ppUL69, which transactivates expression from the *ie1/ie2* promoter-enhancer, particularly when accompanied with ppUL82 (Winkler and Staminger, 1996).

Two immediate-early proteins 1 and 2 (IE1 and IE2) are the most prominent gene products expressed at immediate-early times post-infection in both viruses. In HCMV their transcripts originate from the UL122-123 gene region, which encodes a family of regulatory proteins called the major immediate early (MIE) proteins as a result of alternative splicing (Stenberg RM, 1996). Therefore the IE1 and IE2 proteins share amino acid sequences present in common exons. Homologous genes (M122 and M123)

are identified in MCMV. The encoded proteins are designated as IE1 and IE3 respectively (Keil et al., 1987). The *ie1* gene is expressed more abundantly compared to *ie2* gene in both viruses, and encodes a protein with a molecular weight of 72-kD in HCMV and 89-kD in MCMV (Stenberg et al., 1989; Keil et al., 1987). The *ie2* gene of HCMV encodes a second regulatory protein (IE2) with a molecular weight of 86-kD (Stenberg et al., 1989). Its MCMV counterpart (IE3), encoded by the *ie3* gene, has a molecular weight of 88-kD (Messerle et al., 1992). In both viruses these proteins are phosphorylated nuclear proteins, and both function to transactivate cellular and viral promoters. The MCMV *ie2* gene is unique to this virus. It is located at the opposite end of the enhancer sequence and is transcribed from a separate promoter in the opposite direction (Messerle et al., 1991). The MCMV *ie2* gene encodes a regulatory protein of 43-kD, which is dispensable for viral replication in cultured fibroblasts as well as for replication and latency in the mouse (Cardin et al., 1995). Additional minor immediate early proteins expressed from the HCMV MIE region via differential splicing are 55-kD (IE55) and 18-kD (IE18). These proteins are differentially expressed in non-fibroblast cells (Kerry et al., 1995). The last protein expressed from the HCMV MIE gene locus is actually expressed at late times post-infection and encodes a protein with a molecular weight of 40-kD (IE2-L40). The IE2-L40 protein contains a DNA binding domain identical to the IE86 functional domain, and has a *trans*-acting function in gene regulation (Puchtler and Stamminger, 1991) (Jenkins et al., 1994).

The regulatory functions of the nuclear phosphoproteins IE1 and IE2 of HCMV have been extensively studied. These proteins exhibit both positive and negative regulation of viral gene expression. The IE1 (IE72) protein has two major functions,

including activation of the *ie1/ie2* promoter-enhancer (Mocarski et al., 1996), and regulation of early and late viral genes (Greaves and Mocarski, 1998). The IE2 (IE86) protein carries out two distinct major functions. It represses MIE promoter function as well as the function of the US3 regulatory unit, and it controls the switch from immediate early to early and late gene expression (reviewed by Mocarski, in press, 2001). IE1 (IE72) and IE2 (IE86) cooperate with each other to transactivate the subsequent cascade of viral gene expression. IE2 is a sequence-specific DNA binding protein defined as a promiscuous transactivator (Hagameier et al., 1992). It is able to activate homologous and heterologous promoters containing a TATA box. Direct interaction between IE86 and TFIID may play a role in this effect; however, upstream transcription factors are essential for this activation (Jupp et al., 1993). IE86 also interacts with other components of the basic cellular transcription machinery, such as TFIIB (Caswell et al., 1993), as well as other transcription factors, such as CREB (Lang et al., 1995), CBP (Schwartz et al., 1996), and c-*jun* (Scully et al., 1995).

Other HCMV immediate-early proteins exhibit regulatory functions. The IRS1/TRS1 proteins augment activation of viral gene expression by IE1 and IE2, as well as co-operating with ppUL69 in activating the MIE and IRS/TRS promoters (Romanowski and Shenk, 1997). The UL36-38 gene cluster encodes a number of proteins translated from differentially spliced transcripts (Tenney and Colberg-Poley, 1991). These proteins regulate transcription of the hsp70 gene synergistically with another regulatory immediate early protein US3. They are also required for lytic replication of the virus (Colberg-Poley, 1996). The UL37 protein contributes to the activation of the viral early genes (Colberg-Poley et al., 1998). The positional and

functional homologues of these regulatory proteins have been identified within the MCMV genome, with the exception of the US3 transcription unit. In addition, the m142-m143 immediate-early genes have been recently identified within the MCMV genome. The pm142 and pm143 proteins are function as transcriptional transactivators of the MCMV MIE promoter. They also cooperate with the MCMV IE1 and IE3 proteins to activate the early *e1* promoter. (Dalton et al., manuscript in preparation). Many of these IE regulatory proteins are expressed in significant amounts again at late times post-infection, and are packaged as part of the tegument of progeny virions. This pattern of expression ensures the availability of these necessary viral regulatory proteins for the next round of progeny virus replication.

Regulation and expression of the early genes and replication of viral DNA

Once the immediate-early proteins are expressed and are functional, they initiate and regulate the expression of early genes. Multiple studies on the regulation of early CMV promoters have demonstrated that both the IE1 and IE2 proteins are required for maximal promoter activation (Chang et al., 1989; Depto and Stenberg, 1989; Staprans et al., 1988; Stenberg et al., 1990; Garcia-Ramirez et al., 2000). In addition, many cellular proteins, such as ATF (Kerry et al., 1997), USF (Klucher and Spector, 1988), CREB (Lang, et al., 1995), AP-1 (Wade et al., 1992), and E2F (Staprans and Spector, 1986) in combination with the IE1/IE2 proteins regulate the expression of HCMV early promoters. The early genes are further divided into three subclasses based on the regulation of mRNA expression relative to the time of viral DNA synthesis (Stenberg, 1993). The first subclass comprises those genes that are transcribed early and repressed at late times after infection, the second subclass represents those genes that are expressed at equal levels at

early and late times, and the last subclass designates those genes that are expressed at low levels at early times and upregulated after viral DNA synthesis is completed (reviewed by Mocarski, in press, 2001).

Many HCMV and homologous MCMV early gene products are involved in the replication of viral DNA. These include the DNA polymerase (UL54 and M54), the single-stranded DNA binding protein (UL57 and M57), the DNA polymerase processivity factor (UL 44 and M44), and three subunits of the helicase-primase complex (UL105 [M105], UL102 [M102], and UL70 [M70]) (Anders and McCue, 1996), (Rawlinson et al., 1996).

Of the homologies that have been currently identified between HCMV and MCMV, one group has been extensively characterized. A family of early phosphoproteins, indirectly involved in DNA replication, is expressed from the HCMV UL112-113 gene region. Differentially spliced transcripts of 2.1-, 2.2-, 2.5-, and 2.6-kb arise from a single promoter, and are translated into four phosphoproteins of 50-, 43-, 84- and 34-kD, respectively (Spector, 1996). These proteins are regulating the expression of core DNA replication genes (Iskendarian et al., 1996; (Pari and Anders, 1993; Pari, Kacica et al., 1993) and organizing viral DNA replication compartments (Penfold and Mocarski, 1997). The MCMV M112-113 gene region encodes a homologous family of proteins. One fully spliced transcript of 2.6-kb is expressed at early times from this gene region, called *e1*. This transcript generates three antigenically related proteins of 36-, 37-, and 38-kD in size (Buhler et al., 1990). These proteins are homologous HCMV UL112-113 gene products, which are required for *ori*-Lyt dependent viral DNA replication (Pari and Anders, 1993). Two additional early proteins of 33- and 87-kD are expressed from

unspliced and alternatively spliced transcripts, respectively, and are detected by polyclonal antisera (Ciocco-Schmitt et al., manuscript in preparation). Once all the proteins required for replication of viral DNA are expressed and functional, viral DNA is replicated by a rolling circle mechanism, and results in concatamers of unit-length viral DNA.

Expression of late genes, assembly and egress

Viral DNA replication denotes a transition in the replication cycle from the early to the late phase. Genes that are expressed upon completion of viral DNA replication are designated late genes. The majority of late genes encode structural proteins of the virion, including those of the capsid, tegument, and envelope described above. Only a small number of late gene products have been characterized. Among those are the UL94 true late gene of HCMV (Wing et al., 1996), as well as the IE2-L40 protein expressed from the MIE region, both of which have suggested regulatory functions. Further studies are needed to conclusively address the functions of these proteins.

A group of late proteins whose function has been better defined includes the structural glycoproteins of the viral envelope. Although the UL55 gene is transcribed at early-late times post-infection, its protein product, gB, is synthesized at late times. True late time kinetics is characteristic of the UL75 (gH), UL115 (gL), UL74 (gO), UL100 (gM), and UL73 (gN) gene products. Functional and positional homologs of these late structural glycoproteins have been identified in MCMV (Rawlinson et al., 1996).

During the late phase of CMV infection, newly synthesized capsid proteins are transported back into the nucleus and form empty capsids there (Kasamatsu and Nakanishi, 1998). Replicated viral DNA is then cleaved from concatamers and is packaged into these pre-formed nucleocapsids. Several studies have showed that many tegument proteins, including pp150, pp28, pp65, and some envelope glycoproteins gB, and gH, accumulate in a stable juxtanuclear virally induced structure, suggesting a cytoplasmic site of viral assembly (Sanchez, Greis, et al., 2000). However, the site of intracellular trafficking and final envelopment remains highly controversial. Two recent studies have demonstrated the presence of viral RNA (Bresnahan and Shenk, 2000), as well as cellular RNA (Greijer et al., 2000) in the tegument, but not in the nucleocapsid fraction of progeny virions. These findings again indicate that only nucleocapsids are fully assembled in the nucleus. Furthermore, these studies indicate that the incorporation of cytoplasmic components, such as viral and cellular RNA molecules, into the viral tegument is non-specific. It is believed that viral progeny receive their envelopes at the nuclear membrane, de-envelope in the cytoplasm and then re-envelope by Golgi-derived membranes (Roizman, 2000). Whether the virus follows a single pathway of envelopment at the nuclear membrane and egress, or if it acquires its envelope from other intracellular membranous structures is still unclear.

Murine cytomegalovirus pathogenesis

Susceptibility to murine cytomegalovirus

The pathogenicity of MCMV infection depends on the strain, the age and the immune status of the animal, as well as the preparation of the virus, and its dose and

route of administration. Inbred mouse strains exhibit different levels of susceptibility to MCMV infection. This susceptibility is partially determined by the genes of the major histocompatibility complex (MHC), called H-2 genes in mice. Mice of H-2^d and H-2^b haplotypes, such as BALB/c and C57BL/6 respectively, develop severe morbidity and mortality upon MCMV infection, where strains of mice with H-2^k haplotypes (C3H and CBA) are more resistant to MCMV (Merser and Spector, 1986; Scalzo et al., 1990). Non-H-2 genes are also implicated as determinants in MCMV susceptibility. These can include genes encoding cell surface molecules influencing the MCMV attachment/penetration step, or factors affecting early, innate immune responses (Lathbury et al., 1996).

The genetic background of the mouse also influences the levels and profiles of MCMV-induced cytokines with direct or indirect antiviral activities. For example, partial resistance to MCMV is determined by the autosomal dominant *cmv-1* mouse gene, whose protein product restricts the level of MCMV replication in the spleen, by enhancing cytolytic activity of natural killer (NK) cells (reviewed by Campbell, 1999). NK cells represent a major component of innate immunity, controlling early HCMV and MCMV replication *in vivo*. In order to surpass this defense mechanism, both HCMV and MCMV encode MHC class I homologs, which couple with the NK cell receptor and serve as negative stimuli of NK cytolytic activity (Kleijnen et al., 1997).

MCMV is highly pathogenic in newborn mice (up to three weeks old) with immature immune systems (Fitzgerald et al., 1990). It also causes severe disease in immunosuppressed mice such as that induced by γ-irradiation, or mice with genetically

altered immune responses such as those with severe combined immunodeficiency (SCID) syndrome (Schmader et al., 1995).

Preparation of infectious virus stocks also influences its virulence. Tissue-culture-passaged virus is dramatically less virulent compared to salivary-gland-passaged MCMV. This can be partially explained by the fact that MCMV grown in cultured fibroblasts consists primarily of multi-capsidated virions, which are enveloped infectious particles containing from 2 to 20 capsids (Weiland et al., 1986), (Cavanaugh, unpubl data). It is assumed these large aggregates are probably phagocytized (especially when infecting macrophages) and are not able to deliver infection into the target cell whereas salivary gland-passaged MCMV consists of predominantly single capsid enveloped virions, successfully delivering viral DNA into the cell for a fully productive infection.

Acute murine cytomegalovirus infections and clearance mechanisms

Acute MCMV infection quickly disseminates to the spleen and liver of the susceptible mice. Hepatocytes are the primary cell type replicating the virus in liver, and splenic macrophages are the major primary cells of the spleen supporting MCMV replication (Papadimitrou et al., 1984; Reynolds et al., 1993). All other organs (salivary gland, lung, bone marrow) of infected animals represent secondary sites of viral replication, and become infected as a result of virus spreading via the blood (Stoddart et al., 1994).

Antiviral cytokines, directly or indirectly, control early MCMV (as well as HCMV) infection until a specific T-cell response develops to clear CMV from target organs. An early inflammatory response is provided mainly by activated macrophages

and, subsequently, by natural killer (NK) cells (Heise and Virgin, 1995; Orange and Biron, 1996a,b). It is not fully understood whether tissue monocytes/macrophages represent the first barrier in CMV organ infection, and primary CMV infection induces their activation and subsequent involvement in the early inflammatory responses, or whether monocytes become infected as they infiltrate primary sites of infection, where they maturate and differentiate and thus create more permissive conditions for CMV replication. Regardless, activated macrophages produce interferon alpha/beta (IFN α/β), which enhances the cytotoxicity of natural killer (NK) cells, as well as tumor necrosis factor alpha (TNF-α) and interleukin-12 (IL-12), which induce interferon gamma (IFN-γ) production by NK cells. IL-6, also produced by macrophages, has direct antiviral activity (reviewed by Campbell, 1999).

In addition, investigation of intraperitoneal MCMV infection demonstrated an increase in CD3+ CD4- CD8- (double negative; DN) T cell receptor alpha/beta (TCR α/β) cells in the peritoneal cavity, spleen and liver of infected animals. The peritoneal DN TCR α/β T cells expressed IFN-γ, TNF-α, early T cell activation-1 (Eta-1), and monocyte chemoattractant protein 1 (MCP-1) on day five postinfection in all three tested organs (Hossain et al., 2000). This suggests that DN TCR α/β T cells were activated and may have an antiviral effect through producing IFN-γ and some macrophage-activating factors during the early phase of MCMV infection. Together these early innate immune reactions limit initial viral replication, and provide time for the development of an acquired immune response.

In order to completely resolve an acute CMV infection, the host (human or mouse) must develop a specific T lymphocyte mediated response (Koszinowski et al.,

1990). MCMV is usually cleared by four weeks after infection mainly as the result of robust specific CD8+ T-cell (CTL) response from all organs except the salivary glands (Koszinowski, 1991). Studies with inbred mice have demonstrated that the CD8+ T cell response is required for direct cytotoxicity in susceptible strains of mice, whereas in non-susceptible mouse strains, natural killer (NK) cells appear to have this function (Lathbury et al., 1996). However, independent of genetic background CD4+ T cells are responsible for the delayed type hypersensitivity and antibody responses, as well as for virus clearance in the salivary glands (Lathbury et al., 1996). Adoptive transfer of CD8+ T cells specific for two immunodominant nonapeptides, originating from the MCMV IE1 (M123) protein and the MCMV gp34 (m04) early protein, provide protection from lethal challenge, greatly reducing viral replication in the lungs and other organs (Fernandez et al., 1999; Holtappels et al., 2000). Both CD4+ and CD8+ T lymphocytes are responsible for IFN-γ production, which occurs early after acute infection and persists for about one year in the spleen of infected animals (Shanley et al., 2001).

Clearance of the virus from the salivary glands is significantly delayed and relies largely on the presence of functional CD4+ T-cells (Koszinowski et al., 1993). These CD4+ specific T-cells of the Th1 subset eliminate MCMV from the salivary glands by direct cytotoxicity and by release of antiviral cytokines, IFN-γ and TNF-α (Lathbury et al., 1996; Lucin et al., 1994; Lucin et al., 1992). Antibodies play an insignificant role in clearing primary viral infection, however, they limit spread of the virus *in vivo* once it reactivates from latency (Jonjic et al., 1994).

Cytomegalovirus latency

Following primary infection MCMV and HCMV establish latency. Extensive studies have been done to understand reactivation mechanisms of both latent HCMV and MCMV (reviewed by Mocarski, in press, 2001). *In vivo* studies of latently infected mice demonstrated that latent DNA was harbored in the salivary glands, adrenal glands, spleen, kidney, heart, and lungs (Balthesen et al., 1993), with a particularly high load of latent viral genome and higher rate of recurrence in the lungs (Kruz et al., 1997).

A variety of cells within these organs is known to harbor latent HCMV and MCMV genomes. Monocyte/macrophage and bone marrow hematopoietic cells harbor latent MCMV DNA (Mitchell et al., 1996; Pollock et al., 1997). Recent studies by Reddehase et al. (1999) demonstrated that during latency, focal and stochastic MIEP activity is found to selectively generate IE1, but not IE3 transcripts in the lungs. Moreover, reactivation was triggered upon hematoablative, immunoreductive treatment, but recurrence of the virus was reached only in a few foci of latently infected lungs, indicating the existence of several control points in the transition from MCMV latency to recurrence (Kurz and Reddehase, 1999).

Latent HCMV DNA is carried by granulocyte-macrophage progenitors (Maciejewski et al., 1992; Kondo et al., 1995). Recent studies have demonstrated that both CD34+ progenitor cells and monocytes could be infected with HCMV, and virus can be recovered when these cells were induced to terminally differentiate (Maciejewski and Jeor, 1999). These results were further confirmed when HCMV DNA and a limited number of viral transcripts were detected in *in vivo* infected hematopoietic progenitors, and infectious virus was obtained from macrophages originating from monocytes of

normal seropositive blood donors upon *in vitro* differentiation (Maciejewski and Jeor, 1999).

Cells of myeloid lineage (particularly macrophages expressing dendritic cell markers) are also known to carry latent viral DNA, and reactivate HCMV upon allogeneic stimulation (Soderberg-Naucler et al. 1997). The generation of HCMV-permissive allogeneically-stimulated monocyte-derived macrophages is dependent on appropriate stimuli from both CD4+ and CD8+ T cells. Particularly the presence of IFN-γ, but not of IL-1, IL-2, TNF-α, or granulocyte-macrophage colony stimulating factor (GM-CSF), is critical for the generation of these macrophages (Soderberg-Naucler et al., 1998). Cytokine-induced reactivation likely occurs naturally in selective tissues during normal immune responses. However, human monocyte-like lymphocytes (U937) and peripheral blood cells (HL60) did not reactivate HCMV upon stimulation with 12-O-tetradacanoyl phorbol 13-acetate (TPA) (Lee et al., 1999). Likewise, mature unstimulated macrophages treated with IFN-γ were incapable of reactivating latent HCMV. Therefore, relative degrees of monocyte cell differentiation appear to be another important factor for reactivation of latent HCMV and its ability to start a fully productive infection.

In addition, macrophages generated upon allogeneic stimulation of HCMV latently infected monocytes, actively express a specific type of MHC molecules, called HLA-G (Onno et al., 2000). While HLA-G surface expression is upregulated, classical MHC class I molecules are partially downregulated by HCMV. The same pattern of MHC class I expression was found in vivo, expressed by bronchoalveolar macrophages collected from patients suffering from acute HCMV pneumonitis. The correlation

between macrophage activation (due to primary infection or reactivated latent infection) and modulated expression of MHC class I molecules could be an additional mechanism that helps HCMV to subvert host defenses. Taken together, these studies indicate that monocyte/macrophage cells are important for CMV pathogenesis and, particularly, for viral latency (Sinzger et al., 1996).

Genetic approaches to assessing mechanisms of cytomegalovirus pathogenesis

In spite of the advances in our overall understanding of CMV pathogenesis, a limited number of molecular mechanisms have been well defined for identifying how the virus regulates cell or tissue tropism. The HCMV and MCMV genes are classified into two groups: essential and non-essential based on their requirement for replication of the virus in cultured fibroblasts. Essential genes are those genes which affect one of the necessary steps in viral replication, such as attachment, fusion, penetration, replication of viral DNA, assembly of the infectious viral particle or egress. Mutations within these genes are detrimental for the ability of the virus to propagate both *in vitro* and *in vivo.*

On the contrary, non-essential genes can be deleted or mutated in the virus, but have no effect on the ability of the virus to grow in cultured fibroblasts. This phenomenon allows one to genetically manipulate these genes and to study their effects on the *in vitro* and *in vivo* replication of the virus (Sweet, 1999). Many of the identified non-essential genes are highly conserved among different herpesviruses and can be further divided based on their involvement in either regulation of host-virus interactions and immune evasion or in tissue and organ specific infectivity.

Non-essential cytomegalovirus genes regulating viral-specific immunity

The CMV genes that function to regulate viral-specific immunity do so in many different ways. They can interfere with cytokine/chemokine-mediated events in antiviral immunity, alter the expression of cell surface recognition molecules, or regulate immune surveillance mechanisms.

Chemokine and chemokine receptor homologs

Functional homologs of cellular chemokines or chemokine receptors are identified in a number of herpesviruses, suggesting that the subversion of the host's chemokine response contributes to the pathogenesis of these viruses. Chemokines (chemoattractant cytokines) are small secreted proteins, which play a major role in mobilization and activation of blood leukocytes. Chemokines differ from classical chemoattractants and most other cytokines by remarkably conserved sequences, indicating their common origin. Chemokines are divided into two families based on the content of the conserved motif. The alpha (α)-chemokine family members contain conserved cysteine residues separated by an intervening amino acid (CXC). Alpha chemokines are largely produced by activated mononuclear phagocytes, as well as some tissue cells such as endothelial cells and fibroblasts, and are responsible for neutrophils attraction. Beta (β)-chemokines are characterized by the presence of two adjacent cysteine residues (CC). This group of chemokines is produced mainly by activated T cells and acts upon mononuclear inflammatory cells, attracting monocytes, basophils, and eosinophils. Chemokines bind to specific receptors on the target cells. The receptors belong to the family of seven trans-membrane alpha-helical proteins. There is no cross-

competition for binding between the two different classes of chemokines (reviewed by Horuk, 1994; reviewed by Murphy, 1994).

Homologs for both types of chemokines, as well as the chemokine receptors, are identified in herpesviruses. For example, the HCMV UL146 gene encodes a potent alpha (CXC) chemokine, which binds with high affinity to CXC receptor 1 (CXCR1) but not CXCR2 (Penfold et al., 1999). The UL146 protein induces calcium mobilization, chemotaxis, and degranulation of neutrophils. In addition, the UL146 protein attracts neutrophils, which may provide efficient additional dissemination mechanisms during HCMV acute infection.

Two related proteins encoded by the m131 and m129 genes of MCMV with high homology to the CC (β) family chemokines, arise from alternatively spliced late transcripts (Fleming et al., 1999). Those proteins, called murine cytomegalovirus chemokine 1 and 2 (MCK-1 and MCK-2), induce calcium signaling and adherence in murine peritoneal macrophages. Mutant MCMV deleted of m131 and m129 replicated similarly to wild type MCMV in cultured fibroblasts and during the first 2 to 3 days following *in vivo* infection. However the inflammatory response elicited by m131/m129 mutant MCMV was significantly reduced compare to wild type MCMV infection. This mutant also failed to establish a high-titer infection in salivary glands, suggesting that MCK-1 and MCK-2 possess pro-inflammatory properties *in vivo* and are important for the dissemination of MCMV to the salivary gland or for the infection within this organ (Fleming et al., 1999). In addition, cells expressing human chemokine receptor CCR3 and the human macrophage THP1 cell line are also responsive to MCK-1. This suggests that MCK-1 may act as an agonist promoting leukocyte migration to initial sites of

infection and attracting monocytes or macrophages, which efficiently disseminate virus in the host (MacDonald et al., 1997; MacDonald et al., 1999; Saederup et al., 1999).

The US28 non-essential early gene encodes a beta-chemokine receptor that induces calcium influx in HCMV-infected cells upon ligand binding (Vieira et al., 1998). This causes a significant reduction in concentration of RANTES in the medium of infected cells. Expression of US28 in the presence of CC chemokines including RANTES or MCP-1 is sufficient to promote migration of smooth muscle cells (SMCs) by both chemokinesis and chemotaxis, indicating the possible involvement of the US28 gene product in the development of vascular disease associated with HCMV infection (Streblow et al., 1999). Similar functions are described for the beta-chemokine receptor homolog encoded by the U51 gene of human herpesvirus-6 (HHV-6). The U51 gene stably expressed in cell lines shows specific binding of the CC chemokine RANTES and competitive binding to other beta-chemokines (Milne et al., 2000). In epithelial cells secreting RANTES, U51 expression results in transcriptional down-regulation of that chemokine secretion. This kind of chemokine regulation can modulate a protective inflammatory response to aid the spread of the virus by immune evasion.

Regulation of major histocompatibility complex expression

At least four HCMV and three MCMV gene products that regulate MHC class I molecule expression have been identified to date. HCMV US2 and US11 act similarly by dislocating the MHC class I heavy chain from the endoplasmic reticulum (ER) and transferring it through the Sec61 complex to the proteosome for destruction (Wiertz et al., 1996; Wiertz, Tortorella et al., 1996; Jones and Sun, 1997). US3 of HCMV impairs the

maturation and transport of MHC class I molecules to the surface of infected cells at immediate early times post-infection (Jones et al., 1996). The HCMV US6 gene encodes an ER resident protein that inhibits peptide translocation into the ER for MHC class I assembly by binding to the TAP1/TAP2 translocation complex (Hengel et al., 1996). In MCMV, the m152 gene encodes a glycoprotein named gp40, which prevents the transport of the MHC class I molecule through the Golgi to the surface of the cell (Ziegler et al., 1997). Transmembrane glycoprotein of 48 kilodaltons (gp48), encoded by the MCMV m06 gene, forms a tight complex with β2-microglobulin-associated MHC class I molecules in the ER through its cytoplasmic domain, and reroutes MHC class I complex into the endolysosome for rapid proteolytic degradation (Reusch et al., 1999). Interestingly, the m04 gene product of MCMV, glycoprotein of 34 kilodalton (gp34), counteracts the MHC class I retention function of m152 and m06-directed degradation of mature MHC class I complexes by escorting these complexes to the surface of infected cells. This can possibly decrease the susceptibility of the infected cells to recognition by natural killer cells at the early stages of infection (Kleijnen et al., 1996).

Both human and murine CMV encode class I MHC heavy-chain homologs (UL18 and m144 respectively). The well-characterized HCMV UL18 gene product contains a groove analogous to a similar structure in MHC molecules that function as the binding site for peptides derived from both endogenous and foreign proteins (Fahnestock et a., 1995). However, the m144 gene contains a substantial deletion within the sequence encoding the groove domain, and therefore is incapable of peptide binding (Chapman and Bjorkman, 1998). Nevertheless, the stable heavy chain-β2-microglobulin complex

formed by the m144 protein confers protection from NK cell effector function in the absence of target cell MHC class I expression (Cretney et al., 1999).

Regulation of MHC class II molecule expression by MCMV was discovered during the studies of inefficient MCMV clearance from the salivary glands (Heise et al., 1998). Clearance of MCMV from the salivary glands is significantly delayed compared to other organs and it requires specific CD4+ T cells and IFN-γ. The MCMV infection apparently inhibits IFN-γ-induced presentation of the MHC class II-restricted peptides by the differentiated macrophages to CD4+ T cells via impairment of MHC class II cell surface expression. Similarly, HCMV US2 protein causes degradation of two essential proteins in the MHC class II antigen presentation pathway: HLA-DR-alpha and DM-alpha (Tomazin et al., 1999). More specifically, studies by Miller et al. (1998) revealed a defect in IFN-γ signal transduction, which was associated with a striking decrease in Janus kinase 1 (Jak1) levels (Miller et al., 1998). This modulation of antigen presentation may contribute to the capacity of MCMV to evade immune control and persist in the salivary glands of the infected host for prolonged periods of time.

Non-essential cytomegalovirus genes influencing viral tropism

Furthermore there are non-essential genes influencing viral tropism. Recently two distinct functions were described for the MCMV M45 gene product. Lembo et al. (2000) showed that M45 encodes a homolog of the R1 subunit of the cellular ribonucleotide reductase (RNR). RNR activity is essential for the replication of either viral or cellular DNA in infected cells. Interestingly, in MCMV infected cells the cellular R2 subunit preferentially complexing with the R1 subunit encoded by the MCMV M45

gene to reconstitute a new RNR activity (Lembo et al., 2000). Intriguingly, the M45 mutant MCMV replicates like wild type virus in fibroblasts, but poorly in macrophages and does not replicate in endothelial cells (Brune et al., 2000). Specifically, endothelial cells infected with the M45 mutant virus rapidly undergo apoptosis. The inability of the M45 mutant to grow in endothelial cells suggests that the M45 gene product encodes, or activates, an inhibitor of apoptosis, and that its physiological expression is essential for virus replication in endothelial cells.

The MCMV M33 gene, which is co-linear with HCMV UL33 as well as U12 of human herpesvirus 6 and 7 (HHV6 and HHV7), encodes a G protein-coupled receptor (GCR) homolog that plays an important role in the dissemination to or replication in the salivary glands (Davis-Poynter et al., 1997). A similar function was assigned to the R33 gene product of rat CMV (Beisser et al., 1998). Another non-essential gene influencing MCMV pathogenesis in the salivary glands is the sgg1 (M133) gene, which is critical for high levels of viral replication in this organ (Lagenaur et al., 1994). The MCMV M83 and M84 genes are the putative homologs of the HCMV tegument phosphoprotein pp65 (UL83). Mutations in these genes cause attenuated growth of MCMV in spleen, liver, and kidney. In addition, growth of a recombinant MCMV deleted of M83 is severely restricted in the salivary glands and lungs (Morello et al., 1999). The MCMV M43 gene belongs to the US22 gene family and encodes a protein whose function is dispensable for viral growth in cultured fibroblasts *in vitro* as well as in the lungs, spleen, liver, and kidneys of susceptible mice. However, M43 is a determinant for MCMV growth in the salivary gland (Xiao et al., 2000).

Apparently, many non-essential gene products influence dissemination to or replication in certain organs; however, the molecular mechanisms causing these effects are largely unknown. Thus, study of non-essential genes and the function of their protein products will lead to a better understanding of viral pathogenesis. This knowledge will expedite the development of preventive vaccines against primary CMV infection and/or drugs suppressing reactivation of the virus from a latent stage.

US22 Gene family

The US22 family of genes is found in all betaherpesviruses, particularly in HCMV (Chee et al., 1990), MCMV (Rawlinson et al., 1996), HHV-6 (Efstathiou et al., 1992), and HHV-7 (Nicholas, 1996). Table 2 shows known homologies of the MCMV genes to their HCMV counterparts.

TABLE 2

Homology between MCMV and HCMV US22 family genes

MCMV	HCMV
M23[a]	UL23[b]
m23.1	
M24	UL24
m25.1	UL23
m25.2	UL22
M36	UL36
M36Ex2	UL36Ex2
M36Ex1	UL36Ex1
M43	UL43 (P)[c]
M128Ex3	US22
M139	US22
M140	US23
M141	US24
m142	US26
m143	US23

[a] M and m denote MCMV homologous genes with or without sequence similarity, respectively.
[b] UL and US indicate HCMV genes within unique long and unique short regions of the genome.
[c] Positional homolog.

Members of the US22 gene family are characterized by short stretches of hydrophobic and charged residues within four conserved motifs (Kouzarides et al., 1988; Nicholas, 1996). The functions of most of the US22 family genes are unknown; however the products of certain genes such as HCMV TRS1/IRS1 (Iskenderian et al., 1996) HCMV UL36 (Colberg-Poley et al., 1992), MCMV M128 (Cardin et al., 1995), m142-m143 (Dalton, unpubl. data), and some genes within the US22 homology region in HHV-6 (Nicholas et al., 1994), are transcriptional transactivators. Some of the US22 genes are non-essential for viral replication in tissue culture, like TRS1/IRS1 of HCMV, M128, M43 and M139-M141 of MCMV (Jones et al., 1992; Cardin et al., 1995; Xiao et al., 2000; Cavanaugh et al., 1995). This suggests that the proteins encoded by those genes are either involved in regulation of viral pathogenesis or their function is compensated by another viral protein(s), most likely the other member(s) of the same gene family.

MCMV contains twelve US22 family genes that are localized in two clusters toward the left (M23, M24, M25.1, and m25.2) and right (M139-m143) ends of the genome (Fig.1).

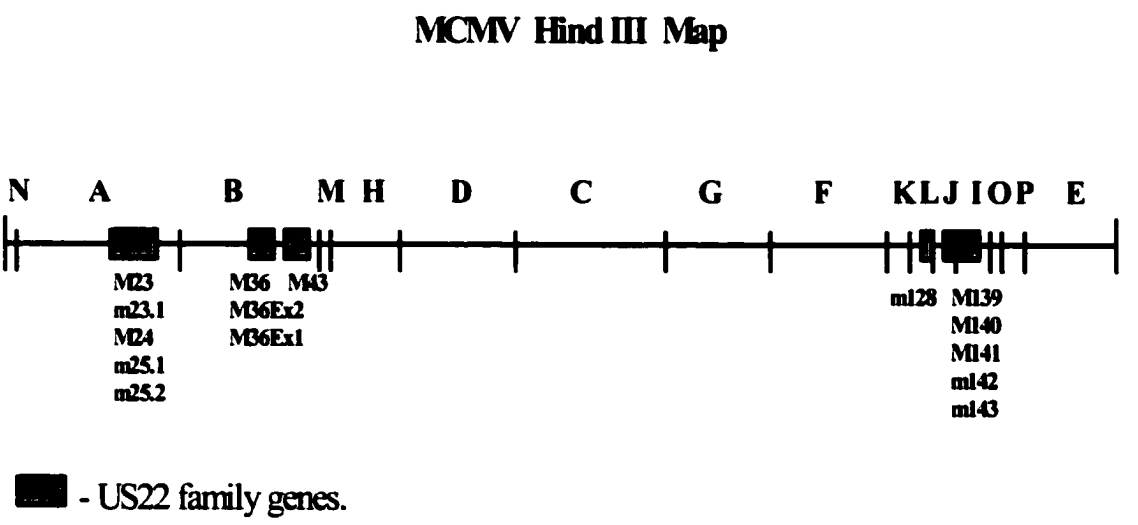


FIG. 1. Map of MCMV (strain Smith). The *Hind*III restriction fragments are indicated. The location of the US22 family genes within the genome are indicated as shaded boxes.

The remaining three US22 family genes are distributed throughout the genome (M36, M43, and M128).

Long-term interest in our laboratory concentrates on the MCMV US22 family genes. We analyzed the expression of most of these MCMV US22 genes in NIH 3T3 fibroblasts. The results showed that M23, M24, M25.1, m25.2, and M36 are not expressed to detectable levels by northern blot analysis (Karabekian, unpubl. data). However, M139, M140, M141, m142, and m143 genes are abundantly expressed in infected fibroblasts and macrophages. Characterization of transcripts originating from these MCMV US22 genes revealed another similarity between HCMV and MCMV US22 family genes. They are expressed with either immediate early (M128, m142, m143) or early kinetics (M139, M140, M141) (Hanson, Dalton et al., 1999). Figure 2 shows the results of transcript mapping of the M139-M141 region.

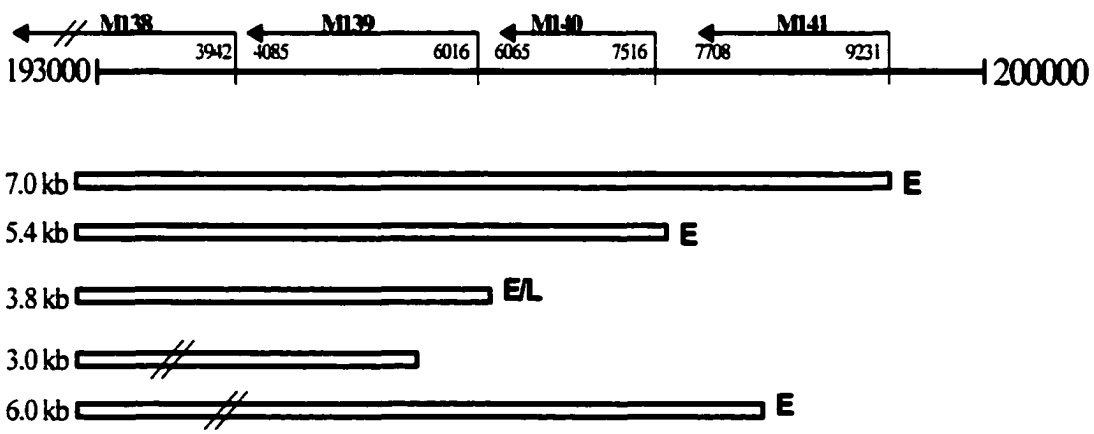


FIG. 2. Transcripts originating from the MCMV M139-M141 gene region. The fragment of MCMV genome from 193000 to 200000 is depicted on the top with the arrows and numbers indicating the start and stop codons of M139-M141 ORFs. Detected transcripts are shown as open boxes. The predicted positions and sizes of early (E) and early/late (E/L) viral RNA are denoted (adapted from Hanson et al. 1999, Virol. 260:156-164). The kinetics of the 3.0kb transcript is not yet determined.

Three abundant and two less abundant 3' co-terminal transcripts are expressed from the M139-M141 gene region with early or early-late kinetics. All five transcripts are expressed from right to left direction and share the same polyadenylation signal located within the M138 gene. However, the stop codons within large readthrough transcripts are probably utilized during translation to yield proteins of the sizes predicted by the ORFs.

The 3.8- and 3.0-kb transcripts map to the M139 region with no evidence of splicing. The 3.8 kb transcript is predicted to start at the position 196016 (Rawlinson et al., 1996). The presence of two potential start sites with an ATG at position 195766 and an ATG at position 195667 supports the hypothesis that the smaller transcript is expressed from a separate promoter within the larger ORF. In addition, DNA sequence analysis showed the presence of a potential TATA box within the proximity of those start sites, although expression of M139, M140, and M141 genes are controlled by TATA-less promoters (Hanson et al., in press). There is one abundant transcript of 5.4-kb corresponding to the M140 gene. The two largest transcripts of 7-kb and 6-kb originate within the M141 gene region. The 7-kb abundant transcript contains the sequence for expression of the full length M141 protein, and the less abundant 6-kb transcript covers about a third of the M141 coding sequence and may represent either a splice variant of M141, or use of an alternative start site (Hanson, Dalton et al., 1999).

Sequence analysis of M139-M141 ORFs reveled the presence of all four US22 characteristic motifs in each one of these genes (Fig. 3).

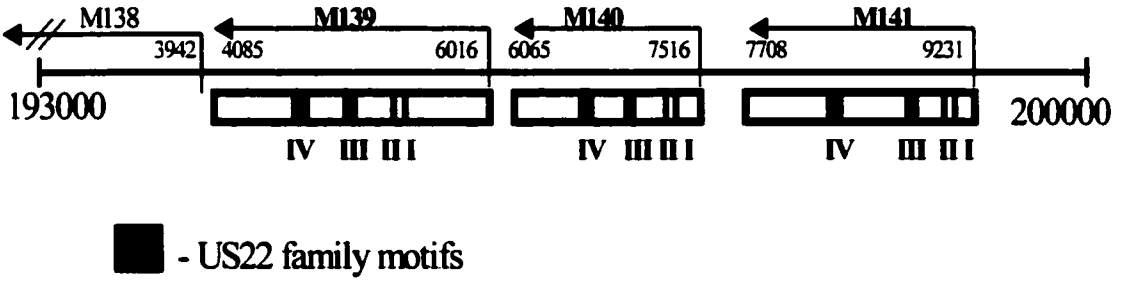


FIG. 3. Map of the M139-M141 gene region and predicted protein products. The fragment of MCMV is depicted on the top with the arrows and numbers indicating the start and stop codons of M139-M141 ORFs. Corresponding predicted protein products are shown as open boxes. The positions of US22 family motifs (I, II, III, IV) are indicated.

To proceed with characterization of translated proteins, rabbit polyclonal antisera specific for each gene product were generated (see Materials and Methods). Western blot analysis of MCMV infected fibroblasts and macrophages demonstrated that two proteins of 72- and 61-kD are detected by the anti-M139 (α-M139) antisera, a 56-kD protein is identified by anti-M140 (α-M140) antisera, and a 52-kD protein is recognized by anti-M141 (α-M141) antisera. Kinetics of expression of M139, M140 and M141 proteins demonstrated that all detected proteins were expressed at early times and accumulated through late times postinfection similar to the kinetics of transcription within this gene region (Hanson et al., in press). This correlated expression of both the transcripts and the proteins suggests a cooperative function that is consistent with the phenotype of deletion viruses as described below. The M139, M140 and M141 gene products localize to both the nucleus and cytoplasm in wild-type infected cells. Table 3 summarizes the results of transcript mapping and western blot analysis.

TABLE 3
Characterization of transcripts and translational products of the MCMV M139, M140. and M141 genes.

Gene	Sizes of specific transcripts (in kb)	Protein size(s) predicted by the ORFs (in kD)	Observed protein size(s) in western blot analysis (in kD)
M139	3.8 3.0	71.8	72 61
M140	5.4	55.8	56
M141	7.0 6.0	57.2	52

Our laboratory found unique phenotypes associated with mutations within M139-m143 genes. One or both of m142 and m143 immediate early genes are essential for viral replication even in NIH 3T3 fibroblasts (Cavanaugh et al., 1996; Menard et al, 2000). However, deletion of M139-M141 alters replication of the virus in macrophages *in vitro* and macrophage-rich organs *in vivo* (Cavanaugh et al., 1996; Hanson et al., 1999).

More specifically, recombinant MCMV RV10, lacking genes M139, M140, and M141, replicates up to 1,000-fold less efficiently in immortalized IC-21 macrophages compared to wild type virus in one- or multi-step growth curves. Expression of IE genes in macrophages upon RV10 (ΔM139-141) infection is significantly reduced which suggests that the block in replication of this mutant virus in macrophages occurs at the earliest stages in the virus life cycle: virus entry or immediate early gene expression (Hanson et al., 1999). When tested *in vivo,* RV10 (ΔM139-141) replicated poorly (if at all) in spleens of intact immunocompetent Balb/c mice, signifying that M139-M141 proteins are required for MCMV replication in the spleen. In this organ, tissue

macrophages are a site of MCMV replication (Stoddart et al., 1994). Indeed, when splenic macrophages were depleted in vivo prior to RV10 infection, the mutant virus replicated to near wild-type levels, indicating replication competency in other cell types. A high degree of attenuation due to deletion of M139-M141 is demonstrated by the fact that RV10 does not kill (within 100 days) SCID mice that are devoid of mature, functional T and B cells (Hanson et al., 1999). Therefore, the M139-M141 gene products influence macrophage specific early events in the replication cycle of MCMV.

Additional viral mutants containing mutations within M139, M140, and M141 genes alone or in combination were generated to assess the effect of a single gene on growth of the virus (Fig. 4). The mutations within M139 and M140 genes were generated by deletion of a coding fragment and insertion of an *e1*-β glucuronidase cassette (*e1*-β-glu) at the same site, and resulting viruses were named RV13 (ΔM139) and RV14 (ΔM140) respectively. The M141 gene was mutated by insertion of the β-glu reporter cassette into M141 coding region, generating RV11 (ΔM141) recombinant MCMV. The RV12 (ΔM139-140) virus lacks both M139 and M140 genes, which are replaced with *e1*-β-glu.

Because messages expressed from the M139-M141 gene region are 3' co-terminal, it was important to determine if a mutation within a single gene alter the transcription of the neighboring genes. Northern blot analysis verified that transcription of a mutated gene was ablated in the cells infected with corresponding recombinant MCMV. However transcription from the neighboring genes in the same cells was not altered (Hanson et al., in press). Subsequent protein analysis demonstrated that steady state levels of both M139 proteins, p75M139 and p61M139, were not affected by

mutations in either the M140 or M141 gene (Hanson et al., in press). Similarly, deletion of M139 did not alter the steady state levels of M140 or M141 proteins (pM140 and pM141 respectively). Interestingly, deletion of M140 resulted in a significant decrease in steady state levels of pM141 in both fibroblasts and macrophages (Hanson et al., in press).

Growth properties of all recombinant MCMV were assessed both *in vitro,* in multiple- and single-step growth analysis in NIH3T3 fibroblasts and IC-21 macrophages, and *in vivo,* in the spleen and liver of BALB/c mice (Hanson et al., in press). RV12 (ΔM139-140) grew like the triple deletion mutant virus both *in vitro* and *in vivo.* Interestingly, the RV13 (ΔM139), which lacks expression of both M139 proteins, but has unaltered expression of the pM140 and pM141, exhibited a wild-type-like phenotype.

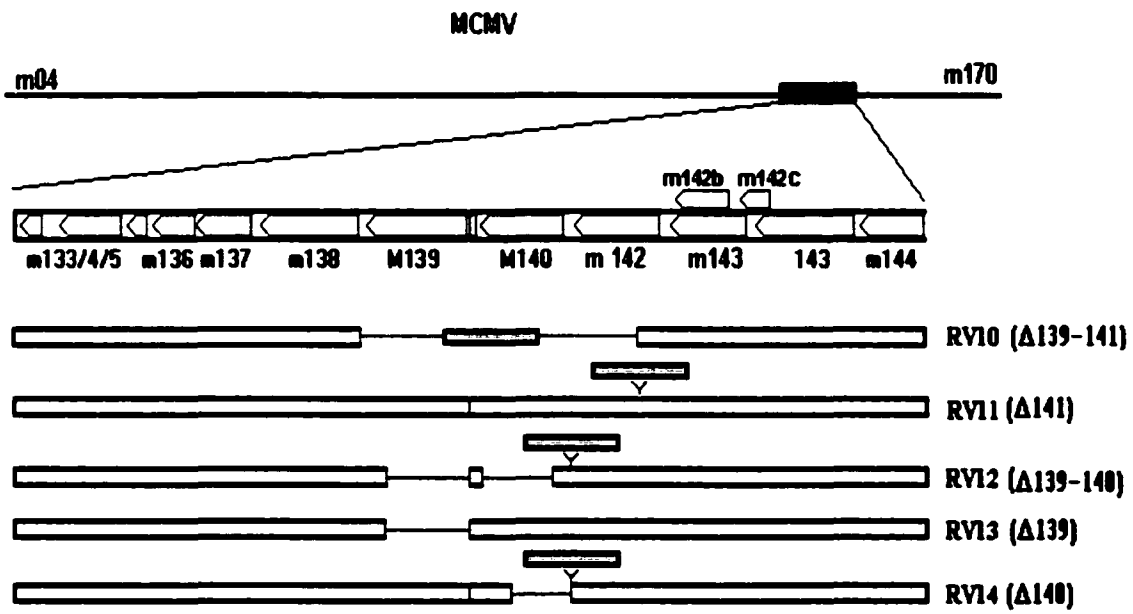


FIG. 4. Maps of recombinant MCMV. The black box indicates wild-type MCMV sequence within the HindIII-J and –I fragments of the MCMV genome. The positions of the MCMV genes including the M139, M140, and M141 are indicated as wide arrows. Solid lines denote deleted sequences. The gray boxes indicate the locations of the e1-β-glu cassette.

However, RV14 (ΔM140) expressing WT levels of M139 proteins, but decreased levels of pM141, replicated identically to RV10 (ΔM139-141) and RV12 (ΔM139-140), indicating an important function for pM140. Lastly, RV11 (ΔM141), expressing M139 and M140 proteins, demonstrated intermediate phenotype both *in vitro* and *in vivo*. Table 4 reflects maximal virus titers of the recombinant MCMV relative to the titers following wild type MCMV infection. These results indicate that M140 and M141 proteins are essential for replication of the MCMV in cultured macrophages and spleens of infected animals. Interestingly, M139 gene products appear not to influence MCMV growth, at least, in the conditions tested.

TABLE 4

Growth of WT recombinant MCMV *in vitro* and *in vivo*

Virus	Mutation	Replication *in vitro*		Replication *in vivo*	
		NIH3T3	IC-21	spleen	liver
WT MCMV	none	++++	++++	++++	++++
RV10	Δ139–141 (193984-198832)	++++	+	—	++++
RV13	Δ139 (195847-4371)	++++	++++	++++	++++
RV14	Δ140 (197626-6820)	++++	+	—	++++
RV11	Δ141 Insertion @198832	++++	+++	++	++++
RV12	Δ139–140 (195847-4371 + 197355-6050)	++++	+	N/D	N/D

Many possible functions can be assigned to these proteins that will explain the mutant phenotypes. One or more of these proteins may

1) affect transcription and/or translation of other pivotal viral genes,
2) compensate for a function of another viral protein, which are normally suppressed in the macrophage environment, but which is required for efficient viral replication within these cells,
3) alter the expression of cellular proteins expressed specifically in macrophages and required for robust virus replication *in vivo*,
4) complement a cellular protein required for MCMV replication and typically present in macrophages in sub-optimal levels,
5) manipulate macrophage-specific innate immune mechanisms, thus influence immune evasion.

However, the molecular mechanism of action of these proteins, alone or in combinations remains to be identified. The function of a protein often follows the structure and could not be completely understood without the characterization of the protein. Immunoprecipitation analysis is generally a more sensitive assay compared to western blotting, which was previously used to describe these proteins. It also provides the means to determine the rate of synthesis, stability of the protein of interest, as well as any interactions with other proteins. Therefore, we decided to use this experimental approach to further characterize M139, M140 and M141 gene products and identify critical interactions among these proteins. This will assist in elucidating the function of these gene products in regulation of MCMV replication in macrophages and macrophage-rich organs in mice.

CHAPTER II

SPECIFIC OBJECTIVES

The overall objective of this study was to characterize proteins originating from the US22 MCMV M139-M141 gene region. The characterization of these proteins is vital because it will lead to elucidation of the function(s) of these gene products in regulation of MCMV replication in macrophages and macrophage-populated target organs in mice, as the M139-141 gene products have been shown to influence this aspect of viral pathogenesis (Hanson et al., 1999). The results revealed not only the molecular weights of the expressed proteins, but also the existence of these proteins as complexes in MCMV-infected cells. In addition to physical interactions, the ability of the M140 gene product to functionally stabilize the M141 protein was revealed.

The specific objectives of this project were:

1) To characterize the expression of the proteins immunoprecipitating with antibodies specific to M139, M140 and M141 proteins. Immunoprecipitation experiments were performed with NIH 3T3 fibroblasts and IC-21 macrophages infected with wild type (WT) MCMV. The results revealed the number and the molecular weight of proteins precipitated by the α-M139, α-M140- and α-M141 polyclonal antisera in both fibroblasts and macrophages. The quality of generated antibodies was assessed for potential crossreactivity.

2) To test complex formation by the M139, M140 and M141 gene products. Complex formation by the M139, M140, and M141 proteins were tested by a series of experiments

including immunoprecipitations of denatured cell lysates compared to those performed under non-denatured conditions, sequential immunoprecipitations, and a combination of immunoprecipitation and western blot analyses.

3) To assess expression of the M139, M140, and M141 from mutant viruses deleted of one or more genes within this region. The origin of the complexed proteins was verified by immunoprecipitation analysis of cells infected with wild type or recombinant MCMV containing mutations within the M139, M140, or M141 genes, individually or in combinations. Use of the MCMV deletion mutants in pulse-chase analyses also revealed that the M140 protein, which complexes with the M141 gene product, confers stability to the M141 protein.

4) To verify the complex formed by the M140 and M141 proteins. The formation of the complex by the M140 and M141 proteins was proven by immunoprecipitation analysis of the cells infected with a mutant MCMV expressing only the M140 and M141 genes. Additional immunoprecipitation analysis of cells transiently expressing these genes as well as analysis of in vitro co-transcribed/translated M140 and M141 proteins was also performed to verify that this complex is formed in the absence of other viral proteins.

5) To determine a role of the M141 protein in complex formation. The involvement of the M141 protein in interactions with M139 and M140 proteins was addressed in experiments combining sequential immunoprecipitation and denaturing of precipitated

proteins. The results indicated that the M141 protein exists as both a free and complexed form.

6) To examine a direct interaction between M139 gene products and the proteins co-precipitating with them. The involvement of the M139 proteins in formation of the complex with the M140-M141 oligomer was addressed by immunoprecipitation analysis of *in vitro* transcribed/translated M139, M140 and M141 proteins alone or in combinations. These results were further confirmed by similar immunoprecipitations of M139, M140, or M141 proteins transiently expressed in NIH3T3 fibroblasts. The data indicated that at least a portion of the M139 proteins might associate with the M140-M141 protein complex.

CHAPTER III

MATERIALS AND METHODS

Cells

NIH 3T3 cells, a contact-inhibited murine fibroblast cell line from the American Type Culture Collection (ATCC, Rockville, MD), were propagated in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Herndon, VA) supplemented with 10% heat inactivated bovine calf serum (Hyclone Laboratories, Logan, UT) and 2 mM L-glutamine (Mediatech, Herndon, VA).

IC-21 cells, an SV40-transformed murine peritoneal macrophages cell line from C57BL/6 mice (Mauel and Defendi, 1971), were obtained from the ATCC, and were propagated in RPMI 1640 media (Mediatech) supplemented with 10% heat inactivated fetal calf serum and 2 mM L-glutamine.

Viruses

The Smith strain of murine cytomegalovirus (MCMV) was obtained from the American Type Culture Collection. Stocks of virus were prepared in, and titered on, NIH3T3 fibroblasts, as previously described (Campbell et al., 1989).

Mutant viruses were generated by homologous recombination as previously described elsewhere (Hanson et al, in press; Hanson et al., 1999; Cavanaugh et al., 1995). In order to generate a recombinant virus with a site-specific mutation within a gene of interest, an appropriate recombination plasmid was constructed in each case. The plasmids for generation of MCMV RV10 (ΔM139-141), and RV14 (ΔM140) contained a deletion (from nucleotides 193984 to 198832, and from nucleotides 197626 to 197682,

respectively) within the gene of interest, and an insertion of a β-glucuronidase (β-glu) cassette, as a marker gene, surrounded by WT sequences on both sides. The plasmid for generation of MCMV RV11 (ΔM141) mutant virus contained an insertion (at position 198832) of the β-glu cassette into the M141 gene. MCMV RV13 (ΔM139) was generated during the rescue of the virus with deletions in both M139 and M140 genes (MCMV RV12). It contains a deletion from nucleotides 194371 to 195847, and does not contain the β-glu cassette. Revertant viruses, RV10REV, RV12REV, RV14REV, contain the wild type sequences introduced back into the RV10 (ΔM139-141), RV12 (ΔM139-140) and RV14 (ΔM140) genomes, respectively, by homologous recombination. These viruses replicate like WT MCMV both *in vitro* and *in vivo* (Hanson et al., 1999; Hanson et al., in press). All recombinant MCMVs were generated by cotransfecting infectious MCMV DNA and linearized recombination plasmids of interest into NIH3T3 fibroblasts as described previously (Cavanaugh et al., 1995; Hanson et el., 1999). Blue plaques produced by the recombinant viruses containing the β-glu cassette or white plaques produced by the revertants and RV13 (ΔM139), identified by addition of substrate for the β-glu enzyme (5-bromo-4-o-3-indolyl-β-D-glucuronide), were isolated and plaque purified in at least five rounds of purification. All mutations were confirmed by Southern blot analysis. The infections described in this study were done at a multiplicity of infection (MOI) of one plaque forming unit/cell (PFU/cell).

Antibodies

Polyclonal antisera were generated against recombinant M139, M140, and M141 proteins. Recombinant proteins were obtained by cloning an appropriate MCMV

genomic fragment into a bacterial expression vector. The M139 expression vector was generated by cloning the 2.3 kb HindIII to EcoRI fragment of MCMV (195850–193532) into the pTrcHis (A) vector (Invitrogen, Carlsbad, CA) resulting in the pHisA139 plasmid. For the expression of the M140 encoded protein, the 1.4 kb HindIII to SalI fragment (195850–197271) was also cloned into pTrcHis (A) vector resulting in the pHisA140 plasmid. For generation of M141 specific antibodies, a 1.2 kb SstI to BglII fragment (198832–197629) was cloned into the pTrcHis (A) vector resulting in pHisA141 (see Fig. 5).

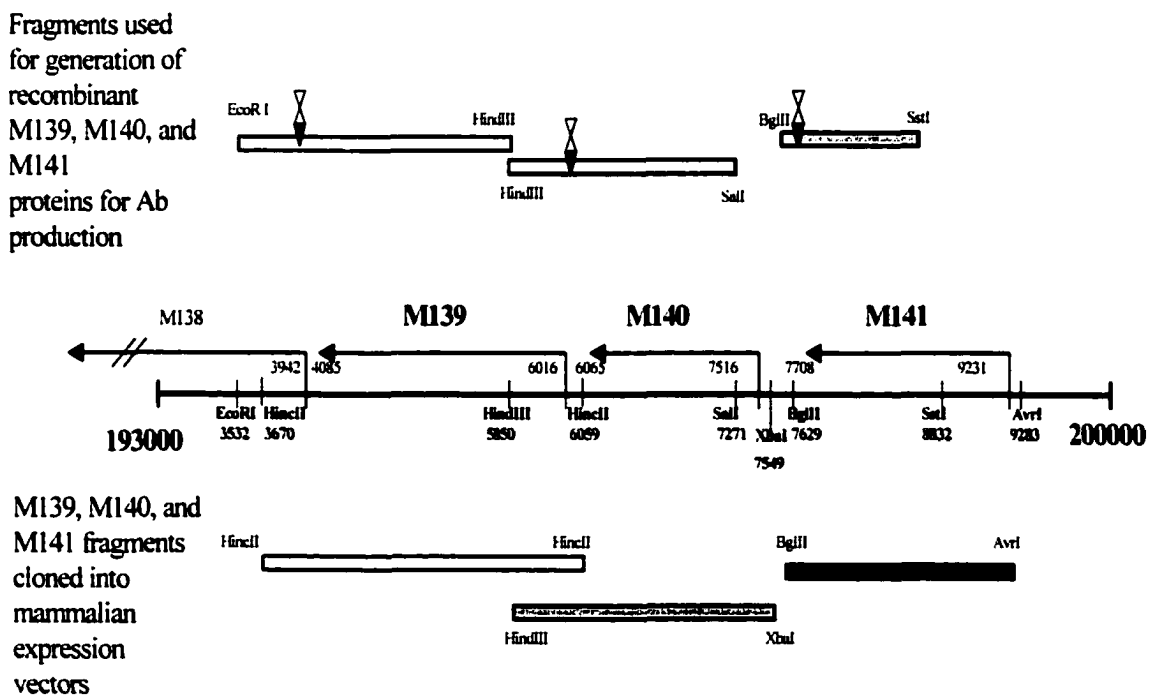


FIG. 5. Map of the MCMV M139-M141 gene region and fragments used in cloning. Location and direction of transcription of the M139, M140 and M141 genes are indicated by the arrows. Predicted start and stop sites for each gene are denoted. Fragments used for the generation of recombinant proteins for antibody production and mammalian expression vectors are depicted on the top and the bottom of the map respectively. Restriction sites utilized in cloning procedures are indicated. The stop sites (▼) of each gene that lie within fragments used for generation of recombinant proteins are denoted.

These plasmids were transformed into Top 10 *E.coli* (Invitrogen, Carlsbad, CA) and bacterial cultures were induced with the lactose analog, isopropyl-β-D-thiogalactosidase (IPTG), for four hours in order to up-regulate expression of the pM139-His, pM140-His, or pM141-His fusion proteins driven by the *lac*O regulated *trc* promoter. The expression of target proteins was tested by western blot analysis, using anti-His antibodies (Qiagen Inc. Valencia, CA). The pM139-His, pM140-His, and pM141-His recombinant proteins were purified using the Xpress™ purification system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Purified fusion proteins were used to generate rabbit polyclonal antisera (Cocalico, Reamstown, PA). Two individual rabbits in each case produced antiserums against recombinant proteins. Polyclonal antiserum against the MCMV E1 protein was obtained from Dr. Gina Ciocco-Schmitt (Easter Virginia Medical School, Norfolk, VA) and used in immunoprecipitation reactions testing the equal levels of infection among different viruses.

To generate horseradish peroxidase conjugated anti-M139 and anti-M140 antibodies, the IgG fraction was first purified from the corresponding rabbit polyclonal antisera. The α-M139 and α-M140 IgG were purified using ImmunoPure IgG (Protein A) Purification kit (Pierce, Rockford, IL) according to the manufacturer's protocol. In brief, 1 ml of α-M139 and α-M140 polyclonal antisera (about 60 mg total protein) was diluted 1:10 with ImmunoPure IgG binding buffer. All 10 ml of each antisera were loaded onto separate protein A agarose columns and allowed to flow through. Each column was washed with 15 ml of ImmunoPure IgG binding buffer. Then the IgG fractions were eluted in 5 ml of ImmunoPure elution buffer. Subsequently, each eluate was desalted by running through a separate Excellulose column. Desalted IgGs of the α-

M139 and α-M140 were eluted in three 1 ml fractions of phosphate buffered saline (PBS pH 7.5) from the Excellulose column. Eluted antibodies were quantitated by spectrophotometry (Beckman DU-7000 spectrophotometer). Aliquots of 30 μg from each eluted fraction were resolved by SDS-PAGE (Fig. 6, lanes 4,5,6) together with unpurified whole α-M140 rabbit polyclonal antisera (Fig. 6, lane 2) and recombinant rabbit IgG (Sigma, St. Louis, MO) (Fig. 6, lane 3), and visualized by Coomassie blue staining.

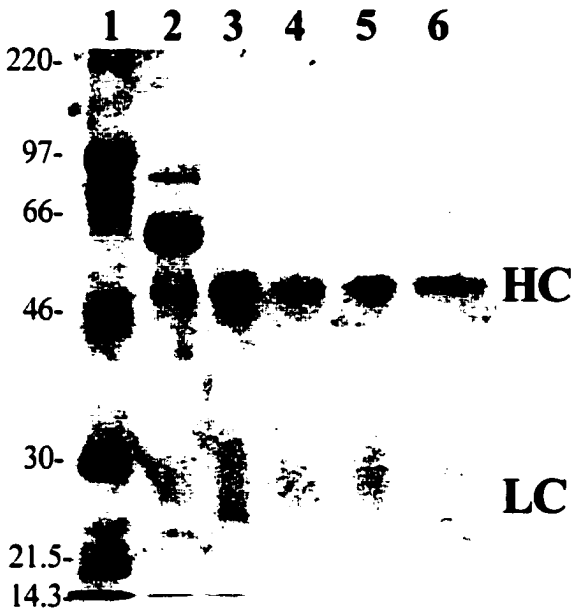


FIG. 6. Purification of the IgG from whole rabbit serum. Figure is a Coomassie stained gel of the α-M140 whole antisera and purified IgG fractions. 30 μg of total protein was loaded to each lane. Lane 1: molecular weight markers (Amersham Life Science, Arlington Heights, IL), Lane 2: unpurified whole α-M140 rabbit polyclonal antisera, Lane 3: recombinant rabbit IgG, Lane 4-6: Fractions 1-3 of purified α-M140 IgG, respectively. HC and HL denotes heavy and light chains of purified IgG molecules.

Subsequently, 3 ml of α-M139 (654 μg/ml) and α-M140 (630 μg/ml) IgG were sent to Genosys by Sigma (Woodlands, TX) for HRP conjugation. The ability of

conjugated antibodies to recognize pM139 and pM140 was tested in western blot analysis of the MCMV-infected cell lysates (Fig. 7).

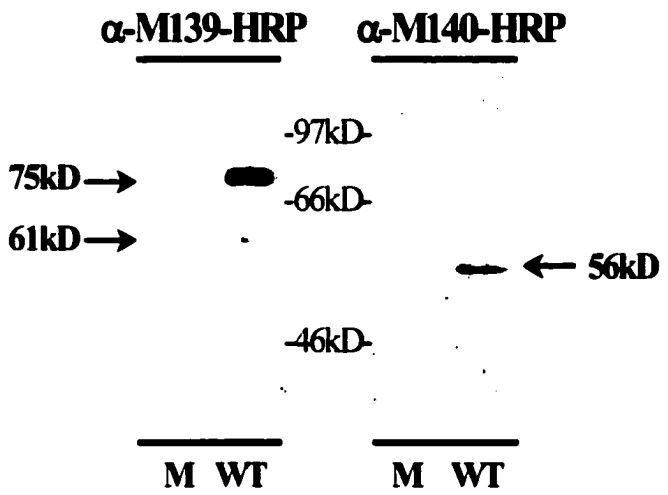


FIG. 7. Anti- M139-HRP and anti-M140-HRP antibodies specifically recognize MCMV proteins. Duplicates of mock (M)- and WT MCMV (WT)-infected NIH3T3 fibroblast lysates were resolved by the 12.5%SDS-PAGE (40 μl/lane). The gel was blotted to a nitrocellulose membrane. Immunoblots were probed with either α-M139-HRP or α-M140-HRP and detected by ECL Western Blotting Detection Reagents. Positions of molecular weight makers are indicated in the center. Sizes of the detected proteins are indicated in bold on the left and the right sides of the blots.

Plasmids

Plasmid generation was conducted by standard methods (Maniatis et al., 1987), and restriction endonuclease digestions were done according to manufacturer's suggestions (Promega, Madison, WI; New England Biolabs, Beverly, MA). The plasmids used for construct generation, in vitro transcription/translation reactions, and transfection studies were prepared and purified using the Quantum Prep Plasmid Maxiprep Kit (Bio-Rad, Hercules, CA).

Mammalian expression vectors containing the M139, M140, or M141 gene were generated in order to express each gene individually in the absence of other viral proteins. The M139 mammalian expression vector was generated by cloning the 2.4-kb HincII fragment (193670–196059) into the EcoRV site of the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA), resulting in the plasmid called pM139/3.1(+). The M140 mammalian expression vector was constructed by cloning the 1.7-kb HindIII to XbaI fragment (195850-197549) into appropriate sites of the pcDNA3.1Zeo (-) vector (Invitrogen, Carlsbad CA), resulting in the plasmid called pM140-Zeo. Similarly, the M141 mammalian expression vector was generated by cloning the 1.6-kb AvrII to BglII fragment (197629-199283) into the XbaI and BamHI sites of the pcDNA3.1Zeo (-) vector respectively (Invitrogen, Carlsbad CA), producing the pM141/Zeo (-) plasmid. The vectors were treated with calf intestine phosphatase (Boehringer Mannheim, Indianapolis, IN) for 2-4 hours following appropriate endonuclease digestion. All genomic fragments and vectors were separated on the 5% polyacrylamide gel and then eluted. Purified DNA fragments were ligated by T4 DNA ligase (New England Biolabs, Beverly, MA) in 1:3 molar ratio of vector to insert. The pM139-His plasmid was generated by cloning the 2.3 kb HindIII to EcoRI fragment (195850-193532, respectively) into corresponding sites of the pcDNA3.1/His B (Invitrogen, Carlsbad, CA) mammalian expression vector. The M139 coding sequence was cloned in frame with the vector sequence encoding a histidine tag at the N-terminus. The cloned fragment of the M139 gene is missing the first 162 base pairs, however the expressed fusion protein (74kD) has a similar molecular weight to the native M139 protein (75 kD) due to the expression of the tag. Both proteins characteristic of the M139 gene (74- and 61-kD)

were expressed from this plasmid following transient transfections, and were detectable in western blots probed with α-M139 Ab. Transfections of mammalian expression vectors into NIH3T3 cells were performed as described below. Figure 5 illustrates the genomic fragments cloned into the mammalian expression vectors.

Procedures

In vitro transcription and translation reactions

In vitro transcription/translation reactions were conducted using the TnT Coupled Reticulocyte Lysate Systems (Promega, Madison, WI) in order to verify the products of the M139, M140, and M141 genes. Each reaction was assembled in a total volume of 50 µl from the following components: 25 µl of rabbit reticulocyte lysate, 2 µl of reaction buffer, 1 µl of RNA polymerase, 1 µl of 1 mM amino acid mixture without methionine. One microliter of RNasin ribonuclease inhibitor (40 U/µl, Promega, Madison, WI) was added to each reaction mix. A total of 1 µg of template DNA and 20 µCi of [^{35}S] methionine (1,000 Ci/mmol at 10 mCi/ml, DuPont NEN, Wilmington, DE) were added to each reaction mix. The reactions were incubated at 30^{0}C for 90 minutes. Next, the expression of radiolabeled transcribed/translated products was tested by visualization. For that, 2 µl of each lysate was mixed with 10 µl of 2X sodium dodecyl sulfate (SDS) protein loading buffer (5 mM Tris, 4% SDS, 2% sucrose, 0.01% brome-phenol blue, 5% beta-mercaptoethanol), boiled for 5 minutes and loaded on a 12.5% polyacrylamide gel. Gels were fixed (10% acetic acid, 25% Methanol) for 1 hour and treated with rapid autoradiography enhancer, Enlightning (DuPont NEN, Wilmington, DE), for 30 minutes.

Subsequently, gels were dried (SDG2000 Digital Slab Gel Dryer, Savant Instruments, Inc., Holbrook, NY) and exposed to X-ray film (Fuji RX) to obtain autoradiographs.

To immunoprecipitate *in vitro* transcribed/translated proteins, 2.5 µl of each lysate was diluted 10-fold in 10S buffer (50 mM Hepes, [pH 7.2], 250 mM NaCl, 0.3% NP-40, 0.1% Triton X-100, 0.005% SDS, 10 mM $NaPO_4$ [pH 7.0], 1 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitoi [DTT]) and incubated for 1 hour with 5 µl of the appropriate antibody at room temperature with constant rocking. Then 30 µl of 50% slurry of protein agarose A beads in standard immunoprecipitation lysis buffer (see description below) was added to each sample and incubated for 2 more hours at room temperature with constant rocking. Immune complexes were washed and processed in accordance with the standard immunoprecipitation protocol described below.

Standard immunoprecipitation analysis

In order to characterize the M139, M140, and M141 gene products from MCMV-infected cells, immunoprecipitations were performed. NIH3T3 fibroblasts ($1.5x10^6$) or IC-21 macrophages ($2x10^6$) were seeded into 100 mm tissue culture dishes 24 hours prior to virus infection. Cells were inoculated with wild type (WT) MCMV or recombinant viruses as indicated, at an MOI of 1 PFU/cell. Mock-infected cells received an equal volume of culture media. Next, between 19-22 hours post-infection, cell monolayers were washed twice with warm Tris-buffered saline (TBS, 30 mM Tris, 150 mM NaCl, pH 7.4) and starved for one hour in methionine-cysteine free DMEM (Mediatech, Herndon, VA). Following starvation cells were exposed to methionine-cysteine free

media supplemented with ^{35}S-methionine-cysteine labeling mix (1175.0 Ci/mmol at 11 mCi/ml, EasyTaq™ Express Protein Labeling Mix, DuPont NEN, Wilmington, DE), at the concentration of 100μCi/ml. Four hours later, cell monolayers were washed twice with cold TBS to remove the unincorporated labeling materials and lysed in standard immunoprecipitation (IP) lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5% deoxycholate [DOC], 1 mM PMSF, 10 μg/ml aprotinin). Cellular lysates were cleared from insoluble fractions by centrifugation and used directly in immunoprecipitations.

Equal amounts of labeled cell lysates (300 μl) were incubated with 5μl of undiluted α-M139, α-M140, or α-M141 serum, or preimmune serum as a negative control for 2 hours at 4°C with constant rocking. The protein A agarose matrix (Roch Molecular Boichemicals, Indianapolis, IN) was washed 3 times with standard IP buffer, and subsequently, 60 μl of 50% slurry of protein A agarose (resuspended in standard IP lysis buffer) was added to each sample. The samples were incubated between 6 and 24 hours at 4°C with constant rocking allowing time for the immobilization of generated immune complexes. These immune complexes were pelleted by centrifugation and washed three times with high salt concentration SNNTE (5% sucrose, 500 mM NaCl, 1% NP-40, 50 mM Tris, 5 mM EDTA) buffer, and three times with a high detergent concentration radioimmunoprecipitaion assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1% Triton-X100, 1% DOC). After pelleting, immune complexes were resuspended in 2X loading buffer, boiled for 5 minutes, and electrophoresed on 12.5% polyacrylamide gels. Following electrophoresis, the gels were fixed (10% acetic acid,

25% Methanol), treated with autoradiography enhancer (Enlightning, DuPont NEN, Wilmington, DE), dried and exposed to X-ray film (Fuji RX).

Immunoprecipitation analysis of denatured cell lysates

In some experiments proteins from infected cells were denatured prior to immunoprecipitations. First, infected cells were harvested in standard IP lysis buffer and clarified from an insoluble fraction by centrifugation. A 10% SDS stock solution was added to the prepared cell lysate to reach a final concentration of 1% SDS (example: 50 µl of 10% SDS to 450 µl of lysate). The SDS-containing samples were boiled for 10 min. Denatured lysates were diluted 10-fold with NET-GEL buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.5], 0.05% NP-40, and 0.25% gelatin) and used directly in an immunoprecipitation reaction as described above.

Sequential immunoprecipitation analysis

In some experiments, proteins immunoprecipitated from infected cells were solubilized and re-immunoprecipitated with α-M139, α-M140, or α-M141 antisera. Infected cells were radiolabeled, harvested, processed and immunoprecipitated according to the standard IP protocol to obtain insoluble immune complexes bound to protein A agarose beads. Subsequently, the supernatants were aspirated and saved for the next IP or discarded. Immune complexes bound to protein A agarose beads were resuspended in 150 µl of 1% SDS-PBS and boiled for 10 min to dissociate immune complexes from protein A agarose beads and immunoprecipitating antibodies. Then, 1ml of IP lysis buffer containing 0.1% of bovine serum albumin (BSA) was added to each of the SDS-

treated samples. Diluted samples were centrifuged to spin down protein A agarose beads and the aqueous phase was transferred to a set of fresh tubes. Equal aliquots (about 330 μl) of denatured and diluted samples were used directly in a second immunoprecipitation reaction with the appropriate antibody. The obtained immune complexes were processed as described above.

Combined immunoprecipitation/western blot analysis

As an alternative to sequential immunoprecipitations, precipitated proteins were subjected to western blot analysis in some experiments. Two million NIH3T3 fibroblasts were infected with either WT MCMV, or RV11 (ΔM141), or RV13 (ΔM139), or RV14 (ΔM140) mutants with MOI of 1 PFU/cell or mock-infected. Unlabeled cell lysates were harvested in standard IP lysis buffer 22-24 hours post-infection and clarified from the cellular debris. One milliliter of each lysate was immunoprecipitated with α-M139, α-M140 or α-M141 antibody, processed, washed and resolved by SDS-PAGE in duplicate as described in the standard immunoprecipitation protocol above. Subsequently, the gels were electroblotted to nitrocellulose membranes (Boehringer Mannheim, Indianapolis, IN), and treated with blocking solution (5% dried milk, 0.1% Tween 20, 10 mM Tris, 150 mM NaCl) overnight at 4^0C. Next, the blots were probed with either α-M139-HRP or α-M140 HRP antibody diluted 1:100 in blocking solution. Western blots were detected by chemiluminesence using ECL Western Blotting Detection Reagents (Amersham Pharamacia Biotech, Piscataway, NJ) on X-ray film (Fuji RX).

Transient transfections or transfections/superinfections of the cells for subsequent immunoprecipitation analysis

Some experiments required immunoprecipitations of proteins not from MCMV-infected cells but from cells transiently expressing the M139, M140, or M141 proteins. Transfections were performed using the Cytofectene (Bio-Rad, Hercules, CA) transfection reagent, according to the manufacturer's suggestions. NIH3T3 fibroblasts were seeded into 60 mm tissue culture dishes ($3x10^5$ cells/plate) 20-24 hours prior to transfection. DNA-Cytofectene mixes were prepared for each transfection as follows: DNA of interest (total of 9 ug) was combined with serum free DMEM in a total of 100µl. Twelve microliters of Cytofectene was mixed with 88 µl of serum free DMEM and added to each DNA mix. The assembled mixes (200 µl/transfection) were incubated overnight at 4^0C. On the next day, 2.5 ml of warm complete NIH3T3 media was added to each DNA preparation. Cell monolayers were washed 2 times with warm PBS (2 ml/wash) and 2.7 ml of DNA-Cytofectene-complete media mix was added to each plate. The plates were incubated for at least 5 hours at 37°C and then 1.3 ml of complete media was added to each plate and incubated overnight at 37°C. The following day, the cells were washed two times with warm PBS, trypsinized, counted, seeded into 100 mm dishes and incubated overnight at 37°C. Transfection efficiency was monitored by assessing the percentage of cells transfected with an unrelated plasmid expressing the green fluorescent protein conducted in parallel transfection experiments. Consistently, 5 to 10% of the cells were transfected when 9 µg of DNA was used, and 1 to 5% efficiency was detected following transfection of 3µg of DNA. Twenty-four hours later the transfected cells were either labeled with [^{35}S] methionine-cysteine labeling mix or superinfected with WT

MCMV or RV13 (ΔM139) MCMV at an MOI of 1 PFU/cell. Superinfected cells were labeled 19-23 hours post-infection. Radiolabelings and subsequent immunoprecipitations were conducted as described in the standard IP protocol above.

Pulse-chase analysis

Stability of the M139, M140, and M141 proteins expressed from WT and mutant viruses were analyzed in pulse-chase experiments. Two million NIH3T3 fibroblasts were infected with either RV10REV, or RV11 (ΔM141), or RV13 (ΔM139), or RV14 (ΔM140) mutants at an MOI of 1 PFU/cell. Nineteen hours post-infection, cells were starved of methionine and cysteine for 1 h and then radiolabeled with 450 μCi/ml of [^{35}S] methionine and cysteine protein labeling mix for 2 h in a total of 5 ml. The unincorporated labeling materials were washed 3 times with warm complete culture medium without radiolabels and labeled proteins were chased with the same medium for indicated times. The cell lysates were harvested in 1 ml of standard immunoprecipitation lysis buffer, clarified from cellular debris, and used in immunoprecipitation reactions with either α-M139, α-M140, or α-M141 polyclonal antisera overnight at 4^{0}C with constant rocking. Next, 60 μl of 50% slurry of protein A agarose was added to each sample for at least 4 h. Immunoprecipitated complexes were washed and processed as described previously.

CHAPTER IV

RESULTS

Identification of protein profiles immunoprecipitating with anti-M139, anti-M140, and anti-M141 antibodies from WT MCMV infected fibroblasts and macrophages

Immunoprecipitation is a sensitive assay used for protein analysis. It provides a means to purify a protein in its non-denatured form and, when combined with radiolabeling of the protein, can reveal any precursors or post-translationaly processed forms of the protein. In addition, due to the sensitivity of this assay, low abundance related proteins could be detected. Immunoprecipitations also allow detection of other protein(s) directly interacting with the protein of interest. Thus, we decided to immunoprecipitate the MCMV M139, M140, and M141 proteins from infected cell lysates in order to further characterize these proteins, which have an important function in MCMV pathogenesis. These proteins influence the ability of MCMV to replicate in cultured macrophages and in macrophage rich organs in mice (Hanson, et al., 1999)

Immunoprecipitation (IP) analysis, thoroughly described in the Material and Methods section, was used as a basic experimental approach for this study. Certain modifications were introduced to that protocol depending on the posed question. Previous analysis of the expression of M139, M140, M141 genes demonstrated that all transcripts, detected by northern blotting, and proteins, detected by western blotting, were expressed with early-late kinetics (Hanson, Dalton et al. 1999, Hanson et al., in press). Therefore, all immunoprecipitations described throughout this study were conducted at late times post-infection. Fibroblasts and macrophages were infected with WT MCMV

or mock-infected. Nineteen hours after infection the cells were starved in methionine- and cysteine-free (Met-Cyst-free) Dulbecco's modification of Eagle's medium (DMEM) for one hour in order to enhance the incorporation of radiolabeled amino acids, and then labeled for four hours with ^{35}S Met-Cyst in the same medium. Each protein of interest contains comparable number of methionines and cysteines, which provided a similar degree of labeling. More specifically, the full-length M139 protein has 12 methionines and 12 cysteines, the M140 protein contains 15 methionines and 14 cysteines, and M141 gene encodes for 11 methionines and 16 cysteines. Cell lysates were harvested at 24 hours post infection, cleared from insoluble fraction, and immunoprecipitated with anti-M139 (α-M139), anti-M140 (α-M140), or anti-M141 (α-M140) antibodies (Abs), or preimmune serum as a negative control (Fig. 8A). All the samples were resolved by 12.5% SDS-PAGE. Subsequently the gels were dried and exposed to X-ray film to obtain autoradiographs (Fig. 8B).

Interestingly, five bands were detected in immunoprecipitants of NIH3T3 fibroblasts and IC-21 macrophages infected with WT MCMV using α-M139 serum. Two of these proteins, 75-kD and 61-kD, were the same size as those detected by western blot analysis although the larger protein was previously sized as 72-kD (Hanson et al, in press). The use of radioactive molecular weight markers allowed us to make a more accurate determination of the size of the larger protein as 75-kD. The 61-kD protein was detected in lower amounts compared to the 75-kD species. Three additional proteins of 98-kD, 56-kD and 52-kD were observed. The lower molecular weight species were equally copious, however the 98-kD protein was visualized in significantly lower amounts.

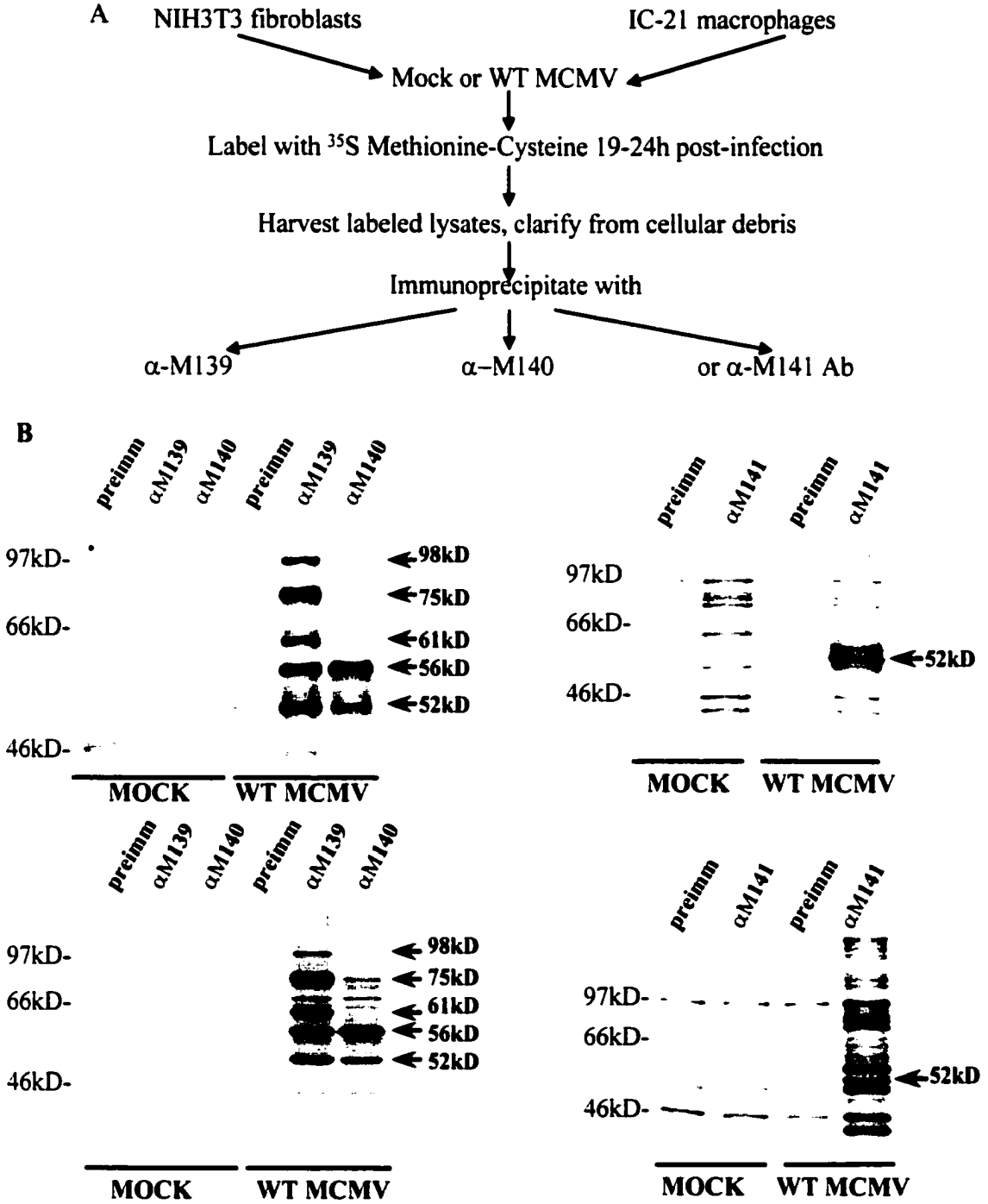


FIG. 8. Immunoprecipitation analysis of the M139, M140, and M141 gene products. (A) An overview of experimental procedures. (B) Autoradiographs of immunoprecipitations (IP) of WT- or mock-infected NIH3T3 fibroblasts (**top panel**) or IC-21 macrophages (**bottom panel**) obtained with rabbit α-M139, α-M140, α-M141 antisera or preimmune sera. Positions of molecular weight makers are indicated on the left side of each autoradiograph. Sizes of the detected proteins are indicated in bold on the right.

Two bands were visualized in a similar assay using α-M140 antibody. The 56-kD band is the same size as the one detected by western blot. The second protein, not detected by western blot was 52-kD and was visualized in lower amounts. Intriguingly, the two lowest molecular weight bands of 56- and 52-kD were immunoprecipitated by both α-M139 and α-M140 antibodies. Lastly, only one protein of 52-kD was detected by α-M141 antibody in both immunoprecipitation and western blot analyses.

In this thesis, the 75-kD protein from the M139 gene will be referred to as p75M139 and the 61-kD protein from the same gene as p61M139. Moreover, pM139 will be used to represent both the 75- and 61-kD proteins expressed from M139 gene. Similarly, the proteins expressed from the M140 and M141 genes will be denoted as pM140 and pM141, respectively.

The characteristic patterns of resolved proteins (98-, 75-, 61-, 56-, and 52-kD for α-M139 IP; 56-and 52-kD for α-M140 IP; and 52-kD for α-M141 IP) were consistent and independent from different WT MCMV stock preparations used in the experiments (Fig. 9). These results were also reproducible and independent of antiserum produced by two individual rabbits against M139, M140, or M141 recombinant proteins (data not shown).

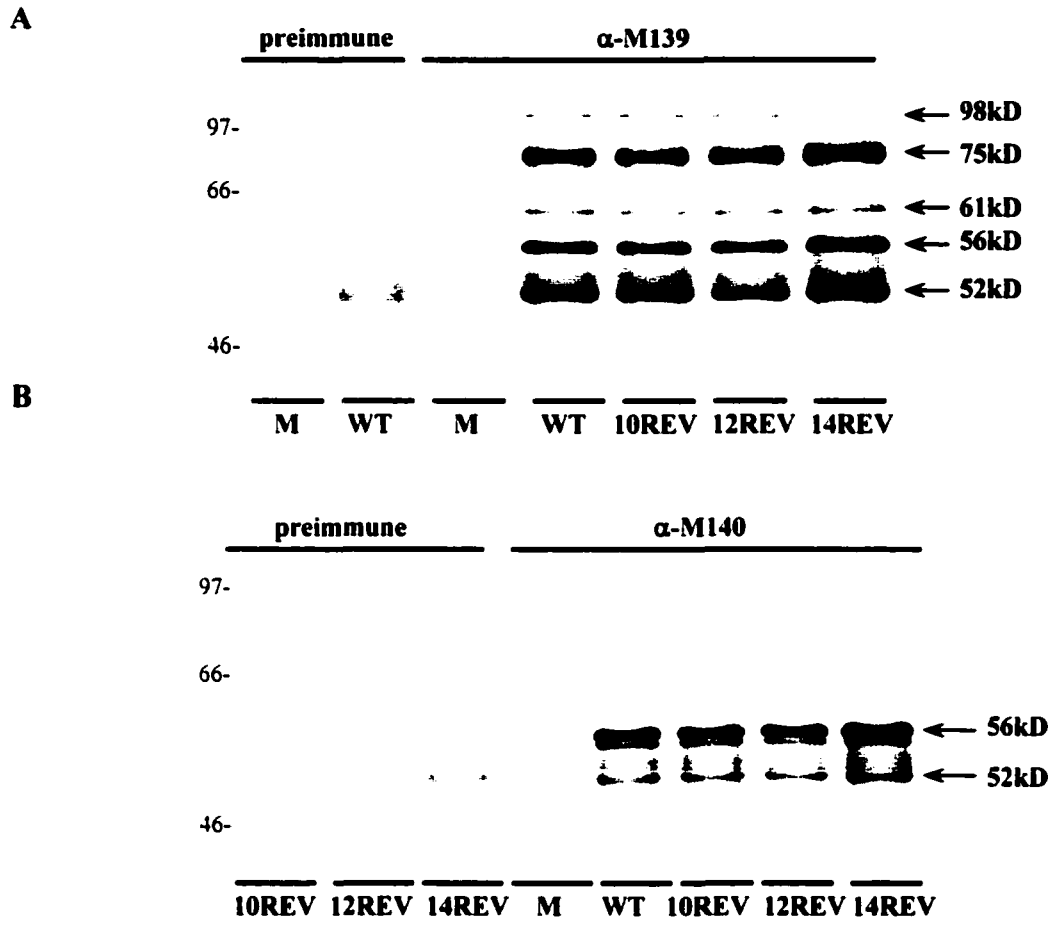


FIG. 9. Immunoprecipitation analysis of WT and revertant MCMV infected cells using (A) α-M139 or (B) α-M140 antibodies. NIH3T3 fibroblasts were infected with the indicated viruses, labeled and immunoprecipitated as described in Materials and methods. M designates mock-infected cells. Molecular weight markers and the sizes of detected proteins are depicted of the left and right sides of the autoradiographs, respectively.

Molecular weights of the most abundant identified proteins of 75-kD, 56-kD, and 52-kD correlate with those predicted by analysis of the MCMV open reading frame (ORF) corresponding to M139, M140, and M141 and those detected by western blot analyses of WT-infected cell lysates (see Table 3). However, because the messages in this region are 3' co-terminal (see Fig. 2), all of the detected transcripts are larger than

open reading frames (ORFs), which code for these proteins. This indicates that during translation appropriate internal stop signal is used by the ribosomes to produce a single protein corresponding to each gene. However this assumption needs further experimental proof. Nevertheless, the discrepancies in protein profiles detected by the immunoprecipitation analysis compared to previous western blotting suggests either one of three possibilities:

a) crossreactivity among the antibodies, because all three proteins belong to the US22 gene family and share four common motifs. It is note worthy, however, that the level of homology among those proteins is low, or
b) complex formation among the M139, M140, and M141 proteins, or
c) interaction of each protein with other unrelated viral or cellular proteins of similar molecular weights.

Assessment of the quality of generated antibodies for potential crossreactivity

As was mentioned above, the M139, M140, and M141 proteins are members of the US22 family, containing four characteristic motifs (see Fig. 5). Although the overall amino acid sequence homology among these proteins is low, crossreactivity between antibodies raised to these proteins is still possible.

To test the quality of the generated antibodies for specificity and potential crossreactivity we utilized the following experimental approach. The M139, M140, and M141 proteins were expressed from a mammalian expression vector in *in vitro* transcription/translation reactions. The resulting radioactively labeled proteins were

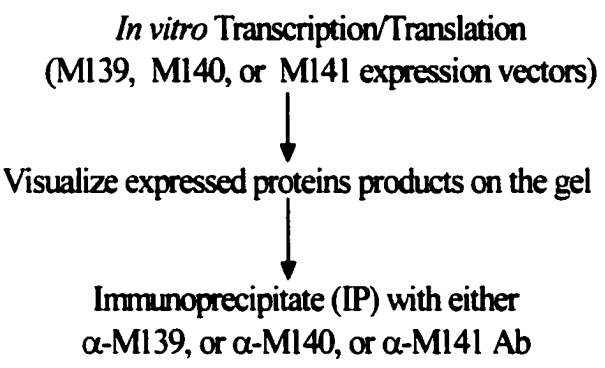
visualized by SDS-PAGE and a portion of the lysates was used in immunoprecipitation reactions such that each protein was precipitated by all three antibodies (Fig. 10A).

Two proteins of 75- and 61-kD were expressed from the mammalian expression vector, pM139/3.1(+), which contained the full-length M139 gene (Fig. 10B, top panel). This suggests that the 75- and 61-kD proteins observed in infected cells arise from the M139 gene. Similarly, a single protein of 56- or 52-kD was expressed from the mammalian expression vector, which contained the full-length M140 or M141 gene, pM140/Zeo (-), and pM141/Zeo (-) respectively. These findings indicate the origin of the pM140 and pM141 in infected cells. The sizes of the *in vitro* transcribed/translated proteins from the constructs expressing each open reading frame (ORF) individually correlated with those detected by western blot analysis of the WT-infected cell lysates.

The α-M139 serum immunoprecipitated two proteins expressed from the M139 gene of 75- and 61-kD as expected. Both the α-M140 and α-M141 sera precipitated a single protein of 56- or 52-kD respectively from the *in vitro* transcription/translation lysates expressing either the M140 or M141 gene product. Thus, we conclude that polyclonal antibodies generated against recombinant M139, M140 and M141 proteins recognize these specific viral proteins.

Detected levels of crossreactivity of the antibodies to the singly expressed heterologous protein were minimal. For example, p75M139 and p61M139 were abundantly immunoprecipitated by α-M139 sera and only negligibly immunoprecipitated by the α-M140 or α-M141 sera. Likewise, the α-M140 antisera precipitated only the pM140, but not M139 or M141 proteins. Similar results were obtained with immunoprecipitations of pM141 by the α-M141 sera (Fig. 10B, bottom panel).

A



B

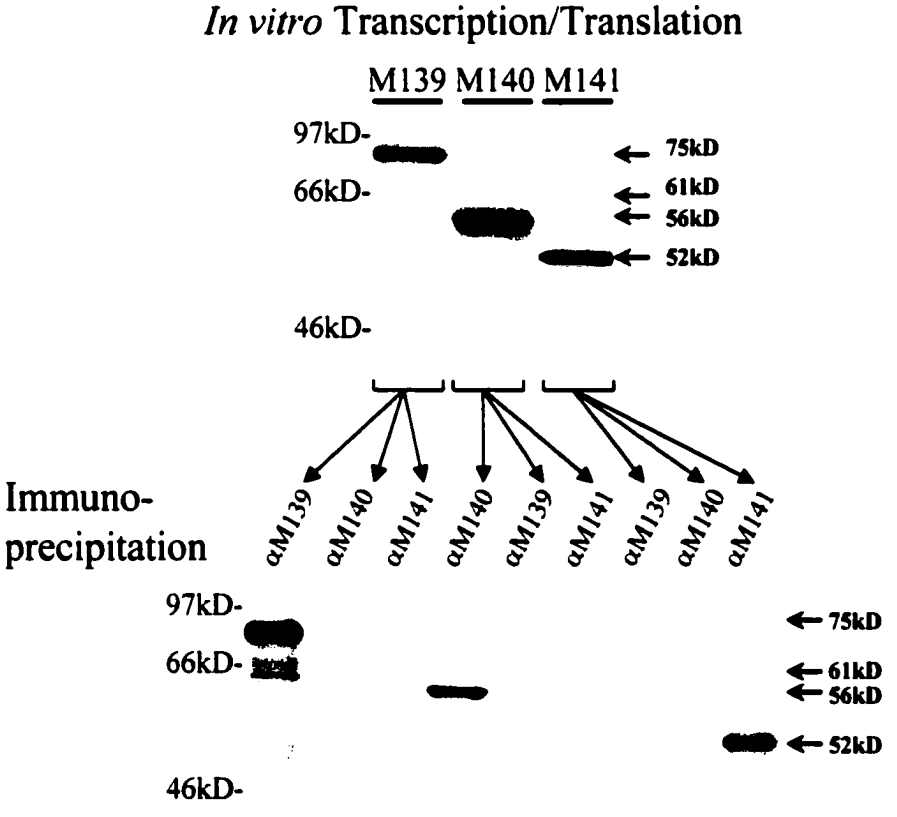


FIG. 10. *In vitro* expression and immunoprecipitation of pM139, pM140, and pM141. (A) An overview of experimental procedures. (B) M139, M140, and M141 proteins were expressed and run on 12.5% acrylamide gel to visualize expressed products (**top panel**). Each lysate was then diluted and used in immunoprecipitation reaction with the antibody indicated. Immune complexes were washed, boiled and run on the 12.5% acrylamide gel. Dried gels were exposed to X-ray film to obtain an autoradiograph (**bottom panel**). Positions of molecular weight makers are indicated on the left side of each autoradiograph. Sizes of the detected proteins are indicated in bold on the right.

These data suggest that M139 and M140 proteins are interacting with other proteins such as

a) viral proteins from within, or outside of, the M139-M141 region, or

b) cellular proteins either constitutively expressed, or induced by viral infection. Furthermore, because two out of the three additional proteins co-immunoprecipitated with α-M139 sera were identical to ones precipitated with α-M140 (56-and 52-kD) and the one protein immunoprecipitated with α-M141 sera is of 52-kD, it was logical to suggest that these proteins form a complex. If pM139, pM140 and pM141 form a complex in infected cells, then antibodies to each of these proteins should immunoprecipitate identical protein profiles, unless the quaternary structure of the complex is such that the antigenic epitopes of the underlying protein are hindered from the specific antibody by other proteins. Interestingly, the 98-kD protein was not detected in these experiments, suggesting that this protein originates from other non-related viral or cellular genes. As a first step in understanding these interactions, we decided to test if pM139, pM140 and pM141 are indeed involved in a complex formed by these proteins alone, or with other viral or cellular proteins.

Assessment of the complex formation by the M139, M140 and M141 proteins.

To provide evidence that pM139, pM140 and pM141 form a complex among themselves or other unrelated proteins, we designed three complimentary approaches:

a) immunoprecipitation of denatured versus non-denatured infected cell lysates

b) sequential immunoprecipitations

c) combination of immunoprecipitation followed by western blot analysis.

One of the biggest advantages of immunoprecipitation analysis is the ability to detect (co-immunoprecipitate) all the proteins directly interacting with the protein of interest when the cell lysates are harvested under non-denatured conditions. Understandably, if one wants to detect which of the immunoprecipitating proteins are specific for a given antibody, and which proteins are co-immunoprecipitating, the cell lysate should first be denatured in order to disrupt existing complexes, and then used in immunoprecipitation analysis. The proteins recaptured from the denatured cell lysates will reveal those specific for the antibody used in the analysis, and undetected proteins would correspond to those directly interacting with identified protein(s). We applied this rational to prove our hypothesis that pM139 and pM140 are parts of a complex or complexes. The procedure, described in details in Materials and Methods section, is outlined in Figure 11A.

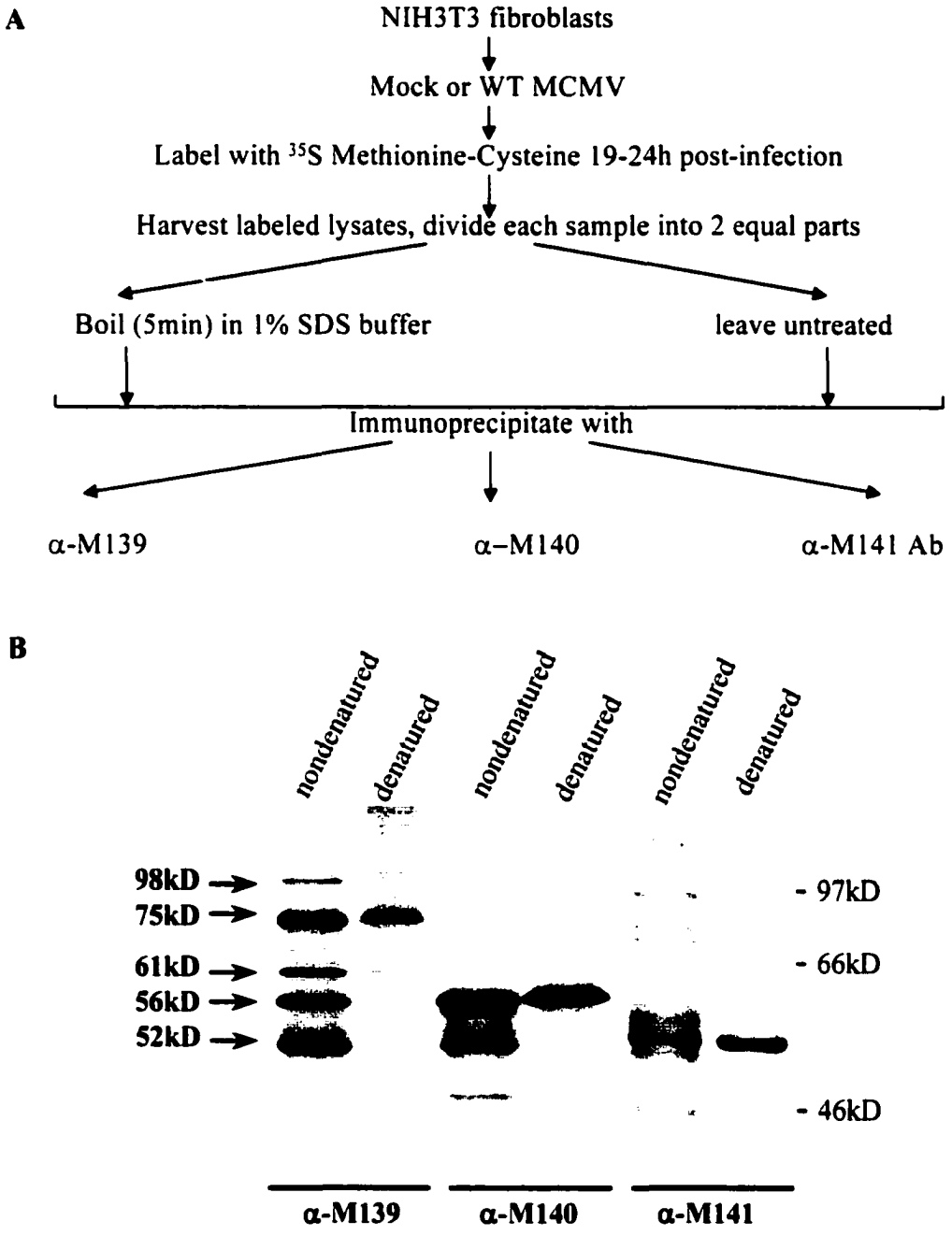


FIG. 11. Immunoprecipitation of denatured infected cell proteins compared to non-denatured proteins. (A). An overview of experimental procedures. (B) Non-denatured and denatured lysates were IP-ed with the indicated antibody and run on 12.5% acrylamide gel. Dried gels were exposed to X-ray film to obtain an autoradiograph. Positions of molecular weight makers are indicated on the right side of the autoradiograph. Sizes of the detected proteins are indicated in bold on the left.

The prototypic protein profiles were resolved with α-M139, α-M140, or α-M141 specific antibodies immunoprecipitating these proteins from non-denatured cell lysates. However, upon denaturing, the three additional bands (98-, 56-, 52-kD), undetectable by western blotting, were no longer detected in the α-M139 immunoprecipitated profile. Likewise, the one band (52-kD) undetectable by western blotting was no longer immunoprecipitated by the α-M140 antibody from denatured lysates (Fig. 10B). The 52-kD protein was precipitated by α-M141 Ab from non-denatured cell lysates as well as from denatured cell lysates, previously detected by western blot analysis. Therefore, the co-precipitation depended on the integrity of native proteins in non-denatured form. Collectively these data support the hypothesis of complex formation by M139 proteins and pM140.

The second approach to confirm complex formation consisted of sequential immunoprecipitations. It also served to verify the origin of the co-immunoprecipitating bands in WT-infected cells. NIH3T3 fibroblasts were infected with wild type MCMV, radiolabeled at late times post infection, harvested, and immunoprecipitated with either α-M139 or α-M140 Abs. Next, precipitated immune complexes were resuspended in phosphate buffered saline (PBS) supplemented with SDS to a final concentration of 1% and denatured by boiling for five minutes. Denatured supernatants were separated from the Protein A agarose beads, diluted 10-fold, divided into equal aliquots, and re-immunoprecipitated with either α-M139, α-M140, or α-M141 specific antibodies (Fig. 12).

The resulting autoradiograph showed that the α-M139 antibody co-precipitates the M140 and M141 proteins, whereas the α-M140 antibody co-precipitates only the

M141 protein. These data suggest that either one or both M139 proteins directly interact with M140 and M141 gene products and that pM140 forms a complex with pM141. However, it is still unclear if the M140-M141 complex is a prerequisite for p75M139 and p61M139 association with this complex.

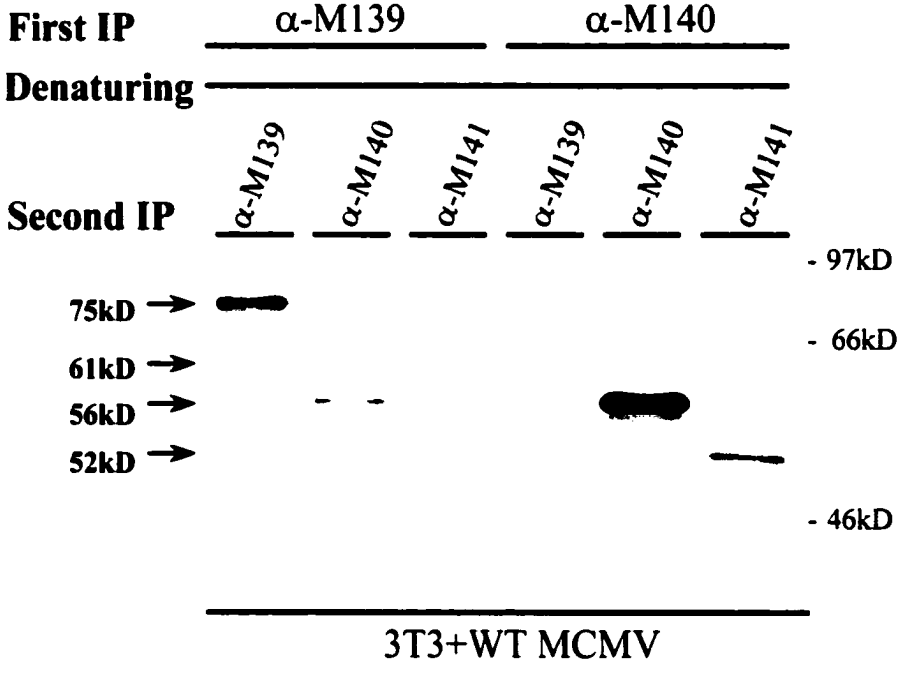


FIG. 12. Sequential immunoprecipitation analysis. Positions of molecular weight markers are indicated on the right side of the autoradiograph. Sizes of the detected proteins are indicated in bold on the left.

It is also evident from these data that α-M140 Ab does not precipitate the M139 proteins. Again, this may be due to quaternary structure of the complex, which masks antigenic epitopes.

The third approach in assessing complex formation among M139 proteins, pM140, and pM141 was based on the notion that unlabeled immunoprecipitated proteins can be subsequently detected by western blot analysis. The technical difficulty of this

analysis was the fact that the immuniprecipitating antibodies and the primary antibody used in western blot analysis are of the same species (rabbit). As a result, the secondary goat-anti-rabbit-HRP antibodies, required to detect the viral specific proteins during western blot analysis, will also bind to the precipitating antibody, shadowing the specific signal being sought. Therefore, in order to perform this analysis we generated horseradish peroxidase conjugated antibodies, anti-M139-HRP (α-M139-HRP) and anti-M140-HRP (α-M140-HRP) to avoid background detection of rabbit polyclonal antibodies used on immunoprecipitation step.

Specifically, NIH3T3 fibroblasts were infected with WT MCMV or mock infected. At 20 hours post-infection unlabeled cell lysates were harvested and immunoprecipitated with α-M139, α-M140, or α-M141 specific antibodies. Obtained immune complexes were washed and duplicate sets of samples were resolved by SDS-PAGE. Subsequently the proteins were electroblotted to the membrane and probed with either anti-M139-HRP or anti-M140-HRP conjugated antibodies (Fig. 13A,B).

The α-M139 Ab precipitated both M139 proteins of 75- and 61-kD, which were visualized by the subsequent western blot analysis probed with α-M139-HRP. Once again, the α-M139 antibody co-precipitated pM140 detected by western blotting probed with α-M140-HRP. As expected, pM140 was detected in immunoprecipitations followed by western blotting using the α-M140 Abs in both the IP and western blotting steps. These results corroborate those of the sequential immunoprecipitation experiments. Namely, α-M139 antiserum co-precipitated pM140, but α-M140 antisera did not co-precipitate either of the two M139 proteins.

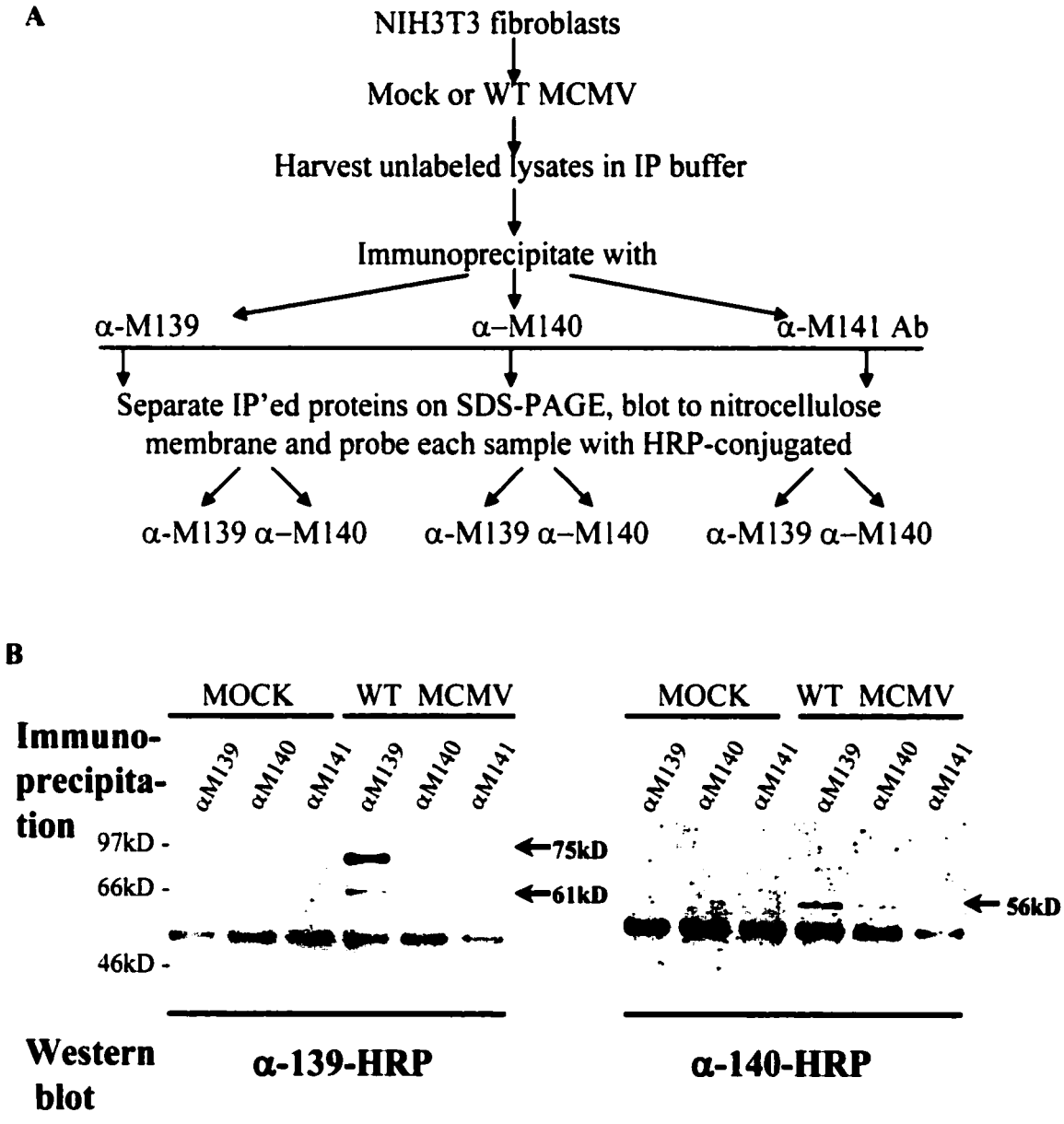


FIG. 13. Immunoprecipitation followed by western blot analysis. (A) An overview of experimental procedures. (B) Unlabeled mock- or WT-infected cell lysates were harvested and immunoprecipitated with either α-M139, or α-M140, or α-M141 antisera. Immune complexes were washed, boiled and run on a 12.5% acrylamide gel for further western blot analysis. The western blots were probed with either anti-M139-HRP or anti-M140-HRP conjugated antibodies. Positions of molecular weight makers are indicated on the left. Sizes of the detected proteins are indicated in bold on the right.

We suspect that pM141 was co-precipitated with M139 and M140 proteins in α-M139 and α-M140 immunoprecipitations, respectively. However we could not prove these speculations because first, we did not have α-M141-HRP conjugated antibody, and second, the 53-kD heavy chain of rabbit IgG would mask detection of the 52-kD pM141. The rabbit IgG heavy chain is detected in this assay, probably, because of non-specific interaction of rabbit HRP-conjugated antibodies with excess amounts of rabbit immunoprecipitating antibodies regardless of specificity, which becomes detectable upon addition of immunofluorescent reagents. Unexpectedly, this phenomenon served as an internal control for protein loading, which appears to be relatively uniform among the samples. There were not any specific bands detected in the mock-infected cell lysates.

Collectively, these three described experiments suggest that one or both M139 proteins complex with pM140 and pM141, and the M140 gene product directly interacts with pM141. However, the discrepancies in protein profiles immunoprecipitating with different antibodies still need to be explained, because all antibodies do not co-precipitate all the components of the predicted larger complex. Is there more that one complex? Do these proteins exist in both free and complexed forms? Is there crossreactivity of an antibody with a complex? We began to approach all these questions first by determining the involvement of pM141 in the interaction with M139 and M140 gene products.

Identification of the role of the M141 protein in complex formation.

The α-M141 antibody immunoprecipitated a protein of 52-kD from both MCMV-infected fibroblasts and macrophages. Interestingly, this protein profile is identical in western blot and immunoprecipitation analysis, however the levels of detected protein in

western blot are much higher (Hanson et al., in press) compared to immunoprecipitations, although neither of these assays is quantitative. In addition, pM141 is abundantly co-immunoprecipitated by both α-M139 and α-M140 Abs, although the α-M141 antisera do not co-precipitate M139 or M140 proteins.

To explain these results, we propose that pM141 is buried inside one or more complexes in its native non-denatured form and therefore is not accessible to the α-M140 antibody. However, newly synthesized free form of pM141 that yet is not incorporated into potential complexes remains accessible to the α-M140 antibody. More specifically, the complex configuration hides antigenic epitopes of the M141 protein from the α-M141 antibody to the level that the antibody is not able to interact with complexed pM141. Therefore, α-M141 antibody is not able to co-precipitate those proteins directly interacting with the M141 gene product. If our hypothesis is true, then the pM141-depleted infected cell lysates should still contain pM141 as a part of the complex with pM139 and pM140 proteins, together or separately. Indirectly this hypothesis is supported by the fact that once the complexes are denatured and the components are resolved (as it is in western blot analysis), pM141 is detected at similar levels compared to p75M139, p61M139, and pM140 (Hanson et al., in press).

To test this hypothesis further we designed the following experimental approach. NIH3T3 fibroblasts were infected with WT MCMV or mock-infected. These cells were radiolabeled between 19 and 23 hours post-infection, harvested, and the lysates were immunoprecipitated with α-M141 antibody as described in Materials and Methods.

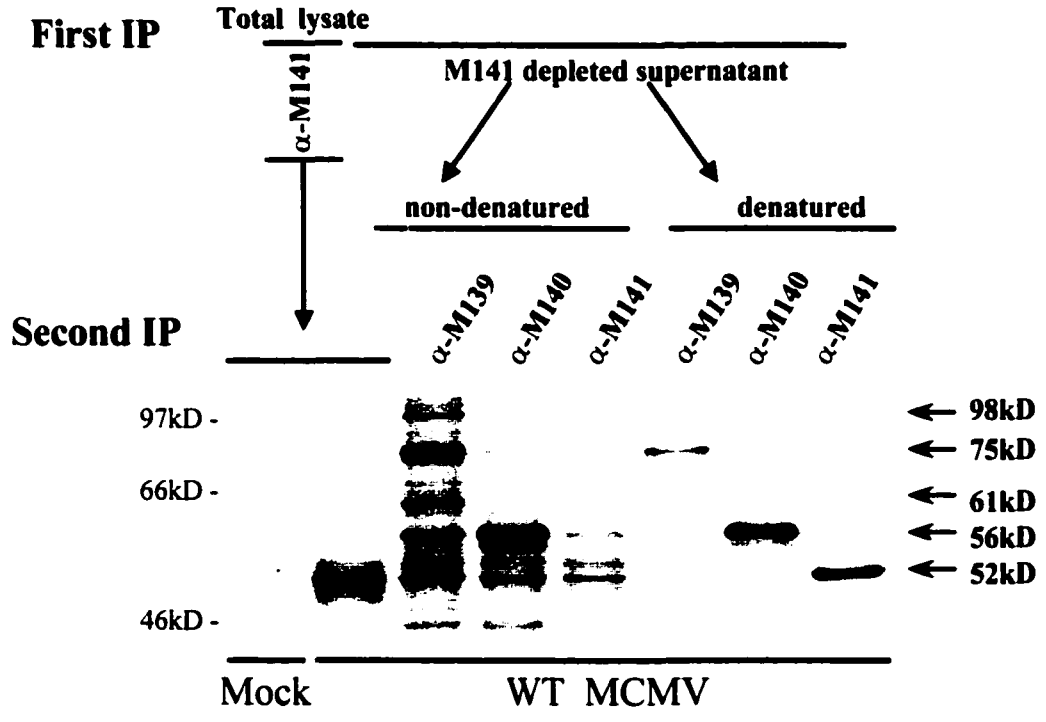


FIG. 14. Identification of M141 protein as a part of one or more complexes. Infected NIH3T3 fibroblasts were radiolabeled, harvested, and immunoprecipitated (IP) with anti-M141 antisera. The beads with immunoprecipitated M141 protein were set aside and the M141-depleted supernatant was divided into two parts and was either used directly in the second IP reaction or denatured (boiled 5 min in 1% SDS buffer) and then used in the second IP reaction. The antibodies used are indicated. All immune complexes obtained after first and second IPs were processed identically and according to previously described protocol. Positions of molecular weight makers are indicated on the left. Sizes of the detected proteins are indicated in bold on the right.

Following the first IP, the beads with immunoprecipitated M141 protein were set aside and the M141-depleted supernatant was divided into two parts. One part was used directly in the second IP reaction to prove that any assembled complex(es) remained in the supernatant fraction and that the complex(es) still contained M141 protein. The other identical part was denatured (boiled 5 min in 1% SDS buffer) and then used in the second IP reaction to identify the parts of the complex(es) (Fig. 14).

The 52-kD protein was precipitated by α-M141 Ab from infected cells lysates and no proteins were detected in mock-infected cells in the first round of

immunoprecipitations, reproducing previous results. Interestingly, when pM141-depleted supernatant was re-immunoprecipitated with α-M139 and α-M140 Abs, the prototypical protein profiles, including M141 protein, were recovered in both cases (Fig. 14). This proves that complexes do indeed remain in the supernatants after α-M141 immunoprecipitation, and that the α-M141 antibody is not able to precipitate the complexes despite the fact that pM141 is still a component of the complex. Moreover, α-M139 antibodies recaptured both M139 proteins from the same supernatants following denaturing. Similarly, the α-M140 antibodies immunoprecipitated a single 56-kD protein from identically treated supernatants. As expected, much lower amounts of pM141 was immunoprecipitated from the pM141-depleted supernatants in the following round. However, the levels of pM141 increased dramatically subsequent to denaturing of the identical supernatants. We hypothesize that denaturing releases previously complexed pM141 and makes it again assessable to α-M141 antibody in perfect agreement with our hypothesis. Following the changes in the levels of detected M141 protein we postulate that the M141 gene product is present in infected cells:

a) in a free form which is immunoprecipitable by α-M141 antibody,

b) as a part of a complex with pM140 immunoprecipitable mainly by α-M140 Ab, and possibly,

c) as a component of pM140-pM141 oligomer complexed with M139 protein(s), which is immunoprecipitated by α-M139 Ab.

Our model predicts that the complex(es) hinders the epitopes of the pM141; therefore α-M141 antibody interacts predominantly with the free uncomplexed form of the M141 protein.

At this time we propose a working hypothesis explaining our results. The M141 protein is the first to initiate the complex. After pM141 is synthesized it is associated with the M140 protein generating the M140-M141 oligomer. Then one or both M139 proteins associate with the preexisting oligomer generating the larger complex (Fig. 15).

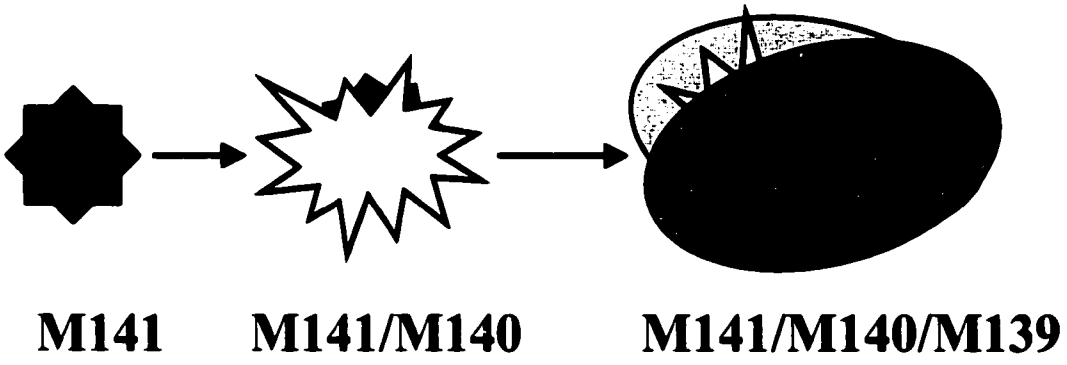


FIG. 15. Possible interaction among the M139, M140 and M141 gene products.

It is likely that a distinct viral or cellular protein of 98-kD is then able to interact with this complicated structure without affecting the ability of α-M139 Ab to immunoprecipitate the complex. At each step the underlying protein is being hidden from the appropriate antibody, which explains the differences in resolved protein profiles.

Identification of the complexed proteins using a genetic approach

As stated above, the M139, M140 and M141 genes belong to the US22 gene family. They are expressed with the same early-late kinetics, and messages originating from the M139-M141 region are 3'co-terminal. To this point we have also demonstrated that the M139, M140 and M141 gene products interact with each other. Taken together

this information indicated a possible cooperation or interdependence of function of these proteins.

In our laboratory a repertoire of recombinant MCMV was generated by homologous recombination. These viruses contain mutations within M139, M140 and M141 genes separately and in combinations. A unique phenotype was found when all three genes were deleted in terms of replication of mutant virus in macrophages. The MCMV ΔM139-141 replicates 100-1000 times less efficiently in IC-21 macrophages, although it grows like WT in cultured fibroblasts (Hanson, et al., 1999). These effects were confirmed *in vivo* when ΔM139-141 virus was found to be highly attenuated in spleen tissue of susceptible mice and was not lethal for SCID mice. The attenuation depended on the inability of the mutant virus to replicate in macrophages *in vivo* because macrophage depletion prior to infection restored the ability of the virus to replicate in the spleens of infected animals to the levels comparable to WT replication (Hanson et al., 1999).

Studies with single gene mutants demonstrated that the recombinant virus containing mutation within the M139 gene exhibited a wild type-like phenotype *in vitro* and *in vivo*. In contrast, the M140 deletion mutant replicated like a ΔM139-141 virus. It was tempting to conclude that the M140 protein is the one that influences viral replication in macrophages both *in vitro* and *in vivo*, but analysis of the growth properties of the M141 mutant reproducibly resulted in an intermediate phenotype for replication in macrophages *in vitro* and spleen tissue *in vivo*. Transcription analysis showed that introduced mutations altered exclusively expression from the mutated gene, and did not affect transcription from neighboring genes. However, deletion of M140 resulted in a

significant decrease in pM141 steady state levels, without alterations in levels of M139 proteins (Hanson et al., in press). The dependence of pM141 on pM140 for stability is consistent with our hypothesis that these two proteins form a complex.

We utilized these mutant viruses to provide a genetic approach to demonstrate the origin of the immunoprecipitated proteins. NIH3T3 fibroblasts were infected with WT MCMV or with the described recombinant viruses (see Introduction section). Infected cells were radiolabeled between 19-23 hours post-infection and harvested as described in Materials and Methods. Each lysate was immunoprecipitated with α-M139, α-M140 and α-M141 Abs or with preimmune sera as a negative control. Immune complexes were processed and resolved by SDS-PAGE as described in Materials and Methods (Fig. 16A).

The prototypical protein profiles were reproduced in immunoprecipitation of WT MCMV-infected cells. Five proteins were immunoprecipitated with α-M139 Ab. Once again the 75-, 56-, and 52-kD proteins were more abundant than the 98-and 61-kD proteins. Two abundant proteins (56- and 52-kD) were immunoprecipitated by the α-M140 Ab with slight prevalence of the 56-kD protein (Fig. 16B, top panel). Lastly, a weak signal was immunoprecipitated by α-M141, identifying one protein of 52-kD.

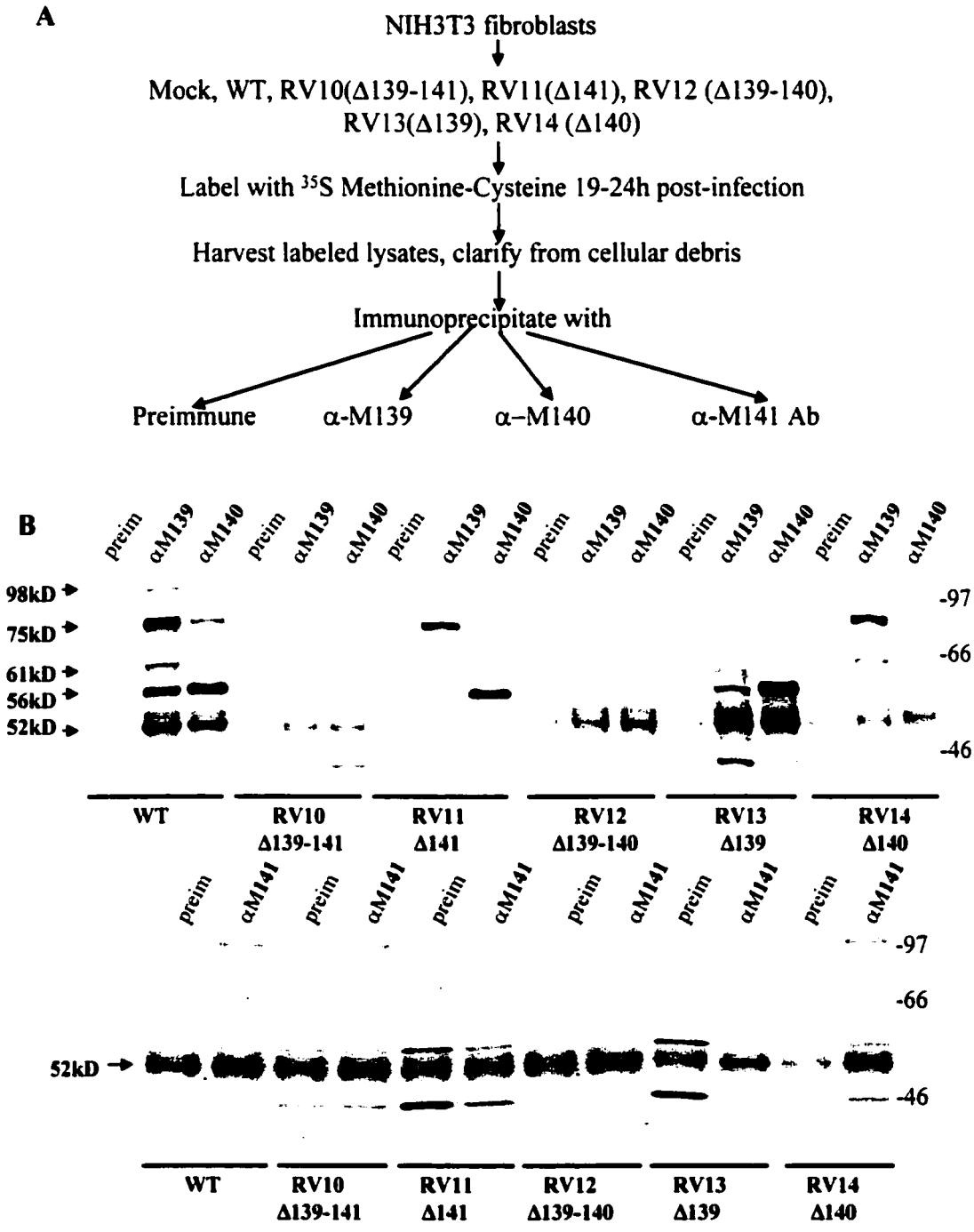


FIG 16. Identification of the complexed proteins using a genetic approach. (A) An overview of experimental procedures. (B) NIH3T3 fibroblasts were infected with either WT or recombinant MCMV as indicated. Cells were radiolabeled, lysed, harvested and immunoprecipitated as described. The positions of molecular weight makers, sizes of detected proteins, and antibodies used are indicated. Δ represents a mutation within the indicated gene.

Detection of pM141 is complicated by two facts. First, the specific signal is initially weak, and second, an unidentified protein non-specifically interacts with rabbit sera regardless of specificity or immunity and co-migrates with pM141, which creates a wide blurred background. Moreover in the absence of the M141 protein the α-M141 antisera interact more strongly with other non-specific proteins of about 46- and 54-kD. However, side-by-side comparison of the bands immunoprecipitated with preimmune sera and α-M141 sera demonstrate the presence or the absence of pM141 (Fig. 16B, bottom panel).

As expected, none of the proteins were detected in RV10 (ΔM139-141)-infected cell lysates. In RV11 (ΔM141)-infected cell lysates the α-M139 Ab precipitated both the 75- and 61-kD proteins. The three additional co-precipitating bands disappeared from the typical α-M139 specific protein profile. Similarly, the α-M140 Ab precipitated one 56-kD protein without co-precipitating the 52-kD protein. As anticipated, no protein was immunoprecipitated by α-M141 Ab under these conditions. Together this verifies that the M141 gene is the origin of the 52-kD protein. It is also suggests that pM141 is necessary for the interaction between M139 proteins and pM140. Because RV12 contains a deletion of both M139 and M140 genes, none of these specific proteins were immunoprecipitated by the appropriate antibody.

Both proteins originating from the M139 gene were absent following immunoprecipitation of cells infected with RV13 (ΔM139) using α-M139 antiserum. Importantly, this genetically confirms the origin of these proteins. Unexpectedly, the same antibody precipitated both the 56- and 52-kD proteins corresponding to the M140 and M141 genes products, respectively. The same two proteins were precipitated by the

α-M140 serum, and one of them (52-kD) was also abundantly immunoprecipitated by the α-M141 Ab. These data indicated that the pM140-pM141 oligomer exists in the cell without the M139 protein(s) and also raises the possibility that the α-M139 antibody crossreacts with the pM140-pM141 complex.

Lastly, in RV14 (ΔM140)-infected cells, only the two M139 proteins were precipitated by the α-M139 serum, identical to immunoprecipitation results of RV11 infection. As expected, the 56-kD band disappeared from both α-M139 and α-M140 immunoprecipitations, verifying that the M140 gene is the origin of that protein. The M141 protein was not co-precipitated by the α-M139 antibody, which suggests that pM141 does not complex with M139 proteins in the absence of pM140. The data obtained from RV14 (ΔM140) immunoprecipitations indicate that in the absence of pM140, M139 gene products do not interact with pM141, although the M139 and M141 proteins are expressed.

Collectively analyzing the results of immunoprecipitation from the cells infected with the different mutant viruses we concluded that:

1. the M139 ORF encodes the 75- and 61-kD protein. In the absence of either the M140 or M141 gene, the α-M139 Ab immunoprecipitates only these two proteins. However, the α-M139 Ab appears to cross-react with complexed M140-M141 proteins. More specifically, α-M139 Ab reproducibly immunoprecipitates M140 and M141 proteins from RV13 (Δ139) infected cells. We conclude that the structure of the pM140-pM141 complex creates a cross-reactive epitope not present in either native protein.

2. the M140 ORF encodes the 56kDa protein. The α-M140 Ab co-precipitates the M141 protein.
3. the M141 ORF encodes the 52kDa protein and the α-M141 Ab does not co-precipitate additional proteins.
4. in the absence of the M139 gene, the 75kDa and 61kDa proteins are not expressed; however the 56kDa and 52kDa proteins are abundant.

To prove that differences in the amount of the detected protein levels were not due to differences in levels of infection, we immunoprecipitated a fraction of each lysate with an unrelated rabbit polyclonal antisera to an early protein, E1, expressed from the M112-113 gene region. The resulting autoradiographs demonstrated similar levels of E1 proteins (33-, 36-, 38-, 87-kD) in all lysates, verifying comparable levels of infection among the different mutants (Fig. 17).

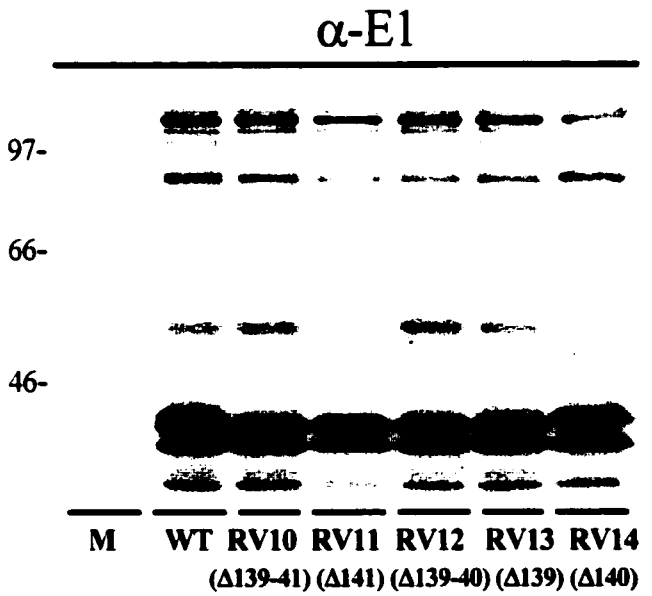


FIG. 17. Immunoprecipitation analysis of WT and mutant MCMVinfected cell lysates using α-E1 rabbit polyclonal antisera. Cells were infected with the indicated viruses. M represents mock-infected cells. One hundred microliters of each lysate (described above) was immunoprecipitated and visualized in accordance with standard IP protocol. Δ represents a mutation within the indicated gene.

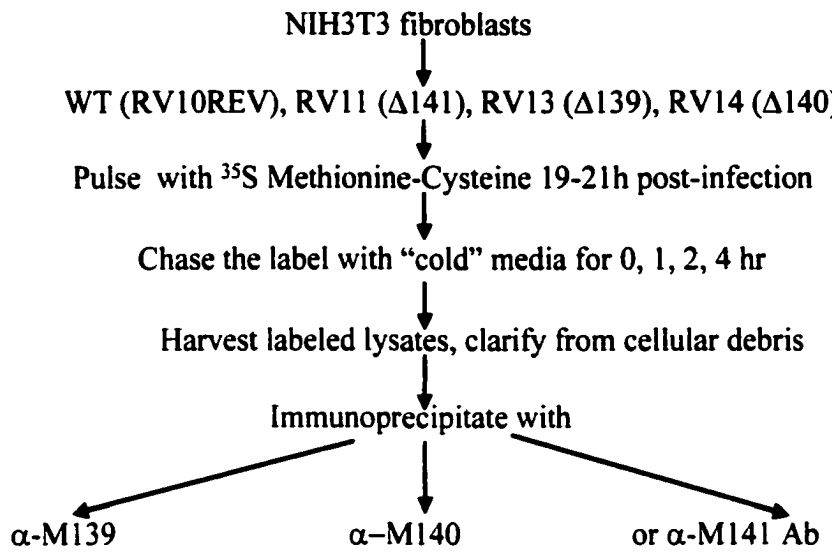
Analysis of the stability of M139, M140 and M141 gene products

To this point, it became evident that the M140 protein forms a complex with pM141. Previously, a decrease in the steady state levels of pM141 was observed in the absence of pM140 detected by western blot analysis of RV14 (ΔM140)-infected fibroblasts and macrophages (Hanson et al., in press). These observations incite the following question: is pM140 required to stabilize the M141 protein and generate the pM140-pM141 complex with a distinct function in MCMV pathogenesis?

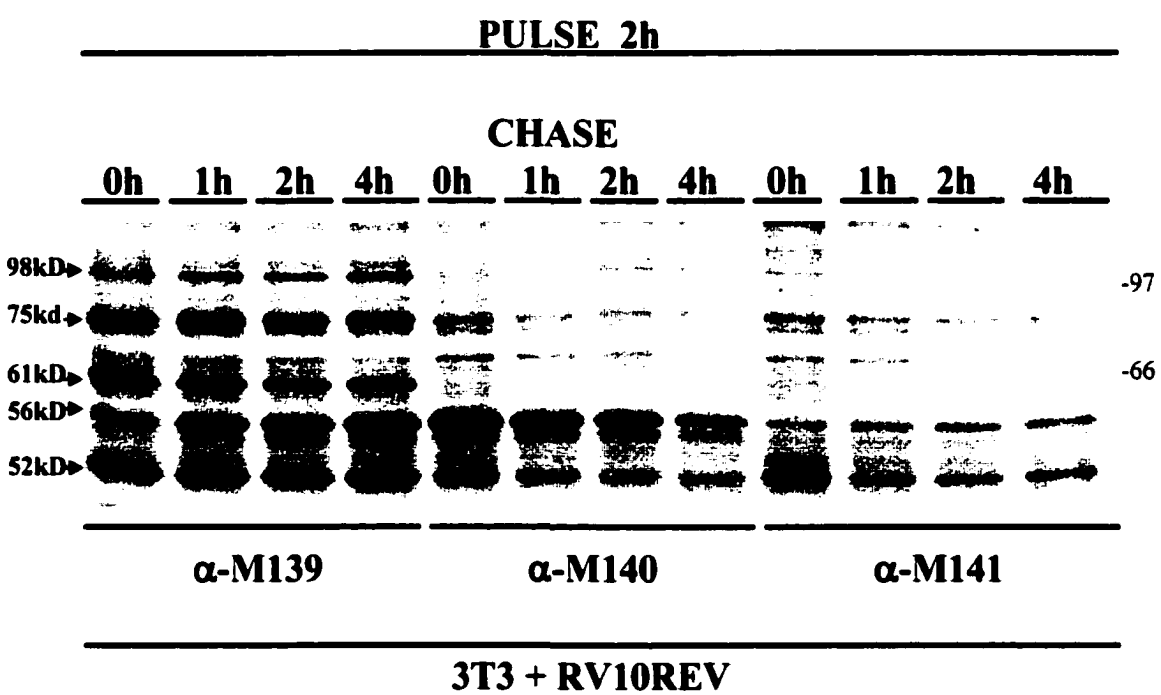
To assess this possibility, we analyzed the stability of these proteins. We conducted the pulse-chase analysis in cells infected with recombinant viruses containing a single mutation within either M139, or M140, or M141 gene, or RV10REV. RV10REV is the revertant virus containing wild type sequence reinserted into the RV10 virus (deleted of the M139-M141 gene region) by homologous recombination. The RV10REV virus has been shown to have wild type growth and replication characteristics (Hanson et al., 1999). Pulse-chase analysis allows one to determine the half-life of newly synthesized proteins and, indirectly, the rate of complex formation.

NIH3T3 fibroblasts were infected with the MCMV single deletion mutants (Fig. 18 C, D, E) or with RV10REV (Fig 18 B). Nineteen hours post-infection, cells were starved of methionine and cysteine for 1 h and then radiolabeled with 450 μCi of [^{35}S] methionine and cysteine protein labeling mix per ml for 2 h. Labeled proteins were chased with complete medium for the indicated times. The cell lysates were harvested, clarified from cellular debris, and immunoprecipitated with α-M139, α-m140, or α-M141 Abs.

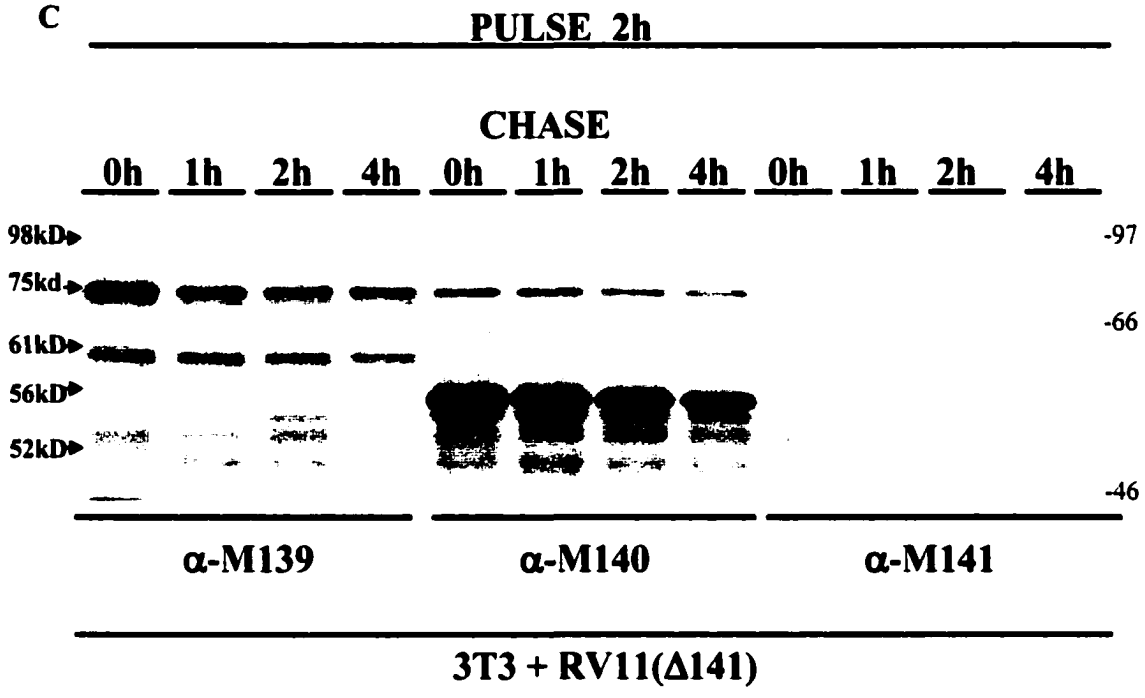
A



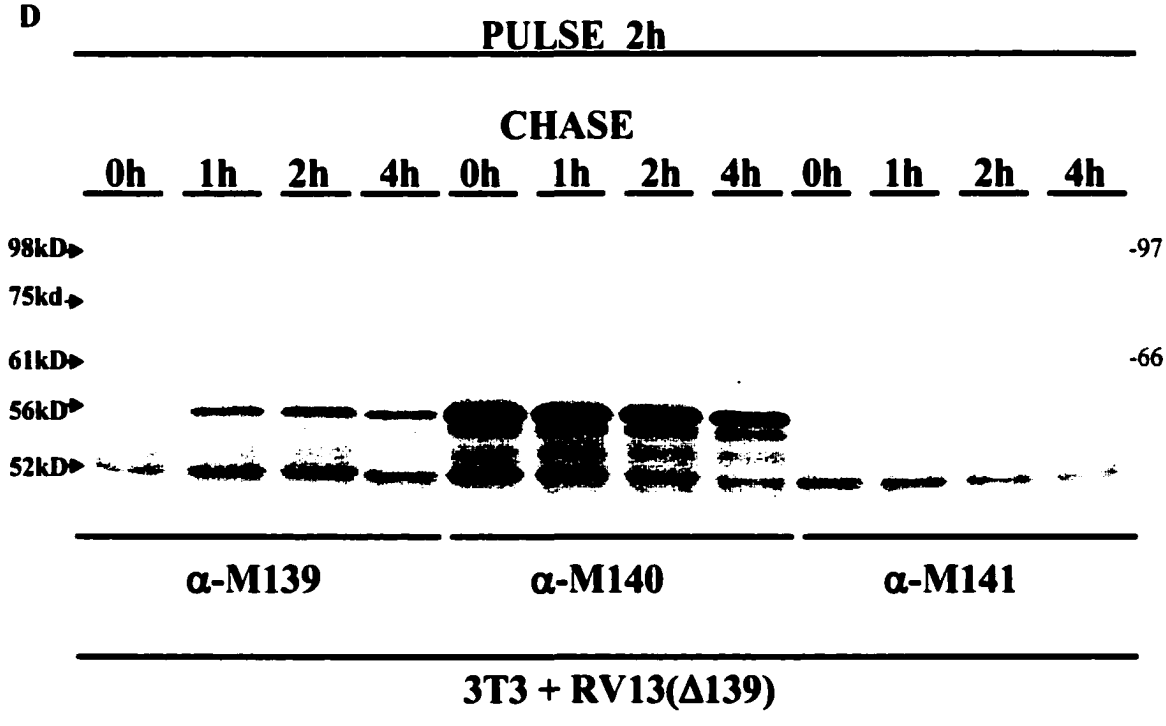
B



C



D



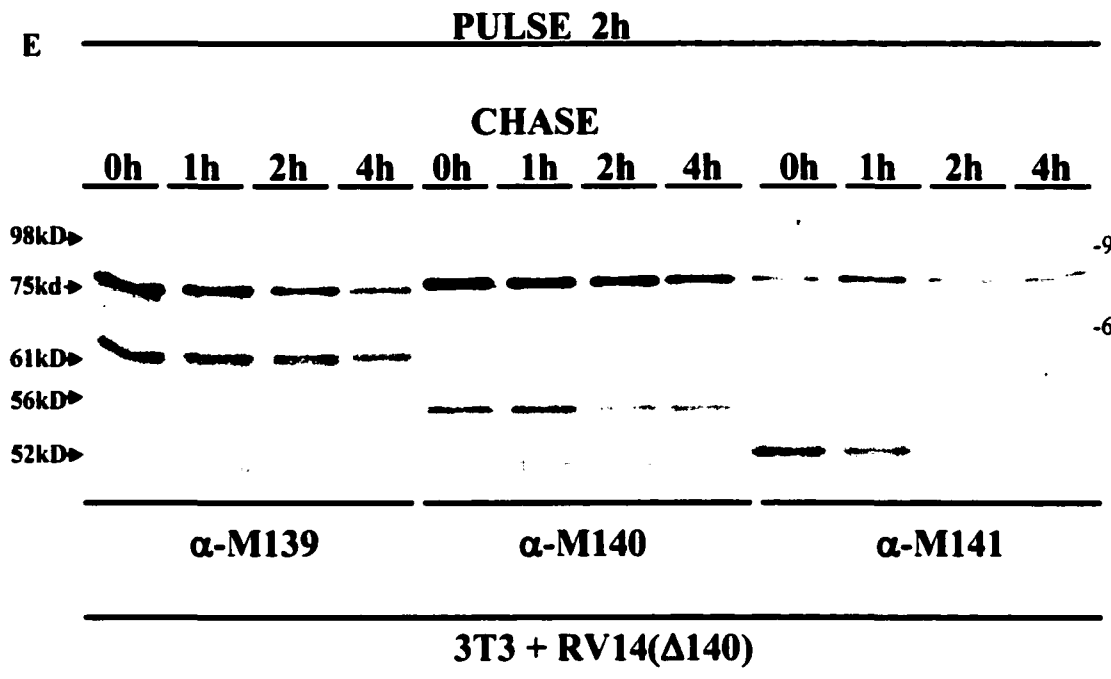


FIG. 18. Pulse-chase analysis. (A) An overview of experimental procedures. NIH3T3 fibroblasts were infected with RV10Rev (B), RV11(ΔM141) (C), RV13(ΔM139) (D), RV14 (ΔM140) (E). Infected cell were labeled for 2 hours, and chased for indicated periods of time. Cell lysates were harvested and immunoprecipitated with the indicated Ab. Immune complexes were processed as described in Materials and Methods. Positions of molecular weight makers are indicated on the right. Protein sizes are indicated in bold on the left. Δ represents a mutation within the indicated gene.

Immunoprecipitated complexes were washed, resuspended, and run on 12.5% acrylamide gel. The gels were dried and exposed to X-ray film to obtain autoradiographs (Fig.18A).

Pulse-chase analysis of wild type MCMV (RV10REV)-infected cells reproduced previous results and prototypical protein profiles were recovered with all three antibodies (Fig. 18B). Five proteins immunoprecipitating with the α-M139 Ab were equally (if not better) detectable from 0 to 4 hours post-chase; therefore the half-life of these five proteins is greater than 4 hours.

The α-M140 Ab precipitated both pM140 and pM141. At 0 hour post-chase the 56-kD band corresponding to pM140 is highly predominant; however, with time the

density of this band decreases and becomes comparable to the intensity of the pM141 specific band (52-kD). The half-life of the pM140 appears to be about 2 hours. It is not clear from these data how much of the newly synthesized pM140 is in the free form, and how much of it is in a complexed form. It is also possible that the free and complexed forms have different half-lives.

The α-M141 antibody again precipitated one prominent protein of 52-kD at 0 hours post-chase. The estimated half-life of the pM141 is 2 hours in the presence of the M139 and M140 proteins. Interestingly, the same antibody co-precipitated increasing amounts of the pM140-specific band over the course of the chase (Fig. 18B). These data support our hypothesis that pM140 and pM141 form a complex, and may explain why the co-precipitating pM140 band was not detectable before. It is possible that the α-M141 Ab has a higher affinity to the free, uncomplexed form of pM141; however, over time, all of the pM141 complexes with the M140 protein. In the absence of free pM141, the α-M141 Ab may be able to coprecipitate the pM140. In fact, the levels of pM140-pM141 oligomer detected by both α-M140 and α-M141 antibodies at 4 hours post-chase are very similar. Conversely, none of the M139 proteins become detectable in either α-M140 or α-M141 immunoprecipitated profiles.

In the cells infected with RV11 (ΔM141) (Figure 18C), the M139 and M140 proteins demonstrated a rate of degradation similar to that detected in RV10REV-infected cells. The pM141 was absent in correlation with the mutation of RV11 (ΔM141) recombinant virus. This indicates that the stability of the M139 and M140 gene products do not depend on the presence of pM141. A protein closely migrated with p75M139 was non-specifically detected by both the α-M140 and α-M141 antisera. This was non-

reproducible phenomenon, characteristic to only one of the two antisera developed for each protein.

In RV13 (ΔM139)-infected cells (Fig.18D), the M140 and M141 proteins were abundantly expressed and their rate of degradation were similar to those seen in RV10REV-infected cells, even in the absence of pM139. Therefore, the stability of pM140 was not influenced by the M139 proteins. Again, pM140 and pM141 were able to form a complex, readily detected by α-M140 and to a lesser extent by α-M141 at 4 hour post-chase. Although both of the M139 proteins were absent, the α-M139 Ab once again immunoprecipitated the pM140-pM141 complex with the levels increasing over the time of the chase. This again raised a concern that α-M139 Ab crossreacts with the complexed form of pM140 and pM141. Apparently this complex develops over time, and eventually becomes recognizable by α-M139 Ab.

Finally, when the cells were infected with RV14 (ΔM140) the M139 proteins were detected following α-M139 immunoprecipitation and their rate of degradation was similar to that of WT MCMV-infected cells (Fig. 18E). Once again a non-specific 76-77kD protein was detected by the α-M140 and α-M141 antibodies. As expected, pM140 was not expressed. Interestingly, pM141 was abundantly expressed and readily immunoprecipitated with α-M141 at 0 hour post-chase; however, this protein was quickly degraded, and became almost undetectable by 4 hours post-chase. Densitometrical analysis of the relative intensity of the bands demonstrated that the half-life of M141 was about 1 hour in RV14 (ΔM140)-infected cells, compared to 2 hours in RV10REV- or RV13 (ΔM139)-infected cells. Moreover, by 4 hours post-chase, only 10% of the initial pM141 remained in the RV14-infected cells, in contrast to 40% of the

labeled M141 protein remaining in RV10REV-or RV13 (ΔM139)-infected cells (Fig. 19). This strongly suggests that the stability of the M141 protein depends on the presence of pM140, most likely because these two proteins form a complex, which renders pM141 more stable.

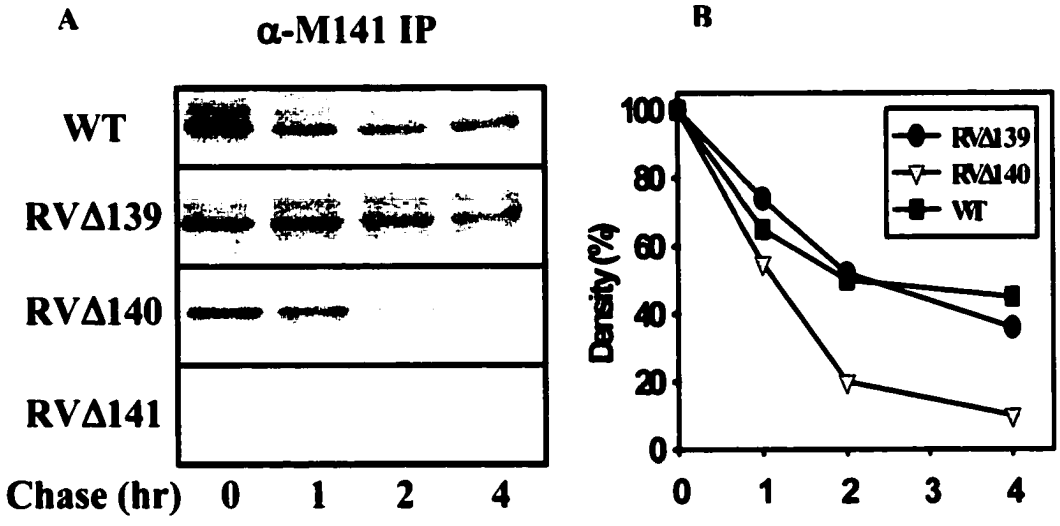


FIG. 19. The relative intensity of pM141-specific bands in cells infected with single deletion MCMV mutants. (A) Autoradiographs of α-M141 pulse-chase analysis of cells infected with indicated viruses, revealing changes in pM141 levels. (B) The plot reflecting relative density of pM141-specific bands in autoradiographs in panel A. Hours post-chase are indicated on the bottom. Δ represents a mutation within the indicated gene.

The pulse-chase analysis of the proteins immunoprecipitated by α-M139 antibody from RV13 (ΔM139)-infected cells corroborated our previous speculation that the α-M139 antiserum cross-reacts with the pM140-pM141 complex. Therefore we developed an alternative hypothesis for possible interactions among the M139, M140, and M141 gene products. Previously we hypothesized that two complexes were immunoprecipitated by α-M140 Ab and by α-M139 Ab sequentially. First the pM140-pM141 complex forms and then association of the M139 proteins with the pM140-pM141 oligomer results in the larger complex among the M139, M140 and M141

proteins. However, an alternative hypothesis is that the M139 proteins do not complex with the pM140-pM141 complex. Instead, this complex is immunoprecipitated by the α-M139 Ab due to a cross-reactivity this antibody with the pM140-pM141 hetero-oligomer. Nevertheless, our previous and now alternative hypotheses may not be mutually exclusive, because the largest 98-kD protein is brought down by α-M139 Ab only from WT-infected cell lysates, and the presence of both M139 proteins, pM140, and pM141 seem to be a prerequisite for that interaction (Fig. 20).

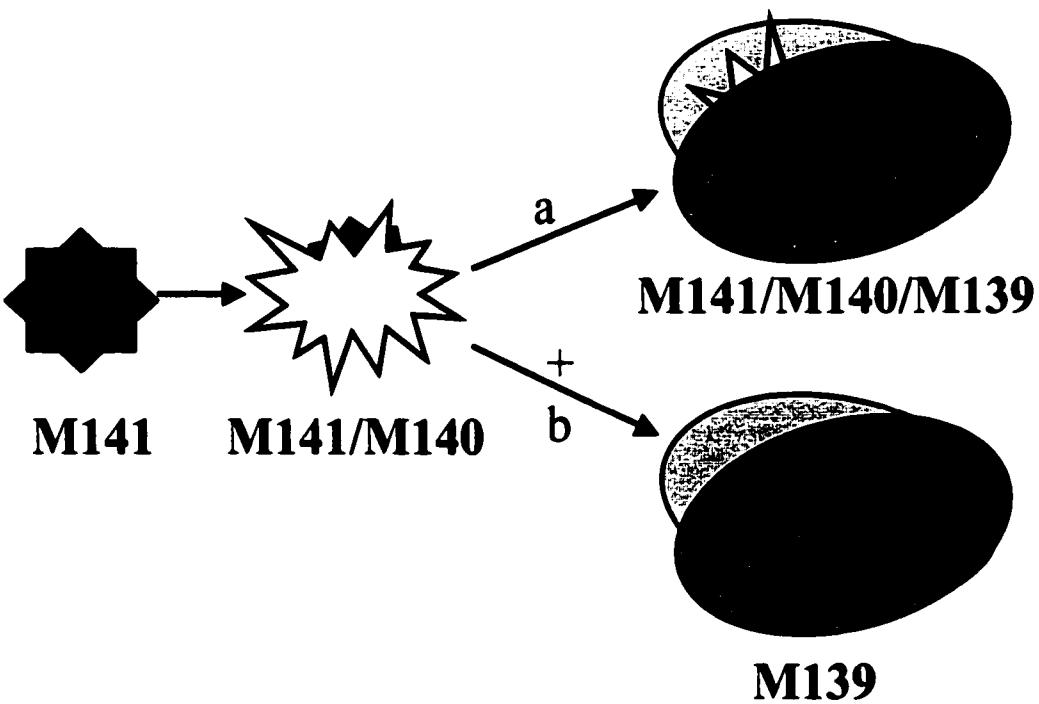


FIG. 20. Two alternatives in interactions among the M139, M140 and M141 gene products.

The last series of experiments were designed to distinguish between the two proposed hypotheses to explain the interaction of the pM140-pM141 complex with pM139 and another 98-kD protein.

Characterization of the interactions between the pM140-pM141 complex and other proteins

If the α-M139 Ab crossreacts with and therefore immunoprecipitates the pM140-pM141 complex, then the pM140 specific band will be visualized in western blotting following α-M139 immunoprecipitation of RV13 (ΔM139) infected cells. To assess this NIH3T3 fibroblasts were infected with RV13 (ΔM139), or with RV14 (ΔM140) or RV11 (ΔM141) mutant MCMV to serve as controls for cross-reactivity with singularly expressed M140 or M141 proteins. The unlabeled lysates were harvested at late times post-infection, and immunoprecipitated with α-M139 Ab. Immune complexes were washed and duplicate samples resolved by SDS-PAGE. The gel was blotted to the membrane and probed with either α-M139-HRP or α-M140-HRP (Fig. 21A).

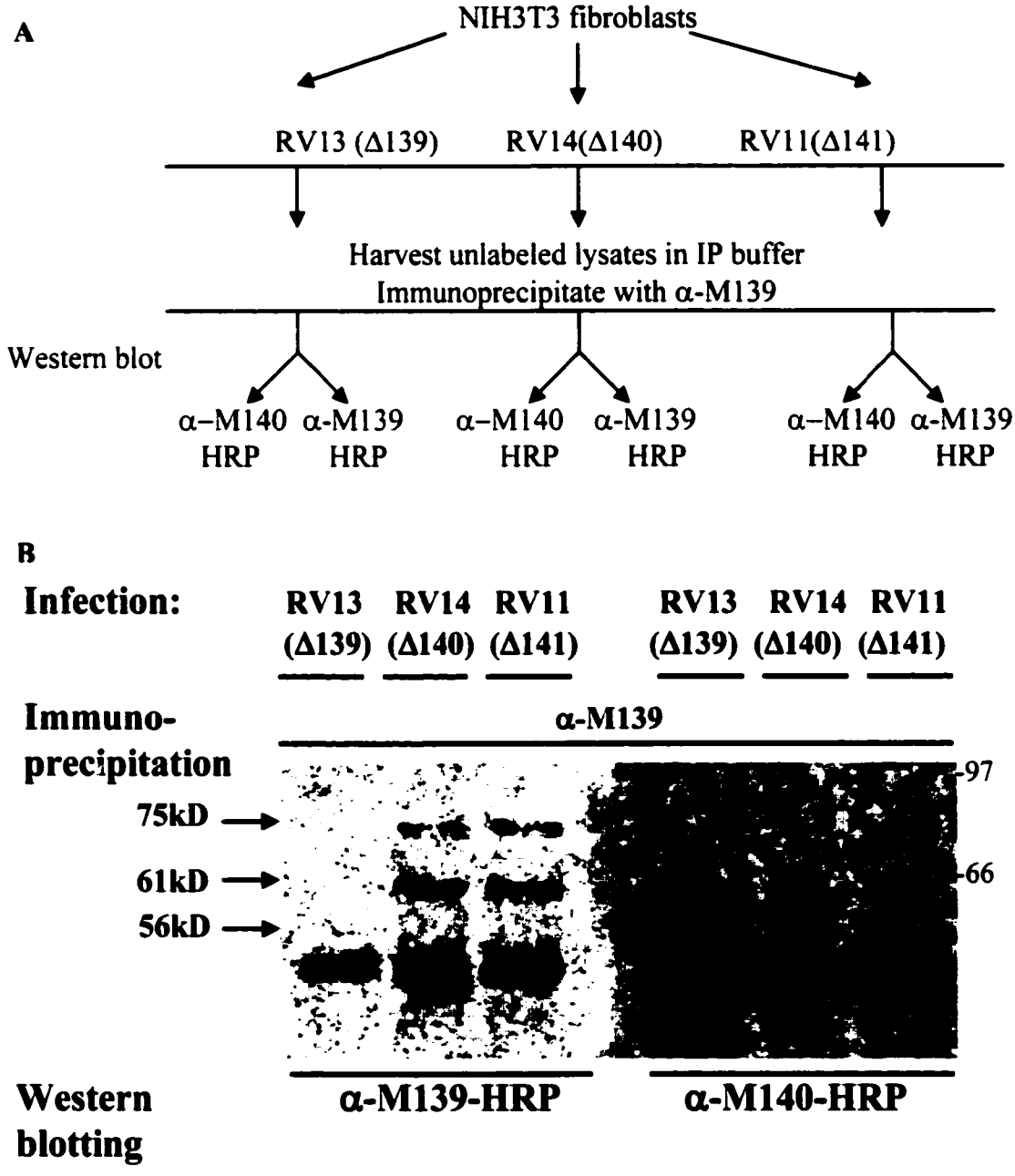


FIG. 21. α-M139 immunoprecipitation followed by western blot analysis. (A) An overview of experimental procedures. (B) NIH 3T3 fibroblasts were infected with mutant viruses as indicated, harvested and immunoprecipitated with α-M139 antisera. Immune complexes were washed, boiled and run on a 12.5% acrylamide gel for further western blot analysis. The western blots were probed with either anti-M139-HRP or anti-M140-HRP conjugated antibodies. Positions of molecular weight makers are indicated on the right. Sizes of detected proteins are indicated in bold on the left. Δ represents a mutation within the indicated gene.

As expected, both M139 proteins were immunoprecipitated from the RV14 (ΔM140) and RV11 (ΔM141) infected cells and absent from cell lysates infected with RV13, as was demonstrated by the subsequent α-M139-HRP western blot analysis. Interestingly, the pM140 specific band was detected by α-M140-HRP western blotting following α-M139 immunoprecipitation of RV13 (ΔM139)-infected cells, but not RV14 (ΔM140)- or RV11 (ΔM141)-infected cells (Fig. 20B). These data indicate that α-M139 Ab does not immunoprecipitate free form (or homo-oligomers) of pM140 or pM141, but rather crossreacts with the pM140-pM141 hetero-oligomer. Crossreactivity of the α-M139 Ab does not exclude the possibility that pM139 proteins also directly interact with the pM140-pM141 complex. Perhaps the two latter proteins are immunoprecipitated by α-M139 Ab from WT MCMV-infected cells not only because of the identified crossreactivity, but also because all of these proteins generate another larger complex.

The use of a transiently expressed epitope tagged M139 expression vector as a source of M139 proteins in combination with superinfection by either WT MCMV or RV13 (ΔM139) MCMV allowed us to assess these possibilities more directly. Specifically, NIH3T3 fibroblasts were transiently transfected with a vector expressing N-terminally His-tagged pM139, or the empty vector. Forty-eight hours after transfection the cells were superinfected with either WT MCMV or RV13 (ΔM139). These cells were radiolabeled between 19-23 hours post-infection, and the label was chased for two hours, allowing time for the labeled proteins to form complexes. WT MCMV-superinfected cell lysates were harvested and immunoprecipitated with either α-M139 or α-His antibodies, whereas RV13 (ΔM139)-superinfected cells were immunoprecipitated only with α-M139 Ab, to served as control for the ability of His-tagged pM139 to interact with the α-M139

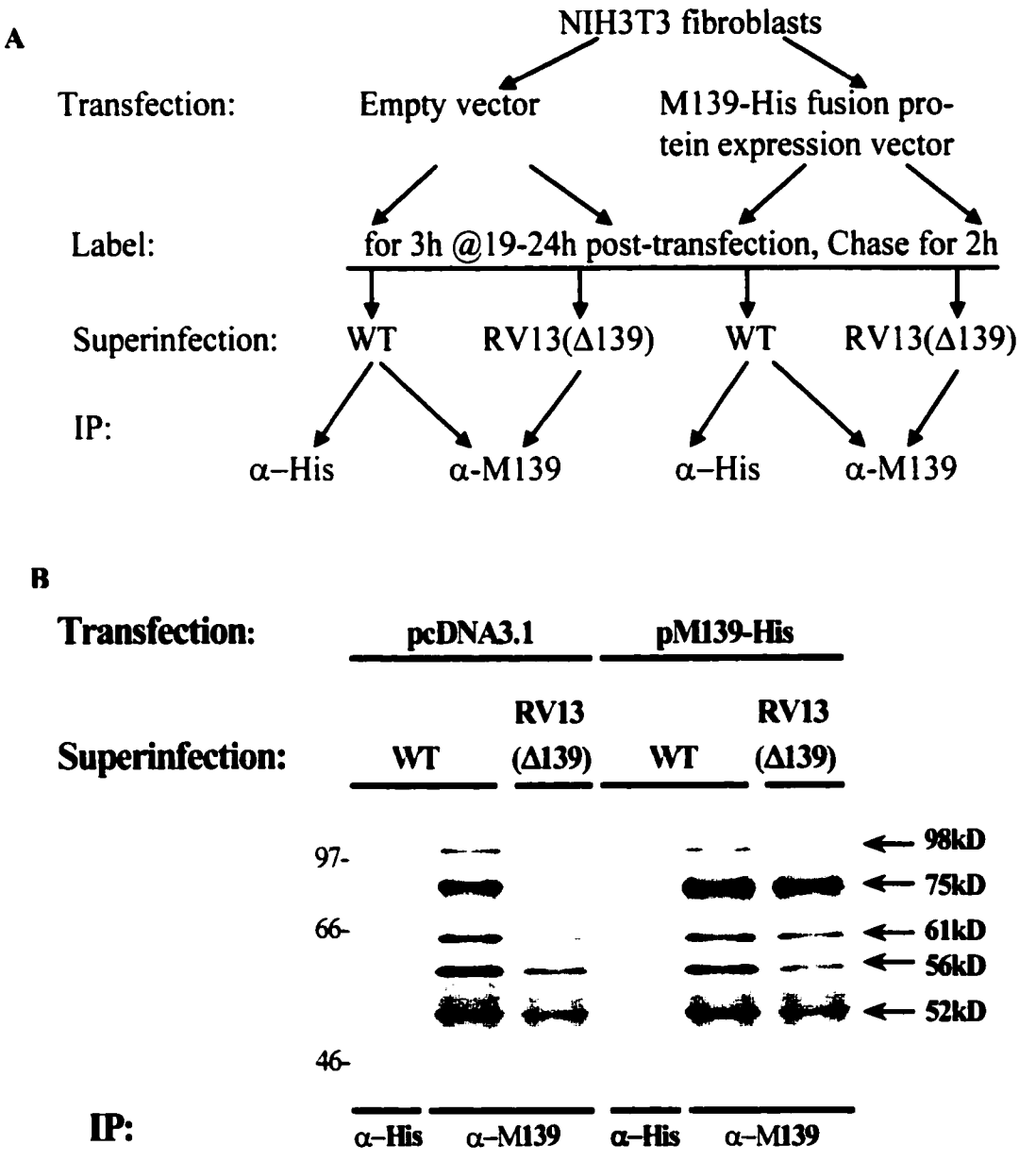


FIG. 22. Immunoprecipitation analysis of transiently transfected-superinfected cell lysates. (A) An overview of experimental procedures. (B) NIH 3T3 fibroblasts were transfected with His-M139 or pcDNA3.1 expression vectors. Cells were superinfected with WT or RV13 MCMV as indicated. Radiolabeled cell lysates were immunoprecipitated with the antibodies indicated. Immune complexes were washed, boiled and run on a 12.5% acrylamide gel. Positions of molecular weight makers are indicated on the left. Sizes of the detected proteins are indicated in bold on the right.

antibody (Fig. 22B). As expected, the α-His antibody did not detect a specific signal in the cells transfected with the empty vector and superinfected with WT MCMV. The prototypical protein profile was immunoprecipitated by the α-M139 antibody from identical lysates. The pM140-pM141 complex was immunoprecipitated by the same antibody from cells transfected with the empty vector and superinfected with RV13 (ΔM139) mutant virus, again indicating the crossreactivity of α-M139 Ab with the pM140-pM141 oligomer. A faint non-specific band similar to 61-kD protein was detected in this immunoprecipitation, which appears non-reproducibly with only one of two α-M139 antisera.

In cells transfected with the vector expressing pM139 fused to a histidine tag at the N-terminus and then superinfected with WT MCMV, the α-His antibody immunoprecipitated only one protein of 75-kD, despite that fact that all five proteins were present in those lysates, as proven by simultaneous immunoprecipitation with α-M139 Ab. These results suggest that a) no other proteins co-precipitate with p75M139 and b) only the 75-kD protein is expressing the histidine tag, indirectly indicating that the 61-kD protein most likely is not a splice variant. There are three potential reasons why the anti-His antibody failed to co-precipitate the pM140-pM141 complex. It is possible that the

1) 61-kD protein is the one responsible for the interaction of the pM140-pM141 complex with pM139 proteins,
2) N-terminally expressed His-tag is buried deep in the complex, therefore not providing enough interaction with the α-His Ab for sufficient immunoprecipitation or

3) M139 proteins do not interact with the pM140-pM141 complex.

The levels of the 75- and 61-kD proteins precipitated with α-M139 Ab from pM139-His transfected and WT MCMV-superinfected cells reflects those expressed collectively from the vector and those synthesized by the WT virus. Whereas in RV13 (ΔM139)-superinfected cells, the 75-and 61-kD band represent the proteins transiently expressed from the vector only. The detection of the 61-kD band is marginal because of the presence of a non-specific band of similar size being immunoprecipitated from RV13 (ΔM139)-superinfected cells. Nevertheless, side-by side comparison of the p75M139 and p61M139 immunoprecipitated from the RV13 (ΔM139)-superinfected cells indicate that both proteins are expressed from the M139-His mammalian expression vector, and also that the His-tag does not interfere with interaction of the expressed proteins with the α-M139 Ab.

Our final approach to assess the interactions between the M139 proteins and the pM140-pM141 complex consisted of transient expression of p75M139, p61M139, pM140, and pM141 *in vitro* (in rabbit reticulocyte lysates), or *in vivo* (in NIH3T3 fibroblasts) followed by immunoprecipitation analysis. This gave us the opportunity to study the interactions among these proteins in the absence of other viral proteins and assess if they were able to form a complex under those conditions.

First, the proteins were expressed *in vitro* using the rabbit reticulocyte lysate system. The products were visualized on the gel, and then an aliquot of each lysate was used in immunoprecipitation with α-M139, α-M140, or α-M141 Abs (Fig. 23). All proteins were expressed in *in vitro* transcription/translation reactions (Fig. 23, top panel). The M140 and M141 proteins were abundantly expressed. When co-expressed with

M140, the M139 proteins and pM141 were expressed in relatively small amounts, possibly due to the number of exclusively methionine residues (12 in full-length M139 gene) in p75M139 and p61M139 coding sequences, because only methionine labeling was used in *in vitro* transcription/translation reactions (see Materials and Methods).

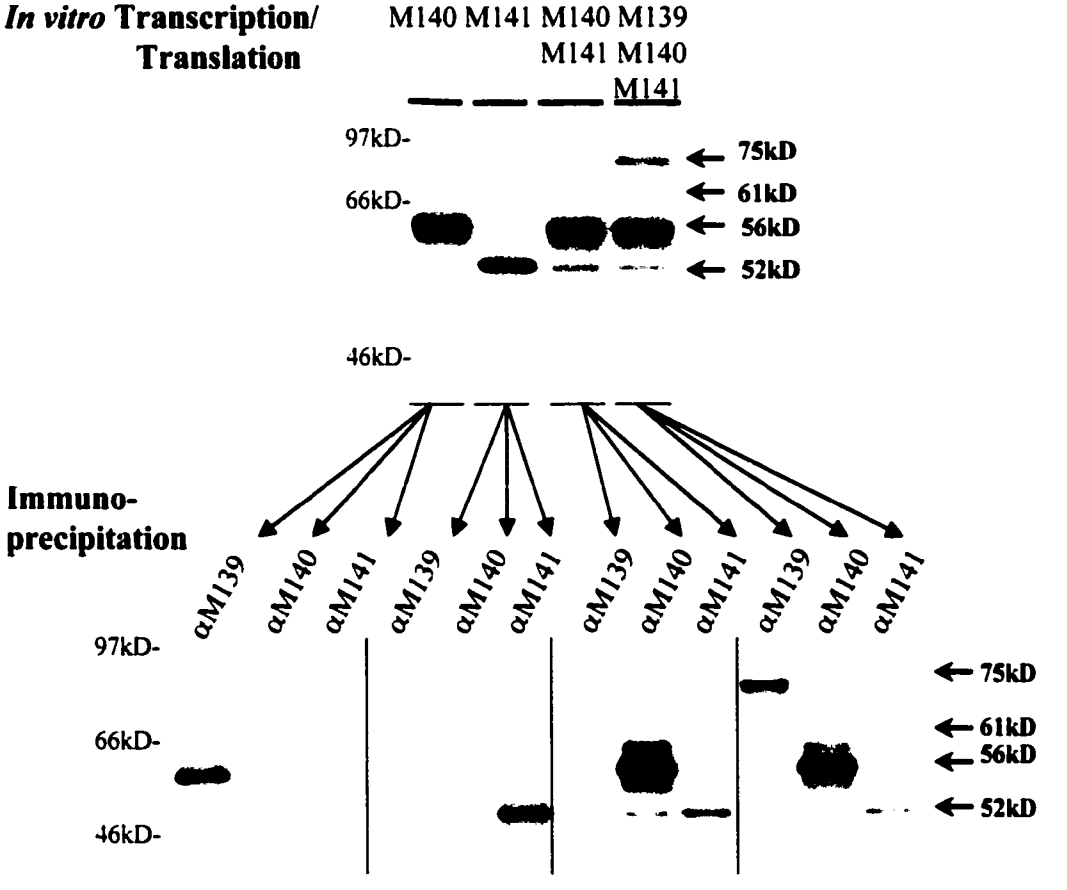


FIG. 23. *In vitro* analysis of interactions among the pM139, pM140, and pM141. M139, M140, and M141 proteins were expressed and run on 12.5% acrylamide gel to visualize expressed products (**top panel**). Each lysate was then diluted and used in immunoprecipitation reaction with the antibody indicated. Immune complexes were washed, boiled and run on the 12.5% acrylamide gel. Dried gels were exposed to X-ray film to obtain an autoradiograph (**bottom panel**). Positions of molecular weight makers are indicated on the left side of each autoradiograph. Sizes of the detected proteins are indicated in bold on the right.

Immunoprecipitations of the lysates expressing either pM140 or pM141 alone confirmed the specificity of the antibodies to the appropriate protein and minimal

crossreactivity among them (Fig. 23, bottom panel). Based on our previous results indicating that the α-M139 Ab cross-reacts with the pM140-pM141 complex, it was surprising that the α-M139 Ab did not precipitate either the M140 or M141 gene products from lysates where both proteins were co-expressed. However, when all the proteins were co-expressed, the α-M139 Ab immunoprecipitated the 75- and 61-kD (visualized better on the longer exposure autoradiograph) proteins, along with pM140 and pM141, indicating complexing of one or both M139 proteins with the M140 and M141 gene products (Fig. 23, bottom panel). Complexing may not be optimal under these non-physiological conditions, which may explain the low levels of co-precipitated pM140 and pM141.

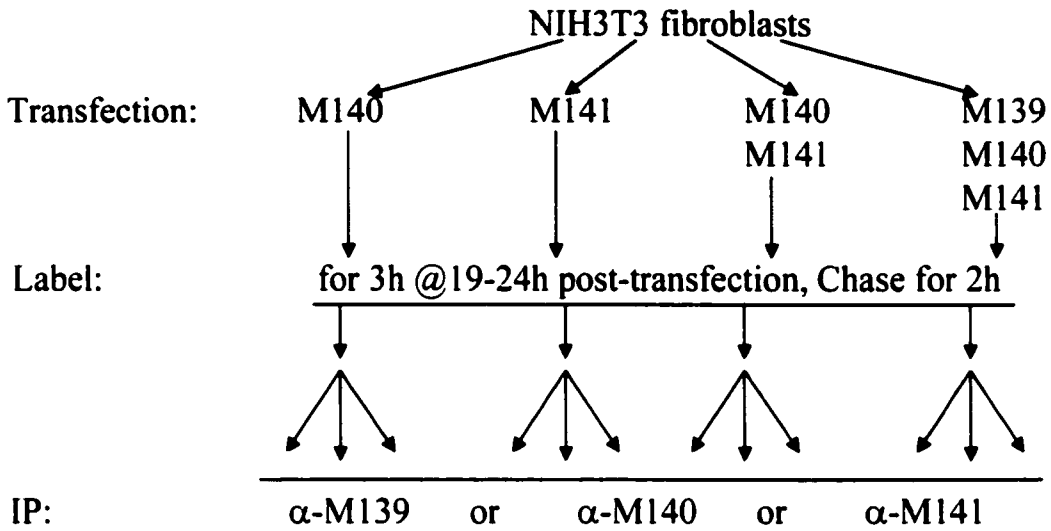
The pM140-pM141 complex was also precipitated with either α-M140 or α-M141 antibodies whenever the two genes were co-expressed in the absence and the presence of pM139. These data support the concept that pM140 forms a complex with pM141, in the absence and the presence of M139 proteins.

The fact that all co-precipitating, and therefore assumed to be complexed, proteins were detected at low levels can be explained by the non-physiological conditions of rabbit reticulocyte lysates, which perhaps do not support stable maintenance of the generated complexes. Alternatively, complex formation may require conditions present only in the microenvironment of the cell.

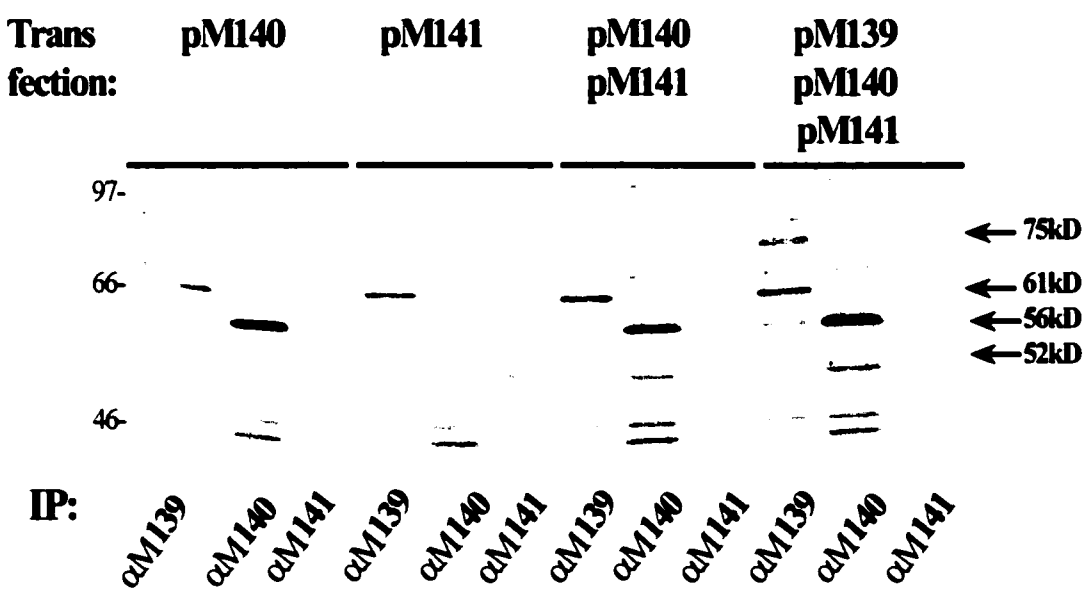
Lastly, immunoprecipitations of NIH3T3 fibroblasts transiently expressing pM139, pM140 and pM141 alone or in combinations provided a means to assess a) direct interactions between the pM140-pM141 complex and M139 proteins, b) efficiency of these proteins to form a complex in the absence of other viral proteins, and c)

crossreactivity of the α-M139 Ab with the pM140-pM141 complex in physiologically relevant conditions. Therefore, NIH3T3 fibroblasts were transiently transfected with the same mammalian expression vectors, encoding pM139, pM140 or pM141. Forty-eight hours post-transfection the cells were radiolabeled for 3 hours, and the label was chased for 2 hour to allow time for the formation of complex(s) by the newly synthesized, labeled proteins. Lysates were harvested and immunoprecipitated with one of the three antibodies (Fig. 24A)

A



B



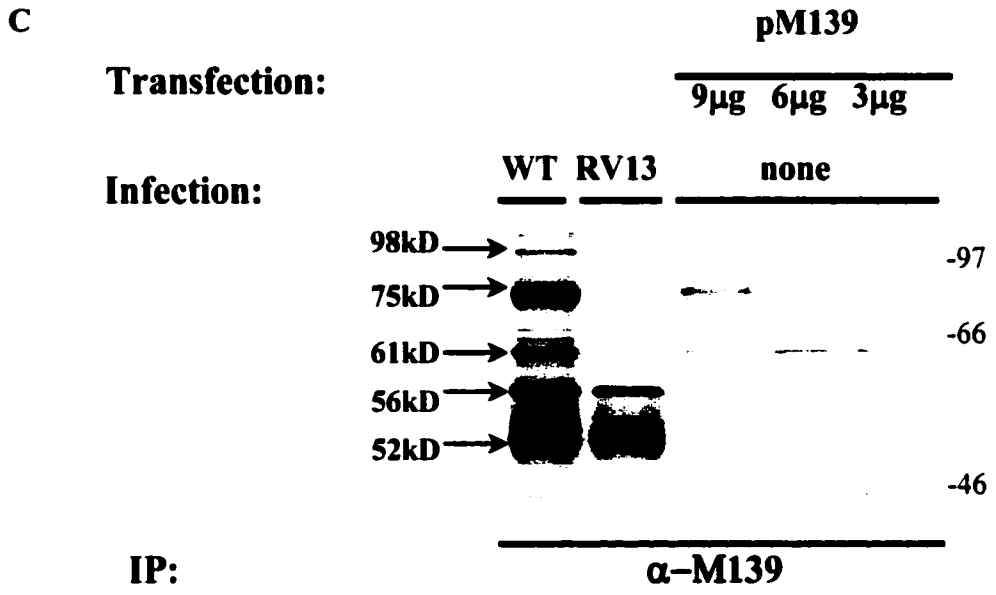


FIG. 24. Analysis of interactions among the pM139, pM140, and pM141 in the absence of other viral proteins. (A) An overview of experimental procedures. (B) NIH 3T3 fibroblasts were transfected with M139, M140 and M141 expressing vectors, radiolabeled and chased for indicated amounts of time. Cell lysates were immunoprecipitated with the antibodies indicated. (C) NIH3T3 fibroblasts were infected with WT or RV139 (Δ139) or transfected with 9-, 6-, or 3-μg of pM139/3.1(+) expression vector. Cells were radiolabeled and immunoprecipitated with α-M139 Ab. Immune complexes were washed, boiled and resolved by SDS-PAGE. The positions of molecular weight makers and sizes of detected proteins are indicated.

Immunoprecipitation of the cells transiently transfected with the M140 expression vector resulted in detection of the 56-kD band only by α-M140 antibody. Similarly, the 52-kD band specific to pM141 was immunoprecipitated only by α-M141 Ab from the cells transfected with the M141 expression vector alone. Again, the α-M139 Ab did not immunoprecipitate the pM140-pM141 complex from the cells transiently expressing these two proteins. However, both proteins were immunoprecipitated by the α-M140 antibody from the cells cotransfected with M140 and M141 expression vectors. Interestingly, when all three expression vectors were used in cotransfection, the pM140-

pM141 complex was immunoprecipitated by the α-M139 Ab, α-M140 Ab, and to a lesser extent, by the α-M141 Ab (Fig. 24B), similar to results obtained from immunoprecipitations of *in vitro* transcribed/translated proteins. These data again contradict the hypothesis that the α-M139 Ab merely crossreacts with pM140-pM141 complex, and rather indicates complexing among pM139, pM140 and pM141. Nevertheless, the reproducible immunoprecipitations of the pM140-pM141 complex from the RV13 (ΔM139)-infected cells clearly indicate a crossreactivity of the α-M139 Ab to the pM140-pM141 complex (Fig. 24C). It is possible that other viral proteins are required to augment the interaction of the pM140-pM141 complex with the α-M139 Ab, and transient expression of the M139 proteins complements that function in the absence of other viral proteins.

Detection of the p75M139 and especially the p61M139 specific signals in transfected cells was difficult to discern because two similarly sized, non-specific proteins were reproducibly immunoprecipitated with the α-M139 antibody (Fig.24B, C). Expression of these proteins appeared to be independent from the DNA content of the transfected expression vectors and is probably a result of transfection manipulations, which non-specifically up-regulate many cellular genes. However, an increased density of the 75-kD band immunoprecipitated from the cells co-transfected with M139 expression vector, compared with those transfected with either M140 or M141 expression vectors alone indicated the presence of p75M139 in those lysates. In addition, control experiments were conducted in order to demonstrate the levels of the specific pM139 signal from the cells transiently transfected with 9-, 6-, and 3-μg compared to the signal detected in WT-infected cells (Fig. 24C). The 75-kD band was easily detectable by α-

M139 immunoprecipitation following transfection of 9 μg of the pM139/3.1(+), however its intensity decreases in correlation with the amount of transfected DNA. The detected 61-kD band appears to be non-specific, because its density does not change with the amount of transfected DNA. It is unfortunate that this non-specific band co-migrates with the 61-kD protein also expressed from the M139 gene and overshadows the specific signal. In co-transfection experiments, the total amount of DNA was limited to 9 μg per transfection; therefore, pM139 (3μg) was minimally detected. Further analysis is needed to determine the role of pM139 proteins in formation of the complex with the pM140-pM141 oligomer, or in augmentation of the interactions of the α-M139 Ab with the pM140-pM141 complex.

Data from pulse-chase experiments indicates that all proteins expressed from the M139-M141 gene region exist in the infected cell in a free form immediately after synthesis, but only the pM141 is detected in this state. Whether or not these proteins are functional in a free form remains to be determined. Furthermore, the results presented in this study demonstrate that pM141 forms a complex with the M140 gene product. However, is not obvious how the pM140-pM141 complex interacts with the M139 gene products. There are three alternative potential interactions among the M139 proteins and pM140-pM141 oligomer, which could not be clearly defined by the results of this project (Fig. 25).

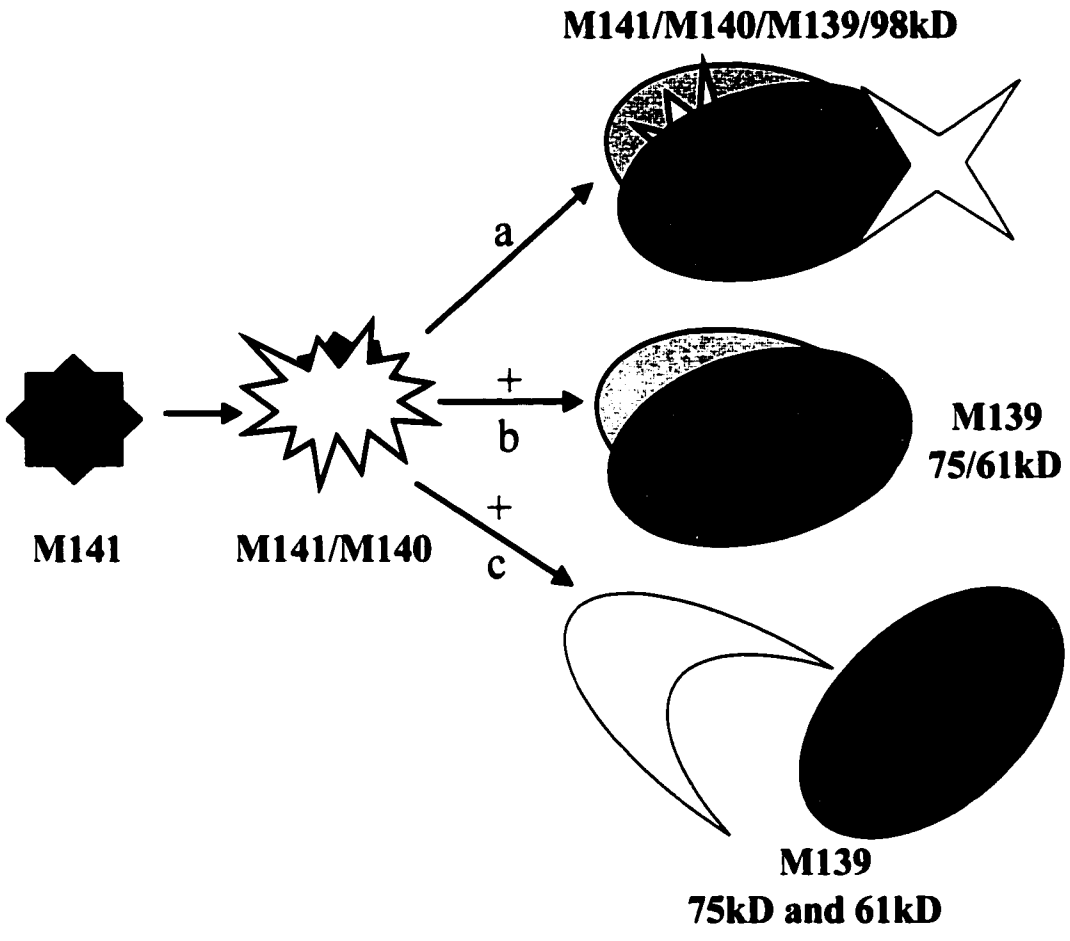


FIG. 25. Possible interactions among the pM140-pM141 complex and M139 gene products.

The first possibility is the progression of the pM140-pM141 complex into a larger one, formed by the addition of the two M139 proteins and the unidentified 98-kD protein (Fig. 25a). It is also possible that M139 gene products interact with each other, but do not complex with the pM140-pM141 oligomer (Fig. 25b). The third and final possibility is the presence of M139 proteins separate from each other, in addition to the pM140-pM141 complex (Fig. 25c). In all three cases the α-M139 Ab will immunoprecipitate all the proteins expressed from the M139-M141 gene region, because of the nature and the characteristics of this antibody.

CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

Monocytes/macrophages are one of the key cell types infected by CMV during an acute stage of infection (Soderberg-Naucler et al., 1998). CMV replicates within these cells upon cellular activation and differentiation. Infected monocytes/macrophages disseminate the virus within the host. These cells harbor latent viral DNA (Mitchell et al., 1996), which reactivates following immunosuppression and allogeneic stimulation (Kurz and Reddehase 1999). Moreover, these cells also participate in innate immune responses to MCMV infection by secreting chemokines and pro-inflammatory cytokines (reviewed by Michelson, 1997). Therefore, it is critical to understand how viral proteins regulate the replication of CMV in monocytes/macrophages.

The studies in our laboratory have demonstrated that the deletion of the MCMV US22 family genes M139, M140 and M141 results in a phenotype profoundly different from WT virus. The MCMV mutant lacking these three genes replicates poorly in cultured macrophages and exhibits attenuated growth in macrophage rich organs of infected mice (Hanson et al., 1999). This study was aimed to characterize the proteins expressed from the M139-M141 genes and to identify potential interactions among them in order to elucidate the ways they influence macrophage-specific replication of MCMV.

Initial characterization of the proteins expressed from the M139-M141 gene region was done by western blot analyses using rabbit polyclonal antibodies generated against recombinant M139, M140 and M141 proteins. The α-M139 antisera detected two proteins sized as 72- and 61-kD. A single protein of 56-kD was identified by the α-

M140 antisera and a 52-kD protein was detected by the α-M141 antisera (Hanson et al., in press). In this study, immunoprecipitation analyses of these proteins, for the first time, revealed a more complex pattern of expression.

Characterization of the M139 gene products

A total of five proteins was immunoprecipitated by the α-M139 antisera. Two of them, p75M139 and p61M139 were identical to those detected by western blot, although a slight correction was applied to the size of the larger protein.

We initially considered that p61M139 might be a degradation product of the p75M139. However, the presence of proteinase inhibitors (PMSF and Aprotinin) in the immunoprecipitation lysis buffer rendered it unlikely that p61M139 is a product of p75M139. More notably, the stoichiometry of the 75- and 61-kD bands, immunoprecipitated by the α-M139 Ab during pulse-chase analysis of WT MCMV-infected cell lysates, did not change between 0 and 4 hours post-chase (Fig. 18B). These data strongly indicated that p61M139 is not a proteolytically processed product of p75M139.

Use of the M139 deletion mutant proved that both p75M139 and p61M139 were expressed from the same gene. In particular, neither one of these proteins were detected in the lysates of the cells infected with RV13 (ΔM139) (Fig. 16B, 18D). In addition, the ability of the α-M139 polyclonal sera to precipitate both proteins indicated that these proteins are transcribed in the same absolute open reading frame and subsequently share common protein sequences.

Results from our study suggested that p75M139 and p61M139 are expressed from distinct messages regulated by separate promoters within the M139 gene. There are two messages (3.8- and 3.0-kb) that map to the M139 gene and likely originate from alternative transcriptional start sites (Hanson, Dalton et al., 1999). Expression of the 3.8-kb message is predicted to start at position 196016 and is regulated by a TATA-less promoter (Rawlinson et al., 1996). Use of the first translation initiation codon in this message is predicted to generate a 75-kD protein. However, a second potential initiation codon has also been identified which if utilized would result in a 61-kD protein. There are two additional potential translation initiation sites at position 195766 and 195667 where the 3.0-kb transcript is predicted to start. The transcript expressed from either one of these translation initiation sites, would lack the upstream initiation codon for the 75-kD protein, but would include the downstream initiation codon of the predicted 61-kD protein. We favor the hypothesis that the 3.0-kb transcript originates at one of these two sites, and encodes the 61-kD protein, as opposed to the possibility that both the 75- and 61-kD proteins are expressed from the larger, 3.8-kb transcript using the alternative translation initiation sites. Additional experiments, such as nuclease protection assays, are needed to conclusively identify the start site of the 3.0-kb transcript. Subsequently, *in vitro* transcription/translation analysis using a temple DNA containing either a full-length M139 gene or a truncated sequence sufficient to encode only 3.0 message, will be necessary to identify the 75- and 61-kD proteins as products of the 3.8- and 3.0-kb transcripts.

It is also possible that the smaller protein is translated from a splice variant of the larger transcript; however, immunoprecipitations of transiently expressed His-tagged

M139 protein argue against this hypothesis. These studies demonstrated that both proteins (75- and 61-kD) were produced from the mammalian expression vector containing M139 sequence fused at its amino-terminus to the histidine tag coding sequence. However, only the 75-kD protein was detected by α-His antibody (Fig. 22). If the 61-kD protein was a splice product of this gene then, most likely, both the 75- and 61-kD proteins would contain the His tag and thus be detected by the α-His antibody in western blot analyses.

Finally, the use of an alternative translation start site is also a possible explanation for the 61-kD protein. However, the inequivalent stoichiometry of the 75- and 61-kD proteins detected in both western blot analyses and immunoprecipitations (which correlates with the relative levels of the 3.8- and 3.0-kb messages) suggests that both proteins are expressed from separate, rather than the same messages. Both proteins are predicted to originate from the same absolute reading frame; therefore, to assess this possibility, a point mutation can be introduced into the second (and third) translation initiation site so that this mutation would not abrogate the transcription of the larger message. Alternatively, analysis of an internal promoter, regulating expression of the 3.0-kb transcript, can reveal those regulatory sequences that could be mutated to inhibit expression of the 3.0-kb transcript, but not interfere with transcription of the larger 3.8-kb message. The proteins expressed from this kind of mutated constructs will definitively determine the origin of the 61-kD protein. Another approach to resolve these questions would be to generate a peptide antibody to sequences predicted to be unique to the 75-kD protein, or amino acid sequencing.

Interaction of the M139 gene products with other proteins

Assessment of the quality of generated antibodies

Three additional proteins of 98-, 56- and 52-kD co-precipitated with pM139 from the WT MCMV-infected cells using α-M139 antibody (Fig. 8B). The two latter proteins (56- and 52-kD) were identified as products of the M140 and M141 genes respectively. A simple explanation for this finding is that the M139 antisera cross-reacted with the three co-precipitating proteins. However, immunoprecipitation analyses of singularly expressed M139, M140 or M141 proteins in *in vitro* transcription/translation reactions proved that the α-M139, α-M140, and α-M141 antisera minimally cross-react with proteins encoded by the other genes from the M139-M141 region (Fig. 10). This conclusion is consistent with the fact that there is low sequence homology among the three genes.

Although the α-M139 antisera do not cross-react with pM140 and pM141 expressed as single proteins, there is evidence that this antibody does cross-react with the complexed form of pM140 and pM141. In particular, pM140 and pM141 were repeatedly immunoprecipitated by the α-M139 antisera in the absence of pM139 (Fig. 16B, 18B,D, 22B). Therefore, we hypothesized that the α-M139 antisera cross-reacts with the pM140-pM141 complex. This can be explained by the fact that complexed proteins acquire conformational changes that affect tertiary structure of these proteins, which differs from the one that each protein has when expressed alone. We tested this hypothesis by combining immunoprecipitation analysis of unlabeled recombinant MCMV-infected cells with subsequent western blotting (Fig 21A). These analyses demonstrated that pM140-pM141 complex was immunoprecipitated from RV13

(ΔM139)-infected cells by the α-M139 Ab, and detected by α-M140-HRP (Fig. 21B). However the pM140 was not detected in western blot analysis of immunoprecipitants from either RV14 (ΔM140)- or RV11 (ΔM141)-infected cells. This strongly suggested that the α-M139 antisera cross-react with the pM140-pM141 complex, and again demonstrated that the same antisera do not cross-react with the free form of pM140 or pM141.

Interactions between pM140-pM141 complex and M139 gene products

In spite of the above findings, our data do not rule out the possibility that pM139 (p75M139 and/or p61M139) complex with pM140-pM141. Moreover, an α-M139 antibody-specific protein profile (total of 5 proteins) was immunoprecipitable exclusively from WT MCMV-infected cells where all the M139-M141 proteins were expressed. This finding supports the hypothesis that M139 proteins directly interact with pM140 and pM141 (either as a complex or as distinct proteins) and this interaction is a prerequisite for association of the 98-kD protein. Therefore, only in WT MCMV-infected cells do the α-M139 antisera immunoprecipitate all the components of this large complex.

The scope of this study did not provide enough information to determine whether p75M139, p61M139, or both are involved in the complex. The M139 specific antisera interact with both proteins expressed from the M139 gene. Therefore both proteins will be immunoprecipitated by this antibody regardless of which one of them directly interacts with other proteins. However, one of the described experiments indirectly addressed this question. Immunoprecipitation analysis using α-M139 antibody revealed the presence of both M139 proteins along with the M140 and M141 proteins in the cells transiently

expressing M139 proteins from the vector containing M139 gene fused to the histidine tag coding sequence and superinfected with the RV13 (ΔM139) (Fig. 22B). Immunoprecipitations of the same cell lysates using α-His antibody demonstrated that only the p75M139 expressed the histidine tag (encoded on the N-terminus) since p61M139 was not immunoprecipitated by the α-His antibody. This provided an opportunity to test the ability of the p75M139 to form a complex with other proteins. Interestingly, no other proteins co-precipitated with the transiently expressed p75M139-His. There are two possible explanations for this finding.

The first possibility is that the N-terminal histidine tag interferes with the ability of p75M139 to form a complex with other proteins. To address this possibility the tag could be expressed on the C-terminus, and similar experiments should be conducted. If proteins co-precipitate with C-terminally tagged p75M139 it would prove the ability of that protein to form a complex and demonstrate that the N-terminus of p75M139 is involved in protein-protein interactions. Alternatively, another antibody could be generated against the unique part of p75M139. The use of this antibody would allow immunoprecipitation exclusively of the 75-kD protein and those protein(s) which complex with it.

The second explanation for N-terminally His-tagged p75M139 not being able to co-precipitate any other proteins is that it is not involved in direct interactions with other proteins. This leaves the possibility that p61M139 is responsible for complexing with other proteins that are immunoprecipitated by the α-M139 antisera. To address this question one could generate a mammalian expression vector that contains a truncated M139 sequence encoding only the p61M139 protein. Then one would transfect that

construct into cells, and superinfect those cells with RV13 (ΔM139) recombinant MCMV. This combination would provide all viral proteins (including transiently expressed p61M139) except p75M139. Immunoprecipitations of these cell lysates with α-M139 polyclonal antisera would demonstrate whether the 61-kD protein is the one involved and possibly responsible for the interactions with other proteins.

Nevertheless, the fact that the α-M139 antisera cross-react with the pM140-pM141 complex brought a question about physical interaction between pM139 (either one or both) and the pM140-pM141 complex. We addressed this question by immunoprecipitating proteins expressed from M139, M140 and M141 genes in *in vitro* transcription/translation reactions, or in fibroblasts transiently transfected with the same mammalian expression vectors. Surprisingly, pM140 and pM141 co-expressed in *in vitro* transcription/translation reaction were not immunoprecipitated by the α-M139 Ab, which contradicts previous results and the hypothesis that α-M139 Ab cross-reacts with pM140-pM141 complex (Fig. 23). Importantly both pM140 and pM141 were immunoprecipitated together by either α-M140 or α-M141 antisera. Furthermore, when all three genes were co-expressed α-M139 antibody precipitated both p75M139 and p61M139 but again failed to immunoprecipitate pM140 and pM141. This indicated that either pM139 did not complex with the pM140-pM141 hetero-oligomer, or the pM140 did not form a complex with pM141, which appears to be required for subsequent pM139 association. The absence of a cross-linking reagent in these reactions supporting maintenance of generated complexes, as well as expression of these MCMV proteins in non-physiological conditions could possibly explain these unexpected results.

The last approach to test direct interaction between the pM140-pM141 oligomer and M139 proteins relied on analyses of interactions among these proteins transiently expressed in mouse fibroblasts. Interestingly, both proteins of the pM140-pM141 oligomer were immunoprecipitated only by the α-M140 or α-M141 antisera but not by the α-M139 antibody. These results once again conflicted with our previous finding of cross-reactivity of the α-M139 antisera with the pM140-pM141 complex. However, when M139 was cotransfected into the cells along with the M140 and M141 genes, the pM140 and pM141 were precipitated by all three antisera. This supports the hypothesis that pM139 directly interacts with the pM140-pM141 oligomer. Nevertheless, these results are not consistent with immunoprecipitation analysis of the RV13 (ΔM139)-infected cells. Therefore, more studies will be needed to resolve these discrepancies. In addition, the 98-kD protein was not recovered in α-M139 immunoprecipitations following cotransfections of the three genes from the M139-M141 gene region into the cells, indicating that viral infection is needed in order for that protein to be expressed and co-precipitated (Fig.22B).

Characterization of the 98-kD protein

The 98-kD protein reproducibly co-precipitated with M139 proteins exclusively from WT MCMV-infected fibroblasts and macrophages. Immunoprecipitations of cells infected with mutant MCMV lacking an expression of M139-M141 genes, alone or in combination, resulted in loss of detection of this band (Fig. 16B). Interestingly, when expression of pM139, pM140 and pM141 was reconstituted by transiently expressing M139 proteins (from a vector containing histidine tag fused to the N-terminus of the

M139 gene) and superinfecting with RV13 (ΔM139), the 98-kD band was still undetectable following α-M139 immunoprecipitations (Fig. 22B). This unexpected result again can be explained by the fact that the histidine tag might interfere with direct interaction of p75M139 with the 98-kD protein. In addition, the finding the α-His antibody did not immunoprecipitate any components of the complex except p75M139, supports the same hypothesis.

Alternatively, the 98-kD protein might be a cellular protein, which is upregulated exclusively by WT MCMV infection. These questions can be addressed by the methods similar to those that were proposed above to identify which of the two M139 proteins are involved in complex formation, or if both of them are required for this interaction.

In order to identify the 98-kD protein, it could be co-immunoprecipitated with α-M139 antisera and be eluted from the gel. The eluted protein could be sequenced and the sequence of the coding gene could be deduced essentially as was previously described (Huber et al., 1998). Comparison of the deduced DNA sequence with known viral or mouse cellular genes might result in identification of a gene encoding the 98-kD protein.

In any case we did not encounter circumstances where pM139, or a possible complex with either of the M139 proteins, correlated with a phenotype different from the WT virus. In fact, RV13 (ΔM139) replicates like WT MCMV in cultured fibroblasts and macrophages, and in the spleens of acutely infected mice. This could be attributed to several factors. First, we did not test the growth of the virus in cells such as epithelial or endothelial cells, which are naturally permissive for MCMV infection. Second, replication of WT and recombinant MCMV were tested only in two organs of mice, spleen and liver. Third, we did not conduct studies of reactivation of latent virus. It is

possible that the function(s) of pM139 would be revealed in the course of these experiments. However, mutations within either the M140 or M141 gene result in dramatically different phenotypes both *in vitro* and *in vivo* from those of WT MCMV (Table 4). Therefore, we concentrated on characterization of the M140 and M141 gene products and the interaction between them.

Identification of M140 and M141 proteins

The origins of the 56- and 52-kD bands were identified by immunoprecipitation analysis of *in vitro* transcribed/translated proteins expressed from mammalian expression vectors encoding M140 and M141 genes respectively (Fig. 10). Subsequently, the M140 and M141 genes were verified as the sources of 56- and 52-kD proteins respectively through immunoprecipitation analysis of RV14 (ΔM140)- and RV11 (ΔM141)-infected cells (Fig. 16). These two bands were co-immunoprecipitated by the α-M140 antibody from the WT MCMV-infected cells (Fig 8B). Interestingly, the α-M141 antibody immunoprecipitated only one band of 52-kD from the same lysates (Fig. 8B). However, during pulse-chase analysis, at 4 hours post-chase the same antibody was able to co-precipitate the 56-kD band in addition to the M141 specific 52-kD band (Fig. 18B,D). Therefore, we concluded that M140 and M141 proteins form a complex.

Although immunoprecipitation analysis used as a basic tool of this study is not quantitative, a reproducible stoichiometry of precipitated bands suggest the concept that all proteins expressed from the M139-M141 genes exist in both free and complexed forms. Our conclusions were based on the fact that all these proteins contain similar number of methionine and cysteine residues, which ensures similar levels of

radiolabeling during synthesis. More specifically, the M139 gene encodes for 12 methionines and 12 cysteines, the M140 contains 15 methionines and 14 cysteines within its sequence, and M141 has 11 methionines and 16 cysteines within the coding sequence.

The difference in detection of exclusively free forms of pM139 compared to free and complexed forms can be seen in autoradiographs of α-M139 immunoprecipitation analyses of WT MCMV- and RV14 (ΔM140)- or RV11 (ΔM141)-infected cells (Fig. 16B). The free and complexed forms of pM140 are immunoprecipitated by the α-M140 Ab immediately after labeling, resulting in a much more intense pM140 specific signal compared to the pM141 signal (Fig. 8, 11, 14). However, with time, most of this newly synthesized protein is incorporated into the complex with pM141, and at 4 hours post-chase both proteins are precipitated at almost equal amounts (Fig.16B). The same appears to be true for the M141 protein. The newly synthesized free form is immunoprecipitated by the α-M141 antibody but, in contrast to pM140 and pM139, when pM141 is integrated into the complex, the antigenic epitopes become hidden from the α-M141 antibody.

The role of the M141 protein in complex formation

The hypothesis that pM141 is buried deep in at least one complex was proven in experiments where WT MCMV-infected cell lysates were immunoprecipitated with α-M141 Ab and these lysates were re-immunoprecipitated with α-M139 or α-M140 antisera to visualize complexed pM141 (Fig. 14). Reproducing previous results, one band of 52-kD was immunoprecipitated from total lysates by the α-M141 Ab. A faint 56-kD band specific to the M140 gene product was also visualized. As predicted, re-

immunoprecipitation of pM141-depleted supernatants recovered all five proteins typically precipitating with α-M139 Ab, whereas the two expected proteins were usually immunoprecipitated by the α-M140 Ab. The components of these complexes were identified as pM139, pM140 or pM141 during re-immunoprecipitations of the same lysates, which were denatured prior to incubation with α-M139, α-M140 or α-M141 antisera respectively. These data convincingly proved that almost all of the pM141 is enclosed in one or more complexes and in this form pM141 remains relatively inaccessible to the α-M141anisera. Whether pM141 directly interacts with M139 gene products in the absence of pM140, or whether the pM140-pM141 complex is a prerequisite for that interaction remains to be determined. Immunoprecipitation analysis of the cells transiently expressing pM139 and pM141 will answer these questions.

Interaction between M140 and M141 gene products

A direct interaction between pM140 and pM141 was demonstrated throughout this study rather convincingly by the following results:

1. the α-M140 immunoprecipitations of denatured WT MCMV-infected cell lysates detected only one 56-kD protein whereas both the 56- and 52-kD proteins were detectable in immunoprecipitations of non-denatured cell lysates (Fig. 11). This proved that pM141 (52-kD) was associated with pM140 (56-kD) and therefore was co-precipitated with the 56-kD protein exclusively from non-denatured lysates.
2. sequential immunoprecipitations of WT MCMV-infected cell lysates demonstrated that the 52-kD protein was co-precipitated with pM140 (56-

kD) by the α-M140 antibody in the first immunoprecipitation reaction and was later visualized by subsequent re-immunoprecipitation using the α-M141antibody (Fig. 12).

3. following co-expression of pM140 and pM141 in either *in vitro* transcription/translation reaction or in transiently transfected cells, both proteins were immunoprecipitated with antibodies specific to either the M140 or M141 gene product. This demonstrated the ability of the two proteins to form a complex in the absence of other cellular or viral proteins (Fig. 23, 24B)
4. immunoprecipitations of RV14 (ΔM140)-and RV11 (ΔM141)-infected cells demonstrated the absence of the corresponding coprecipitating protein, due to absence of the pM140-pM141 complex (Fig. 16B).

The number of monomers forming this complex and how many molecules of each protein in particular are involved in the pM140-pM141 interaction is not known, although a similar density of the two bands precipitated at 4 hours post-chase by all three antibodies suggests a one-to-one ratio (Fig 16B). Regardless, we refer to it as the "pM140-pM141 hetero-oligomer" alternatively to the "complex".

Immunoprecipitations of RV13 (ΔM139) -infected cell lysates (either during standard radiolabeling or pulse-chase analyses) provided additional proof that pM140 and pM141 form a complex and they do so even in the absence of M139 proteins (Fig. 16D). Interestingly, both of these proteins were precipitated not only by antibodies specific for the M140 and M141 proteins but also by the α-M139 antisera, due to potential crossreactivity.

Another significant discovery was made during pulse-chase analysis of the cells infected with the RV14 (ΔM140) recombinant MCMV. No pM140 was expressed in these cells, and both M139 proteins were synthesized, as expected. The pM141 was expressed and immunoprecipitated by the α-M141 Ab in the amount similar to those in WT MCMV-infected cells. However, the half-life of pM141 was dramatically decreased to 1 hour, compared to 2 hours in WT-or RV13 (ΔM139)-infected cells. As a result in RV14 (ΔM140)-infected cells, the M140 protein is absent due to the mutation within the M140 gene and M141 protein is, at least, undetectable because of its instability in the absence of pM140.

Biological significance of interaction between the M140 and M141 gene products.

As was mentioned above, RV14 (ΔM140) replicates to 2-3 $\log_{10}$ lower titers in cultured macrophages even following high multiplicity of infection. This mutant also does not replicate in spleens of infected mice (Hanson et al., in press). The RV11 (ΔM141) mutant is characterized by a distinct, yet significantly less dramatic phenotype. More specifically, the RV11 (ΔM141) MCMV replicates to 1-1.5 $\log_{10}$ lower titers in cultured macrophages and to 2-2.5 $\log_{10}$ lower titers in spleens of infected mice. Interestingly, pM140 is expressed in RV11 (ΔM141)-infected cells to levels similar to those detected in WT MCMV-infected cells and with an analogous half-life. At this time, knowing that pM140 and pM141 form a complex and that the stability of pM141 depends on the presence of pM140, we propose that pM141 possesses a distinct function influencing macrophage-specific pathogenesis. Moreover, pM141 can deliver this

function only when complexed with the M140 protein. This might be attributed to the fact that pM140 stabilizes pM141.

Stabilization of the M141 protein may not be the only function of the pM140. The fact that the M141 mutant (RV11) expressing high levels of pM140, reproducibly exhibits an intermediate phenotype in replication both *in vitro* and *in vivo* compared to WT and RV14 (ΔM140) MCMV indirectly suggests that pM140 has another function influencing macrophage-specific viral pathogenesis independent from stabilization of pM141. Whether pM140 has a function redundant to pM141, or it can form a homo-oligomer that can partially compensate for the function of the pM140-pM141 complex or interact with pM139 and the 98-kD protein to accomplish the same objective, remains to be determined.

It is well known that many viral proteins complex with cellular counterparts to deliver their function (Caswell et al., 1993; Jupp et al., 1993; Lang et al., 1995; Schwartz, Helmich, and Spector, 1996; Scully et al., 1995). Moreover, structural proteins of the viral envelope form complexes (gC-I, gC-II, and gC-III) in order to function (see Structure of the Virion). Characterization of interactions between pM140 and pM141 provides another example where formation of a complex by two distinct viral proteins results in a unique function influencing macrophage-specific replication of MCMV both *in vitro* and *in vivo*.

Further studies should be aimed toward mapping the interaction domains within pM140 and pm141. This can be achieved by analysis of the interactions between M140 and M141 gene products expressed from constructs, containing certain deletions within M140 and M141 coding sequences. Subsequently, the key amino acids can be identified

within the interaction domains, using point mutations, which would alter protein expression and, as a result, abrogate the interaction. These studies will also allow determining if pM140 or pM141 have a separate unique function, as well as whether or not these proteins have redundant (or at least complementing) functions under circumstances when both proteins are expressed but are not forming a complex.

As was mentioned above, phenotypically *in vitro* and *in vivo* RV14 (ΔM140) infection is indistinguishable from the RV10 (ΔM139-M141) MCMV (Hanson et al., 1999: Hanson, et al., in press). Interestingly, the analysis of viral gene expression demonstrated that in RV10 (ΔM139-M141)-infected macrophages the expression of the immediate-early genes is decreased whereas in RV14 (ΔM140)-infected cells the IE genes are expressed to the levels comparable with those induced by WT MCMV infection (Hanson et al., in press). This phenomenon taken together with early-late expression kinetics of the M140 and M141 proteins suggests that pM140-pM141 complex influence macrophage-specific protein synthesis shut-off mechanism, because both of these proteins are affected by the mutation within the M140 gene.

CHAPTER VI

CONCLUSIONS

Cytomegalovirus (CMV) is a large and complex ubiquitous herpesvirus. It causes morbidity and even mortality in neonates, and severe disease in immunocompromised individuals. CMV is characterized by complicated pathogenicity, which involves acute, chronic, and latent infections. More than 200 predicted open reading frames (ORFs) are encoded by CMV's double-stranded DNA genome. Many of those genes are nonessential for CMV replication in tissue culture and their protein products are involved in regulating CMV pathogenesis and host-virus interactions.

Murine CMV (MCMV) serves as a model for human CMV (HCMV) disease and allows one to analyze the function of viral proteins in the context of a natural host. We identified three genes (M139, M140, and M141) within the MCMV genome, which regulate viral expression in macrophages and mice, but are nonessential for replication of the virus in fibroblasts. These genes are members of the US22 gene family with respect to HCMV homology. There is no function assigned to the proteins encoded by these genes. However, deletion of M139, M140, and M141 significantly curtails growth of MCMV in macrophages in vitro and in macrophage-dense target organs in vivo (Hanson et al. 1999, J.Virol. 73(7): 5970-80). Therefore, M139, M140, and/or M141 gene products likely affect tissue specific viral infectivity. The purpose of this study was to characterize these proteins (pM139, pM140, and pM141) with respect to kinetics of their expression, stability, and complexing with themselves and other viral or cellular proteins. Fine characterization of these proteins and the interactions which they are involved will

provide initial information about the function of these three gene products in regulation of MCMV expression in macrophages and macrophages-rich target organs in mice.

Our approach to characterizing the M139, M140, and M141 proteins consisted of generating polyclonal antibodies (Ab) specific for each protein, and using them in extensive analyses such as western blotting or immunoprecipitation (IP) of cells infected with wild type MCMV or recombinant mutant viruses. In order to generate pM139, pM140, and pM141 specific antibodies, genomic fragments coding the appropriate protein were cloned into bacterial expression vectors. Next, the recombinant proteins were expressed, purified, and injected into rabbits to produce specific polyclonal antiserum. A different set of expression vectors was generated to test the antibodies for the purpose of crossreactivity, and for further use in the study. These mammalian expression vectors contain the genomic fragment coding for either pM139, or pM140, or pM141 full-length proteins. Expression form these vectors was tested positively in *in vitro* transcription/translation reactions. Subsequently, each protein (M139, M140, and M141) expressed form these vectors was immunoprecipitated by anti-M139, anti-M140 and anti-M141 antibodies. The antibodies proved to be specific and immunoprecipitated only one appropriate protein. For example, anti-M139 IP visualized only the M139 proteins but not pM140 or pM141. Analogous results were obtained in anti-M140 and anti-M141 immunoprecipitations.

The kinetics of expression of the M139, M140, and M141 proteins was initially examined by western blot analysis, which showed that these proteins are abundant at steady state levels, and are expressed at early and late times during an MCMV infection in both NIH3T3 fibroblasts and IC-21 macrophages. Western blot analyses also revealed

that the anti-M139 Ab detects two proteins of 75 kD and 61 kD; anti-M140 Ab detects one protein of 56 kD; anti-M141 Ab detects one protein of 52 kD.

However, immunoprecipitation analyses of pM139, pM140, and pM141 reveal additional proteins co-precipitating with pM139 and pM140. Whereas α-M139 Ab detected two proteins in Western blot analysis, five bands were immunoprecipitated with the same Ab. Likewise, two proteins were immunoprecipitated with α-M140 Ab, although the same Ab detected only one protein in Western blot analysis. Interestingly only one protein was detected by α-M141 Ab in both immunoprecipitation and western blot analysis, but the levels of immunoprecipitated protein were much lower than those detected in Western blot analysis. Therefore we propose that M139 and M140 proteins are interacting with other proteins originating from either the M139-M141 gene region, or other viral or cellular genes.

A series of experiments including immunoprecipitation of non-denatured versus denatured lysates, sequential IPs, and combination of IP followed by western blot analysis confirmed that α-M139 Ab co precipitates M140 and M141 proteins, whereas α-M140 Ab co precipitates only the M141 protein and α-M141 Ab did not coprecipitate any additional proteins. The fact that each antibody was immunoprecipitating a unique protein profile can be explained by the quaternary structure of the pM139, pM140 and pM141 complex. When these proteins are complexed epitopes of underlying protein(s) may be masked thus making these proteins inaccessible for the specific Ab. Reimmunoprecipitations of pM141-depleted supernatants resolved the issue of the pM141 involvement in complex formation. It was shown that pM141 exists in infected

cells in a free form that is accessible by its Ab, and also as a component of one or more complexes which hinders the epitopes of the pM141.

Using a genetic approach and immunoprecipitations of cell lysates infected with recombinant MCMV containing mutations in M139, M140, and M141 genes alone or in combination, the origins of identified proteins were confirmed *in vivo*. Pulse-chase analyses were done to determine the stability of the proteins. It was determined that the stability of pM141 depends on the presence of pM140, but not pM139.

A final series of experiments were design to distinguished if all three proteins complex with each other or coprecipitate by virtue of antibody crossreactivity. The results demonstrated that indeed the M140 and M141 proteins form a stable complex. It remains to be determined if the two M139 proteins form a heterodimer or exist separately within an infected cell. Given the complexity of viral pathogenesis and the fact that pM139, pM140, and pM141 proteins are dispensable for viral replication in tissue culture, it is possible that each single protein as well as the complex(s) they form may have a distinct function which influences tissue specific infectivity.

REFERENCES

Anders, D. G., and McCue, L. A. (1996). The human cytomegalovirus genes and proteins required for DNA synthesis. *Intervirology* **39**(5-6), 378-88.

Angulo, A., Messerle, M., Koszinowski, U. H., and Ghazal, P. (1998). Enhancer requirement for murine cytomegalovirus growth and genetic complementation by the human cytomegalovirus enhancer. *J. Virol.* **72**(11), 8502-9.

Balthesen, M., Messerle, M., and Reddehase, M. J. (1993). Lungs are a major organ site of cytomegalovirus latency and recurrence. *J. Virol.* **67**(9), 5360-6.

Battista, M. C., Bergamini, G., Boccuni, M. C., Campanini, F., Ripalti, A., and Landini, M. P. (1999). Expression and characterization of a novel structural protein of human cytomegalovirus, pUL25. *J. Virol.* **73**(5), 3800-9.

Beisser, P. S., Vink, C., Van Dam, J. G., Grauls, G., Vanherle, S. J., and Bruggeman, C. A. (1998). The R33 G protein-coupled receptor gene of rat cytomegalovirus plays an essential role in the pathogenesis of viral infection. *J. Virol.* **72**(3), 2352-63.

Bhella, D., Rixon, F. J., and Dargan, D. J. (2000). Cryomicroscopy of human cytomegalovirus virions reveals more densely packed genomic DNA than in herpes simplex virus type 1. *J. Mol. Biol.* **295**(2), 155-61.

Biegalke, B. J. (1999). Human cytomegalovirus US3 gene expression is regulated by a complex network of positive and negative regulators. *Virology* **261**(2), 155-64.

Boldogh, I., AbuBakar, S., Deng, C. Z., and Albrecht, T. (1991). Transcriptional activation of cellular oncogenes fos, jun, and myc by human cytomegalovirus. *J. Virol.* **65**(3), 1568-71.

Boyle, K. A., Pietropaolo, R. L., and Compton, T. (1999). Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway. *Mol. Cell. Biol.* **19**(5), 3607-13.

Bresnahan, W. A., and Shenk, T. (2000). A subset of viral transcripts packaged within human cytomegalovirus particles. *Science* **288**(5475), 2373-6.

Britt, W. J., and C. A. Alford (1996). Cytomegalovirus. *In* "Fields Virology" (B. N. Fields, Knipe, D. M., Howley, P. M., Ed.). Lippinocott-Raven, Philadelphia.

Britt, W. J., and Mach, M. (1996). Human cytomegalovirus glycoproteins. *Intervirology* **39**(5-6), 401-12.

Brune, W., Menard, C., Heesemann, J., and Koszinowski, U. H. (2001). A ribonucleotide reductase homolog of cytomegalovirus and endothelial cell tropism. *Science* **291**(5502), 303-5.

Buhler, B., Keil, G. M., Weiland, F., and Koszinowski, U. H. (1990). Characterization of the murine cytomegalovirus early transcription unit e1 that is induced by immediate-early proteins. *J. Virol.* **64**(5), 1907-19.

Campbell, A. E. (1999). Murine cytomegalovirus. *In* "Persistent Viral Infections" (R. Ahmed, Chen, I., Ed.). John Wisley & Son, New York, NY.

Cardin, R. D., Abenes, G. B., Stoddart, C. A., and Mocarski, E. S. (1995). Murine cytomegalovirus IE2, an activator of gene expression, is dispensable for growth and latency in mice. *Virology* **209**(1), 236-41.

Caswell, R., Hagemeier, C., Chiou, C. J., Hayward, G., Kouzarides, T., and Sinclair, J. (1993). The human cytomegalovirus 86K immediate early (IE) 2 protein requires the basic region of the TATA-box binding protein (TBP) for binding, and

interacts with TBP and transcription factor TFIIB via regions of IE2 required for transcriptional regulation. *J. Gen. Virol.* **74**(Pt 12), 2691-8.

Cavanaugh, V. J., Stenberg, R. M., Staley, T. L., Virgin, H. W. t., MacDonald, M. R., Paetzold, S., Farrell, H. E., Rawlinson, W. D., and Campbell, A. E. (1996). Murine cytomegalovirus with a deletion of genes spanning HindIII-J and - I displays altered cell and tissue tropism. *J. Virol.* **70**(3), 1365-74.

Chang, C. P., Malone, C. L., and Stinski, M. F. (1989). A human cytomegalovirus early gene has three inducible promoters that are regulated differentially at various times after infection. *J. Virol.* **63**(1), 281-90.

Chang, C. P., Vesole, D. H., Nelson, J., Oldstone, M. B., and Stinski, M. F. (1989). Identification and expression of a human cytomegalovirus early glycoprotein. *J. Virol.* **63**(8), 3330-7.

Chang, Y. N., Jeang, K. T., Chiou, C. J., Chan, Y. J., Pizzorno, M., and Hayward, G. S. (1993). Identification of a large bent DNA domain and binding sites for serum response factor adjacent to the NFI repeat cluster and enhancer region in the major IE94 promoter from simian cytomegalovirus. *J. Virol.* **67**(1), 516-29.

Chapman, T. L., and Bjorkman, P. J. (1998). Characterization of a murine cytomegalovirus class I major histocompatibility complex (MHC) homolog: comparison to MHC molecules and to the human cytomegalovirus MHC homolog. *J. Virol.* **72**(1), 460-6.

Chau, N. H., Vanson, C. D., and Kerry, J. A. (1999). Transcriptional regulation of the human cytomegalovirus US11 early gene. *J. Virol.* **73**(2), 863-70.

Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A., 3rd, Kouzarides, T., Martignetti, J. A., and et al. (1990). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154,** 125-69.

Chen, D. H., Jiang, H., Lee, M., Liu, F., and Zhou, Z. H. (1999). Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus. *Virology* **260**(1), 10-6.

Colberg-Poley, A. M. (1996). Functional roles of immediate early proteins encoded by the human cytomegalovirus UL36-38, UL115-119, TRS1/IRS1 and US3 loci. *Intervirology* **39**(5-6), 350-60.

Colberg-Poley, A. M., Huang, L., Soltero, V. E., Iskenderian, A. C., Schumacher, R. F., and Anders, D. G. (1998). The acidic domain of pUL37x1 and gpUL37 plays a key role in transactivation of HCMV DNA replication gene promoter constructions. *Virology* **246**(2), 400-8.

Colberg-Poley, A. M., Santomenna, L. D., Harlow, P. P., Benfield, P. A., and Tenney, D. J. (1992). Human cytomegalovirus US3 and UL36-38 immediate-early proteins regulate gene expression. *J. Virol.* **66**(1), 95-105.

Compton, T., Nowlin, D. M., and Cooper, N. R. (1993). Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. *Virology* **193**(2), 834-41.

Cranmer, L. D., Clark, C., and Spector, D. H. (1994). Cloning, characterization, and expression of the murine cytomegalovirus homologue of the human cytomegalovirus 28-kDa matrix phosphoprotein (UL99). *Virology* **205**(2), 417-29.

Cretney, E., Degli-Esposti, M. A., Densley, E. H., Farrell, H. E., Davis-Poynter, N. J., and Smyth, M. J. (1999). m144, a murine cytomegalovirus (MCMV)-encoded major histocompatibility complex class I homologue, confers tumor resistance to natural killer cell-mediated rejection. *J. Exp. Med.* **190**(3), 435-44.

Davis-Poynter, N. J., Lynch, D. M., Vally, H., Shellam, G. R., Rawlinson, W. D., Barrell, B. G., and Farrell, H. E. (1997). Identification and characterization of a G protein-coupled receptor homolog encoded by murine cytomegalovirus. *J. Virol.* **71**(2), 1521-9.

Depto, A. S., and Stenberg, R. M. (1989). Regulated expression of the human cytomegalovirus pp65 gene: octamer sequence in the promoter is required for activation by viral gene products. *J. Virol.* **63**(3), 1232-8.

Dorsch-Hasler, K., Keil, G. M., Weber, F., Jasin, M., Schaffner, W., and Koszinowski, U. H. (1985). A long and complex enhancer activates transcription of the gene coding for the highly abundant immediate early mRNA in murine cytomegalovirus. *Proc. Natl. Acad. Sci. U S A* **82**(24), 8325-9.

Efstathiou, S., Lawrence, G. L., Brown, C. M., and Barrell, B. G. (1992). Identification of homologues to the human cytomegalovirus US22 gene family in human herpesvirus 6. *J. Gen. Virol.* **73**(Pt 7), 1661-71.

Fahnestock, M. L., Johnson, J. L., Feldman, R. M., Neveu, J. M., Lane, W. S., and Bjorkman, P. J. (1995). The MHC class I homolog encoded by human cytomegalovirus binds endogenous peptides. *Immunity* **3**(5), 583-90.

Fernandez, J. A., Zavala, F., and Tsuji, M. (1999). Phenotypic and functional characterization of CD8(+) T cell clones specific for a mouse cytomegalovirus epitope. *Virology* **255**(1), 40-9.

Fish, K. N., Depto, A. S., Moses, A. V., Britt, W., and Nelson, J. A. (1995). Growth kinetics of human cytomegalovirus are altered in monocyte- derived macrophages. *J. Virol.* **69**(6), 3737-43.

Fitzgerald, N. A., Papadimitriou, J. M., and Shellam, G. R. (1990). Cytomegalovirus-induced pneumonitis and myocarditis in newborn mice. A model for perinatal human cytomegalovirus infection. *Arch. Virol.* **115**(1-2), 75-88.

Fleming, P., Davis-Poynter, N., Degli-Esposti, M., Densley, E., Papadimitriou, J., Shellam, G., and Farrell, H. (1999). The murine cytomegalovirus chemokine homolog, m131/129, is a determinant of viral pathogenicity. *J. Virol.* **73**(8), 6800-9.

Garcia-Ramirez, J. J., Ruchti, F., Huang, H., Simmen, K., Angulo, A., and Ghazal, P. (2001). Dominance of virus over host factors in cross-species activation of human cytomegalovirus early gene expression. *J. Virol.* **75**(1), 26-35.

Goldmacher, V. S., Bartle, L. M., Skaletskaya, A., Dionne, C. A., Kedersha, N. L., Vater, C. A., Han, J., Lutz, R. J., Watanabe, S., McFarland, E. D., Kieff, E. D., Mocarski, E. S., and Chittenden, T. (1999). A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. *Proc. Natl. Acad. Sci. U S A* **96**(22), 12536-41.

Gonczol, E., deTaisne, C., Hirka, G., Berencsi, K., Lin, W. C., Paoletti, E., and Plotkin, S. (1991). High expression of human cytomegalovirus (HCMV)-gB protein in

cells infected with a vaccinia-gB recombinant: the importance of the gB protein in HCMV immunity. *Vaccine* **9**(9), 631-7.

Greaves, R. F., and Mocarski, E. S. (1998). Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus ie1 mutant. *J. Virol.* **72**(1), 366-79.

Greijer, A. E., Dekkers, C. A., and Middeldorp, J. M. (2000). Human cytomegalovirus virions differentially incorporate viral and host cell RNA during the assembly process. *J. Virol.* **74**(19), 9078-82.

Gretch, D. R., Kari, B., Rasmussen, L., Gehrz, R. C., and Stinski, M. F. (1988). Identification and characterization of three distinct families of glycoprotein complexes in the envelopes of human cytomegalovirus. *J. Virol.* **62**(3), 875-81.

Griffiths, P. D., Clark, D. A., and Emery, V. C. (2000). Betaherpesviruses in transplant recipients. *J. Antimicrob. Chemothr.* **45 Suppl T3**, 29-34.

Hagemeier, C., Walker, S., Caswell, R., Kouzarides, T., and Sinclair, J. (1992). The human cytomegalovirus 80-kilodalton but not the 72-kilodalton immediate-early protein transactivates heterologous promoters in a TATA box-dependent mechanism and interacts directly with TFIID. *J. Virol.* **66**(7), 4452-6.

Hanson, L. K., Dalton, B. L., Karabekian, Z., Farrell, H. E., Rawlinson, W. D., Stenberg, R. M., and Campbell, A. E. (1999a). Transcriptional analysis of the murine cytomegalovirus HindIII-I region: identification of a novel immediate-early gene region. *Virology* **260**(1), 156-64.

Hanson, L. K., Slater, J. S., Karabekian, Z., Virgin, H. W. t., Biron, C. A., Ruzek, M. C., van Rooijen, N., Ciavarra, R. P., Stenberg, R. M., and Campbell, A. E. (1999b).

Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis. *J. Virol.* **73**(7), 5970-80.

Heise, M. T., Connick, M., and Virgin, H. W. (1998). Murine cytomegalovirus inhibits interferon gamma-induced antigen presentation to CD4 T cells by macrophages via regulation of expression of major histocompatibility complex class II-associated genes. *J. Exp. Med.* **187**(7), 1037-46.

Heise, M. T., and Virgin, H. W. t. (1995). The T-cell-independent role of gamma interferon and tumor necrosis factor alpha in macrophage activation during murine cytomegalovirus and herpes simplex virus infections. *J. Virol.* **69**(2), 904-9.

Hengel, H., Flohr, T., Hammerling, G. J., Koszinowski, U. H., and Momburg, F. (1996). Human cytomegalovirus inhibits peptide translocation into the endoplasmic reticulum for MHC class I assembly. *J. Gen. Virol.* **77**(Pt 9), 2287-96.

Holtappels, R., Thomas, D., Podlech, J., Geginat, G., Steffens, H. P., and Reddehase, M. J. (2000). The putative natural killer decoy early gene m04 (gp34) of murine cytomegalovirus encodes an antigenic peptide recognized by protective antiviral CD8 T cells. *J. Virol.* **74**(4), 1871-84.

Horuk, R. (1994). Molecular properties of the chemokine receptor family. *Trends Pharmacol. Sci.* **15**(5), 159-65.

Hossain, M. S., Takimoto, H., Ninomiya, T., Yoshida, H., Kishihara, K., Matsuzaki, G., Kimura, G., and Nomoto, K. (2000). Characterization of CD4- CD8- CD3+ T-cell receptor-alphabeta+ T cells in murine cytomegalovirus infection. *Immunology* **101**(1), 19-29.

Huber, M. T., and Compton, T. (1998). The human cytomegalovirus UL74 gene encodes the third component of the glycoprotein H-glycoprotein L-containing envelope complex. *J. Virol.* **72**(10), 8191-7.

Iskenderian, A. C., Huang, L., Reilly, A., Stenberg, R. M., and Anders, D. G. (1996). Four of eleven loci required for transient complementation of human cytomegalovirus DNA replication cooperate to activate expression of replication genes. *J. Virol.* **70**(1), 383-92.

Jahn, G., Stenglein, S., Riegler, S., Einsele, H., and Sinzger, C. (1999). Human cytomegalovirus infection of immature dendritic cells and macrophages. *intervirology* **42**(5-6), 365-72.

Jenkins, D. E., Martens, C. L., and Mocarski, E. S. (1994). Human cytomegalovirus late protein encoded by ie2: a trans-activator as well as a repressor of gene expression. *J. Gen. Virol.* **75**(Pt 9), 2337-48.

Jones, T. R., and Muzithras, V. P. (1992). A cluster of dispensable genes within the human cytomegalovirus genome short component: IRS1, US1 through US5, and the US6 family. *J. Virol.* **66**(4), 2541-6.

Jones, T. R., Muzithras, V. P., and Gluzman, Y. (1991). Replacement mutagenesis of the human cytomegalovirus genome: US10 and US11 gene products are nonessential. *J. Virol.* **65**(11), 5860-72.

Jones, T. R., and Sun, L. (1997). Human cytomegalovirus US2 destabilizes major histocompatibility complex class I heavy chains. *J. Virol.* **71**(4), 2970-9.

Jones, T. R., Wiertz, E. J., Sun, L., Fish, K. N., Nelson, J. A., and Ploegh, H. L. (1996). Human cytomegalovirus US3 impairs transport and maturation of major

histocompatibility complex class I heavy chains. *Proc. Natl. Acad. Sci. U S A* **93**(21), 11327-33.

Jonjic, S., Pavic, I., Polic, B., Crnkovic, I., Lucin, P., and Koszinowski, U. H. (1994). Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. *J. Exp. Med.* **179**(5), 1713-7.

Jupp, R., Hoffmann, S., Stenberg, R. M., Nelson, J. A., and Ghazal, P. (1993). Human cytomegalovirus IE86 protein interacts with promoter-bound TATA- binding protein via a specific region distinct from the autorepression domain. *J. Virol.* **67**(12), 7539-46.

Kari, B., and Gehrz, R. (1993). Structure, composition and heparin binding properties of a human cytomegalovirus glycoprotein complex designated gC-II. *J. Gen. Virol.* **74**(Pt 2), 255-64.

Kari, B., Li, W., Cooper, J., Goertz, R., and Radeke, B. (1994). The human cytomegalovirus UL100 gene encodes the gC-II glycoproteins recognized by group 2 monoclonal antibodies. *J. Gen. Virol.* **75**(Pt 11), 3081-6.

Kasamatsu, H., and Nakanishi, A. (1998). How do animal DNA viruses get to the nucleus? *Annu. Rev. Microb.* **52**, 627-86.

Kaye, J. F., Gompels, U. A., and Minson, A. C. (1992). Glycoprotein H of human cytomegalovirus (HCMV) forms a stable complex with the HCMV UL115 gene product. *J. Gen. Virol.* **73**(Pt 10), 2693-8.

Keil, G. M., Ebeling-Keil, A., and Koszinowski, U. H. (1987). Sequence and structural organization of murine cytomegalovirus immediate-early gene 1. *J. Virol.* **61**(6), 1901-8.

Kerry, J. A., Priddy, M. A., Kohler, C. P., Staley, T. L., Weber, D., Jones, T. R., and Stenberg, R. M. (1997a). Translational regulation of the human cytomegalovirus pp28 (UL99) late gene. *J. Virol.* **71**(2), 981-7.

Kerry, J. A., Priddy, M. A., Staley, T. L., Jones, T. R., and Stenberg, R. M. (1997b). The role of ATF in regulating the human cytomegalovirus DNA polymerase (UL54) promoter during viral infection. *J. Virol.* **71**(3), 2120-6.

Kerry, J. A., Sehgal, A., Barlow, S. W., Cavanaugh, V. J., Fish, K., Nelson, J. A., and Stenberg, R. M. (1995). Isolation and characterization of a low-abundance splice variant from the human cytomegalovirus major immediate-early gene region. *J. Virol.* **69**(6), 3868-72.

Kleijnen, M. F., Huppa, J. B., Lucin, P., Mukherjee, S., Farrell, H., Campbell, A. E., Koszinowski, U. H., Hill, A. B., and Ploegh, H. L. (1997). A mouse cytomegalovirus glycoprotein, gp34, forms a complex with folded class I MHC molecules in the ER which is not retained but is transported to the cell surface. *Embo. J.* **16**(4), 685-94.

Klucher, K. M., Rabert, D. K., and Spector, D. H. (1989). Sequences in the human cytomegalovirus 2.7-kilobase RNA promoter which mediate its regulation as an early gene. *J. Virol.* **63**(12), 5334-43.

Kondo, K., Xu, J., and Mocarski, E. S. (1996). Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc. Natl. Acad. Sci. U S A* **93**(20), 11137-42.

Koszinowski, U. H. (1991). Molecular aspects of immune recognition of cytomegalovirus. *Transplant. Proc.* **23**(3 Suppl 3), 70-3, discussion 74.

Koszinowski, U. H., Del Val, M., and Reddehase, M. J. (1990). Cellular and molecular basis of the protective immune response to cytomegalovirus infection. *Curr. Top. Microbiol. Immunol.* **154,** 189-220.

Koszinowski, U. H., Reddehase M.J., and Jonjic S. (1993). "The role of T-lymphocyte subsets in the control of cytomegalovirus infection." Viruses and cellular immune responce (B. D. Thomas, Ed.) Marcel Dekker, Inc., New York, N.Y.

Koszinowski, U. H., Reddehase, M. J., and Jonjic, S. (1991). The role of CD4 and CD8 T cells in viral infections. *Curr. Opin. Immunol.***3**(4), 471-5.

Kouzarides, T., Bankier, A. T., Satchwell, S. C., Preddy, E., and Barrell, B. G. (1988). An immediate early gene of human cytomegalovirus encodes a potential membrane glycoprotein. *Virology* **165**(1), 151-64.

Kurz, S., Steffens, H. P., Mayer, A., Harris, J. R., and Reddehase, M. J. (1997). Latency versus persistence or intermittent recurrences: evidence for a latent state of murine cytomegalovirus in the lungs. *J. Virol.* **71**(4), 2980-7.

Kurz, S. K., Rapp, M., Steffens, H. P., Grzimek, N. K., Schmalz, S., and Reddehase, M. J. (1999). Focal transcriptional activity of murine cytomegalovirus during latency in the lungs. *J. Virol.* **73**(1), 482-94.

Kurz, S. K., and Reddehase, M. J. (1999). Patchwork pattern of transcriptional reactivation in the lungs indicates sequential checkpoints in the transition from murine cytomegalovirus latency to recurrence. *J. Virol.* **73**(10), 8612-22.

Lagenaur, L. A., Manning, W. C., Vieira, J., Martens, C. L., and Mocarski, E. S. (1994). Structure and function of the murine cytomegalovirus sgg1 gene: a determinant of viral growth in salivary gland acinar cells. *J. Virol.* **68**(12), 7717-27.

Lang, D., Gebert, S., Arlt, H., and Stamminger, T. (1995). Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB. *J. Virol.* **69**(10), 6030-7.

Lathbury, L. J., Allan, J. E., Shellam, G. R., and Scalzo, A. A. (1996). Effect of host genotype in determining the relative roles of natural killer cells and T cells in mediating protection against murine cytomegalovirus infection. *J. Gen. Virol.* **77**(Pt 10), 2605-13.

Lee, C. H., Lee, G. C., Chan, Y. J., Chiou, C. J., Ahn, J. H., and Hayward, G. S. (1999). Factors affecting human cytomegalovirus gene expression in human monocyte cell lines. *Mol. Cells* **9**(1), 37-44.

Lembo, D., Gribaudo, G., Hofer, A., Riera, L., Cornaglia, M., Mondo, A., Angeretti, A., Gariglio, M., Thelander, L., and Landolfo, S. (2000). Expression of an altered ribonucleotide reductase activity associated with the replication of murine cytomegalovirus in quiescent fibroblasts. *J. Virol.* **74**(24), 11557-65.

Li, F., Yin, M., Van Dam, J. G., Grauls, G., Rozing, J., and Bruggeman, C. A. (1998). Cytomegalovirus infection enhances the neointima formation in rat aortic allografts: effect of major histocompatibility complex class I and class II antigen differences. *Transplantation* **65**(10), 1298-304.

Liu, B., and Stinski, M. F. (1992). Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements. *J. Virol.* **66**(7), 4434-44.

Ljungman, P., Aschan, J., Lewensohn-Fuchs, I., Carlens, S., Larsson, K., Lonnqvist, B., Mattsson, J., Sparrelid, E., Winiarski, J., and Ringden, O. (1998). Results of

different strategies for reducing cytomegalovirus-associated mortality in allogeneic stem cell transplant recipients. *Transplantation* **66**(10), 1330-4.

Lucin, P., Jonjic, S., Messerle, M., Polic, B., Hengel, H., and Koszinowski, U. H. (1994). Late phase inhibition of murine cytomegalovirus replication by synergistic action of interferon-gamma and tumour necrosis factor. *J. Gen. Virol.* **75**(Pt 1), 101-10.

Lucin, P., Pavic, I., Polic, B., Jonjic, S., and Koszinowski, U. H. (1992). Gamma interferon-dependent clearance of cytomegalovirus infection in salivary glands. *J. Virol.* **66**(4), 1977-84.

MacDonald, M. R., Burney, M. W., Resnick, S. B., and Virgin, H. I. (1999). Spliced mRNA encoding the murine cytomegalovirus chemokine homolog predicts a beta chemokine of novel structure. *J. Virol.* **73**(5), 3682-91.

MacDonald, M. R., Li, X. Y., and Virgin, H. W. t. (1997). Late expression of a beta chemokine homolog by murine cytomegalovirus. *J. Virol.* **71**(2), 1671-8.

Mach, M., Kropff, B., Dal Monte, P., and Britt, W. (2000). Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73). *J. Virol.* **74**(24), 11881-92.

Maciejewski, J. P., Bruening, E. E., Donahue, R. E., Mocarski, E. S., Young, N. S., and St Jeor, S. C. (1992). Infection of hematopoietic progenitor cells by human cytomegalovirus. *Blood* **80**(1), 170-8.

Maciejewski, J. P., and St Jeor, S. C. (1999). Human cytomegalovirus infection of human hematopoietic progenitor cells. *Leuk. Lymphoma* **33**(1-2), 1-13.

Meier, J. L., and Stinski, M. F. (1996). Regulation of human cytomegalovirus immediate-early gene expression. *Intervirology* **39**(5-6), 331-42.

Menard, C., Brune W., Wagner M., Koszinowski U.H. (2000). Characterization of the US22-homolog gene family in the Mouse Cytomegalovirus (MCMV). *25-th International Herpesvirus Workshop, Portland, Oregon, USA.*

Mercer, J. A., and Spector, D. H. (1986). Pathogenesis of acute murine cytomegalovirus infection in resistant and susceptible strains of mice. *J. Virol.* **57**(2), 497-504.

Messerle, M., Buhler, B., Keil, G. M., and Koszinowski, U. H. (1992). Structural organization, expression, and functional characterization of the murine cytomegalovirus immediate-early gene 3. *J. Virol.* **66**(1), 27-36.

Messerle, M., Keil, G. M., and Koszinowski, U. H. (1991). Structure and expression of murine cytomegalovirus immediate-early gene 2. *J. Virol.* **65**(3), 1638-43.

Michelson, S. (1997). Interaction of human cytomegalovirus with monocytes/macrophages: a love- hate relationship. *Pathol. Biol. (Paris)* **45**(2), 146-58.

Miller, D. M., Rahill, B. M., Boss, J. M., Lairmore, M. D., Durbin, J. E., Waldman, J. W., and Sedmak, D. D. (1998). Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. *J. Exp. Med.* **187**(5), 675-83.

Milne, R. S., Mattick, C., Nicholson, L., Devaraj, P., Alcami, A., and Gompels, U. A. (2000). RANTES binding and down-regulation by a novel human herpesvirus-6 beta chemokine receptor. *J. Immunol.* **164**(5), 2396-404.

Mitchell, B. M., Leung, A., and Stevens, J. G. (1996). Murine cytomegalovirus DNA in peripheral blood of latently infected mice is detectable only in monocytes and polymorphonuclear leukocytes. *Virology* **223**(1), 198-207.

Mocarski, E. S. (1996). Cytomegaloviruses and their replication. *In* "Fields Virology" (B. N. Fields, Knipe D. M., Howley P. M., Ed.). Lippincot-Raven, Philadelphia.

Mocarski, E. S. (2001 in press). Cytomegaloviruses and their replication. *In* "Fields Virology".

Mocarski, E. S., Jr., Abenes, G. B., Manning, W. C., Sambucetti, L. C., and Cherrington, J. M. (1990). Molecular genetic analysis of cytomegalovirus gene regulation in growth, persistence and latency. *Curr. Top. Microbiol. Immunol.* **154,** 47-74.

Mocarski, E. S., Kemble, G. W., Lyle, J. M., and Greaves, R. F. (1996). A deletion mutant in the human cytomegalovirus gene encoding IE1(491aa) is replication defective due to a failure in autoregulation. *Proc. Natl. Acad. Sci. U S A* **93**(21), 11321-6.

Morello, C. S., Cranmer, L. D., and Spector, D. H. (1999). In vivo replication, latency, and immunogenicity of murine cytomegalovirus mutants with deletions in the M83 and M84 genes, the putative homologs of human cytomegalovirus pp65 (UL83). *J. Virol.* **73**(9), 7678-93.

Morello, C. S., Cranmer, L. D., and Spector, D. H. (2000). Suppression of murine cytomegalovirus (MCMV) replication with a DNA vaccine encoding MCMV M84 (a homolog of human cytomegalovirus pp65). *J. Virol.* **74**(8), 3696-708.

Munch, K., Messerle, M., Plachter, B., and Koszinowski, U. H. (1992). An acidic region of the 89K murine cytomegalovirus immediate early protein interacts with DNA. *J. Gen. Virol.* **73**(Pt 3), 499-506.

Murphy, P. M. (1994). The molecular biology of leukocyte chemoattractant receptors. *Annu. Rev. Immunol.* **12,** 593-633.

Nguyen, Q., Champlin, R., Giralt, S., Rolston, K., Raad, I., Jacobson, K., Ippoliti, C., Hecht, D., Tarrand, J., Luna, M., and Whimbey, E. (1999). Late cytomegalovirus pneumonia in adult allogeneic blood and marrow transplant recipients. *Clin. Infect. Dis.* **28**(3), 618-23.

Nicholas, J. (1996). Determination and analysis of the complete nucleotide sequence of human herpesvirus. *J. Virol.* **70**(9), 5975-89.

Onno, M., Pangault, C., Le Friec, G., Guilloux, V., Andre, P., and Fauchet, R. (2000). Modulation of HLA-G antigens expression by human cytomegalovirus: specific induction in activated macrophages harboring human cytomegalovirus infection. *J. Immunol.* **164**(12), 6426-34.

Orange, J. S., and Biron, C. A. (1996a). An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *J. Immunol.* **156**(3), 1138-42.

Orange, J. S., and Biron, C. A. (1996b). Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *J. Immunol.* **156**(12), 4746-56.

Pande, H., Campo, K., Shanley, J. D., Creeger, E. S., Artishevsky, A., Gallez-Hawkins, G., and Zaia, J. A. (1991). Characterization of a 52K protein of murine cytomegalovirus and its immunological cross-reactivity with the DNA-binding protein ICP36 of human cytomegalovirus. *J. Gen. Virol.* **72**(Pt 6), 1421-7.

Papadimitriou, J. M., Shellam, G. R., and Robertson, T. A. (1984). An ultrastructural investigation of cytomegalovirus replication in murine hepatocytes. *J. Gen. Virol.* **65**(Pt 11), 1979-90.

Pari, G. S., and Anders, D. G. (1993). Eleven loci encoding trans-acting factors are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA replication. *J. Virol.* **67**(12), 6979-88.

Pari, G. S., Kacica, M. A., and Anders, D. G. (1993). Open reading frames UL44, IRS1/TRS1, and UL36-38 are required for transient complementation of human cytomegalovirus oriLyt-dependent DNA synthesis. *J. Virol.* **67**(5), 2575-82.

Penfold, M. E., Dairaghi, D. J., Duke, G. M., Saederup, N., Mocarski, E. S., Kemble, G. W., and Schall, T. J. (1999). Cytomegalovirus encodes a potent alpha chemokine. *Proc. Natl. Acad. Sci. U S A* **96**(17), 9839-44.

Penfold, M. E., and Mocarski, E. S. (1997). Formation of cytomegalovirus DNA replication compartments defined by localization of viral proteins and DNA synthesis. *Virology* **239**(1), 46-61.

Pollock, J. L., Presti, R. M., Paetzold, S., and Virgin, H. W. t. (1997). Latent murine cytomegalovirus infection in macrophages. *Virology* **227**(1), 168-79.

Puchtler, E., and Stamminger, T. (1991). An inducible promoter mediates abundant expression from the immediate- early 2 gene region of human cytomegalovirus at late times after infection. *J. Virol.* **65**(11), 6301-6.

Rawlinson, W. D., Farrell, H. E., and Barrell, B. G. (1996). Analysis of the complete DNA sequence of murine cytomegalovirus. *J. Virol.* **70**(12), 8833-49.

Reddehase, M. J., Balthesen, M., Rapp, M., Jonjic, S., Pavic, I., and Koszinowski, U. H. (1994). The conditions of primary infection define the load of latent viral genome in organs and the risk of recurrent cytomegalovirus disease. *J. Exp. Med.* **179**(1), 185-93.

Reusch, U., Muranyi, W., Lucin, P., Burgert, H. G., Hengel, H., and Koszinowski, U. H. (1999). A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation. *Embo. J.* **18**(4), 1081-91.

Reynolds, R. P., Rahija, R. J., Schenkman, D. I., and Richter, C. B. (1993). Experimental murine cytomegalovirus infection in severe combined immunodeficient mice. *Lab. Anim. Sci.* **43**(4), 291-5.

Roizman, B. (2000). Redefining virology. *Science* **288**(5475), 2327-8.

Romanowski, M. J., and Shenk, T. (1997). Characterization of the human cytomegalovirus irs1 and trs1 genes: a second immediate-early transcription unit within irs1 whose product antagonizes transcriptional activation. *J. Virol.* **71**(2), 1485-96.

Saederup, N., Lin, Y. C., Dairaghi, D. J., Schall, T. J., and Mocarski, E. S. (1999). Cytomegalovirus-encoded beta chemokine promotes monocyte-associated viremia in the host. *Proc. Natl. Acad. Sci. U S A* **96**(19), 10881-6.

Samaniego, L. A., Tevethia, M. J., and Spector, D. J. (1994). The human cytomegalovirus 86-kilodalton immediate-early 2 protein: synthesis as a precursor polypeptide and interaction with a 75- kilodalton protein of probable viral origin. *J. Virol.* **68**(2), 720-9.

Sanchez, V., Greis, K. D., Sztul, E., and Britt, W. J. (2000). Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly. *J. Virol.* **74**(2), 975-86.

Sanchez, V., Sztul, E., and Britt, W. J. (2000). Human cytomegalovirus pp28 (UL99) localizes to a cytoplasmic compartment which overlaps the endoplasmic reticulum-golgi-intermediate compartment. *J. Virol.* **74**(8), 3842-51.

Scalzo, A. A., Fitzgerald, N. A., Simmons, A., La Vista, A. B., and Shellam, G. R. (1990). Cmv-1, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J. Exp. Med.* **171**(5), 1469-83.

Schmader, K., Henry, S. C., Rahija, R. J., Yu, Y., Daley, G. G., and Hamilton, J. D. (1995). Mouse cytomegalovirus reactivation in severe combined immune deficient mice after implantation of latently infected salivary gland. *J. Infect. Dis.* **172**(2), 531-4.

Schwartz, R., Helmich, B., and Spector, D. H. (1996). CREB and CREB-binding proteins play an important role in the IE2 86- kilodalton protein-mediated transactivation of the human cytomegalovirus 2.2-kilobase RNA promoter. *J. Virol.* **70**(10), 6955-66.

Scully, A. L., Sommer, M. H., Schwartz, R., and Spector, D. H. (1995). The human cytomegalovirus IE2 86-kilodalton protein interacts with an early gene promoter via site-specific DNA binding and protein-protein associations. *J. Virol.* **69**(10), 6533-40.

Shamu, C. E., Story, C. M., Rapoport, T. A., and Ploegh, H. L. (1999). The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. *J Cell Biol* **147**(1), 45-58.

Shanley, J. D., Shanley, J. A., Albert, G., and Biegel, D. (2001). Characterization of virus-induced interferon-gamma responses in mice previously infected with murine cytomegalovirus. *J. Infect. Dis.* **183**(5), 697-706.

Sinzger, C., Bissinger, A. L., Viebahn, R., Oettle, H., Radke, C., Schmidt, C. A., and Jahn, G. (1999). Hepatocytes are permissive for human cytomegalovirus infection in human liver cell culture and In vivo. *J. Infect. Dis.* **180**(4), 976-86.

Sinzger, C., and Jahn, G. (1996). Human cytomegalovirus cell tropism and pathogenesis. *Intervirology* **39**(5-6), 302-19.

Sinzger, C., Kahl, M., Laib, K., Klingel, K., Rieger, P., Plachter, B., and Jahn, G. (2000). Tropism of human cytomegalovirus for endothelial cells is determined by a post-entry step dependent on efficient translocation to the nucleus. *J. Gen. Virol.* **81 Pt 12**, 3021-35.

Soderberg-Naucler, C., Fish, K. N., and Nelson, J. A. (1997a). Interferon-gamma and tumor necrosis factor-alpha specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines. *J. Clin. Invest.* **100**(12), 3154-63.

Soderberg-Naucler, C., Fish, K. N., and Nelson, J. A. (1997b). Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* **91**(1), 119-26.

Soderberg-Naucler, C., Fish, K. N., and Nelson, J. A. (1998). Growth of human cytomegalovirus in primary macrophages. *Methods* **16**(1), 126-38.

Spector, D. H. (1996). Activation and regulation of human cytomegalovirus early genes. *Intervirology* **39**(5-6), 361-77.

Staczek, J. (1990). Animal cytomegaloviruses. *Microbiol. Rev.* **54**(3), 247-65.

Staprans, S. I., Rabert, D. K., and Spector, D. H. (1988). Identification of sequence requirements and trans-acting functions necessary for regulated expression of a human cytomegalovirus early gene. *J. Virol.* **62**(9), 3463-73.

Staprans, S. I., and Spector, D. H. (1986). 2.2-kilobase class of early transcripts encoded by cell-related sequences in human cytomegalovirus strain AD169. *J. Virol.* **57**(2), 591-602.

Stenberg, R. M. (1993). Immediate-early genes of human cytomegalovirus:organization and function. *In* "Frontiers of virology 2, Molecular aspects of Human Cytomegalovirus diseases", pp. 330-359. Springer-Verlag Berlin Heidelberg, Berlin, Germany.

Stenberg, R. M. (1996). The human cytomegalovirus major immediate-early gene. *Intervirology* **39**(5-6), 343-9.

Stenberg, R. M., Depto, A. S., Fortney, J., and Nelson, J. A. (1989). Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. *J. Virol.* **63**(6), 2699-708.

Stenberg, R. M., Fortney, J., Barlow, S. W., Magrane, B. P., Nelson, J. A., and Ghazal, P. (1990). Promoter-specific trans activation and repression by human

cytomegalovirus immediate-early proteins involves common and unique protein domains. *J. Virol.* **64**(4), 1556-65.

Stoddart, C. A., Cardin, R. D., Boname, J. M., Manning, W. C., Abenes, G. B., and Mocarski, E. S. (1994). Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. *J. Virol.* **68**(10), 6243-53.

Streblow, D. N., Soderberg-Naucler, C., Vieira, J., Smith, P., Wakabayashi, E., Ruchti, F., Mattison, K., Altschuler, Y., and Nelson, J. A. (1999). The human cytomegalovirus chemokine receptor US28 mediates vascular smooth muscle cell migration. *Cell* **99**(5), 511-20.

Sweet, C. (1999). The pathogenicity of cytomegalovirus. *FEMS. Microbiol. Rev.* **23**(4), 457-82.

Tenney, D. J., and Colberg-Poley, A. M. (1991). Human cytomegalovirus UL36-38 and US3 immediate-early genes: temporally regulated expression of nuclear, cytoplasmic, and polysome-associated transcripts during infection. *J. Virol.* **65**(12), 6724-34.

Thale, R., Lucin, P., Schneider, K., Eggers, M., and Koszinowski, U. H. (1994). Identification and expression of a murine cytomegalovirus early gene coding for an Fc receptor. *J. Virol.* **68**(12), 7757-65.

Thale, R., Szepan, U., Hengel, H., Geginat, G., Lucin, P., and Koszinowski, U. H. (1995). Identification of the mouse cytomegalovirus genomic region affecting major histocompatibility complex class I molecule transport. *J. Virol.* **69**(10), 6098-105.

Tomazin, R., Boname, J., Hegde, N. R., Lewinsohn, D. M., Altschuler, Y., Jones, T. R., Cresswell, P., Nelson, J. A., Riddell, S. R., and Johnson, D. C. (1999).

Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. *Nat. Med.* **5**(9), 1039-43.

Vieira, J., Schall, T. J., Corey, L., and Geballe, A. P. (1998). Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J. Virol.* **72**(10), 8158-65.

Wade, E. J., Klucher, K. M., and Spector, D. H. (1992). An AP-1 binding site is the predominant cis-acting regulatory element in the 1.2-kilobase early RNA promoter of human cytomegalovirus. *J. Virol.* **66**(4), 2407-17.

Waldman, W. J., Roberts, W. H., Davis, D. H., Williams, M. V., Sedmak, D. D., and Stephens, R. E. (1991). Preservation of natural endothelial cytopathogenicity of cytomegalovirus by propagation in endothelial cells. *Arch. Virol.* **117**(3-4), 143-64.

Weiland, F., Keil, G. M., Reddehase, M. J., and Koszinowski, U. H. (1986). Studies on the morphogenesis of murine cytomegalovirus. *Intervirology* **26**(4), 192-201.

Weston, K. (1988). An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. *Virology* **162**(2), 406-16.

Wiertz, E. J., Jones, T. R., Sun, L., Bogyo, M., Geuze, H. J., and Ploegh, H. L. (1996a). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell* **84**(5), 769-79.

Wiertz, E. J., Tortorella, D., Bogyo, M., Yu, J., Mothes, W., Jones, T. R., Rapoport, T. A., and Ploegh, H. L. (1996b). Sec61-mediated transfer of a membrane protein

from the endoplasmic reticulum to the proteasome for destruction. *Nature* **384**(6608), 432-8.

Wing, B. A., Lee, G. C., and Huang, E. S. (1996). The human cytomegalovirus UL94 open reading frame encodes a conserved herpesvirus capsid/tegument-associated virion protein that is expressed with true late kinetics. *J. Virol.* **70**(6), 3339-45.

Winkler, M., and Stamminger, T. (1996). A specific subform of the human cytomegalovirus transactivator protein pUL69 is contained within the tegument of virus particles. *J. Virol.* **70**(12), 8984-7.

Xiao, J., Tong, T., Zhan, X., Haghjoo, E., and Liu, F. (2000). In vitro and in vivo characterization of a murine cytomegalovirus with a transposon insertional mutation at open reading frame M43. *J. Virol.* **74**(20), 9488-97.

Yurochko, A. D., Hwang, E. S., Rasmussen, L., Keay, S., Pereira, L., and Huang, E. S. (1997). The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-kappaB during infection. *J. Virol.* **71**(7), 5051-9.

Yurochko, A. D., Kowalik, T. F., Huong, S. M., and Huang, E. S. (1995). Human cytomegalovirus upregulates NF-kappa B activity by transactivating the NF-kappa B p105/p50 and p65 promoters. *J. Virol.* **69**(9), 5391-400.

Zanghellini, F., Boppana, S. B., Emery, V. C., Griffiths, P. D., and Pass, R. F. (1999). Asymptomatic primary cytomegalovirus infection: virologic and immunologic features. *J. Infect. Dis.* **180**(3), 702-7.

Zhu, H., Cong, J. P., and Shenk, T. (1997). Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular

RNAs: induction of interferon-responsive RNAs. *Proc. Natl. Acad. Sci. U S A* **94**(25), 13985-90.

Zhu, H., Shen, Y., and Shenk, T. (1995). Human cytomegalovirus IE1 and IE2 proteins block apoptosis. *J. Virol.* **69**(12), 7960-70.

Ziegler, H., Thale, R., Lucin, P., Muranyi, W., Flohr, T., Hengel, H., Farrell, H., Rawlinson, W., and Koszinowski, U. H. (1997). A mouse cytomegalovirus glycoprotein retains MHC class I complexes in the ERGIC/cis-Golgi compartments. *Immunity* **6**(1), 57-66.

VITA

Name: Zaruhi Karabekian

Title: Graduate student
Department of Microbiology and Molecular Cell Biology
Easter Virginia Medical School
Lewis Hall, P.O. Box 1980
Norfolk, VA 23507

Education: **1994-present** Ph.D. Candidate. Biomedical Sciences Ph.D. Program (Cell Biology and Molecular Pathogenesis Track), Eastern Virginia Medical School, Norfolk, Virginia, USA.

1988-1989 Masters of Science (equivalent). Dept. of Botany and Mycology Yerevan State University, Yerevan, Armenia.

1983-1987 Bachelors of Science (equivalent). Dept. of Botany and Mycology Yerevan State University, Yerevan, Armenia.

Awards: **Virology Focal Group Award**, Research Day 2000, Eastern Virginia Medical School
Graduate Student Research Paper Award,
American Society for Microbiology, Virginia Branch Annual Meeting, 1997.
Best Graduate Student Poster Presentation,
Research Day 1997, Eastern Virginia Medical School

Publications:

Hanson L.K., J.S. Slater, Z. Karabekian, M.R. MacDonald, H.W. Virgin IV, Ch.A. Biron, M.C. Ruzek, N. van Rooijen, R.P. Ciavarra, R.M. Stenberg, and A.E. Campbell. Replication of murine cytomegalovirus in differentiated macrophages as a determinant of viral pathogenesis. 1999. Journal of Virology, **73**:5970-5980.

Hanson, L.K., B.L. Dalton, Z Karabekian, H.E. Farrell, W.D. Rawlinson, R.M. Stenberg, and A.E. Campbell. Transcriptional analysis of the murine cytomegalovirus Hind III I region: Identification of a novel immediate early gene region. 1999. Virology, **260**:156-164.

Hanson, L. K., J. S.Slater, Z. Karabekian, G. Ciocco-Schmitt, and A. E. Campbell. Characterization of the murine cytomegalovirus M139, M140, and M141 US22 gene products have multiple roles in viral pathogenesis (in press Journal of Virology, January 2001).