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CHARACTERIZATION OF MURINE CYTOMEGALOVIRUS

m142 AND m143 ESSENTIAL GENE PRODUCTS

by

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Eastern Virginia Medical School and Old Dominion University
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AND OLD DOMINION UNIVERSITY
MAY 2001

Approved by:

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ABSTRACT

CHARACTERIZATION OF MURINE CYTOMEGALOVIRUS m142 AND m143 ESSENTIAL GENE PRODUCTS

Bridget Dalton
Eastern Virginia Medical School and Old Dominion University, 2001
Director: Dr. Ann Campbell

Cytomegalovirus (CMV) is a species-specific virus belonging to the Herpesviridae family. This DNA virus causes severe disease or even death in newborns and immunosuppressed patients. Murine CMV (MCMV) provides an opportunity to study the role of various viral products in replication and pathology in the natural host. Successful replication of CMV in the host depends upon the expression of a cascade of viral genes: immediate early (IE), early (E), and late (L). To date only three MCMV IE proteins have been characterized (IE1, IE2 and IE3). Our laboratory recently identified a novel IE gene region within the Hind III I region of MCMV. These genes, m142 and m143, are members of the US22 gene family of HCMV, some of which are transcriptional transactivators. The m142 and m143 genes are also essential for viral replication in fibroblasts.

The purpose of this dissertation was to characterize the m142 and m143 transcripts and proteins, as well as determine if the gene products function as transcriptional transactivators like some of the other US22 gene family members. Although the m142 and m143 transcripts were present during IE times, levels of these two transcripts increased during early times of viral infection and remained abundant during late times. The genes, m142 and m143, encode a 1.8 and 3.8 kb transcript, respectively. The m142 and m143 proteins (designated pm142 and pm143, respectively) encoded by these transcripts were present by 3 hours post infection and

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remained abundant at 12 and 24 hours post infection. Interestingly, pm142 and pm143 could not be designated as immediate early proteins, because they were not expressed in the presence of drugs that block viral growth at this specific stage within the viral replication cycle. Therefore, the m142 and m143 immediate early genes do not express detectable levels of protein until early times post infection. The pm142 protein is 43 kD protein, and the pm143 protein is 53 kD. The pm142 and pm143 proteins localized to both the nucleus and the cytoplasm at 3, 4 and 24 hours post infection.

In transactivation studies, we tested the ability of pm142 and pm143 to activate the MCMV major immediate early promoter (M122-123) and the early e1 promoter (M112-113). In combination, MCMV pm142 and pm143 transactivated the MCMV major immediate early promoter and enhancer (MIEPE) in a dose dependent manner to at least 3 fold above basal levels. However, individually pm142 or pm143 failed to transactivate the MIEPE. Together, pm142 and pm143 also exhibited noncooperative effects with IE1 and IE3 in the activation of the MCMV MIEPE. In combination, pm142 and pm143 failed to activate the early e1 promoter, but pm142 and pm143 cooperated with IE1 and IE3 to activate this promoter. Activation of the e1 promoter by IE1 and IE3 increased from 30 fold above basal levels in the absence of pm142 and pm143, to as high as 100 fold above basal levels in the presence of pm142 and pm143. This was the first time it has been shown that other MCMV viral proteins assist in IE1/IE3 mediated transactivation of the e1 promoter or any other promoter, and these findings have important implications in MCMV replication.

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This work is dedicated to my daddy Mr. Milton Dalton and my mom Ms. Mary Dalton, for their love, support, encouragement and prayers.

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With sincere gratitude, I thank my mentor Dr. Ann Campbell, for all she has done for me. She has been there to encourage and support me from the very first day I entered the Ph.D. program, until now. She is a role model to me, not only because of her scientific intellect and thoroughness, but also because she is a kind and compassionate person. She has been a good friend, and I hope our friendship will continue to grow in the future. I would also like to thank the members of my guidance committee: Dr. Julie Kerry, Dr. Kenneth Somers, and Dr. Timothy Bos.

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Last but not least, I would like to thank my dad, Mr. Milton Dalton, for teaching me what hard work and discipline means. I would also like to thank my mom, Ms. Mary Dalton, for teaching me that I can accomplish any goal I set for myself.

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CHAPTER I

INTRODUCTION

Cytomegaloviruses

The cytomegaloviruses are members of the herpesvirus family. They share many characteristics with the other herpesviruses such as virion and genome structure and the ability to establish latent and persistent infection (reviewed in Mocarski, 1996). Cytomegaloviruses infect a wide variety of host species including rodents, domestic animals, and primates (Plummer, 1973). The cytomegaloviruses have several distinguishing characteristics. They are highly species specific, display a tropism for the salivary gland, have a relatively long replication cycle, and produce cell enlargement with intranuclear inclusions (Roizman et al., 1981).

Cytomegalovirus Associated Disease

In North America, the prevalence of human cytomegalovirus (HCMV) infection is about 50% of the population in rural areas (reviewed in Mocarski, 1996). In major cities, the prevalence of cytomegalovirus infection is up to 90% (Mocarski, 1996). In immunocompetent individuals, HCMV infection is usually mild or asymptomatic. However, the virus persists in the host for life in a latent st[l]ate, with episodes of spontaneous reactivation. The virus can cause severe disease, and even death in those individuals with suppressed immune systems. Therefore individuals that are at high risk for HCMV disease include the very young, such as the developing fetus or newborn, as well as patients undergoing immunosuppressive therapy for either cancer or organ

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transplantation, or those with immunodeficiency diseases such as AIDS (Mocarski, 1996).

Approximately 0.5-1% of all newborns are born infected with HCMV. However, only one out of ten of these infants demonstrate symptoms at birth. The clinical manifestations of congenital HCMV include brain damage, mental retardation, ocular damage, hepatic disease and pneumonia. Neurologic damage is a symptom of severe disseminated disease (Fjaer et al., 1997; Ahlfors et al., 1999; Dahle et al., 2000; Coats et al., 2000).

Cytomegalovirus is the most important infectious agent affecting organ and bone marrow transplant recipients. At least two-thirds of organ transplant and bone marrow recipients exhibit evidence of HCMV infection within 1-4 months after transplantation (Rubin, 1990; Zaia, 1993; Costa et al., 1999). More serious disease occurs when seronegative recipients receive organs from a seropositive donor, since a primary HCMV infection ensues under conditions in which the transplant recipient may be immunosuppressed. HCMV is also associated with decreased graft survival. It is proposed that rejection of the graft is due to a viral-induced increase in the expression of MHC antigens within the graft, predisposing the graft to rejection. Alternatively molecular mimicry could explain host mediated graft rejection of the infected organ. In this case, HCMV proteins with homology to MHC class 1 molecules within the organ induce an immune response against the transplanted organ (Mocarski, 1996; Borchers et al., 1999). Transplant recipients with HCMV develop a wide variety of clinical diseases. These include prolonged fevers, leukopenia, thrombocytopenia, atypical lymphocytosis

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and elevated hepatic transaminases. Severe HCMV infections are associated with gastrointestinal problems, hepatitis and pneumonia (Ho, 1991).

HCMV is also one of the most important opportunistic infections encountered in AIDS patients. About 90% of AIDS patients develop HCMV infection, and up to 40% may develop vision and/or life threatening HCMV induced disease (Gallant et al., 1992). The most common manifestations of disease include encephalitis, retinitis, colitis and CMV pneumonitis (Roullet, 1999; Chakraborty, 1999; Soloman and Perlman, 1999) . The link between HCMV and AIDS could be due to molecular interactions between HIV and HCMV. The HIV long terminal repeat is transcriptionally activated by the HCMV major immediate early (IE) proteins, and HCMV replication is enhanced by the presence of active HIV infection (Rando et al., 1990; Walker et al., 1992; McCarthy et al., 1998; Yurochko et al., 1999). However to date, the role of HCMV in AIDS is poorly understood.

Cytomegalovirus Pathogenesis

HCMV is usually spread by direct or indirect person to person contact (reviewed in Mocarski, 1996). Sources of the virus include the saliva, urine, cervical and vaginal secretions, semen, breast milk, tears, feces and blood. In individuals with severe disseminated HCMV, virtually all organ systems may be affected. However, the salivary gland, the genitourinary tract, the gastrointestinal tract, the respiratory tract, the central nervous system, and the liver are sites commonly involved in HCMV infections of immunosuppressed individuals. In individuals with a normal immune system, no clinical evidence of infection occurs. However, the virus remains associated with the individual

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for life, either as an asymptomatic productive infection or in a latent state (Mocarski, 1996).

Cellular immune responses are important in curtailing the virus infection and maintaining the virus-host equilibrium in persistent HCMV infection (Alp et al., 1991). Natural killer cells are activated early after viral infection and are a part of the non-specific immune response. During this time, interferon alpha/beta are produced by macrophages, and they represent another non-specific antiviral defense mechanism. Cell mediated immunity involving CD4+ and CD8+ T lymphocytes are responsible for clearing the virus from target organs. The HCMV immediate early 1 (IE1) protein is a target for CD8+ T cells in humans (Alp et al., 1991), as is the equivalent IE1 protein in mice (Reddehase et al., 1987). Several late HCMV proteins are also the target of specific cytotoxic T cell responses. These proteins include pp65, gB, pp150 and pp28 (McLaughlin-Taylor et al., 1994; Hopkins et al., 1996; Li et al., 1997; Gyulai et al., 2000). HCMV IE1 also contains several immunodominant polypeptides, which elicit an antibody response against HCMV (Landini and La Placa, 1991). The CD4+ T cells are important helper cells for antibody and CD8+ responses (Alp et al., 1991). Antibodies may control systemic spread of recurrent infections following reactivation, however they do not participate in clearing virus from target organs (Landini and La Placa, 1991).

HCMV infects several different cell types. These include epithelial, endothelial, mesothelial, fibroblast, and glial cells (Sinzger et al., 1993). Leukocytes circulating in the peripheral blood are also susceptible to CMV infection (Sinzger et al., 1996) and these cells play a central role in viral pathogenesis. More specifically, the monocyte precursor cell or CD34+ bone marrow progenitor cell has been shown to harbor HCMV

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DNA in the form of a circular plasmid (Bolovan-Fritts et al, 1999), under conditions in which transcription from the viral genome is limited. However when these cells are allowed to differentiate in culture, infectious virus can be recovered. Therefore in vivo, the CD34+ bone marrow progenitor cell serves as a reservoir of latent virus and also as a vehicle for disseminating the virus to target organs (Maciejewski et al, 1992; Mendelson et al., 1996; Maciejewski and St Jeor, 1999). The CD34+ cells differentiate into CD33+ cells. Latent infection, the expression of latency associated transcripts, and reactivation of virus upon cellular differentiation has also been demonstrated in cells coexpressing CD33 and CD15 or CD33 and CD14 along with dendritic cell markers (Hahn et al, 1998).

During latency, HCMV viral gene expression is restricted such that only immediate early transcripts are detected. The immediate early transcripts expressed during latency originate from a single locus, the major immediate early gene locus. Two genes are expressed from this locus during latency (ie1 and ie2); however, these transcripts differ significantly from those present during a productive infection. Latent transcripts called sense transcripts are encoded in the same direction as productive phase transcripts but use novel start sites in the HCMV ie1/ie2 promoter region. Other latent transcripts called antisense transcripts are unspliced and are complementary to exons within ie1. Antibody to the proteins encoded by these transcripts are detectable in the serum of seropositive individuals implying that protein products are synthesized from these transcripts during latent or productive infection (Kondo et al., 1994; Kondo and Mocarski, 1996; Landini et al., 2000).

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Factors that cause the latently infected monocyte precursor cell to differentiate into a fully permissive macrophage contribute to HCMV reactivation. When myeloid progenitor cells are experimentally infected, HCMV can be reactivated when these cells are cultured in the presence of interferon gamma, tumor necrosis factor alpha (TNFα), interleukin-4 (IL-4) or granulocyte-macrophage-colony-stimulating factor (GM-CSF) (Hahn et al., 1998). These cells can also reactivate when cultured in the presence of fibroblasts or fibroblast conditioned medium. Naturally infected macrophage progenitor cells can reactivate latent HCMV when allogeneically stimulated. The cytokine interferon-gamma, but not IL-4, TNF or GM-CSF, is essential for reactivation in these cells (Soderberg-Naucler et al., 1998). Therefore, there are differences between experimentally and naturally infected cells.

The murine model has been used to address the role that monocytes and macrophages play in the pathogenesis of cytomegalovirus infection. Since CMV is species specific, MCMV infection offers the opportunity to study CMV disease, protection against it, and conditions for establishing persistent and latent infection in the natural host. In the mouse model, the macrophage progenitor cell not only is one of the first cell types to support MCMV replication during infection, but macrophages also disseminate the virus to the spleen, lung, and liver, and harbor latent MCMV DNA (Collins et al., 1993; Stoddart, 1994; Pollock and Virgin, 1995; Koffron et al., 1998). On the other hand, the infected macrophage triggers the early innate immune response by producing monokines like interferon alpha/beta, TNF alpha, and IL-12. Interferon alpha leads to the activation of NK cells, which produce interferon gamma. Interferon

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gamma activates macrophages which makes them less permissive and limits viral gene expression, and along with IL-12 activates CTLs (Hanson et al., 1999).

As stated previously, monocyte progenitor cells are a source of latent CMV and also serve as a source of CMV dissemination and reactivation (Zhuravskaya et al., 1997). During latency, monocytes harbor viral DNA; however, the presence of infectious virus can not be detected. In mice, macrophages that are latently infected with MCMV contain as many as 10 copies of the MCMV genome per cell. When the latently infected monocyte is cocultured with murine embryonic fibroblast cells, infectious virus is reactivated (Pollock et al., 1997). Latency has been observed in the heart, kidney, liver, lung, spleen, brain, and salivary glands of mice (Collins et al., 1993, Reddehase et al., 1994).

During latency in the lung, transcription from the MCMV major immediate early promoter occurs (Kurz et al., 1999; Kurz and Reddehase, 1999). Under these conditions transcription is restricted to ie1, while the ie3 transcript equivalent to ie2 of HCMV is absent. The transcription of ie1 in the lung tissue during latency is focal and randomly distributed, although the viral genome is evenly distributed throughout the entire organ. Foci that express ie1 may be progressing through several sequentially ordered control points that when passed, will lead to recurrence (Kurz et al., 1999; Kurz and Reddehase, 1999). The switch from ie1 to ie3 precursor mRNA transcriptional processing may trigger this transition. During recurrence, ie1 transcription in the lung is generalized, and the ie3 transcript is present (Kurz et al., 1999; Kurz and Reddehase, 1999).

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The Viral Genome

Human CMV has a very large genome, of over 230 kilobase pairs, encoding over 230 genes (Chee et al., 1990). These genes are numbered sequentially within the viral genome. The genome is arranged in a very complex manner, containing internal reiterated sequences that enable the virus double-stranded DNA to arrange in four genomic isomers. For example, HCMV has sequences from both termini that are repeated in an inverted orientation and juxtaposed internally dividing the genome into a unique long region and a unique short region (UL and US), each flanked by internal repeats. The unique long region of HCMV is flanked by terminal repeat sequences (TRL) and internal repeat sequences (IRL). Likewise, the unique short region of the virus is flanked by terminal repeat sequences (TRS) and internal repeat sequences (IRS) (Jahn and Sinzger, 1996). HCMV genes are named according to their location within the repeat sequences or unique regions of the viral genome as well as their number within the HCMV genome (ex. UL112).

In HCMV the unique components can invert relative to each other during replication giving rise to the four genomic isomers. Virally infected cells can contain any one of the four isomers and each isomer is present at equimolar concentrations (Oram et al., 1982). The MCMV genome however, consists primarily of unique sequences with few repeat sequences. Therefore, no isomers are present in the murine strain. Although the HCMV and MCMV genomes have been completely sequenced, analysis of individual gene function is incomplete. However, HCMV and MCMV genes are characterized as either essential or non-essential for viral growth in cultured cells. Essential genes are those required for virion structure and virus replication in tissue culture, whereas non-

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essential genes are those not required for virus growth in cell culture but may be involved in virus/host interactions in vivo (Mocarski et al., 1990).

The Virion Structure

CMV has an icosahedral virion structure containing the double stranded DNA genome, and is surrounded by a tegument. These components are packaged in a lipid bilayer envelope containing virally encoded glycoproteins (Wright et al., 1964).

The CMV capsid contains seven proteins. These proteins include the major capsid protein (UL86), the minor capsid protein (UL85), the minor capsid binding protein (UL46), the smallest capsid protein (UL48.5), and three distinct assemblin/assembly proteins (UL80.5, UL80, and UL80a). These proteins make up the structure of the capsid or are involved in capsid assembly (Mocarski, 2000).

An array of fibrous, granular proteins called tegument proteins are interfaced between the capsid and the envelope. There are 25 tegument proteins and most of these proteins are phosphorylated and denoted by the prefix "pp". The function of most tegument proteins is unknown. However, several have been shown to function as transcriptional transactivators (Mocarski, 2000). For example, HCMV pp71 (UL82) is a tegument protein, which activates HCMV IE gene expression and increases the infectivity of HCMV DNA (Lui and Stinski, 1992; Baldick et al., 1997; Chau et al., 1999). Recently, several HCMV transcripts called virion RNAs were found to be packaged within the virion (Bresnahan and Shenk, 2000). The function of the putative proteins encoded by these transcripts is unknown (Bresnahan and Shenk, 2000).

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The viral envelope is composed of a lipid bilayer membrane containing eight virally encoded glycoproteins. The viral envelope contains two prominent glycoprotein complexes. One is composed of dimers of gB (UL55), and the other is composed of gH, gL and gO (UL75, UL115, and UL74) (Mocarski, 2000). Several of these glycoproteins are highly conserved among the herpesviruses (gB, gH, gL, and gM). These proteins are involved in virion attachment, penetration, transmission from cell to cell, and fusion of infected cells (Britt and Mach, 1996). These proteins, in particular glycoprotein gB, are also targets of the host humoral immune response (Navarro et al., 1997). The envelope of CMV renders this virus sensitive to low pH, lipid solvents and heat; accordingly, virions have a half life of approximately 60 minutes at 37 degrees Celsius (Vonka and Benyesh-Melnick, 1966).

Cytomegalovirus Attachment/Penetration

The initial event in the viral replication cycle is viral attachment. During viral attachment, the viral envelope protein gB binds to cell surface heparin sulfate (Compton et al., 1993; Navarro et al., 1993; Boyle and Compton, 1998). The initial interaction with heparin sulfate is followed by a more specific interaction between the virus and a cellular receptor. The cellular receptor that mediates this event is unknown. It was proposed that a 30-36 kDa gB-binding cell surface protein is involved in attachment. However when this protein, which has been identified as annexin II, was blocked with annexin II specific antibodies, viral entry was not hampered. These experiments suggest that annexin II is not the true molecule involved in CMV attachment and penetration (Pietropaolo and Compton, 1999).

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After the virus particle has attached to the cell surface, penetration occurs within 5 minutes (Bodaghi et al., 1999). HCMV penetrates the host cell in a pH independent manner mediated by fusion between the viral envelope and the host cell plasma membrane. This event likely involves the complex of envelope glycoproteins gH, gL and gO (Compton et al., 1992). Following viral entry, the viral nucleocapsids enter the nuclear pores and deliver the viral DNA to the nucleus for expression (Bodaghi et al., 1999).

Cytomegalovirus Replication

Cytomegalovirus genes are expressed in three sequential phases: immediate early, early, and late. Immediate early gene expression does not require de novo protein synthesis. The immediate early gene products activate the expression of the early genes and serve to autoregulate their own expression. The early genes encode proteins involved in DNA replication; for example the DNA polymerase, the DNA polymerase accessory protein, and the helicase/primase complex. Other early genes are not required for viral DNA replication, but are involved in viral pathogenesis, tissue tropism and immune evasion. Late genes encode structural proteins involved in packaging the viral genome, such as the tegument proteins and the envelope glycoproteins (Mocarski, 1996).

Drugs have been used to define the three classes of CMV genes expressed in an infected cell. The drugs anisomycin and cycloheximide prevent protein synthesis, thereby allowing expression of only the immediate early genes from a CMV infected cell. Gancyclovir and phosphonoacetic acid prevent viral DNA replication by causing premature nucleic acid chain termination and inhibition of the activity of the viral DNA

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polymerase. Therefore the replication cycle is blocked at the expression of the early genes. Late genes are those that are expressed following viral DNA replication. These genes are expressed when the viral genome is allowed to replicate completely in the absence of drug inhibitors.

Recently DNA microarray technology has been used to study the three classes of viral genes expressed from CMV infected cells. This system allows all HCMV genes to be studied simultaneously. Briefly, a DNA microarray containing sequences from each individual viral open reading frame was printed on glass slides. These viral oligonucleotides were then analyzed in a single hybridization step using flourescently labeled cDNA from virally infected cells as a probe. The viral cDNA was obtained from cells that had been treated with the drug inhibitors mentioned previously. The results displayed a temporal map of both known and previously unknown immediate early (IE), early (E), and late (L) genes of the entire HCMV genome. Since this system allows the study of over 200 genes in a single experiment, it is particularly important when studying large viral genomes like those in HCMV (Chambers et al., 1999).

In MCMV, immediate early genes are expressed 0-3 hours post infection, early genes are expressd 3-12 hours post infection and late genes are expressed 12-24 hours post infection (Keil et al., 1984; Messerle et al., 1991; Messerle et al., 1992). HCMV replication occurs much slower than MCMV replication. The immediate early genes are expressed 0-24 hours, early genes are expressed 24-48 hours and late genes are expressed 48-72 hours post infection.

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HCMV Major Immediate Early Genes

There are five regions within the HCMV genome that are transcribed at immediate early times. These include UL36-38, UL122-123, TRS1-IRS1, UL69 and US3. The majority of the IE transcripts arise from the UL122-123 locus and are referred to as major immediate early proteins (Fig. 1). The major immediate early genes are designated by lower case letters (ex. ie1), while the major immediate early proteins are designated by upper case letters (ex. IE1). Two immediate early proteins (IE55 and IE18) are expressed at low levels by differential splicing from this region and likely influence cell type specificity. Two predominant proteins also originate from this gene region by differential splicing, the 72 kDa IE1 protein and the 86kDa IE2 protein (Stinski et al., 1983; Stenberg et al., 1984; Stenberg et al., 1985; Stenberg et al., 1989; Kerry et al., 1995). The IE1 and IE2 gene products have been implicated in the positive regulation of viral gene expression (Stenberg et al., 1990). IE1 activates expression of the IE1/IE2 enhancer (Stenberg et al., 1990). IE1/IE2 is also required for DNA replication by functioning to activate early proteins required for DNA synthesis (Kerry et al., 1994, Kerry et al., 1996). In addition to its ability to autostimulate its own synthesis, IE1 cooperates with IE2 in the activation of subsequent kinetic classes of gene expression (Stenberg et al., 1996; Colberg-Poley et al., 1992; Fortunato and Spector, 1999). For example, although the UL112-113 promoter is efficiently transactivated by IE2 alone, this activation is augmented by IE1 (Colberg-Poley et al., 1992). IE2 is involved in the shut off of IE1/IE2 gene expression (Stenberg et al., 1990), thought to occur by direct DNA binding to the cis repression sequence (crs). The crs spans the IE1/2 promoter CAP site (Lang and Stamminger, 1993), and IE2 binding interferes with assembly of the

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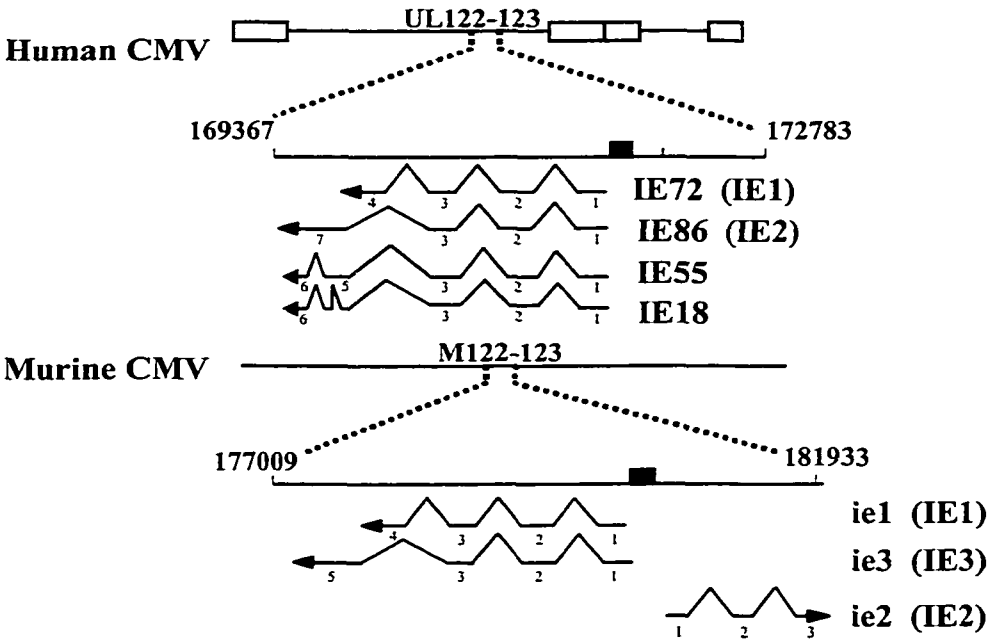

FIG. 1. Structural organization of the human and murine CMV major immediate early gene loci. The HCMV IE early gene region (UL122-123) is located between HCMV sequences 169367-172783. There are four IE mRNAs originating from this region by differential splicing. The MCMV IE gene region (M112-113) is located between MCMV sequences 177009-181933. There are three IE mRNAs originating from this region by differential splicing. The arrows depict transcripts, with the exon number underneath. The black boxes represent the enhancer region (Stenberg et al., 1989; Kerry et al., 1995; Keil et al., 1987). The bold numbers denote the name of the IE protein products.

preinitiation complex (Wu et al., 1993). IE2 is also a promiscuous transactivator of cellular genes, especially when used in conjunction with IE1 (Stenberg et al., 1990). IE2 upregulates the hsp70, c-fos and c-myc promoters but transactivation by IE1 and IE2 together is synergistic (Hagemeier et al., 1992).

The expression of the HCMV immediate early proteins is controlled by a very strong and complex enhancer (ie1/ie2 enhancer) (Fig. 2). This enhancer is active in a cell type and tissue specific manner. In transgenic mice containing the LacZ gene under the

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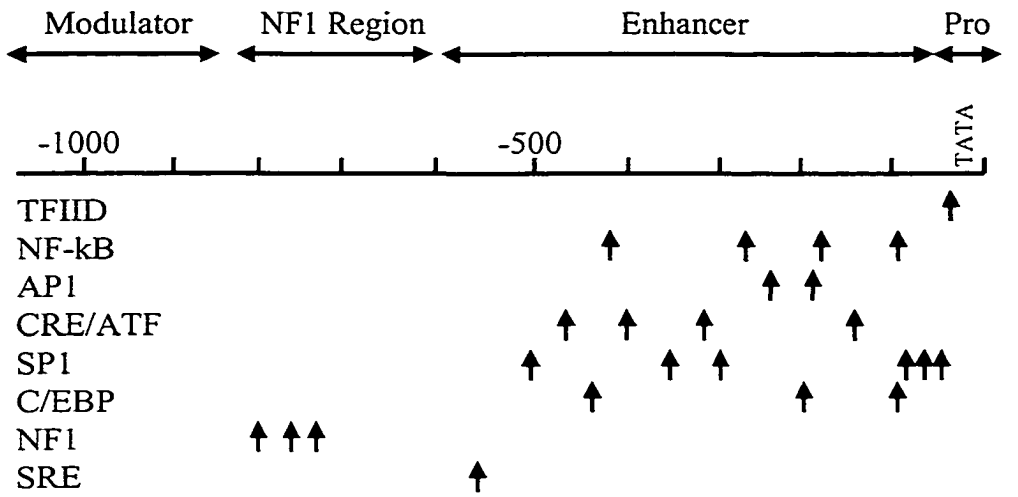

FIG 2. The upstream regulatory domains within the HCMV MIEP. A very strong and complex enhancer controls the expression of the HCMV immediate early genes, and this enhancer is active in a cell type and tissue specific manner. The activity of this promoter is driven by a wide variety of cellular transcription factor binding sites present within the enhancer region. The promoter (pro), enhancer, NF1 and modulator regions are denoted by bidirectional arrows. The transcription factor binding sites are denoted by arrowheads and the region on the promoter to which they bind has been mapped. (Mocarski, 1996).

control of the HCMV major immediate early promoter, expression of LacZ was found in 19 out of 29 tissues. These tissues include the brain, eye, spinal cord, esophagus, stomach, pancreas, kidney, bladder, testes, ovary, spleen, salivary gland, thymus, bone marrow, skin, cartilage and muscle. Distinct cell types within these tissues expressed LacZ. These cell types include retinal cells of the eye, ductile cells of the salivary gland, exocrine cells of the pancreas, mucosal cells of the stomach and intestine, neuronal cells of the brain, muscle fibers, thecal cells of the corpus luteum, and Leydig and sperm cells of the testes (Baskar et al., 1996). The level of differentiation of the infected cell also

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influences the activity of this enhancer (Stamminger et al., 1990; Stein et al., 1993; Chan et al., 1996; Baskar et al., 1996; Shering et al., 1997).

The strength of the HCMV ie1/ie2 enhancer can be attributed to a wide variety of cellular transcription factor binding sites present within the DNA sequence. These transcription factors include CREB/ATF, AP-1, NFkB, SP1, serum response factor and NF1 (Mocarski, 2000). These factors bind to repeat elements, which are referred to by their size (16-, 18-, 19- and 21- bp repeats). The transcription factor YY1 binds to a region in the MIEP called the modulator. YY1 seems to modulate expression from this enhancer by either acting as a repressor or as an activator of transcription (Lui et al., 1994). The modulator however, is not necessary for the transcription of the HCMV IE genes (Meier and Stinski, 1997).

In order to determine the importance of this enhancer, HCMV mutant viruses have been made in which the HCMV major immediate early enhancer has been deleted. Results indicate that the deletion of the enhancer impairs virus growth in human fibroblasts severely at a low multiplicity of infection (MOI). However, at a high MOI, viral growth in human fibroblasts is not inhibited when the MIEP is deleted. Therefore the MIEP is important in augmenting viral IE gene expression under conditions in which few viral DNA copies are present (Meier and Pruessner, 2000).

A HCMV IE1 mutant virus has also been made. This virus is deleted of exon 4 of IE1. In vitro, this virus is unable to replicate in human fibroblast cells at a low MOI. At a low MOI, the IE1 mutant virus also failed to form viral DNA replication compartments and to express the DNA polymerase accessory protein (ppUL44) which is required for viral DNA replication (Greaves and Mocarski, 1998). However, at an MOI of at least

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three plaque forming units, the virus is able to replicate at levels similar to wild-type virus (Mocarski et al., 1996). At a high MOI, the IE1 mutant virus was likely able to replicate due to the presence of virion proteins which have the ability to compensate for the lack of IE1 by transactivating the MIEP.

HCMV Ancillary IE Genes

All of the HCMV ancillary IE proteins also regulate gene expression and interact cooperatively. UL36-38 and US3 immediate early gene products regulate gene expression of the hsp70 promoter, and this expression was synergistically increased when both US3 and UL36-38 were transfected together (Colberg-Poley et al., 1992). HCMV US3 is also involved in immune evasion by blocking the transport of MHC class 1 heavy chains from the Golgi complex through the endoplasmic reticulum (Jones et al., 1996). UL37 is a type 1 integral membrane N-glycoprotein, and is proposed to regulate nuclear gene expression by cell signaling (Zhang et al., 1996). The IE proteins UL36-UL38, IRS1/TRS1, and UL122-123 are required for HCMV ori Lyt DNA replication. These proteins cooperatively regulate the expression of the early proteins involved in DNA replication (Pari and Anders, 1993; Pari et al., 1993; Iskenderian et al., 1996; Kerry et al., 1996).

HCMV Early Regulatory Genes

Early CMV genes are expressed prior to viral DNA replication and encode enzymes required for viral DNA replication. The early proteins involved in DNA replication have been identified due to their homology to herpes simplex virus type 1

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(HSV-1) genes which are essential for HSV-1 replication (McGeoch et al., 1988; Wu et al., 1988). The HCMV early genes involved in viral DNA replication include the DNA polymerase UL54, the polymerase-accessory protein UL44, the single-stranded DNA binding protein UL57, and the proposed subunits of the helicase-primase complex, UL70, UL105 and UL101-102 (Kouzarides et al., 1987; Heilbronn et al., 1987; Anders and Gibson, 1988; Martignetti, 1991; Ertl and Powell, 1992). The viral DNA polymerase (UL54) shares extensive amino acid homology with the DNA polymerase of HSV-1. The HCMV DNA polymerase is found associated with an additional polypeptide in virally infected cells, the DNA polymerase accessory protein (UL44). This protein is the CMV homolog of the DNA polymerase accessory protein of HSV-1 (Ertl and Powell, 1992). The DNA polymerase accessory protein functions to stimulate DNA polymerase activity by increasing the processivity of the DNA polymerase enzyme (Ertl and Powell, 1992). The single stranded DNA binding protein (UL57) binds to single stranded DNA and anchors the polymerase to the replication complex (Anders et al., 1986). The helicase complex (UL70, UL105 and UL101-102) is involved in priming lagging strand synthesis and unwinding DNA at the replication fork (Crute et al., 1989; Martignetti and Barrell, 1991). The sequence of the CMV helicase complex is also similar to the helicase complex of HSV-1. The two remaining proteins, UL84 and UL112-UL113, encode nuclear localized proteins. UL112-UL113 helps to regulate expression of the replication fork proteins (Iskenderian, 1996; Kerry 1996), while UL84 is required for the formation of intranuclear replication compartments (Sarisky and Hayward, 1996; Yamamoto et al., 1998).

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In order for the expression of the viral DNA replication genes and subsequent viral DNA replication to occur, the presence of the immediate early proteins is necessary. The immediate early proteins which are required to complement HCMV DNA replication in transient transfection assays include UL36-38, IRS1/TRS1, and IE1 and IE2 (UL122-123). These proteins are involved in regulating the expression of the early genes required for DNA replication, and they act synergistically in activating the expression of these genes (Iskenderian et al., 1996, Kerry et al., 1996). Not only are the immediate early genes important in activating the early gene promoters, but cellular factors play an important role as well. The cellular proteins ATF-1 and SP-1 assist the immediate early proteins in regulating the DNA polymerase promoter (Kerry et al, 1996; Kerry et al., 1997; Luu and Flores, 1997). The cellular factors CREB and ATF are required for full activation of the US11 and UL112-113 early promoters (Rodems et al., 1998; Chau et al., 1999). The presence of these cellular factors, which work in conjunction with the immediate early proteins in activating viral early promoters, likely influence cell-specific transactivation of viral genes.

HCMV Late Genes

Following early gene expression, viral DNA is replicated in a rolling circle mechanism that yeilds head to tail concatemers of unit length DNA. Following DNA replication, the late genes are expressed. These encode the structural proteins of the virion (see Virion Structure). Since very few late genes have been studied, very little is known about their regulation. The late gene pp65, which encodes the lower-matrix phosphoprotein, has been studied in transient transfection assays. In these assays, the

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pp65 promoter is activated by the IE1 and IE2 proteins, and a 8-base pair sequence appears to be important in the regulation of this promoter (Depto and Stenberg, 1989). The HCMV UL94 gene product, which encodes a virion protein, has been studied as well. These studies indicate that a positive and negative regulatory region is present within the UL94 promoter, and the cellular protein p53 plays a central role in regulating this promoter (Wing et al., 1998). Functional analyses of the pp28 promoter indicate that a 10 base pair sequence is important for promoter activation. This suggests that viral or cellular factors may act through these specific sequences to activate late gene expression (Depto and Stenberg, 1992).

Viral Assembly And Egress

Following late gene expression, the viral DNA is processed into genome length monomers and packaged in the viral capsids in the nucleus. Viral glycoproteins and tegument proteins accumulate and form patches in the nuclear membrane. The capsids then attach to a patch of modified inner lamellae of the nuclear membrane and become enveloped (Radsak et al., 1990). Although the nuclear membrane is the initial site of envelopment, this coat is removed in the cytoplasm (de-envelopment). The virion is then transported in vesicles through the Golgi to the cell surface (Eggers et al., 1992; Whiteley et al., 1999). Re-envelopment occurs as the virion buds from the cellular membrane, which is also modified by viral glycoproteins (Stinski et al., 1979). Therefore, egress from the cell may follow the exocytic pathway although this has not been definitively established and is still under investigation (Mocarski, 1996).

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MCMV As a Model For HCMV Disease

Due to the strict species specificity of the cytomegaloviruses, murine cytomegalovirus (MCMV) has been used as a model for human cytomegalovirus disease. Human and murine cytomegaloviruses share several characteristics. They both contain a large DNA genome of approximately 230 kilobases in length. HCMV and MCMV also have significant similarity in gene families, the presence of short direct and internal repeat sequences, and similar G and C content (particularly within the central part of the genome). Furthermore, the central part of the MCMV genome is colinear and generally homologous to the HCMV genome. The MCMV genes with homology to HCMV are indicated by the uppercase prefix M (ex. M112) while the MCMV genes with minimal sequence similarity to HCMV are indicated by the lowercase prefix m (ex. m40).

The functions of several MCMV gene products can be predicted based on sequence homology with HCMV genes or have been proven based on functional assays (Rawlinson et al., 1996). Finally, both viruses are biologically similar in terms of tissue tropism, latency and reactivation (Mocarski et al., 1990; Campbell, 1999).

MCMV Immediate Early Genes

To date only three MCMV immediate early genes have been characterized (M122, m123 and m128). These are located between map units 0.769 - 0.817, a region roughly colinear with the HCMV immediate early region (Kosinowski et al., 1986; Keil et al., 1987a; Keil et al., 1987b; Messerle et al., 1991; Messerle et al., 1992; Cardin et al., 1995). MCMV ie1 (m123) and MCMV ie3 (M122) genes are expressed from the same promoter from right to left and have a similar splicing pattern as the HCMV IE proteins

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(Fig. 1). MCMV IE1 is composed of exons 1-4, while MCMV IE3 is composed of exons 1-3, and 5 (Keil et al., 1987a; Keil et al., 1987b; Messerle et al., 1992). The MCMV ie2 gene (m128) is transcribed from a separate promoter in the opposite direction of the MCMV ie1/ie3 and is composed of three exons (Keil et al., 1987a; Messerle et al., 1991; Cardin et al., 1995). However, all three of these ie genes share the same strong enhancer (Fig. 1).

The expression of the MCMV immediate early genes is also driven by a strong and complex enhancer (Dorsh-Hasler et al., 1985). This enhancer region contains three long repeats of 181, 180, and 130 base pairs, and included within these repeats are five 51 base pair repeats containing NF-kB binding sites (Sandford and Burns, 1996). These NF-kB binding sites mediate transactivation of the MCMV enhancer by the immediate early protein IE1 (Cherrington and Mocarski, 1989). There are also binding sites for AP1 and CRE, but no binding sites for NF-1, SP1, or YY1 (Sandford and Burns, 1996). Since AP-1 and NFkB binding sites are present, expression from the enhancer is driven by serum and growth factors. This stimulation of the MCMV IE enhancer occurs through a pathway in which p21 ras is involved (Angeretti et al., 1994). Therefore the enhancer is active under conditions in which the cell is metabolically active.

In order to determine the importance of the MCMV major immediate early enhancer, MCMV mutant viruses have been made in which the MCMV immediate early enhancer has been deleted. For these MCMV mutants, viral growth is impaired in mouse fibroblast cells (NIH 3T3 cells). Since viral growth is not inhibited completely, the enhancer is not essential for viral replication but is required for optimal levels of viral infection to occur in permissive cells. When the HCMV enhancer is used to substitute for

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the MCMV enhancer (hybrid MCMV strains), the HCMV enhancer is able to restore growth in fibroblasts to kinetics similar to wild type MCMV (Angulo et al., 1998; Grzimek et al., 1999). When mice are infected with this virus, liver cells are infected to similar levels as wildtype virus. However the mutant virus is attenuated in the spleen, lung, and adrenal gland (Grzimek et al., 1999). This suggests that the MCMV enhancer is active in a tissue specific manner.

The MCMV IE1 gene product is an 89kDa nuclear phosphoprotein analogous to the HCMV IE1 gene product. The MCMV IE1 gene product transactivates the ie1/ie3 promoter and several other promoters such as the SV40 promoter, the early HSV-1 gD promoter, and the NF-kB p105/p50 promoter (Koszinowski et al., 1986; Gribaudo et al., 1996). The MCMV IE3 gene product (88kDa) is analogous to HCMV IE2 and represses the MCMV ie1/ie3 promoter. In transient assays using a construct containing the MCMV MIEP cloned upstream of the chloramphenicol acetyl transferase (CAT) gene, a low level of CAT activity was found with IE1 alone, and this activity was reduced by 5 fold with IE1 and IE3 (Fig. 3) (Messerle et al., 1992, Cardin et al., 1995).

In addition to having sequence similarity to the carboxyl terminus of the HCMV IE2 protein, both MCMV IE3 and HCMV IE2 exhibit functional similarity in activating the expression of early genes. The MCMV IE3 protein is a strong transcriptional transactivator of the murine CMV early promoter e1 (M112-M113). In transient assays using the e1 promoter cloned upstream of CAT, IE3 was able to increase CAT activity by 150 fold. When the e1 promoter-CAT construct was cotransfected with IE1 and IE3, a 200 fold increase in CAT activity was observed. Therefore, together MCMV IE1 and IE3 act synergistically in activating the e1 promoter (Fig. 3) (Messerle et al., 1992).

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Finally, the MCMV IE2 gene product has no obvious counterpart in HCMV, but it too transactivates the MCMV ie1/3, the ie2 promoter and the HSV-1 ICP8 promoter (Cardin et al., 1995). In transient studies of the MIEP using the *Escherichia coli* lacZ gene as a reporter, IE2 was able to activate this promoter 12 fold above basal levels in a dose dependent manner (Cardin et al., 1995). IE2 was able to autoregulate the expression of an IE2 promoter-lacZ construct by 3 fold above basal levels (Cardin et al., 1995).

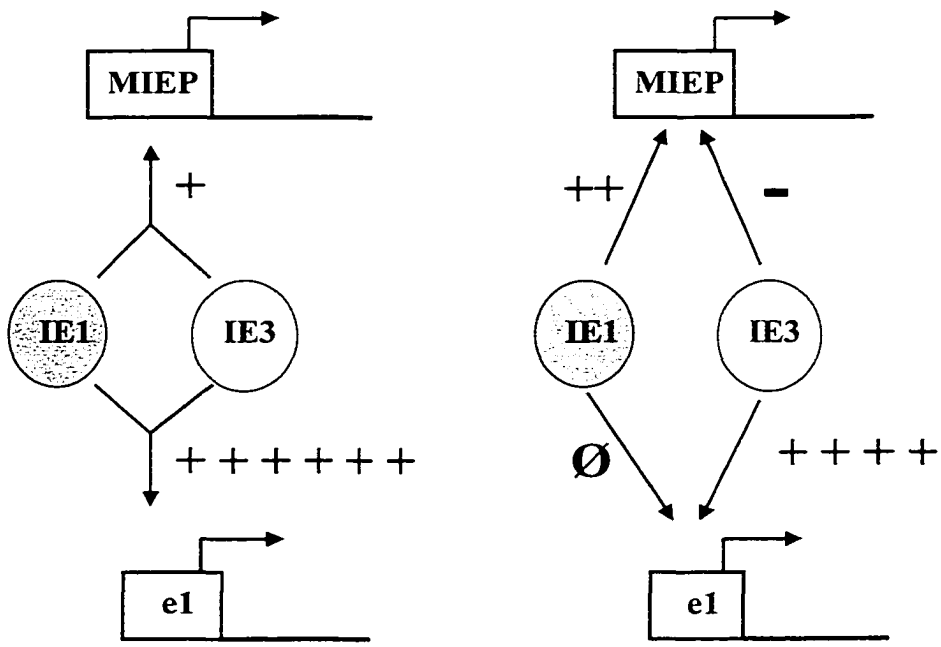

FIG. 3. Model of MCMV IE protein function. Influence of IE1 and IE3 on the MCMV MIEP and the early e1 promoter is depicted. Arrows from the proteins indicate the interaction of the proteins and their subsequent influence on the promoters. The relative levels of activation (+) and repression (-) of the promoters by IE proteins are indicated. The lack of an influence on promoter activation is indicated by Ø (Messerle et al., 1992).

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Like IE1 of HCMV, MCMV IE1 is not necessary for growth in cell culture. When a low MOI was used to infect mouse NIH3T3 fibroblast cells with a MCMV ie1 mutant virus, the IE1 mutant grew significantly slower than wild-type virus. However when the MOI was increased, replication was restored to levels similar to wild type (Messerle et al., 1997). This can be explained by the phenomenon referred to as "multiple hits''. When the MOI is high, other factors are able to compensate for the lack of IE1 in activating the MIEP. Compensating factors could include transactivating proteins within the tegument (Mocarski et al., 1996). Although MCMV IE1 is dispensible for growth in culture, a MCMV mutant deleted of the MIEP and part of exon 1 of ie1/ie3 is replication incompetent in cultured cells (Angulo et al., 1998).

IE2 also is dispensible for growth in NIH3T3 cells. An IE2 deletion mutant displayed similar growth kinetics as wildtype virus in vitro (Cardin et al., 1995; Manning and Mocarski, 1998). In vivo, the IE2 mutant virus grows to similar levels as wild type virus in the spleen, liver, kidney and adrenal glands. In the absence of IE2, the establishment of and reactivation from latency also occurs to levels similar to wildtype virus (Cardin et al., 1995).

MCMV Non-essential Genes

MCMV genes are classified as either non-essential or essential. The essential genes are those required for viral attachment/penetration, viral DNA replication in fibroblast cells, or viral packaging. Non-essential genes are not required for viral DNA replication in fibroblasts, but likely influence tissue tropism and/or viral pathogenesis. Several MCMV non-essential genes have been studied to date. The sgg-1 gene (M133)

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and the G protein-coupled receptor homolog gene (M33) are involved in MCMV growth in the salivary gland (Lagenaur et al., 1994; Davis-Poynter et al., 1997). The chemokine homolog m131 is also involved in infection of the salivary gland by eliciting proinflammatory signals which may be important for dissemination to or replication of MCMV in this organ (Fleming et al., 1999).

Like HCMV, several MCMV non-essential genes interfere with the expression of MHC class 1 molecules on the surface of an infected cell. The m152 gene blocks antigen presentation by preventing the export of MHC class 1 molecules from the endoplasmic reticulum through the Golgi (Zeigler et al., 1997). The m06 gene allows the MHC class 1 molecules to be transported from the endoplasmic reticulum through the Golgi, however the MHC class 1 molecule is not expressed on the surface of the cell but is redirected to the lysosome and rapidly degraded. The mouse gp34 protein (m04), which is also nonessential, counteracts the MHC class 1 inhibition by m152 and m06, and associates with the MHC class 1 molecules as they are transported to the cell surface. This may allow the infected cell to escape recognition by natural killer cells by increasing MHC class 1 levels at the cell surface (Kleijnen et al., 1997). Another MCMV non-essential gene encodes the MCMV IgG Fc receptor (M138).

US22 Gene Family

One gene family likely to influence viral pathogenesis is the US22 gene family. Members of the US22 gene family have been found in HCMV (Weston and Barrell, 1986), MCMV (Rawlinson et al., 1996), HHV-6 (Nicholas and Martin, 1994), and HHV-7 (Nicholas, 1996). These are all betaherpesviruses that infect myeloid and lymphoid

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cells. The functions of many members of the US22 gene family are unknown. However certain members of the family, HCMV TRS1/IRS1and UL36, MCMV ie2, and some ORFs within the region of homology in HHV-6, encode transcriptional transactivators (Cardin et al., 1989; Stasiak and Mocarski, 1992; Nicholas and Martin, 1994; Iskenderian et al., 1996).

Conserved motifs contained within the US22 gene family. US22 family members contain up to four conserved consensus sequences that are referred to as motifs (Kouzarides et al., 1988; Nicholas and Martin, 1994). The US22 motifs I and II are composed of conserved sequences containing hydrophobic residues. Motif I contains the consensus OXOXXPXXW and motif II contains the consensus OOCCXXLXXOG, where O is any hydrophobic residue and X is any residue. The remaining two motifs are less well defined but have stretches of nonpolar residues (Kouzarides et al., 1988)

IRS1/TRS1 of HCMV. IRS1/TRS1 are HCMV US22 family members. IRS1/TRS1 are partially contained within the repeat elements of the short component of the HCMV genome. IRS1 extends from an internal repeat element into the short unique domain while TRS1 extends from a terminal repeat element into the short unique domain. Therefore, synthesis of their mRNAs is controlled by repeated promoters and the two proteins share residues at their amino termini but differ in their carboxyl-terminal sequences. IRS1/TRS1 have duplicating function. Therefore, only one of the two open reading frames is required for transient complementation of ori-Lyt dependent viral DNA synthesis (Jones and Muzithras, 1992). Both mRNA and protein products of TRS1/IRS1

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(pTRS1/pIRS1) can be detected in all phases of the viral replication cycle. pIRS1 and pTRS1 are present in both the nucleus and the cytoplasm during the immediate early and early phases of the viral growth cycle, but are found predominately in the cytoplasm during late phases of viral growth (Romanowski and Shenk, 1997).

As stated earlier, TRS1/IRS1 are required for HCMV ori-Lyt DNA replication. TRS1/IRS1 cooperate with other immediate early regulatory proteins to promote expression of the six replication fork proteins (Pari and Anders, 1993; Pari et al., 1993; Iskenderian, 1996; Kerry et al., 1996). IRS1/TRS1 exhibit little activity as transcriptional transactivators on its own, but with IE1 and IE2 proteins expression can be enhanced from a variety of promoters (Romanowski and Shenk, 1997). For example, both IRS1 and TRS1 genes upregulate the activity of the polymerase accessory protein gene promoter (UL44) in the presence of IE1 and IE2 proteins, but do not transactivate it when transfected alone (Stasiak and Mokarski, 1992).

An additional immediate early gene product is encoded within the IRS1 gene. This gene product called $pIRS1^{263}$ is encoded within the 3' end of the IRS1 gene and is in the same reading frame as the large pIRS1 protein. Expression of the $IRS1^{263}$ gene is controlled by a promoter that resides within the IRS1 open reading frame in the unique short region of the viral genome. $pIRS1^{263}$ accumulates to its highest level during the early phase of HCMV infection. This protein resides in the nucleus and antagonizes the transcriptional activating capability of pTRS1 and pIRS1. $pIRS1^{263}$ may therefore serve as part of a regulatory loop, preventing overexpression of some early or late gene products, optimizing or perhaps slowing the process of viral replication (Romanowski and Shenk, 1997). Whatever its role, the effect of $pIRS1^{263}$ on virus growth in cultured

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fibroblast cells is at best subtle because no clear phenotype was evident for a mutant lacking the IRS1 coding region (Jones and Muzithras, 1992).

pIRS1 and pTRS1 are also present in the HCMV virion. These proteins were not degraded when intact virions were treated with trypsin, suggesting that the proteins are localized inside the viral tegument (Romanowski et al., 1997). In contrast the pIRS1^{263} was not detected in the virions (Romanowski and Shenk, 1997). Virion pIRS1 and pTRS1 can also activate expression of viral immediate early promoters in cooperation with pUL69 but not pUL82 (Romanowski et al., 1997). Both pUL69 and pUL82 are transcriptional regulatory proteins that are components of the HCMV tegument (Liu and Stinski, 1992; Winkler et al., 1994; Winkler and Stamminger, 1996). Therefore pIRS1/TRS1 may assist in the activation of HCMV promoters as the DNA first reaches the nucleus (Romanowski et al., 1997).

Table 1. Homology between MCMV and HCMV US22 family genes.

MCMV	HCMV	Potential Function
M23[a]	UL23[b]	
m23.1		
M24	UL24	
m25.1	UL23	
m25.2	UL22	
M36	UL36	transactivator
M36 Ex2	UL36 Ex2	transactivator
M36 Ex1	UL36 Ex1	transactivator
M43	UL43(P)[c]	
m128 Ex3	US22	transactivator
M139	US22	cell tropism
M140	US23	cell tropism
M141	US24	cell tropism
m142	US26	transactivator
m143	US23	transactivator

[a] M and m denote MCMV genes with and without homology to HCMV genes, respectively.
[b] UL and US indicate HCMV genes within unique long and unique short regions of the genome, respectively.
[c] Homolog to HCMV according to position in the genome. (Rawlinson, 1996).

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MCMV US22 family members. The HCMV US22 family members are contained within the US or the UL region of the genome. In MCMV, several ORFs have homology to the HCMV US22 family members (Table 1). The MCMV US22 family members are arranged in a similar fashion as those found in HCMV, with two clusters of tandemly repeated homologs at either end of the genome (Fig. 4) (Rawlinson et al., 1996). The MCMV US22 family members located at the left side of the genome are more similar to the HCMV UL family members, and include genes M23, m23.1, M24, m25 and M36 (Fig. 4) (Rawlinson et. al., 1996). The MCMV US22 family members at the right side of the genome are more US-like and include genes m128 and M139-m143 (Fig. 4) (Kouzarides et al., 1988; Nicholas and Martin, 1994; Nicholas et al., 1996). Products of the US22 genes M139, M140 and M141 are nonessential for viral replication and are involved in MCMV cell tropism and pathogenesis (Cavanaugh et al., 1996; Hanson et al., 1999; Ménard, 2000). A mutant virus deleted of M139-M141 replicates in NIH 3T3 fibroblasts like wild type virus, but replicates three $\log_{10}$ lower in macrophages in vitro. This virus also replicates poorly in mouse spleen tissue that contains macrophages that are permissive for wildtype MCMV. However, when these splenic macrophages are depleted, replication is restored to wild type levels (Hanson et al., 1999). This suggests that M139, M140 or M141 individually or in combination, is involved in macrophage tropism. Single mutants deleted of M140 or M141 grow 2-3 $\log_{10}$ lower and 2 $\log_{10}$ lower respectively, in macrophages compared to wild type virus (Hanson et al., in press). In fibroblasts M140 and M141 single deletion mutants grow similar to wild type virus. In vivo, the M140 and M141 single deletion mutants are also attenuated for replication in target organs rich in macrophages (Hanson et al., in press). These data suggest that M140

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and M141 genes are both involved in regulating MCMV growth in macrophages. The possibility of M139 involvement in macrophage tropism was eliminated since M139 mutant virus grows similar to wild type virus in macrophages and macrophage rich organs (Hanson et al., in press).

The MCMV US22 family member m128 (ie2) is an immediate early protein that activates its own promoter as well as the IE1/IE3 promoter (Cardin et al., 1995).

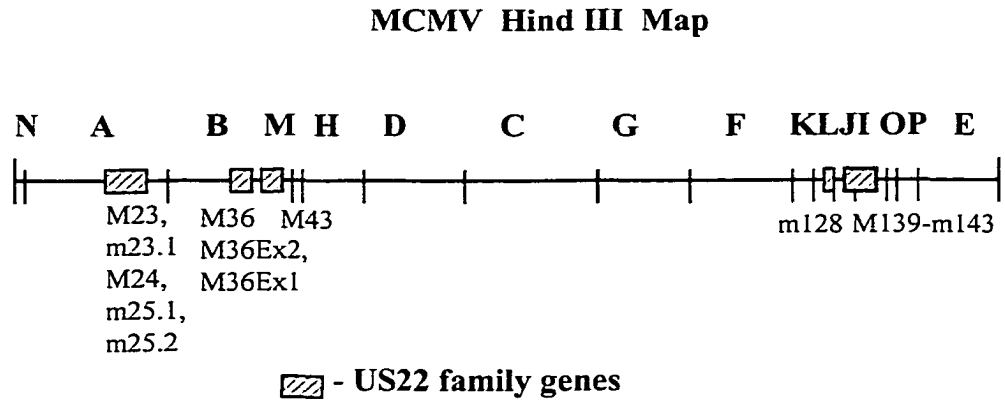

FIG 4. Map of MCMV (Smith strain) denoting location of US22 family genes. Members of the MCMV US22 family influence viral pathogenesis. The location of these genes within the genome is indicated as shaded boxes (Rawlinson, 1996). The US22 family members are arranged with two clusters of tandemly repeated homologs at either end of the genome. US22 family members located to the right side of the genome are more like HCMV US members while the members on the left side of the genome are more similar to HCMV UL family members.

IE2 is dispensible for growth in NIH3T3 cells as well as in the mouse (Cardin et al., 1995; Manning and Mocarski, 1998). M36 has homology to the US22 family member UL36 of HCMV. HCMV UL36, also an immediate early gene product, assists in the transactivation of genes involved in viral DNA replication (Iskenderian, 1996).

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The US22 family members m142-m143 are essential for viral replication in fibroblasts (Cavanaugh, 1996; Ménard et al., 2000). While a virus deleted of M139-M141 is able to grow in fibroblast cells to levels similar to wild type virus, a virus deleted of M139-m143 is helper dependent (Cavanaugh et al., 1996). Also, when MCMV is mutated within m142 or m143, infectious virus does not result upon transfection of cultured cells with mutant viral DNA (Ménard et al., 2000). Collectively, these data suggest that m142 and m143 are essential genes. The genes m142-m143 have homology to the IRS1/TRS1 transactivator genes of HCMV (Rawlinson, et al., 1996). Based on homology, we hypothesize that m142-m143 are functional homologs to IRS1/TRS1. We test this hypothesis in this proposal.

MCMV Immediate Early Genes m142 and m143

US22 family members m142 and m143 have some homology to IRS1/TRS1 of HCMV, and are located within the Hind III I fragment of MCMV (Fig. 4). The m142 transcript corresponds to a 1.8 kb RNA and the m143 transcript corresponds to a 3.8 kb transcript. The transcriptional start site of m142 and m143 genes have been identified by nuclease mapping. For the transcript mapping, a 5' end probe was hybridized to RNA from NIH 3T3 fibroblast cells that were infected for either 3 hours (early times) or 24 hours (late times). Analysis of the m143 transcript revealed two major protected bands of identical size at 3 hours post infection and 24 hours post infection. These bands correspond to the initiation of m143 expression at nucleotides 202686 or 202694. For m142, at early times a protected band was found which corresponds to a start site at nucleotide 200847. However, an additional minor band was found which corresponds to

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a start site at nucleotide 200848 (Fig. 5). This suggests an additional initiation site for m142 expression (Fig. 6). At late times, the same bands were present along with another protected band that may correspond to m142c. The m142c start site was not mapped in these studies (Fig. 6) (Hanson et al., 1999).

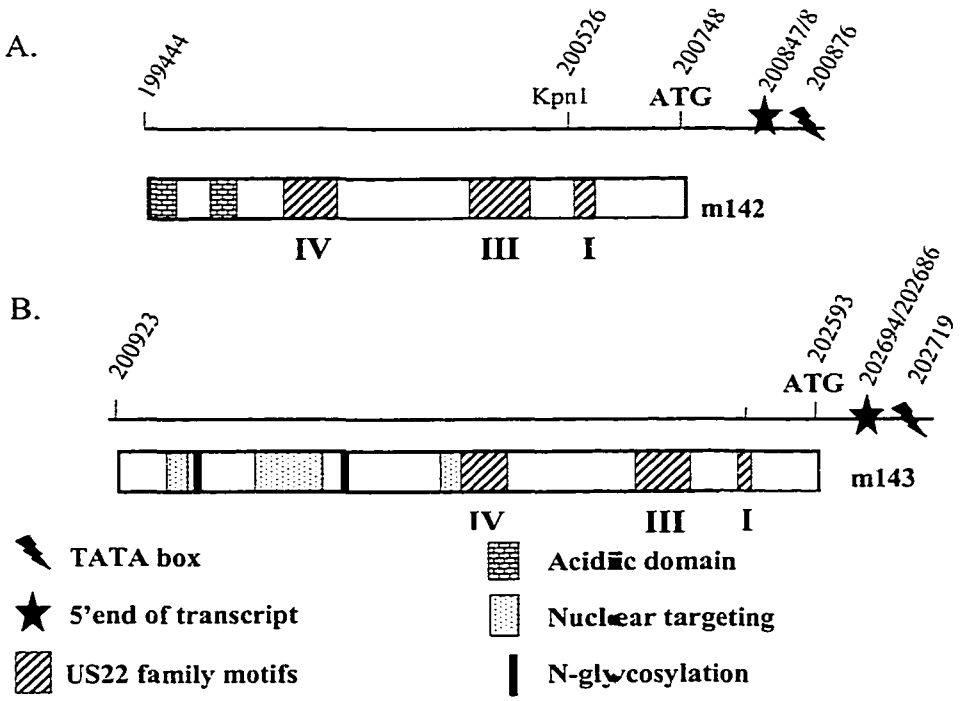

FIG. 5. Map of the m142 and m143 proteins. The location of conserved US22 family motifs, and other predicted motifs are shown for A) m142 and B) m143. The location of the m142 and m143 genes within the MCMV genome are also indicated, with the TATA Box, 5' end of the transcript, and common restriction enzyme sites denoted. Numbers refer to nucleotides within the MCMV genome.

Analysis for protein motifs show that in addition to the US22 motifs, m142 has an acidic domain that is common to all herpesvirus transcriptional transactivators. The ORF

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m143 contains potential bipartite nuclear localization signals, as well as several N-glycosylation sites (Fig. 5).

In summary, genes m142 and m143 are members of the US22 gene family. This gene family is known for its biological importance, by influencing viral pathogenesis. Since m142 and m143 are required for viral DNA replication in fibroblast cells, the role they play during a MCMV infection is a vital one. Thus far, the m142 and m143 transcripts have been identified, and their transcriptional start sites have been mapped. The experiments in this dissertation will give us more knowledge about these essential genes and their function during a MCMV infection.

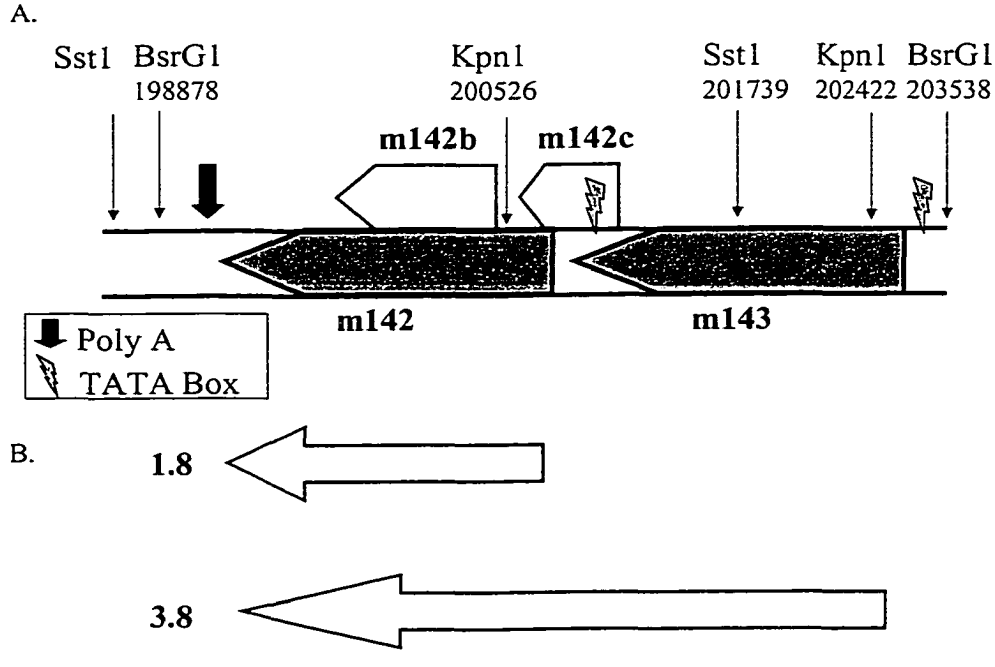

FIG. 6. Transcripts originating from the MCMV m142 and m143 gene region. A. Potential m142 and m143 open reading frames (ORFs) are indicated by closed boxes. Overlapping ORFs are indicated above the closed boxes. The poly A, TATA box, and common restriction sites are denoted. Numbers refer to nucleotides within the MCMV genome. B. The position and sizes of the m142 and m143 transcripts (Hanson et al., 1999).

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CHAPTER II

SUMMARY OF EXPERIMENTAL PLAN

As previously stated, m142 and m143 are members of the US22 gene family of HCMV. Some members of this family, HCMV TRS1/IRS1and UL36, MCMV ie2, and some ORFs within the region of homology in HHV-6, encode transcriptional transactivators (Stasiak and Mocarski, 1992; Nicholas and Martin, 1994; Cardin et al., 1995; Iskenderian et al., 1996). The genes m142-m143 also have homology to HCMV IRS1/TRS1 which are involved in the activation of viral early genes involved in viral DNA replication (Iskenderian et al., 1996). Furthermore, these genes are expressed at one hour post infection which suggests that they may be immediate early genes. Most of the betaherpesvirus immediate early gene products discovered to date function as transcriptional transactivators. Therefore it is likely that m142 and/or m143 are functional homologs of IRS1/TRS1 and function as transcriptional transactivators.

The specific objectives of this study were:

1) To determine to which kinetic class the m142 and m143 genes belong: immediate early, early or late. In order to do this, NIH3T3 cells were infected in the presence of drug inhibitors that block MCMV replication at immediate early, and early times. The drug anisomycin blocks de novo protein synthesis, thus allowing the expression of only the immediate early genes. The drug phosphonoformate prevents viral DNA replication, allowing the expression of only the early genes. Late genes are expressed in the absence of drug inhibitors.

2) To generate polyclonal antisera against m142 and m143 proteins. Since the m142 and the m143 proteins have not been previously described, m142 and m143

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specific antiserum was needed. Therefore, m142 and m143 were cloned separately, in frame with a histidine tag. The histidine tagged m142 and m143 fusion proteins were expressed and then purified using a nickel-chelating column. Following purification the proteins were injected into rabbits for antibody production. Once the m142 and m143 specific rabbit anti-serum was obtained, experiments were done to ensure the specificity of these antibodies.

3) To characterize the m142 and m143 proteins. Since the m142 and m143 proteins have not been previously described, the size and expression kinetics of these proteins were determined. For the kinetics studies, NIH3T3 cells were infected with MCMV at an MOI of 2 for 0, 3, 6, 12, or 24 hours. Next lysates from these infected cells were analyzed by Western blot to determine the molecular weight and time of expression of m142 and m143 proteins.

4) To determine the localization of m142 and m143 proteins within an MCMV infected cell. Since it was proposed that m142 and/or m143 are transcriptional transactivators, it was of great interest to determine if they localize to the nucleus. In order to determine this, immunofluorescence assays were done to localize the m142 and m143 protein products to at least the nucleus or the cytoplasm. These immunofluorescence studies were performed at 24 hours post infection. Cell fractionation experiments allow the separation of the nuclear and cytoplasmic fractions of infected cells. This procedure was also used to determine if m142 and m143 localize to the nucleus or cytoplasm at various times post infection.

5) To determine the ability of m142 and m143 to function as transcriptional transactivators. Gene products m142 and m143 could have several possible functions as

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transcriptional transactivators. The m142 and m143 gene products could a) transactivate the MCMV ie1/ie3 promoter, b) transactivate the promoters of MCMV early genes whose products participate in MCMV DNA replication, c) transactivate other early or late genes whose products do not function in MCMV DNA replication, or d) serve as a co-activator with IE1 and/or IE3 to enhance transcription of MCMV genes. We determined the transactivation function of m142 and m143 by testing their ability, individually and collectively, to activate selective MCMV promoters. First, it was determined if m142 and/or m143 can transactivate the ie1/ie3 promoter. We also determined if m142 and/or m143 might serve as a co-activator of the ie1/ie3 promoter along with IE1 and IE3. Our next goal was to determine if m142 and/or m143 can activate the promoter of the MCMV early gene e1 (M112-M113). The MCMV e1 gene has homology to HCMV UL112-113, which is required for HCMV DNA replication (Pari and Anders, 1993). We also determined if m142 and/or m143 might serve as a co-activator of the e1 promoter along with IE1 and IE3.

Genes m142 and m143 are essential for viral DNA replication in fibroblasts cells. Therefore, these gene products play a vital role in the biology of MCMV. These studies are very important because they characterize these proteins that are necessary for viral growth, as well as assign a potential function to these proteins. This information will provide a foundation for studying the role of these genes in the context of a viral infection. In the future, m142/m143 mutant viruses will be generated for this purpose.

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CHAPTER III

MATERIALS AND METHODS

Cell line

NIH 3T3 cells, a contact-inhibited murine fibroblast cell line from American Type Culture Collection (Rockville, MD), were propagated in Dulbecco's Modified Eagle's Medium (Mediatech, Herndon, VA) supplemented with 10% heat inactivated bovine calf serum (Hyclone Laboratories, Logan, UT) and 1% L-glutamine (Mediatech, Herndon, VA).

Virus

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

Plasmid constructions

Plasmid cloning was conducted by standard methods (Maniatis et al., 1987), and each plasmid was tested by restriction endonuclease digestion to ensure that the correct DNA sequences were inserted. The restriction endonuclease digestions were done according to manufacturer's suggestions. The plasmids to be used for the transfection studies were prepared and purified using cesium chloride/ethidium bromide centrifugation (Current Protocols in Molecular Biology, 1998) or the Quantum Prep Plasmid Maxiprep Kit (Bio-Rad, Hercules, CA).

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Plasmids encoding MCMV immediate early proteins were kindly provided by Dr. Martin Messerle (Messerle et al., 1992). These include plasmid pp89UC, pIE3, and pIE111 that encode the MCMV immediate early proteins IE1, IE3, and IE1+IE3 respectively. The plasmid pMCMV3CAT, which contains the MCMV ie1/ie3 promoter/enhancer sequence upstream of the chloramphenicol acetyl transferase (CAT) gene and the plasmid e1 CAT, which contains the MCMV 112-113 promoter upstream of the CAT gene, were provided by Messerle as well.

Generation of m142/ m143 expression vectors. A construct expressing both m142 and m143 genes, under the control of their natural promoters, was generated for use in transient assays. This construct was made by digesting the Hind III I clone of MCMV with BsrG1 (New England Biolabs, Beverly, MA) and Hind III (Promega, Madison, WI). Next, the 4.6 kb linearized molecule containing m142-m143 was isolated from a 5% acrylamide gel. The plasmid pSVZeo (Invitrogen, Carlsbad, CA) was linearized with Acc65I (MBI Fermentas, Amherst, NY), treated with calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) for an hour, and gel purified. Since DNA digested with the enzymes BsrG1 and Acc65I have compatible ends, the 4.6kb fragment containing m142/m143 was cloned into the Acc65I site of pSVZeo (Invitrogen, Carlsbad, CA). This construct was called Zeo-I4.6.

The construct ZeoI-4.6 was not optimal for use in the transient assays, because of problems with high basal levels of chloramphenicol acetyl transferase (CAT) activity in the CAT assays. Therefore, we generated another construct that expresses m142-m143 under the control of their native promoters. In order to do this, the construct Zeo-I4.6

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was digested with Hind III and EcoR1, and the 4.6 kb insert was isolated from an acrylamide gel. The Hind III and EcoR1 sites are located in the multiple cloning site of the pSVZeo vector (Invitrogen, Carlsbad, CA) on opposite sides of the insert containing m142/m143. The vector pCDNA3.1 was also digested with Hind III and EcoR1, followed by phosphatase treatment for an hour, and gel purification. The 4.6 kb fragment containing m142/m143 was cloned into the Hind III/ EcoR1 site of pCDNA3.1. This construct was called 142/143pCDNA.

Generation of 142pCDNA and 143pCDNA expression vectors. Vectors expressing either m142 (142pCDNA) or m143 (143pCDNA) were also generated for use in transient assays. The 142pCDNA and 143pCDNA constructs were made by polymerase chain reaction. The primers used for the generation of p142 include MCMV sequences 200502-200517 for the forward primer (sequences TCTCCACCCGAATTC CCGCTGCCG) and MCMV sequences 199417-199442 for the reverse primer (sequences CGAGGCGATATCCCGTCCGTCCGTC). These primers were generated by Life Technologies (Rockville, MD). The 142/143pCDNA vector was used as the template DNA for PCR. The PCR product generated with the use of these primers (1.1 kb) was digested with EcoR1 and EcoRV. The expression vector pCDNA3.1 was also digested with EcoR1 and EcoRV, and treated with phosphatase for an hour. The DNA fragments were run on a 5% polyacrylamide gel and eluted. The 1.1 kb fragment containing m142 was then cloned into the pCDNA3.1 vector (Invitrogen, Carlsbad, CA) under the control of the HCMV major immediate early promoter/enhancer (MIEPE), and this construct was called 142pCDNA.

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The 143pCDNA vector was also generated using PCR. The MCMV sequences 202602-202626 were used as the forward primer (sequences TCCGCTCGAATTCGTC CGCCCGTC) and MCMV sequences 200873-200899 were used as the reverse primer (sequences GGGCGGGTGATATCAGAGATGACATG). These primer sequences were also generated by Life Technologies (Rockville, MD). The expression vector 142/143pCDNA was used as the template DNA. The PCR product (1.7 kb) was then digested with EcoR1 and EcoRV. The expression vector pCDNA3.1 (Invitrogen, Carlsbad, CA) was also digested with EcoR1 and EcoRV, and phosphatase treated for an hour. The DNA fragments were run on a 5% polyacrylamide gel and eluted. The 1.7 kb fragment containing m143 was then cloned into the pCDNA3.1 vector (Invitrogen, Carlsbad, CA) under the control of the HCMV MIEPE and this construct was called 143pCDNA.

Generation of m142pBad/HisA and m143pBad/HisA. Constructs were generated that contain the m142 or m143 gene cloned in frame to sequences encoding the Xpress epitope and six histidine tags. These constructs were made using the pBADHisA vector (Invitrogen, Carlsbad, CA) in order to generate histidine-tagged m142 or m143 fusion proteins that would later serve as immunogens for the production of m142 and m143 polyclonal antisera. The polyhistidine tags allow purification of the recombinant fusion protein on a metal-chelating resin and the Xpress epitope permits detection of the recombinant fusion protein by the anti-Xpress antibody. This vector also allows the expression of the inserted gene to be strictly regulated. Expression from the plasmid pBADHisA (Invitrogen, Carlsbad, CA) occurs only in the presence of arabinose.

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In order to generate a construct containing the histidine and Xpress tagged m143 recombinant gene, the plasmid Zeo-I4.6 was digested with Kpn1 (New England Biolabs, Beverly, MA). The plasmid Zeo-I4.6 contains both m142 and m143. Next the digest was run on an acrylamide gel and the 1.8 kb fragment (MCMV sequences 200526-202422) containing the m143 open reading frame was eluted. The vector pBad/His A was digested with Kpn1 (New England Biolabs, Beverly, MA) and treated with calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) for an hour, then gel purified. The 1.8 kb fragment containing m143 was ligated into the pBad/His A vector which was linearized with Kpn1. This construct was called m143pBad/HisA. The vector Zeo-I4.6 was also digested with Kpn1 and EcoR1 (Promega, Madison, WI) in order to generate a construct containing the histidine and Xpress tagged m142 recombinant gene. The 1.6 kb fragment (MCMV sequences 198878-200526) containing m142 was eluted from an acrylamide gel. The vector pBad/HisA was digested with Kpn1 and EcoR1, and treated with calf intestine alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) for an hour, then gel purified. The 1.6 kb fragment (containing m142) was inserted into the pBad/HisA vector which was digested with Kpn1 and EcoR1. This vector was called m142pBad/HisA.

Procedures

Expression from m142pBad/HisA and m143pBad/HisA. The Kpn1-EcoR1 fragment containing m142 (MCMV sequences 198878-200526) or the Kpn1-Kpn1 fragment containing m143 (MCMV sequences 200526-202422) was cloned in frame to sequences encoding six polyhistidine tags and the Xpress epitope using the pBAD/HisA

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vector (see above). These plasmids were used to transform TOP10 *E. coli*. In the presence of arabinose, expression from pBAD/HisA is turned on while in the absence of arabinose very low levels of transcription occur. Since each recombinant gene has different characteristics that may affect optimum expression, it was necessary to vary the arabinose concentration to determine the best condition for optimal expression of m142 and m143. The arabinose concentration was varied over a 10,000 fold range (0.00002%-0.2%).

For both the pm142 and the pm143 TOP10 transformants, the expression studies were performed as follows. Briefly, 2 ml of LB containing 50 μg/ml of ampicillin was inoculated with a single recombinant TOP10 colony and incubated overnight at 37°C with vigorous shaking. The next day, 10 ml of LB containing 50 μg/ml ampicillin was added to five tubes. Each tube was inoculated with 0.1 ml of overnight culture and allowed to grow at 37° C to a 0.5 OD_{600}. Next, 20 % arabinose was added to each tube to give the following final concentrations of arabinose: 0.00002%, 0.0002%, 0.002%, 0.02% and 0.2%. These cultures were grown at 37° C with shaking for 4 hours. From each culture a 1 ml aliquot of cells was taken. Next the cells were pelleted and the supernatant was removed by aspiration. The cell pellet was then frozen at –20° C. Each sample was resuspended in 100 μl of 1 x SDS-PAGE sample buffer and boiled for 5 minutes. Each sample was then analyzed by Western blot using the anti-Xpress antibody (Invitrogen, Carlsbad, CA).

It was found that the optimal concentration for the expression of m142 was 0.2% arabinose and the optimal concentration for the expression m143 was 0.02% arabinose.

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Using the given concentrations of arabinose, 250 ml cultures of the recombinant protein were prepared, and used for recombinant protein purification.

Initially we tried to purify the m142 and m143 protein products using only the Xpress™ purification system (Invitrogen, Carlsbad, CA). The Xpress purification system involves the lysis of Top 10 *E. coli* expressing m142 or m143 protein in guanidinium lysis buffer, removal of insoluble bacterial debris, purification of the recombinant protein using a metal chelating column, and elution of the histidine tagged m142 or m143 protein from the column. These attempts were unsuccessful because the m142 and m143 proteins were insoluble proteins found in inclusion bodies, which were discarded with the bacterial debris. The m142 and m143 proteins were proven to be insoluble proteins when the pellet containing the bacterial debris was solubilized using the Inclusion Body Solubilization Reagent (Pierce, Rockford, IL) and analyzed by Western blot using the anti-Xpress antibody. Both m142 and m143 proteins were detected in the inclusion bodies using this method.

Therefore in order to correct for this problem, we used several different commercial reagents and followed the instructions detailed by the manufacturer. Top 10 *E. coli* expressing m142 or m143 were pelleted, and lysed using the B-PER™ Bacterial Protein Extraction Reagent (Pierce, Rockford, IL). The bacterial lysate was then centrifuged and the precipitated protein was solubilized using the Inclusion Body Solubilization Reagent (Pierce, Rockford, IL). The proteins were then purified using the Xpress™ purification system (Invitrogen, Carlsbad, CA) under denaturing conditions. Using this system, the histidine tagged m142 or m143 fusion proteins were allowed to bind to a metal chelating column, and then eluted from the column. The eluted protein

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was then checked to make sure that m142 or m143 proteins were present by Western blot using the anti-Xpress antibody. As expected, a band of 42 kD was detected for the histidine tagged m142 recombinant protein and a band of 56 kD was detected for the histidine tagged m143 recombinant protein. The purified proteins were then sent to Cocalico Biologicals, Inc. (Reamstown, PA) for rabbit injection. Two rabbits were immunized with the same preparation of recombinant protein.

Western Blot Analysis. Western blots were conducted as previously described (Pari and St. Jeor, 1990). Briefly, NIH3T3 cells were harvested in Western lysis buffer (50 mM Tris, 1% SDS, pH 7.5). The samples were subjected to SDS-PAGE on 12.5% gels, and transferred to nitrocellulose membranes (Boehringer Mannheim, Indianapolis, IN). The blots were treated with protein blocking solution containing 5% milk diluted in 0.1% Tween 20/TBS (10mM Tris, 150 mM NaCl) overnight at 4°C, and then incubated with a primary antibody for one hour at room temperature at a 1:100 dilution in milk (5% milk diluted in 0.1% Tween 20/TBS). The blots were washed three times in 0.1% Tween 20/TBS at room temperature, and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St. Louis, MO) (diluted 1:10,000) in 5% milk for one hour at room temperature. Following three more washes in 0.1% Tween 20/TBS, antibody binding was detected by chemiluminescence using the ECL Western Blotting Detection Kit (Amersham Pharmacia Biotech, Arlington Heights, IL) on Fuji RX film.

DNA Transfection. Transient transfection experiments were done using the Transfast Reagent (Promega, Madison, WI). This reagent utilizes cationic liposomes to

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deliver nucleic acid to eukaryotic cells. Briefly, low passage NIH 3T3 cells were plated the day before transfection in 60 mm dishes at $3x10^5$ cells/dish. The Transfast Reagent (Promega, Madison, WI) was also suspended in nuclease free water the day prior to transfection. To suspend the Transfast, the vial of Transfast Reagent and nuclease free water were warmed to room temperature. The nuclease free water was added to the vial to create a volume of 1mM of cationic lipid component (400 µl per 0.4 mg vial). After adding the nuclease free water, the sample was vortexed vigorously for 10 seconds to suspend the lipid film. The Transfast Reagent was then frozen at –20° C overnight. At the time of transfection, the vial of Transfast Reagent was warmed to room temperature. Serum free media and complete media (containing serum) were warmed to 37° C. The Transfast Reagent/DNA mixture was then prepared. A total of 5 µg of plasmid DNA was added to 2 ml of serum free media and the mixture was vortexed briefly. Next a 1:1 charge ratio of Transfast Reagent to DNA was added to the DNA mixture (15 µl Transfast per 5µg of DNA) and vortexed immediately. The Transfast/DNA mixture was then allowed to incubate at room temperature for 10-15 minutes. After the incubation period, the DNA mixture was vortexed briefly. Next the medium was removed from the cells and the DNA mixture was added. The cells were then allowed to incubate at 37° C for one hour. At the end of the incubation period, the cells were gently overlayed with 4 mls of complete media and returned to the incubator for 48 hours. The overall transfection efficiency using this method was about 10%.

Transfection /Superinfection. Since the NIH3T3 cells transfected using the Transfast reagent did not demonstrate cytopathic effect (CPE) subsequent to MCMV

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infection, transfection/superinfection experiments were done using the Cytofectene reagent (Bio-Rad, Hercules, CA). Approximately $3x10^5$ NIH 3T3 fibroblast cells were seeded into 25 mm tissue culture flasks the day before transfection. Next the plasmid DNA/Cytofectene mix was prepared. First 9 μl of Cytofectene mix was added to 100 μl of Dulbecco's Modified Eagle's Medium (DMEM) per sample. This mix (mix 1) was incubated at room temperature for 10 minutes. Next 6 μg of plasmid DNA was mixed with 100 μl of DMEM (mix 2). Next the Cytofectene solution (mix 1) was added to the plasmid DNA solution (mix 2) and incubated at room temperature for ten minutes. This Cytofectene/DNA mix was then incubated at 4° C overnight.

The following day, the Cytofectene/DNA mix was allowed to reach room temperature. Next 2 ml of complete 3T3 media were added to each Cytofectene/DNA sample and mixed. The cells were washed with sterile PBS and the Cytofectene/DNA mix was added to the cells. The cells were incubated for at least 5 hours at 37° C, the Cytofectene/DNA mix removed, the cells washed with PBS, and NIH 3T3 complete media added. After 24 hours post transfection, the cells were split 1:2 into a 60 mm dish. The following day the cells were infected with the appropriate multiplicity of infection for one hour. Following the infection, complete medium was added to the cells.

Chloramphenicol Acetyl Transferase Assays. Approximately $3x10^5$ NIH 3T3 fibroblast cells were seeded into 60 mm tissue culture dishes the day before transfection. A total of 5 μg of plasmid (including the transcriptional transactivators and MCMV reporter-CAT constructs) were transfected into cells using the Transfast Reagent (Promega, Madison, WI) at the indicated concentration. The empty vector pCDNA3.1

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was used to maintain a constant amount of plasmid DNA per transfection. At 48 hours post-transfection, the cells were harvested. Briefly, the transfected cells were washed twice with 3 mls of Tris Buffered Saline (TBS)(30mM Tris, 150mM NaCl, pH 7.4), then scraped off the plates into 0.4 ml of TBS. Next the cells were pelleted and resuspended in 100 μl of 0.25 M Tris-HCL pH 7.8. Following three rounds of freezing in an ethanol dry ice bath and thawing at 37° C, the cell lysates were centrifuged and the supernatants were transferred to new tubes. The extracts were then heated to 68° C for 10 minutes and the amount of protein per extract was determined using the BCA Protein Assay Kit (Pierce, Rockford, Il). Equal amounts of protein (15 μg of protein per sample) were used in each sample to be analyzed for CAT activity. The standard CAT assay contains 20 μl of extract (containing 15 μg of protein in 0.25 M Tris pH 7.8), 70 μl 0.25 M Tris pH 7.8, 20 μl of 4 mM acetyl coenzyme A, 34 μl of H_20, and 0.1μCi of [^{14}C] chloramphenicol (Amersham Pharmacia Biotech, Piscataway, NJ) in a reaction volume of 125 μl. Reactions were carried out for 30 minutes at 37° C, extracted with ethyl acetate, and the extracted products were dried in a Speed Vacuum Concentrator. Reaction products were resuspended in 20 μl of ethyl acetate, spotted onto silica gel thin layer chromatography plates and the products were separated by ascending chromatography using 95:5 chloroform:methanol (190ml chloroform/10ml methanol). After autoradiography, each form of chloramphenicol was quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The reactions were linear for at least 30 minutes for up to 50% acetylation. When above 50% acetylation occurred during the 30 minute incubation, the assay was repeated using less protein. Experiments were repeated a minimum of three times and the average of three experiments were shown.

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Northern Blot Experiments. Approximately $1x10^6$ NIH 3T3 cells were seeded into 100 mm tissue culture plates the day before infection. The fibroblasts were either mock infected or infected with wild type (WT) MCMV at a multiplicity of 2 PFU per cell. Following virus adsorption at 37° C for one hour, the inoculum was removed, and 10 ml of culture media were added. For immediate early RNA isolation, 100μM anisomycin was added to the cells one hour prior to and during infection. For early RNA isolation, phosphonoformate was added to the culture media at a concentration of 300 μg per ml at the time of infection. For late RNA isolation, RNA was harvested from cultures at 24 hours after infection. Total RNA was harvested using the Qiagen Rneasy kit for total RNA isolation (Qiagen, Chatsworth, CA). Northern blots were conducted as described by Maniatis and coworkers (1987). RNA samples were quantitated at 260 nm, and 5 ug of each sample was loaded into the well of a 1.5% agarose/6.6% formaldehyde gel and electrophoresed overnight at 20 volts. The following day, the gels were rinsed three times in sterile distilled water (10 minutes per wash), and transferred to a positively-charged nylon membrane overnight in 20XSSC (3M NaCl, 300 mM sodium citrate, pH 7.0). In some cases the gels were treated to enhance transfer of large RNA species by partial alkaline hydrolysis. This includes soaking for 30 min in 0.05 M NaOH/1.5 M NaCl followed by neutralization in 0.5 M Tris/1.5 M NaCl (pH 7.5) prior to blotting. After transfer, the membranes were UV cross-linked (150J), and baked at 80° for 30 minutes. The Boehringer Mannheim Genius System (Boehringer Mannheim, Indianapolis, IN) was used to detect hybridized probes of MCMV DNA labeled with digoxygenin-11-dUTP/dATP. Bands were visualized using the chemiluminescent

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substrate disodium3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate which is referred to as CSPD.

Cell Fractionation Experiments. The NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce, Rockford, IL) was used to determine the localization of pm142 and pm143 within an MCMV infected cell. Approximately 1x10^6 NIH 3T3 cells were seeded into 100 mm tissue culture plates the day before infection. The fibroblasts were either mock infected or infected with wild type (WT) MCMV at a multiplicity of 5 PFU per cell. Following virus adsorption at 37° C for one hour, the inoculum was removed, and 10 ml of culture media were added. At the indicated time post infection, the cells were washed with sterile PBS, scraped from the plate, transferred to a 1.5 ml microcentrifuge tube, and pelleted. Next 100 µl of cytoplasmic extraction reagent 1 (CER1) were added to the cell pellet and vortexed vigorously for 15 seconds. The tube was then placed on ice for 10 minutes. Next 11 µl of CER II were added to the tube, vortexed, and placed on ice for 1 minute. After vortexing again, the tube was centrifuged for 5 minutes at maximum speed (16,000XG). The supernatant, which contains the cytoplasmic extract, was then transferred to another tube then placed on ice. The insoluble pellet was then resuspended in 100 µl of nuclear extraction reagent (NER) and vortexed for 15 seconds. The sample was then placed on ice and vortexed for 15 seconds every 10 minutes for a total of 40 minutes. The nuclear fraction was then centrifuged at 16,000XG, and the supernatant, which contains the nuclear fraction, was removed. The samples were stored at –80° C until use.

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The nuclear and cytoplasmic fractions were analyzed by Western blot analysis using m142 and m143 polyclonal antisera as described above. As a control to ensure that minimal nuclear contamination of the cytoplasmic fraction occurred, the antibody against phosphotyrosine phosphatase-PEST (PTP-PEST) was used in the Western blot detection. The PTP-PEST protein is a mouse cytoplasmic protein. The PTP-PEST antibody was kindly provided by Dr. M. Tremblay (Department of Biochemisty, McGill University, Montreal, Canada).

Immunofluorescence. Immunofluorescence was also used to determine the localization of the m142 and m143 proteins. The day prior to infection approximately $1x10^5$ NIH 3T3 cells were seeded per chamber on chambered slides. The next day these cells were either mock infected or infected with MCMV at an MOI of 5 for an hour at 37° C, the viral inoculum removed, complete media added, and the cells incubated for 24 hours at 37° C. After the 24 hour incubation, the growth medium was removed and the cells were washed two times with PBS containing Ca^{2+} and Mg^{2+}. Cells were fixed in 2% paraformaldehyde (diluted in PBS, pH 7.4) for 10 minutes at room temperature. Following fixation, the cells were rinsed twice with Ca^{2+}/ Mg^{2+} free PBS, and permeabilized with PBS containing 0.5% triton X 100 for 8 minutes at room temperature. The slides were stored at 4° C overnight. For the staining procedure, the cells were first rehydrated in 0.1% bovine serum albumin (BSA)/PBS. Next the primary antibody (anti-142 or anti-143) was added to the slides at several dilutions (1:100, 1:1000) in 0.1% BSA/PBS, and the slides incubated for one hour at 37° C. Control chambers were incubated with preimmune serum in 0.1% BSA/PBS at the same dilutions. As a positive

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control, the e1 antibody was used (obtained from Dr. Gina Ciocco-Schmitt, Eastern Virginia Medical School, Norfolk, VA). Following primary antibody, the cells were washed three times with 0.1% BSA/PBS, and incubated with goat anti-rabbit antiserum conjugated with FITC (Sigma, St. Louis, MO) at a 1:100 dilution for 1 hour at 37° C. The slides were washed twice with 0.1% BSA/PBS, once with distilled water, and then air dried. The coverslips were mounted on the slides with mounting fluid and viewed under a confocal microscope (Olympus Fluoview 100). Different Z-series of the images were taken at 1-μM intervals and projected onto a single plane.

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CHAPTER IV

RESULTS

Kinetics of m142 and m143 Transcript Expression

Since m142 and m143 are essential genes, we wanted to determine at what time during viral infection m142 and m143 are expressed. In order to do this, a time course experiment was done. In this experiment RNA was harvested from NIH3T3 infected cells at 0, 1, 3, 6, and 16 hours after infection. This RNA was then analyzed by Northern blot using a DNA probe containing the Sst-Sst fragment of MCMV from this region (Fig. 6) (Hanson et al., 1999).

The m142 and m143 genes were expressed by one hour post infection, and reach maximal levels by three hours post infection. The m142 transcript corresponds to a 1.8 kb RNA and the m143 transcript corresponds to a 3.8 kb RNA (Fig. 6B. and 7). Levels of these transcripts remained abundant at six and sixteen hours post infection (Hanson et al., 1999). It appears that the 1.8 kb m142 transcript may shift to a 1.6 kb RNA species as time progresses, since at late times a 1.6 kb m142 band predominates over the 1.8 kb band (Fig. 7). This could be explained by a variation in the size of the polyadenylation signal. Alternatively, the shorter 1.6 kb transcript may be generated by splicing, since splice donor and acceptor sites are dispersed throughout this gene. However, nuclease mapping would have to be done to confirm this. According to the expected size of the m142b transcript, the 1.6 kb band could also be transcribed from m142b (Fig. 6) (Hanson et al., 1999).

The Sst1-Sst1 probe contains 404 nucleotides of the M141 gene. Therefore, the 7.0 kb M141 transcript was detected as well. The 5.1 kb transcript was expressed from

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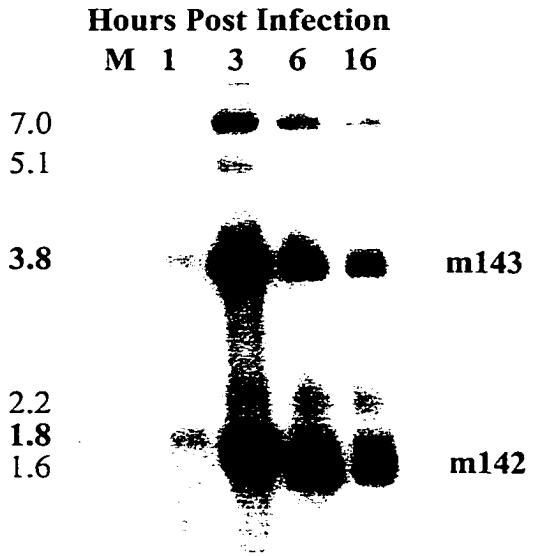

FIG 7. Time course of expression of m142 and m143 transcripts. NIH 3T3 fibroblast cells were either mock infected (M) or infected with 5 PFU/cell of wild type MCMV for the indicated times (hours post infection). RNA was isolated as described in the Materials and Methods. The gel was treated by partial alkaline hydrolysis to enhance transfer of larger RNA species and probed with a labeled probe spanning the m142 and m143 gene region. Numbers to the left denote sizes (Kb) (Hanson et al., 1999).

m144. Although the Sst1-Sst1 probe is outside of the m144 gene region, the m144 transcript was detected because m142, m143 and m144 have 3' coterminal transcripts (Hanson et al., 1996).

m142 and m143 Are Immediate Early Genes

Since m142 and m143 were expressed at one-hour post infection, we speculated that these might be novel immediate early genes not previously identified. By definition, immediate early genes are those expressed in the absence of de novo protein synthesis.

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The drug anisomycin prevents de novo protein synthesis thus allowing the expression of only the immediate early genes during an MCMV infection.

We therefore determined if m142 and m143 are true immediate early genes. In order to do this, NIH 3T3 fibroblast cells were infected in the presence of anisomycin. Under these conditions, both m142 and m143 were detected when a probe containing the Sst 1-Sst 1 fragment of MCMV corresponding to this region was used (Fig. 6A, 8). Therefore, m142 and m143 are true immediate early genes (Fig. 6B, Fig. 8). Under these conditions MCMV IE1 and IE3 are abundantly expressed, while early genes like m144 are absent (data not shown). This proves that the anisomycin treatment was effective in that only the immediate early genes were expressed.

These genes were also expressed in the presence of the viral DNA replication inhibitor phosphonoformate, suggesting that these genes are also expressed at early times. At early times, it was also evident that a 1.6 kb and a 2.2 kb transcript were expressed (Fig. 8) (Hanson et al., 1999). Both m142 and m143 were expressed at late times of MCMV infection as well (24 hours post infection) (Fig. 8). Therefore m142 and m143 are true immediate early genes, and are also expressed at early and late times of MCMV infection.

We were surprised to find that the expression of m142 and m143 is very different from the expression of the other MCMV immediate early genes. For example, the immediate early genes ie1 and ie3 are expressed at abundant levels in the presence of the protein synthesis inhibitor cycloheximide (Messerle et al., 1991). However MCMV IE transcripts ie1 and ie3 are absent when NIH 3T3 cells are infected in the presence of the DNA replication inhibitor phosphonoacetic acid, and therefore do not accumulate at early

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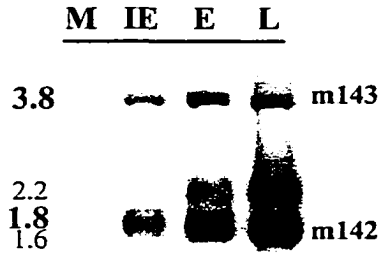

FIG. 8. m142 and m143 are newly identified genes expressed at immediate early times. NIH 3T3 fibroblasts were infected with 2 PFU MCMV/cell and RNA was isolated. Immediate early (IE) RNA was produced in the presence of 100 μM anisomycin, early (E) RNA was produced in the presence of 300 ug/ml of phosphonoformate, and late (L) RNA was harvested 24 hours post infection. A labeled probe spanning the m142 and m143 gene region (Sst1-Sst1) of MCMV Hind III I was used to detect transcripts from this region (see figure 6). The 3.8 kb transcript corresponds to m143; the 1.8 kb transcript corresponds to m142 (Hanson et al., 1999).

times (Messerle et al., 1991; Keil et al., 1987; Koszinowski, 1986). This suggests that unlike IE1 and IE3, m142 and m143 proteins may have important functions not only at immediate early times, but at all stages of viral infection.

The expression kinetics of m142 and m143 are most like the immediate early genes IRS1/TRS1 and UL36-38 of HCMV. In HCMV, the IRS1/TRS1 and UL36-38 genes are expressed in the presence of anisomycin. These genes are also abundantly expressed in the presence of phosphonoacetic acid (Romanowski and Shenk, 1997, Tenney and Colberg-Poley, 1991a, Tenney and Colberg-Poley, 1991b). The IRS1/TRS1 and UL36-38 proteins are involved in ori lyt dependent replication of HCMV, and

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function as transcriptional transactivators. Interestingly, genes m142 and m143 have homology to the HCMV IRS1/TRS1 genes (Rawlinson et al., 1996) suggesting that m142 and m143 gene products may have a similar function.

Characterization of pm142 and pm143

Generation of m142 and m143 Polyclonal Antibodies. The above experiments indicated that m142 and m143 are true immediate early genes in that their transcripts are expressed in the absence of de novo protein synthesis. It was logical to assume that their protein products are also expressed at immediate early times, and like the transcripts, are upregulated at early and late times. Therefore, we proceeded to characterize the expression of the m142 and m143 gene products in MCMV infected fibroblasts. In order to do this, antibodies specific for m142 and m143 protein products were generated. To serve as an immunogen for production of the polyclonal antisera, histidine tagged fusion proteins were made as bacterially expressed proteins. The pBADHisA vector (Invitrogen, Carlsbad,CA) was used to generate these fusion proteins. This vector allows the m142 and m143 protein products to be fused to six histidine residues which comprise a polyhistidine tag and the Xpress epitope. The polyhistidine tags allow purification of the recombinant fusion protein on a metal-chelating resin and the Xpress epitope permits detection of the recombinant fusion protein by the anti-Xpress antibody. This vector also allows the expression of the proteins to be strictly regulated. Expression from the plasmid pBADHis A only occurs in the presence of arabinose.

In order to generate histidine tagged m142 and m143 proteins, the 142 and 143 genes were cloned into the pBADHis A vector. A Kpn1-BsrG1 fragment (nucleotide

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sequences 200526-198878) of the Hind III I genomic region of MCMV was cloned into pBAD/His A in order to express a portion of m142 (Fig. 6). This construct does not include the first 222 bases of the m142 gene, and was called m142pBAD/HisA. Remaining in this construct are US22 motifs III and IV within m142, as well as the acidic domains (Fig. 5). A Kpn1-Kpn1 fragment of the Hind III I genomic region of MCMV (nucleotide sequences 202422-200526) (Fig. 6) was cloned into the pBAD/His A vector in order to express a truncated m143 protein. The truncated m143 gene is missing 171 bases from the 5' end. This construct, which was called m143pBAD/HisA, includes the putative US22 family motifs I, III and IV, the nuclear localization signals, and the N-glycosylation sites within the m143 open reading frame (Fig. 5). These constructs were tested by restriction enzyme digestion to confirm that the correct MCMV sequence was inserted, and that the MCMV sequence was in the proper orientation.

Next it was necessary to determine if the recombinant m142 and m143 proteins were expressed from their respective expression vectors and to determine the optimal concentration of arabinose needed for their expression. In order to do this, m142pBAD/HisA and m143pBAD/HisA were transformed into TOP10 *E. coli.* Next various arabinose concentrations were used to determine if protein expression was induced from these vectors. According to the DNA sequence, the expected size for the m142 recombinant protein was 44 kD. At an arabinose concentration of 0.2 %, a protein of 42 kD was expressed from the m142pBAD/HisA vector (Fig. 9). The predicted size for the histidine tagged m143 was 59 kD. At arabinose concentrations of 0.002% and 0.02%, a 56 kD protein was induced from the m143pBAD/HisA vector (Fig. 9). These

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data indicate that the histidine tagged m142 and m143 proteins are expressed from their respective expression vectors and are similar to their expected size.

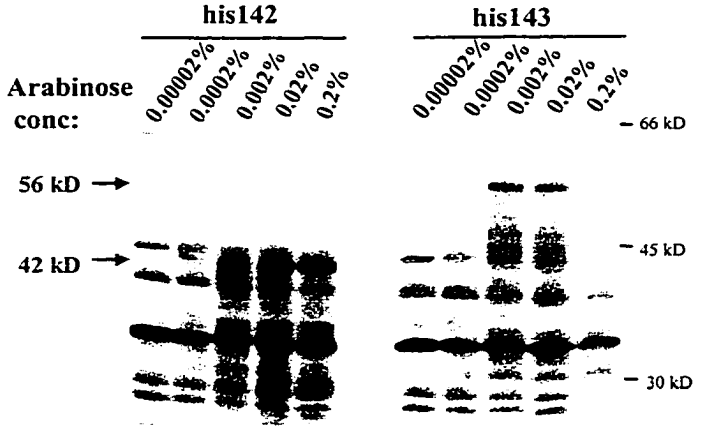

FIG. 9. Expression of the histidine tagged recombinant m142 and m143 proteins. Top10 *E.coli* containing m142BAD/HisA or m143BAD/HisA were incubated with various arabinose concentrations. The bacterial pellet was then lysed and analyzed by Western blot as described in the Materials and Methods using the Anti-Xpress Antibody. Sizes of molecular weight markers are indicated on the right.

Since the histidine tagged m142 and m143 proteins were expressed from their respective expression vectors, our next goal was to purify these proteins from bacterial cells. In order to do this, 250 ml cultures of bacteria containing m142pBAD/HisA and m143pBAD/HisA were grown, induced with arabinose, and used for recombinant protein purification. Several attempts were made to purify the recombinant m142 and m143 proteins from the bacterial lysate using the Invitrogen Xpress Purification System. This method was unsuccessful at first because the m142 and m143 recombinant proteins proved to accumulate in inclusion bodies that were discarded with the bacterial debris. Inclusion bodies are very dense aggregates of insoluble protein that contain most of the

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expressed protein. Our next approach was to lyse the cells, purify the inclusion bodies, solubilize the inclusion bodies, and then purify the recombinant protein. The cells were lysed and the inclusion body was purified using the B-PER Bacterial Protein Extraction Reagent (Pierce, Rockford, IL). This reagent allows gentle and complete lysis of the bacterial cells, and purification of the inclusion bodies. The inclusion bodies were then solubilized using the Inclusion Body Solubilization Reagent (Pierce). The Inclusion Body Solubilization Reagent moves the insoluble inclusion body into solution, thus allowing purification of the recombinant protein. Finally the proteins were purified using the Xpress Purification System (Invitrogen, Carlsbad, CA). In this system the recombinant proteins, which were tagged with six histidines, were purified with a metal chelating column and then eluted from the column. The polyhistidine tag functions as a metal-binding site for affinity purification of the recombinant fusion protein on the metal-chelating resin. The eluted proteins were then checked to make sure that m142 and m143 proteins were present by Western blot analysis using the anti-Xpress antibody. As expected, a band of 42 kD was detected for the histidine tagged m142 recombinant protein and a band of 56 kD was detected for the histidine tagged m143 recombinant protein (data not shown). The purified proteins were then sent to Cocalico Biologicals, Inc. (Reamstown, PA) for rabbit injection. Exsanguination of the rabbits took place following a two month period, which included four immunizations with the purified recombinant protein.

Once the polyclonal antisera were obtained, it was necessary to test them for their ability to recognize the m142 and m143 proteins. MCMV lysates from fibroblast cells that had been either mock infected or infected for 24 hours with MCMV at an MOI of 2

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were used to screen the rabbit serum by Western blot analysis for specificity of the antibody. Specific bands were seen in the infected cell lysates, but not in the mock infected cell lysates when both m142 and m143 antiserum were used. The m142 protein corresponds to a 43 kD band, whereas the m143 protein corresponds to a 53 kD band (Fig. 10). The m142 protein product will be called pm142 and the m143 protein product will be called pm143. The expected size of pm142 according to its DNA sequence is 48 kD, and the expected size of pm143 according to its DNA sequence is 64 kD. The 142 and 143 proteins may be smaller than expected due to rapid post translational modification such as proteolytic processing of the proteins, although there is no evidence that this is taking place. The m142 transcript shifts from 1.8 kb to 1.6 kb as time progresses, suggesting that splicing may occur which could account for the smaller size of this protein. However nuclease mapping studies have not yet been done to confirm this (Hanson et al., 1999). There is also precedence for proteins with a high proportion of basic or charged amino acids to migrate faster or slower than their actual molecular weight on a polyacrylamide gel (Takano et al., 1988). Since both pm142 and pm143 have a high content of charged residues, they could be running faster on the polyacrylamide gel. This phenomenon could explain why pm142 and pm143 appear to be smaller than expected.

Another approach was used to confirm that the 43 and 53 kd proteins originate from ORFs m142 and m143, respectively. This approach utilizes transient transfection of m142 or m143 expression plasmids into cells, followed by Western blot analysis of cell lysates using pm142 and pm143 antisera to determine if pm142 and pm143 are recognized. To generate the m142 and m143 expression plasmids, the full length m142

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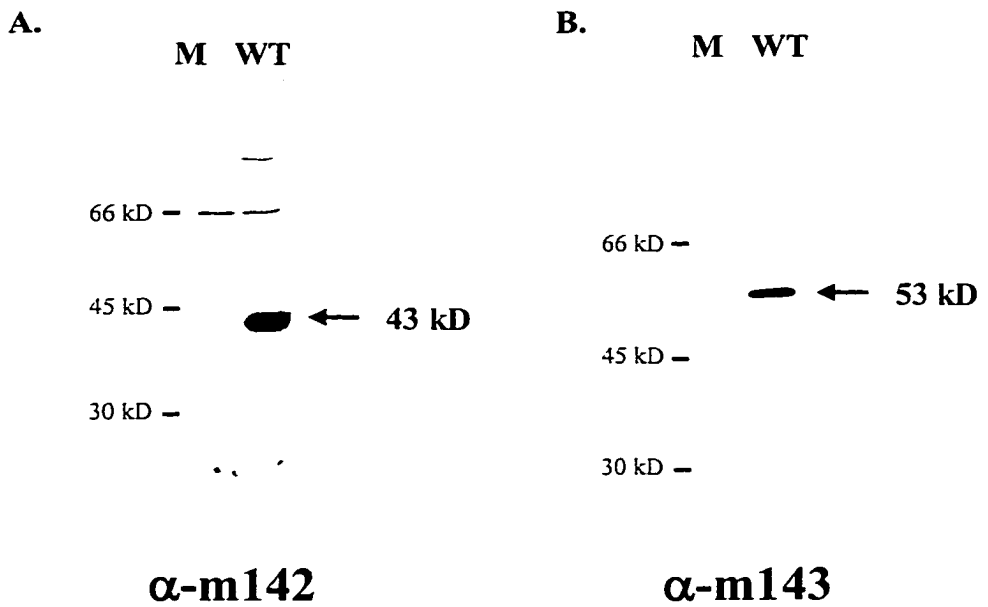

FIG. 10. m142 and m143 polyclonal antisera specifically recognize the pm142 and pm143 proteins, respectively. NIH 3T3 fibroblast cells were mock infected (M) or infected with wildtype MCMV (WT) at an MOI of 2 for 24 hours. Lysates were then analyzed by Western blot (as described in the Materials and Methods) using A.) the pm142 or B.) the pm143 antibody. Sizes of molecular weight markers are indicated on the left.

and m143 genes were cloned into the pCDNA3.1 expression vector using the polymerase chain reaction (PCR). The vector pCDNA3.1 was used because it is designed for high level expression of the gene of interest in mammalian cells. This high level of expression is achieved because of the presence of the HCMV major immediate early gene (UL123) enhancer and promoter. This vector also contains the bovine growth hormone polyadenylation signal and transcription termination sequence to enhance mRNA stability. The expression plasmid 142pCDNA contains the m142 gene and the expression plasmid 143pCDNA contains the m143 gene. NIH 3T3 fibroblast cells were

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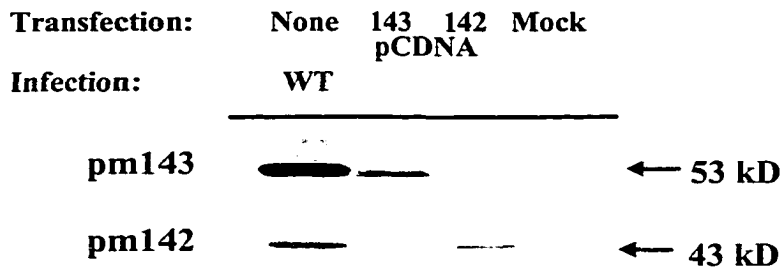

FIG. 11. m142 and m143 polyclonal antisera recognize proteins expressed from the m142 and m143 genes respectively. NIH 3T3 cells were mock transfected or transfected with a plasmid containing the m143 or the m142 gene using the Cytofectine Reagent (Bio-Rad) as described in the Materials and Methods. Lysates were then analyzed by Western blot as described in the Materials and Methods using an antibody mixture containing pm142 and pm143 antisera. Lysates from wild type MCMV infected cells (WT) serve as a positive control.

transfected with the expression plasmid 142pCDNA or the expression plasmid 143pCDNA. Lysates from these cells were analyzed by Western blot using a mixture of pm142 and pm143 antisera. The specificity of the antibodies for the m142 and m143 gene products was confirmed. The antibody against pm142 reacted with a 43 kD protein product of 142pCDNA and likewise the antibody against pm143 reacted with a 53 kD protein product of 143pCDNA in transfected cell lysates (Fig. 11). Therefore, the 43 kD and 53 kD proteins recognized by the pm142 and pm143 antisera are products of the m142 and m143 genes, respectively.

Kinetics of the pm142 and pm143 proteins. As previously described, the m142 and m143 transcripts are present at immediate early times and remain abundant throughout infection. Prior to these studies the m142 and m143 protein products had not

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been identified. Therefore our next goal was to characterize these proteins. Since the 142 and 143 transcripts are present at IE, E, and late times, we also expected to see their respective protein products at immediate early, early, and late times. To determine if this occurs, a time course experiment was done in which fibroblast cells were infected with wild type MCMV at an MOI of 2 for 0, 3, 6, 12 and 24 hours. These lysates were analyzed by Western blot using a mixture of pm142 and pm143 antibodies. The results are shown in Figure 12. The m142 protein product (43 kD) was present at 3 hours post infection as expected, although at low levels. The levels increased between 3 and 6 hours post infection, and remained abundant at 12 and 24 hours post infection. To our surprise, it was found that pm143 (53 kD) was not detected at 3 hours post infection as expected for an IE protein, even after a prolonged exposure of film. However after increasing the MOI to 5 in subsequent experiments, pm143 was also readily detectable at 3 hours post infection (see below). Therefore, these proteins appear to be regulated with similar kinetics.

The kinetics of pm142 and pm143 expression is very different from that of the MCMV IE1 and IE3 proteins. The IE1 and IE3 proteins are present by 2 hours post infection and then gradually decrease at 4, 8, 12, 16 and 24 hours post infection (Messerle et al., 1992). At the protein level, pm142 and pm143 are most similar to the HCMV immediate early proteins IRS1/TRS1. These proteins are expressed at 2 hours post infection and remain throughout the entire HCMV replication cycle at relatively high levels (Romanwoski and Shenk, 1997). The IE proteins IRS1/TRS1 function at early times as transcriptional

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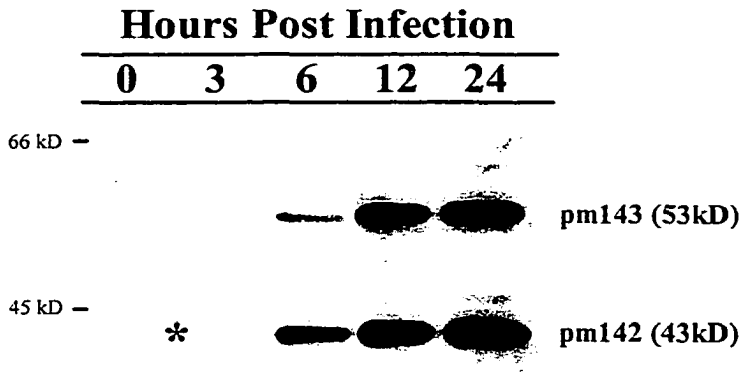

FIG. 12. Kinetics of pm142 and pm143 protein expression. NIH 3T3 fibroblast cells were infected with wild type MCMV at an MOI of 2 for 0, 3, 6, 12, and 24 hours. Lysates were analyzed by Western blot as described in the Materials and Methods using a mixture containing both pm142 and pm143 antibodies. Asterisks denote low levels of pm142 expression.

transactivators of genes required for viral DNA replication. This potential function will be considered for pm142 and pm143.

pm142 and pm143 are not immediate early proteins. Proteins pm142 and pm143 were expressed at 3 hours post infection. Therefore they were expressed at a time that is at the borderline between an immediate early protein and an early protein. The designation immediate early or early cannot be assigned to pm142 and pm143 based on the time course that was performed previously. This designation can only be assigned to pm142 and pm143 by using drug blocks at specific stages within the viral replication cycle. To determine if a protein is expressed at immediate early times, cells must first be infected in the presence of the protein synthesis inhibitor anisomycin. Under these conditions only the transcripts that are made in the absence of de novo protein synthesis

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are expressed, the immediate early transcripts. The cells are then washed to remove the anisomycin, then actinomycin D is added. Actinomycin D prevents additional transcription of early genes from occurring. Therefore, only the immediate early transcripts made previously will be translated into protein, resulting in the expression of only the immediate early proteins.

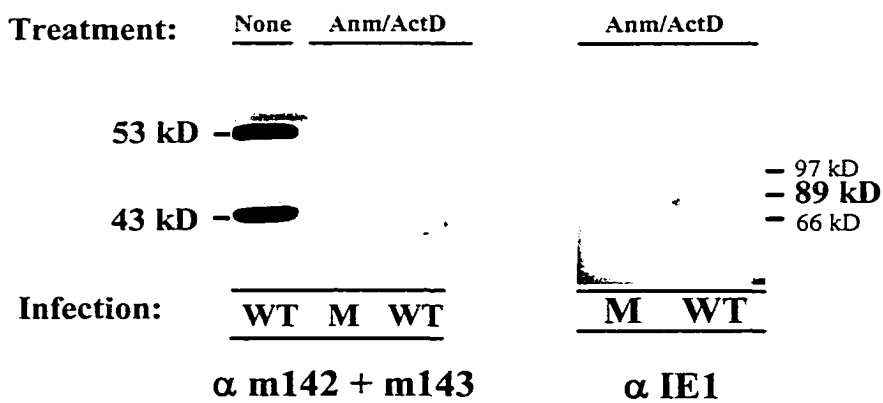

FIG. 13. Immediate early genes m142 and m143 do not encode detectable immediate early proteins. NIH 3T3 fibroblast cells were incubated in the presence of 100 μM anisomycin (Anm) for one hour, then mock infected (M) or infected with wild type MCMV (WT) for 3 hours at an MOI of 5 in the presence of 100 μM anisomycin. The cells were then washed with PBS, then incubated in the presence of 10 μg/ml of actinomycin D (ActD) for 6 hours. The lysates were harvested and analyzed by Western blot using an antibody mixture containing both pm142 and pm143 antibodies. Lysates from cells infected in the absence of drugs serve as a positive control. As a control to confirm the drug blocks were effective, the Anm/ActD lysates were also probed with the IE1 antibody. The IE1 antibody was kindly provided by Ulrich Koszinowski (Department of Microbiology, Federal Research Center for Virus Diseases in Animals, Federal Republic of Germany).

We wanted to determine if pm142 and pm143 are immediate early proteins. In order to do this, NIH 3T3 cells were incubated for an hour in the presence of 100μM anisomycin. Next these cells were either mock infected or infected with MCMV at a

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MOI of 5 in the presence of 100μM anisomycin for 3 hours. The cells were then washed with PBS, and incubated in the presence of 10 μg/ml of actinomycin D for 6 hours. The lysates were harvested and analyzed by Western blot using an antibody mixture containing both pm142 and pm143 antibodies. Neither pm142 nor pm143 was detected in this assay (Fig. 13). The immediate early protein IE1 was detected under these conditions, although at low levels. By definition, pm142 and pm143 are therefore classified as early proteins. Since pm142 and pm143 were present at 3 hours post infection in the protein kinetics experiment, which is borderline between IE and E, these data were not surprising.

The MCMV immediate early proteins IE1 and IE3 should be expressed at abundant levels under the conditions described above (anisomycin/actinomycin D). Since the immediate early transcripts were allowed to accumulate in the presence of the anisomycin, the level of IE protein should be more abundant in the drug treated cells (anisomycin/actinomycin D), than cells not treated with drugs. Since the levels of IE1 in the anisomycin/actinomycin D treated lysates were very low, this indicates that a technical problem may have occurred with this assay. For example, it is possible that the anisomycin was not completely removed. To assess this, lysates from cells infected in the absence of drugs should be used as a control in future experiments.

Our results have shown that genes m142 and m143 express RNA at immediate early times of viral infection. However, pm142 and pm143 are expressed at early times. Therefore we hypothesize that there is translational control of m142 and m143 RNA. One possible reason for translational regulation of pm142 and pm143 is that the proteins may be needed immediately following IE protein expression. The presence of the m142

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and m143 transcripts at IE times could allow the rapid synthesis of these proteins at early times, without the need for m142 and m143 transcription.

Localization of the pm142 and pm143 proteins

As previously stated m142 and m143 are immediate early genes that encode early proteins, and are members of the US22 gene family of HCMV. Although the function of most US22 gene family members is unknown, certain members of the family encode transcriptional transactivators. Also, m142 and m143 are homologous to the IRS1/TRS1 proteins of HCMV, and are regulated similarly to IRS1/TRS1. IRS1/TRS1 are involved in the regulation of genes active during viral DNA replication. Therefore, it is likely that pm142 and pm143 are transcriptional transactivators. In order for pm142 and pm143 to be transcriptional transactivators, they need to localize to the nucleus. Sequence analysis suggests that this is possible for at least pm143, because pm143 has several nuclear targeting signals. In contrast, pm142 does not contain any nuclear localization signals (Fig. 5).

Localization of viral proteins in an MCMV infected cell can be tested in several different ways, for example by immunofluorescence studies. In this case, infected cells are fixed to glass slides then stained with a primary antibody against the protein of interest. Next the primary antibody is replaced with a secondary antibody that will recognize the primary antibody. The secondary antibody is conjugated to a label such as fluorescein isothiocyanate (FITC). This FITC label will provide a colored reagent that can be observed directly under a fluorescent microscope. Alternatively, the protein of interest can be fused to the green fluorescent protein (GFP). In this case GFP serves as a

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reporter molecule for monitoring protein localization in transfected cells in vitro, since the protein fluoresces green when exposed to ultraviolet light. It is possible however that the GFP molecule may mask any nuclear localization signals or alter the structure of the protein of interest, thus interfering with the localization of the protein. Other viral proteins that may be important in the localization of the protein of interest may be absent when using GFP-fusion proteins. This limitation can be overcome however by doing transfections with the GFP fusion plasmid, followed by superinfection with MCMV. Lastly, localization of viral proteins can be tested by performing cell fractionation experiments. In these experiments the nuclear fraction of a cell is separated from the cytoplasmic fraction. These fractions are then analyzed by Western blot using an antibody against the protein of interest to determine localization of the protein.

We decided to use immunofluorescence to determine the localization of the pm142 and pm143 proteins. Immunofluorescence is a very powerful tool, since it allows the localization of the protein of interest to be determined while maintaining the integrity of the infected cell. NIH 3T3 cells were infected with wild type MCMV at an MOI of 5 for 6 hours or 24 hours. Following infection, the cells were fixed with paraformaldehyde then stained with pm142 or pm143 antiserum as the primary antibody (Fig. 14A,B). As a positive control, the antibody against e1, a nuclear localized protein, was used (Fig. 14D). Rabbit preimmune serum was used as a negative control (Fig. 14C). Following primary antibody staining, FITC conjugated goat anti-rabbit antibody was used as the secondary antibody (see Materials and Methods). There was no e1, pm142 or pm143 antibody signal observed when cells were infected for 6 hours (data not shown). This is

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FIG. 14. Immunofluorescense analysis of the pm142 and pm143. NIH 3T3 fibroblasts were infected with MCMV at an MOI of 5 for 24 hours. Immunofluorescent staining was performed as described in the Materials and Methods using A. pm142 antibody or B. pm143 antibody. As a negative control (C.) preimmune serum was used. As a positive control (D.) e1 antibody was kindly provided by Dr. Gina Ciocco-Smitt (Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, VA).

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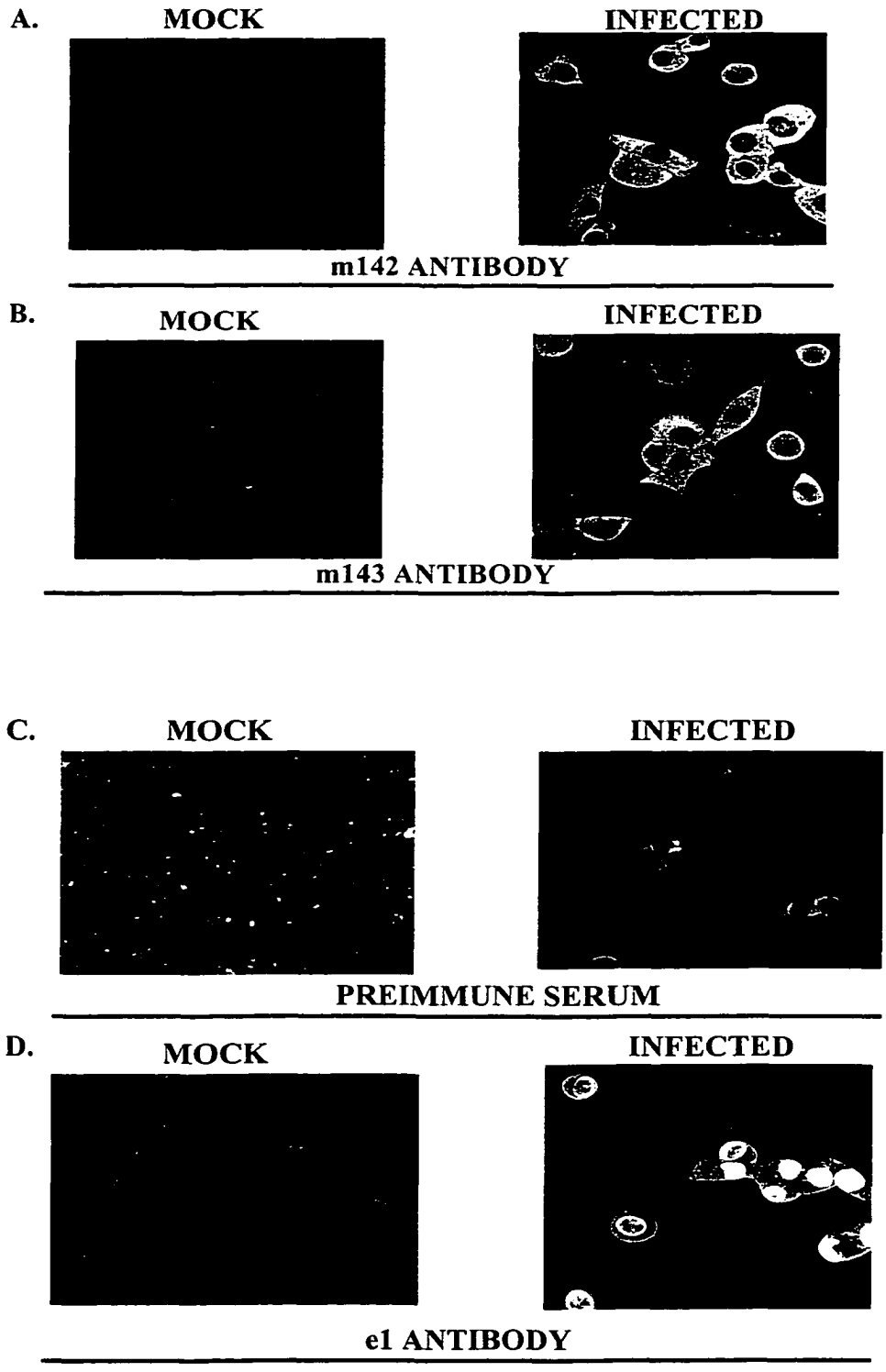

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likely due to the fact that not enough viral protein was present in the infected cell at 6 hours post infection to be detectable in this assay. However at 24 hours post infection, the pm142 protein localized predominantly to the cytoplasm, although low levels of pm142 protein were present in the nucleus (Fig. 14A). It was shown that pm143 also localized to the cytoplasm predominantly, with very small amounts in the nucleus (Fig. 14B). Although the presence of low levels of nuclear pm142 and pm143 were not obvious in figure 14, it was more visible in the cross-sectional images of the infected cells (data not shown). The cross-sectional images allow the cells to be viewed at different depths or sections, thus allowing the nucleus to be viewed without interference from the cytoplasmic components. Figure 14 is a representative picture of all of the sections combined, therefore the nuclear staining that was observed may not be evident in this image.

We also used cell fractionation experiments to determine the localization of pm142 and pm143. These experiments allow the nuclear and the cytoplasmic fraction of infected cells to be separated and analyzed by Western blot with antisera against the protein of interest. This allows the localization of the protein to at least the nuclear or the cytoplasmic fraction to be determined. For the cell fractionation experiments, NIH 3T3 fibroblast cells were infected with MCMV at an MOI of 5 for 3 and 4 hours. Then the NE-PER Nuclear and Cytoplasmic Extraction Reagent was used (Pierce, Rockford, IL) as described in the Materials and Methods. It was determined that pm142 localizes to both the nucleus and the cytoplasm at 3 and 4 hours post infection, and it appears that there is approximately equal distribution of pm142 in both fractions (Fig. 15A). Although in the

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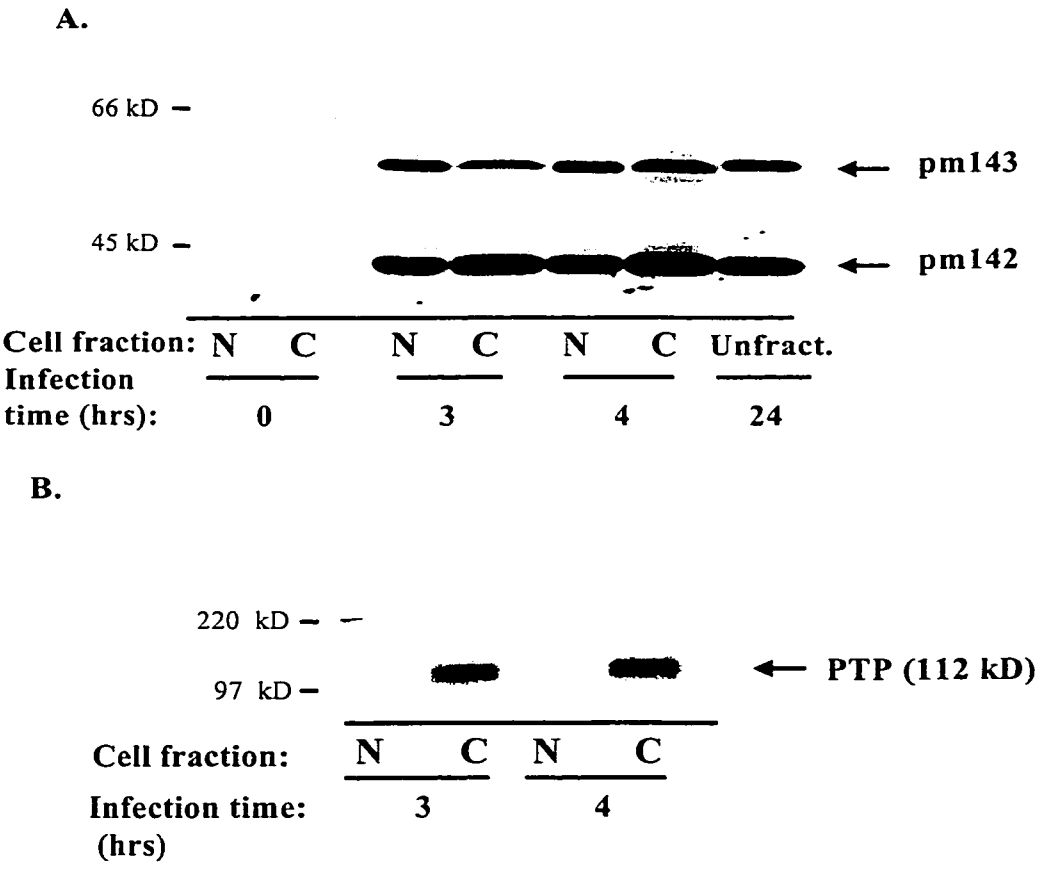

FIG. 15. pm142 and pm143 proteins localize to the nucleus and the cytoplasm at 3 and 4 hours post infection. A). NIH 3T3 fibroblast cells were infected with MCMV at an MOI of 5 for 3 or 4 hours. The NE-Per Nuclear and Cytoplasmic Extraction Reagent (Pierce) was used as described in the Materials and Methods to separate the nuclear (N) and the cytoplasmic (C) fractions of the infected cells. Lysates were then analyzed by Western blot using an antibody mixture containing pm142 and pm143 antisera. Lysates from unfractionated cells infected with MCMV for 24 hours served as a positive control. B). The infected cell lysates from above were analyzed by Western blot using the phosphotyrosine phosphatase (PTP) antibody to assess nuclear contamination by the cytoplasmic fraction.

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kinetics experiment described previously pm143 was not detected when cells were infected with an MOI of 2 for 3 hours, when the MOI was increased to 5 in this experiment, pm143 was readily detected at 3 and 4 hours. Increasing the MOI likely increased the expression levels of the 143 protein, and therefore assisted in its detection. The pm143 protein also localized to both the nucleus and the cytoplasm at approximately equal amounts at 3 and 4 hours post infection (Fig. 15A). As a control to detect the degree of contamination of the nuclear fraction with the cytoplasmic fraction, the phosphotyrosine phosphatase antibody was used to analyze these lysates. The phosphotyrosine phosphatase protein localizes specifically to the cytoplasm. Since the phosphotyrosine phosphatase protein was present almost exclusively in the cytoplasmic fraction, this confirms that minimal cytoplasmic contamination of the nuclear fraction occurred (Fig. 15B).

In parallel to the immunofluorescence studies, we wanted to determine pm142 and pm143 localization at 24 hours post infection. Both pm142 and pm143 were found in the nuclear and cytoplasmic fractions at 24 hours post infection (Fig. 16A) It appears that more pm142 and pm143 protein was present in the cytoplasmic fraction than the nuclear fraction, since equal representative volumes of each sample were analyzed by Western blot. Also the control protein phosphotyrosine phosphatase localized predominately to the cytoplasm, suggesting that minimal cytoplasmic contamination of the nuclear fraction occurred (Fig. 16B).

In summary, cell fractionation studies have shown that both pm142 and pm143 localized to the nucleus and the cytoplasm at 3, 4, and 24 hours post infection. With the immunofluorescence studies, it was shown that at 24 hours post infection pm142 and

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pm143 localized predominantly to the cytoplasm, with low levels of pm142 and pm143 nuclear protein. Our next goal was to determine the ability of pm142 and/or pm143 to transactivate several MCMV promoters. Since pm142 and pm143 are early proteins, we will determine their ability to activate the expression of the early e1 gene that is involved in viral DNA replication (Pari and Anders, 1993). We will also investigate the ability of pm142 and pm143 to activate the MCMV immediate early promoter.

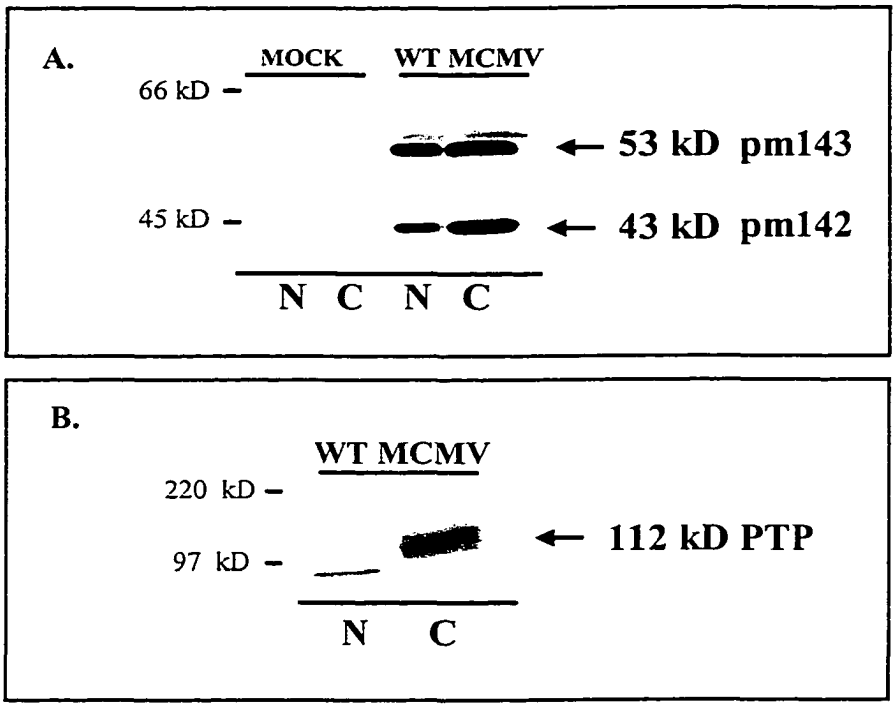

FIG. 16. pm142 and pm143 localize to the nucleus and the cytoplasm at 24 hours post infection. A.) NIH 3T3 fibroblast cells were mock infected or infected with wild type (WT) MCMV at an MOI of 5 for 24 hours. The NE-Per Nuclear and Cytoplasmic Extraction Reagent (Pierce) was used as described in the Materials and Methods to separate the nuclear (N) and the cytoplasmic (C) fractions of the infected cells. The lysates were then analyzed by Western blot using an antibody mixture containing pm142 and pm143 antisera. B). Infected cell lysates from above were analyzed by Western blot using the phosphotyrosine phosphatase (PTP) antibody to assess nuclear contamination by the cytoplasmic fraction.

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Functional Characterization of the pm142 and pm143 proteins

We propose that pm142 and/or pm143 are transcriptional transactivators. As previously stated, pm142 and pm143 are members of the US22 gene family, some of which are transcriptional transactivators. Also, pm142 and pm143 are homologous to the IRS1/TRS1 proteins of HCMV (Rawlinson et al., 1996), and are regulated similarly to IRS1/TRS1. IRS1/TRS1 are involved in the regulation of genes active during viral DNA replication (Pari and Anders, 1993; Pari et al. 1993; Iskenderian, 1996; Kerry et al., 1996). According to the cell fractionation experiments and immunofluorescence analysis, the pm142 and pm143 proteins also localize to the nucleus (Fig. 14A, 14B, 15A, 16A). Therefore we next determined the transactivation capabilities of pm142 and pm143 collectively and individually.

These studies commenced immediately after the m142 and m143 transcripts were identified as immediate early RNAs, but prior to the analysis of their gene products. Since the m142 and m143 transcripts were immediate early, we reasoned that their protein products are likely expressed at immediate early times as well. The other immediate early proteins of MCMV (IE1, IE3, and IE2) are involved in the regulation of the MCMV major immediate early promoter and enhancer (MIEPE). The MCMV IE1 and IE2 proteins positively regulate the MIEPE, while IE3 represses expression from this promoter/enhancer (Koszinwoski et al., 1986; Messerle et al., 1992; Cardin et al., 1995). Therefore, we began our analysis of m142 and m143 function by investigating the ability of pm142 and pm143 to enhance activation of the MIEPE (ie1/ie3 promoter/enhancer).

For these studies, the vector MCMV3CAT (MIEP-CAT) was kindly provided by Dr. Martin Messerle (Department of Virology, University of Ulm, Germany) (Messerle

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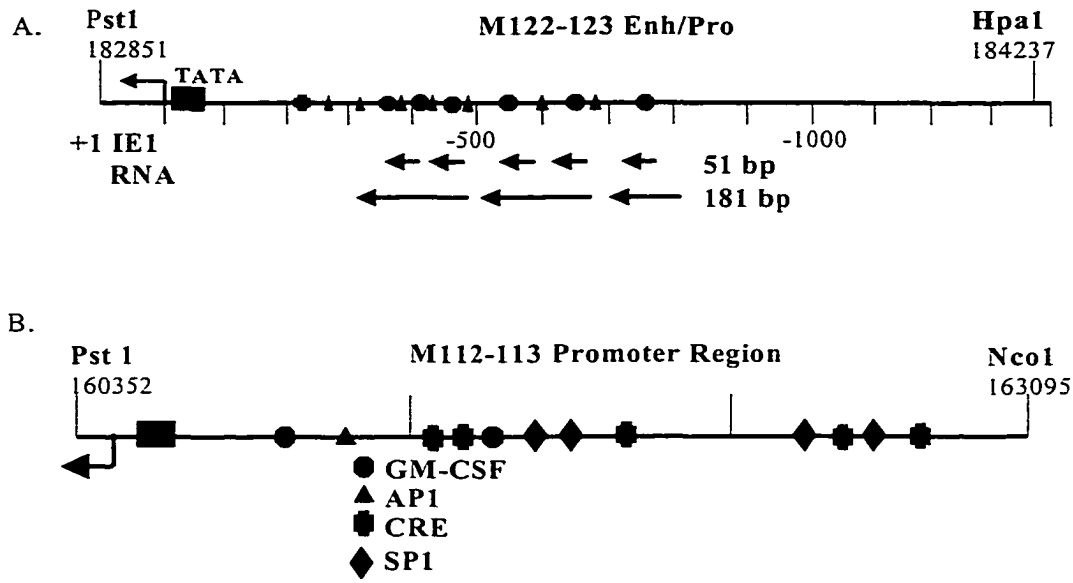

FIG. 17. Schematic representation of the promoter/enhancer regions used in transient assays. A. Schematic representation of the MCMV IE enhancer/promoter (M122-123) used in the transient assays. Structural organization of known consensus binding sites for cellular transcription factors, repeat elements, restriction enzyme sites and TATA box are shown (Dorsch-Hasler et al., 1985; Sanford and Burns, 1996). B. Sequences of the e1 enhancer/promoter (M112-113) used in transient assays. Structural organization of the predicted consensus binding sites for cellular transcription factors. Restriction enzyme sites are denoted.

et al., 1992). This vector contains the entire MCMV major IE enhancer and promoter (M122-123) upstream of the chloramphenicol acetyl transferase gene (CAT). This enhancer/promoter region (MCMV sequences 182851-184237) includes all of the known MIEPE transcription factor binding sites and repeat elements (Fig. 17A) The CAT gene serves as a reporter that allows the amount of transactivation to be determined by CAT assay.

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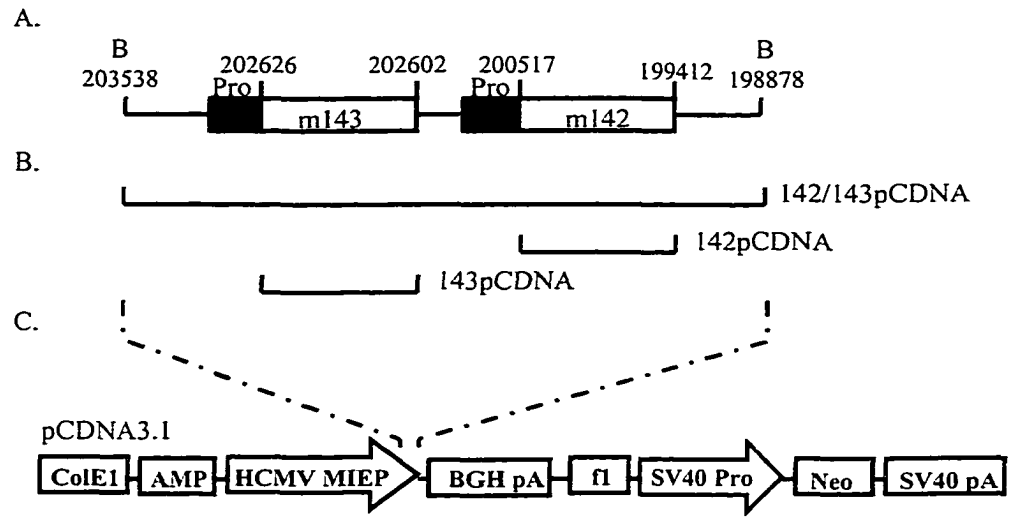

FIG. 18. Description of m142/m143 plasmids used in transient assays. A. Structural organization of the m142-m143 gene region. Location of the promoter sequences (Pro) are depicted by black boxes and restriction enzyme sites are denoted (B; BsrG). Numbers refer to nucleotides within the MCMV genome. B. Map of plasmid inserts containing m142 and/or m143 used in transient assays. C. Map of pCDNA3.1 vector (Invitrogen) that was used to express m142 and/or m143 proteins. Broken lines depict the location in which these genes were cloned (multiple cloning site).

Expression of pm142 and pm143 from the plasmid 142/143pCDNA3.1. To determine if the pm142 and pm143 proteins activate the MCMV MIEPE, a plasmid was generated to express the m142 and m143 genes collectively. The m142 and m143 genes, along with their native promoters, were cloned into the expression vector pCDNA3.1 (Invitrogen) as described in the Materials and Methods. This construct includes MCMV sequences 198878-203538, and contains the entire m142 and m143 open reading frames with their native promoters and polyadenylation signal (Fig. 5, 6A.). The construct that contains both m142 and m143 was called 142/143pCDNA (Fig. 18B.). The pCDNA3.1 vector contains the HCMV major immediate early promoter/enhancer (FIG 18C.), which appears to be necessary for optimal expression of pm142 and pm143 from these

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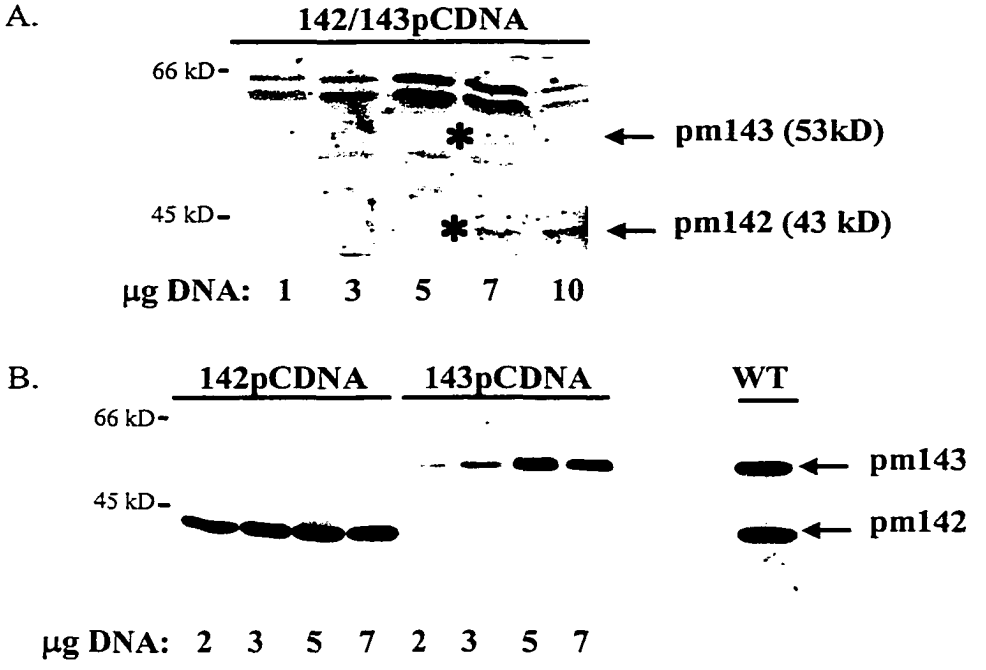

FIG. 19. Proteins pm142 and pm143 are expressed from the plasmids 142/143pCDNA, 142pCDNA and 143pCDNA. A. NIH 3T3 fibroblasts were transfected with 1, 3, 5, 7, or 10 μg of 142/143pCDNA. The cells were then harvested after 48 hours and analyzed by Western blot using a pm142 and pm143 antibody mixture. B. NIH 3T3 fibroblast cells were transfected with 2, 3, 5, or 7 μg of 142pCDNA or 143pCDNA as indicated. Lysates from cells infected with wild type MCMV serve as a positive control. Cell lysates were harvested at 48 hours and analyzed as described above. Asterisks denote low levels of protein expression

sequences, since a vector lacking the HCMV MIEPE did not express pm142 and pm143 to detectable levels by Western blot analysis.

The 142/143pCDNA vector was tested in transient assays to make sure that pm142 and pm143 proteins were expressed from this vector. NIH 3T3 fibroblast cells were transfected with 1, 3, 5, 7, and 10 μg of 142/143pCDNA. The pm142 and pm143 proteins were not detected when 1, 3, or 5 μg of 142/143pCDNA were transfected (FIG.

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19A.). However, the pm142 and pm143 proteins were detected when at least 7 μg of DNA was transfected, although the levels of protein were very low (Fig. 19A.). These promoters may require other viral proteins for efficient activity. This could also be explained by expression of pm142 and pm143 from their native promoters, which may be very weak.

Activation of the ie1/ie3 enhancer/promoter by the pm142 and pm143 proteins. The vector 142/143pCDNA was tested in transient transfections to determine if pm142 and/or pm143 transactivates the MCMV MIEPE. Various amounts of 142/143pCDNA were transfected into NIH 3T3 cells, along with 1 μg of the plasmid MIEP-CAT as described in figure 20. Following transient transfection of the given constructs, cell lysates were analyzed by chloramphenicol acetyl transferase assay as described in the Materials and Methods. When 1.0 μg of the vector 142/143pCDNA was transfected with the MIEP-CAT construct, transactivation of the MCMV MIEPE was minimal (Fig. 20). However when 2.0 or 3.0 μg of 142/143pCDNA was used, the transactivation levels rise to at least 3 fold above basal levels. Although low levels of pm142 and pm143 were expressed under these conditions (Fig. 19A), promoter activity was still increased. Therefore the m142 and/or m143 gene products enhance activation of the MCMV MIEPE. We believe that increasing the concentration of 142/143pCDNA increased the level of pm142 and pm143 expression, thus allowing optimal levels of transactivation to be obtained. Initially we were not surprised by these results, since it was known that immediate early gene products activate the MIEP. However, when it was subsequently determined that pm142 and pm143 are early proteins, the interpretation of these results

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was brought into question, because no other MCMV early proteins have been shown to activate the MIEP.

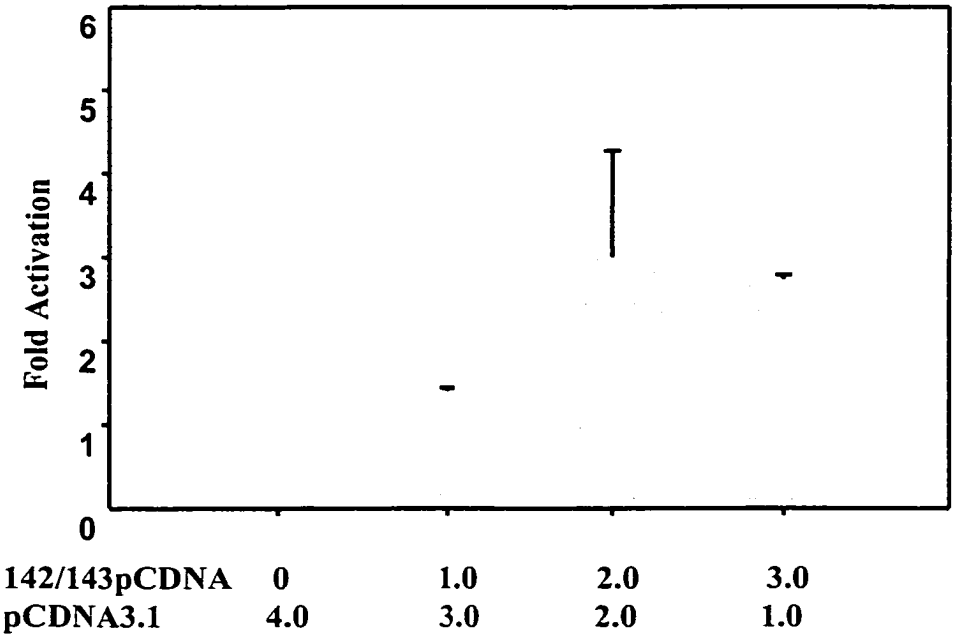

FIG. 20. Activation of the MCMV immediate early promoter/enhancer (MIEP) by IE proteins pm142/pm143. A total of 1.0 μg of reporter plasmid MCMV3CAT (MIEPCAT) was transfected into NIH 3T3 fibroblasts with the empty vector pCDNA3.1 (lane 1), or with the plasmid 142/143pCDNA at 1.0 μg (lane 2), 2.0 μg (lane 3), or 3.0 μg (lane 4). The empty vector pCDNA3.1 (Invitrogen) was used to maintain a constant amount of plasmid DNA per transfection. Cell lysates were harvested 48 hours post transfection and analyzed as described in the Materials and Methods. The transfections were done in duplicate and each experiment was repeated three times. Error bars represent standard deviations.

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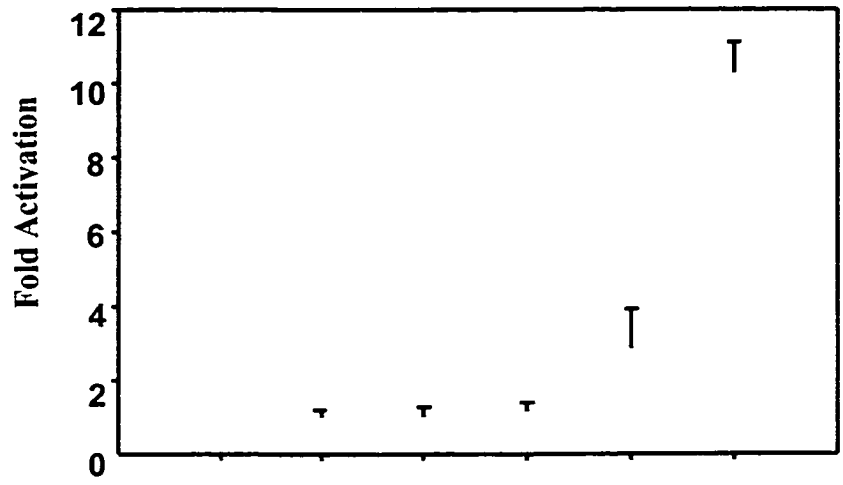

142pCDNA	**0**	**3.0**	**0**	**1.5**	**0**	**0**
143pCDNA	**0**	**0**	**3.0**	**1.5**	**0**	**0**
142/143pCDNA	**0**	**0**	**0**	**0**	**3.0**	**0**
pIE111	**0**	**0**	**0**	**0**	**0**	**1.0**
pCDNA3.1	**4.0**	**1.0**	**1.0**	**1.0**	**1.0**	**3.0**

FIG. 21. Both pm142 and pm143 are required for activation of the MCMV immediate early promoter/enhancer (MIEP). A total of 1.0 μg of the reporter plasmid MCMV3CAT (MIEPCAT) was transfected into NIH 3T3 fibroblasts with the empty vector pCDNA3.1 (lane 1), or with the plasmids 142pCDNA (lane 2), 143pCDNA (lane 3), 142pCDNA and 143pCDNA (lane 4), 142/143pCDNA (lane 5), or pIE111 (lane 6). The empty vector pCDNA3.1 (Invitrogen) was used to maintain a constant amount of plasmid DNA per transfection. The numbers denote the amount of plasmid DNA transfected in μg. Cell lysates were harvested 48 hours post transfection and analyzed as described in the Materials and Methods. The transfections were done in duplicate and each experiment was repeated three times. Error bars represent standard deviations.

Expression of pm142 and pm143 from the plasmid 142pCDNA and 143pCDNA

Since 142/143pCDNA expresses both pm142 and pm143, we were not sure which protein functioned to enhance transcriptional transactivation. Therefore, we wanted to determined if pm142, pm143 or both have the ability to enhance transactivation.

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Expression plasmids containing m142 and m143 alone were cloned into pCDNA3.1 as described in the Materials and Methods. The plasmid 142pCDNA contains the entire m142 sequence (MCMV sequences 199412-200517) but not the m142 promoter. The plasmid 143pCDNA contains the entire m143 sequence, but not the m143 promoter (MCMV sequences 202602-202626) (Fig. 18B). These plasmids were also tested in transient assays to determine if pm142 and pm143 were expressed from their respective expression vectors. NIH 3T3 cells were transfected with 2, 3, 5 and 7 μg of 142pCDNA or 143pCDNA, and cell lysates were analyzed by Western blot analysis using a pm142 and pm143 antibody mixture. Unlike 142/143pCDNA, the levels of protein expressed from 142pCDNA and pm143pCDNA were very high. With as low as 2 μg of 142pCDNA and 143pCDNA, the pm142 and pm143 proteins, respectively, were clearly detectable (Fig. 19A). High levels of protein expression are likely due to the presence of the HCMV MIEP that is located immediately upstream of the m142 and m143 sequence.

Both pm142 and pm143 are required for transactivation of the MCMV MIEPE.

In order to determine if pm142, pm143 or both has the ability to enhance transactivation of the MIEPE, the plasmids containing m142 or m143 alone (called 142pCDNA or 143pCDNA, respectively) were tested in transient transfection assays along with MIEP-CAT. The positive control plasmid expressing both IE1 and IE3 transactivates this promoter 10 fold above basal levels. However, proteins expressed from neither 142pCDNA nor 143pCDNA transactivated the MIEPE alone (Fig. 21). When the construct 142/143pCDNA was transfected with the MIEP-CAT construct, transactivation of 3-4 fold above basal levels was obtained. Therefore both pm142 and pm143 are

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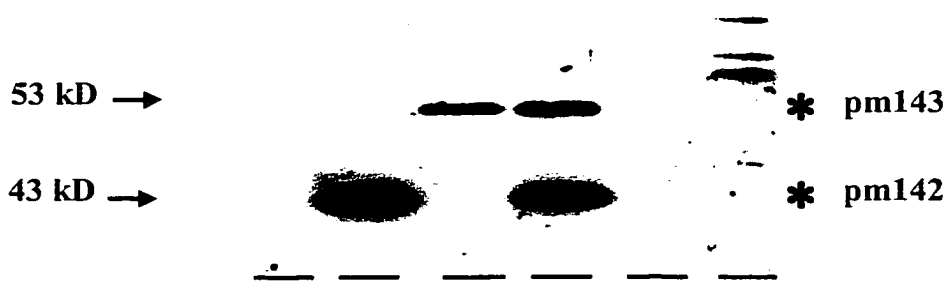
necessary for transactivation of the MIEPE to occur. To our surprise, when both 142pCDNA and 143pCDNA were transfected together, transactivation of the MIEPE above basal levels was not achieved. This could be due to the phenomenon of squelching, since the levels of protein expressed from 142pCDNA and 143pCDNA under these conditions were very high compared to proteins expressed from 142/143pCDNA (Fig. 22). It is also possible that a splice variant may be expressed from 142/143pCDNA. There is some evidence that splicing of the m142 transcript may be occurring since the size of the m142 transcript changes from 1.8 kb to 1.6 kb as time progresses (Fig. 8). Also an alternate transcript may be expressed from 142/143pCDNA (m142b, m142c) that is not expressed from 142pCDNA or 143pCDNA, and that protein product may act as the transcriptional transactivator (Fig. 6A).



142pCDNA	**0**	**3.0**	**0**	**1.5**	**0**	**0**
143pCDNA	**0**	**0**	**3.0**	**1.5**	**0**	**0**
142/143pCDNA	**0**	**0**	**0**	**0**	**3.0**	**3.0**
pIE111	**0**	**0**	**0**	**0**	**0**	**0**
pCDNA3.1	**4.0**	**1.0**	**1.0**	**1.0**	**1.0**	**1.0**

FIG. 22. Elevated expression of pm142 and pm143 may have caused squelching to occur in the MCMV MIEP promoter analysis. NIH 3T3 cells were transfected with the indicated plasmids. The numbers refer to μg of DNA. Cell lysates were harvested 48 hours post transfection and analyzed by Western blot analysis using a pm142 and pm143 antibody mixture. Asterisks denote location of proteins. Last lane includes a darker exposure.

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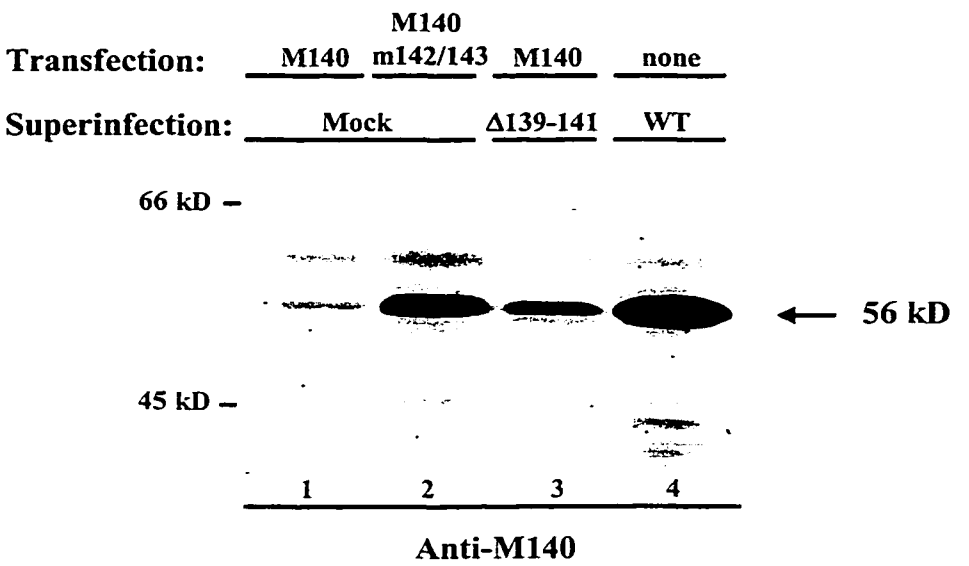

FIG. 23. The m142/m143 gene products enhance expression from the HCMV MIEP. NIH3T3 fibroblasts were transfected with an HCMV-MIEP driven MCMV M140 expression vector (lanes 1 and 3) or cotransfected with the same construct in addition to the m142/143 expression vector (lane 2). Cells were mock-infected (lane 1 and 2) or superinfected with a MCMV triple deletion mutant (Δ139-141)(lane 3). Wild type (WT) infected lysates serve as a positive control (lane 4). Transfection, superinfection and Western blot analyses were conducted as described in the Materials and Methods.

m142 and m143 gene products can transactivate the HCMV MIEPE. An alternate approach was used to test the ability of the pm142 and pm143 to activate the HCMV MIEPE. It has previously been shown that the HCMV MIEPE can substitute for the MCMV MIEP in the context of an MCMV infection (Angulo et al., 1998; Grzimek et al., 1999). In our laboratory we had a construct containing the MCMV M140 gene under the control of the HCMV MIEPE. We reasoned that if pm142 and pm143 are able to activate the MCMV MIEP, they should also be able to transactivate the HCMV MIEP. Therefore, in the presence of pm142 and pm143, M140 protein expression should be

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increased. We cotransfected a M140 expression plasmid with the 142/143pCDNA plasmid into NIH 3T3 cells. We then analyzed the lysates for levels of M140 protein by Western blot analysis using a M140 antibody. Our results show that the levels of M140 were increased when pm142 and pm143 were present compared to M140 alone (Fig. 23). These levels were even higher than those found in cells superinfected with the Δ139-141 mutant virus that contains the full repertoire of IE proteins. Therefore, pm142 and/or pm143 are able to transactivate the HCMV MIEPE. These results were not surprising since it was previously shown that pm142 and pm143 transactivated the MCMV MIEPE. The HCMV and MCMV MIEPE have some sequence homology and therefore pm142 and pm143 proteins may function via similar sequences within both the MCMV and HCMV MIEPE, thus allowing transactivation to occur.

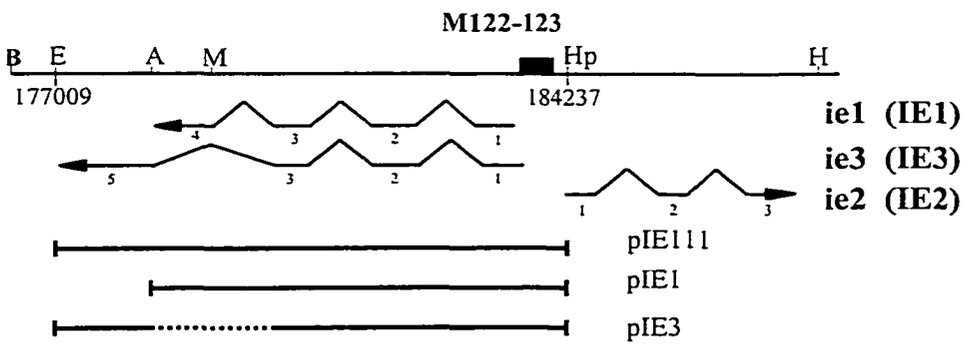

FIG. 24. Structural organization of the MCMV major IE gene loci (M122-123). Map of MCMV major immediate early gene region and useful restriction enzyme sites. The promoter/enhancer region is denoted by a black box. Also included are IE transcripts and plasmids used in transient assays (Messerle et al., 1992). The dotted line denotes deleted sequences and restriction enzyme abbreviations are as follows: B, BamH1; E, EcoR1; A, Aval; M, Msc1; Hp, Hpal; and H, Hind 111.

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Lack of cooperativity between IE1/IE3 and pm142/pm143 in the activation of the MCMV MIEP (M122-123). In HCMV and MCMV, it has been shown that the immediate early proteins can act cooperatively in the activation of some promoters. We wanted to determine if pm142 and pm143 might cooperate with the MCMV IE1 protein in transactivating the MIEP. Therefore, in the next study we used a plasmid that expresses both IE1 and IE3 proteins (pIE111). This vector was kindly provided by Dr. Martin Messerle (Messerle et al., 1992) and is depicted in figure 24. The vector 142/143pCDNA was cotransfected with the MIEP-CAT construct and the plasmid expressing both IE1 and IE3 (pIE111). It has previously been shown that 1 μg of the IE1/IE3 expressing plasmid (pIE111) transactivates the MIEPE to very high levels. The high levels of transactivation can be attributed to IE1, which positively regulates the MIEPE (Kosinowski, 1986). Therefore, in our studies we reduced the amount of pIE111 to 0.25 μg, since IE1 is such a potent transactivator of the MIEPE. We then titrated in increasing amounts of 142/143pCDNA. We found that 0.25 μg of IE1/IE3 alone transactivated the MIEPE to about 1.6 fold above basal levels (Fig. 25). When 142/143pCDNA was added, the levels of transactivation increased in a dose responsive manner. With 2.0 μg of 142/143pCDNA, transactivation by IE1 and IE3 doubled to almost 4 fold above basal levels, and with 3 μg of 142/143pCDNA activation of 5 fold by IE1/IE3 was found. The levels of transactivation by IE1/IE3 and pm142/pm143 were additive, and no cooperativity was observed. These results were not surprising since the other MCMV immediate early proteins exhibit noncooperative effects on the MIEPE as well. For example, IE1 alone transactivates the MIEP, while IE3 alone represses the

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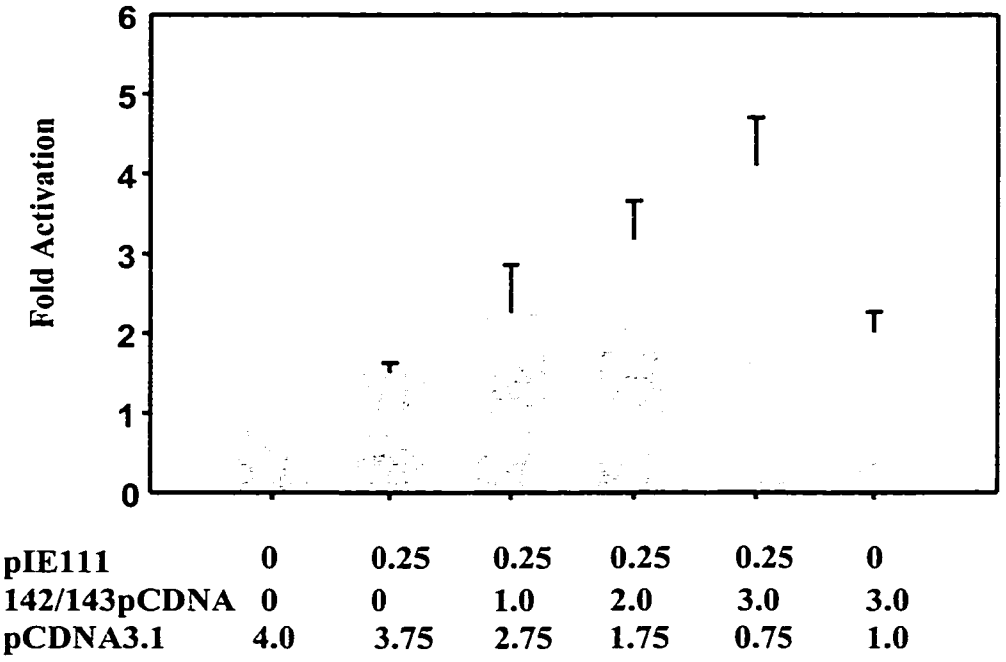

FIG. 25. Additive effects between the proteins pm142/pm143 and IE1/3 in activating the MCMV MIEP. A total of 1.0 μg of the reporter plasmid MCMV3CAT (MIEPCAT) was transfected into NIH 3T3 fibroblasts with the empty vector pCDNA3.1 (lane 1) or with plasmids pIE111 (IE1 and IE3) and/or m142/143pCDNA. The amount of pIE111 DNA was kept constant at 0.25 μg per sample (lanes 2-5) and 142/143pCDNA was titrated in at 1.0 μg (lane 3), 2.0 μg (lane 4), 3.0 μg (lane 5) or 3.0 μg (lane 6). The empty pCDNA3.1 vector was used to maintain a constant amount of plasmid DNA per transfection. Cell lysates were harvested 48 hours post transfection and analyzed as described in the Materials and Methods. The transfections were done in duplicate and each experiment was repeated three times. Error bars represent standard deviations.

MIEP. When IE1 and IE3 are combined, their effects on the MIEP are additive (Messerle et al., 1992).

In this experiment the level of transactivation of the MIEPE by IE1/IE3 and m142/m143 were additive. Since ie1 and ie3 are under the control of their native promoter, the MIEPE, in the ie1/ie3 expression vector (pIE111), it is possible that the

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additive levels of transactivation by m142/m143 and IE1/IE3 result from an increase in the expression of IE1. The proteins m142 and m143 may cause an increase in IE1 expression from pIE111, and IE1 could then transactivate the MIEP-CAT plasmid resulting in an increase in CAT expression. In order to determine if m142/m143 and IE/IE3 contribute to the activation of the MIEP-CAT independently in an additive manner or if m142/m143 are increasing IE1 expression under these conditions, Western blot analyses looking at IE1 levels would have to be done in parallel with the transactivation studies.

pm142 and pm143 fail to activate the early e1 (M112-113) promoter. Since pm142 and pm143 are early proteins, they likely transactivate genes that are expressed at early times. Furthermore, pm142 and pm143 have homology to IRS1-TRS1 of HCMV. IRS1/TRS1 have been shown to activate early genes involved in viral DNA replication. So, we wanted to determine if pm142 and pm143 are able to transactivate early genes involved in DNA replication. In these studies we used the early e1 promoter (M112-113) (Fig. 17B). The e1 gene (M112-113) encodes nuclear localized proteins that are homologous to HCMV UL112-113 gene products required for HCMV ori-Lyt dependent viral DNA replication. The plasmid e1-CAT was kindly provided by Dr. Martin Messerle (Messerle et al., 1992) and includes MCMV sequences 160352-162095 upstream of the CAT gene (Fig. 17B). This promoter contains several predicted consensus binding sites for the cellular transcription factors GM-CSF, AP-1, CRE and SP1. When NIH 3T3 cells were transiently transfected with 1.0 μg of the e1-CAT construct and 1.0 μg of 142/143pCDNA, there was no transactivation (Fig. 26). When

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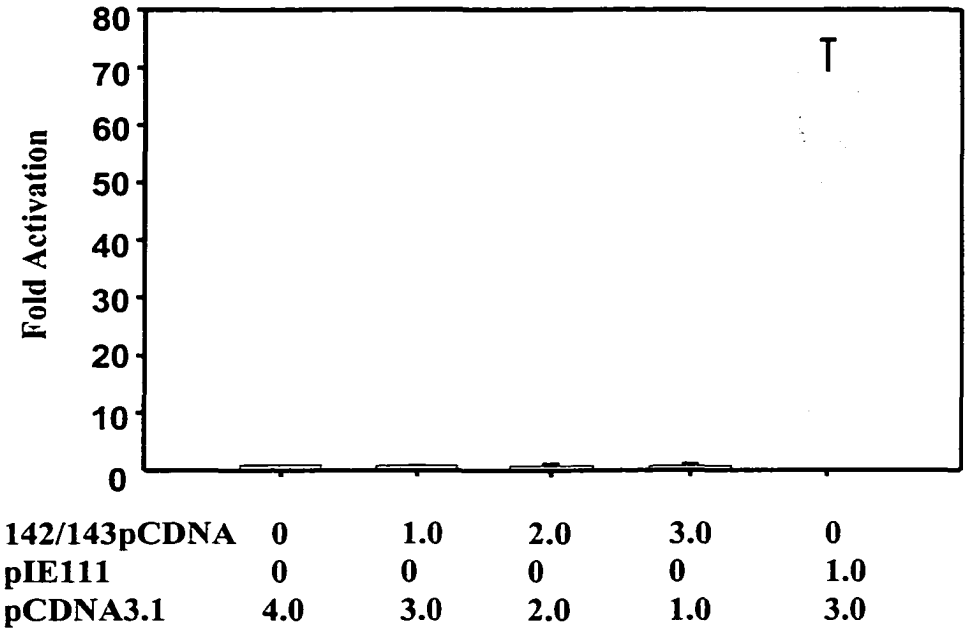

FIG. 26. Failure of the proteins pm142 and pm143 to activate the early e1 promoter. A total of 1.0 μg of the reporter plasmid e1CAT was transfected into NIH 3T3 fibroblasts with the empty vector pCDNA3.1 (lane 1), or with the plasmid 142/143pCDNA at 1.0 μg (lane 2), 2.0 μg (lane 3), or 3.0 μg (lane 4). As a positive control the plasmid pIE111 (IE1 and IE3) was used (lane 5). The empty vector pCDNA3.1 (Invitrogen) was used to maintain a constant amount of plasmid DNA per transfection. Cell lysates were harvested 48 hours post transfection and analyzed as described in the Materials and Methods. The transfections were done in duplicate and each experiment was repeated three times. Error bars represent standard deviations.

142/143pCDNA was increased to 2.0 μg and 3.0 μg, transactivation still did not take place. However, the IE1/IE3 proteins were potent transactivators of this promoter. Therefore, pm142 and/or pm143 do not transactivate the e1 promoter alone.

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pm142 and pm143 proteins cooperate with IE1 and IE3 to transactivate the e1 promoter (M112-113). Since IE1 and IE3 act synergistically to transactivate the e1 promoter (Messerle et al., 1992), we wanted to determine if pm142 and/or pm143 might enhance this phenomenon. In order to do this, we transfected NIH 3T3 cells with 1.0 μg of e1-CAT and 0.25 μg of the ie1/ie3 plasmid (pIE111), along with various amounts of 142/143pCDNA. We used the concentration of 0.25 μg of pIE111 since 1.0 μg of pIE111 transactivated this promoter to very high levels (80% acetylation). Although proteins expressed from 142/143pCDNA did not transactivate e1-CAT in the absence of other viral proteins, pm142 and/or pm143 cooperated with IE1/IE3 in transactivating the e1 promoter (Fig. 27). When e1-CAT was transfected along with 0.25 μg of the IE1/IE3 expression plasmid (pIE111), activation of about 30 fold above basal levels was achieved. When 1.0 μg of the plasmid 142/143pCDNA was added, transactivation increased to about 40 fold above basal levels. The fold activation continued to increase as more 142/143pCDNA was added. Therefore, this response was dose dependent. Activation as high as 80 fold above basal levels was achieved when 3 μg of 142/143pCDNA was added. Therefore pm142 and/or pm143 cooperate with IE1/IE3 in activating the e1 promoter. We were very excited about these findings since this is the first documentation that other MCMV viral proteins assist in IE1/IE3 mediated transactivation of the e1 promoter or any other promoter.

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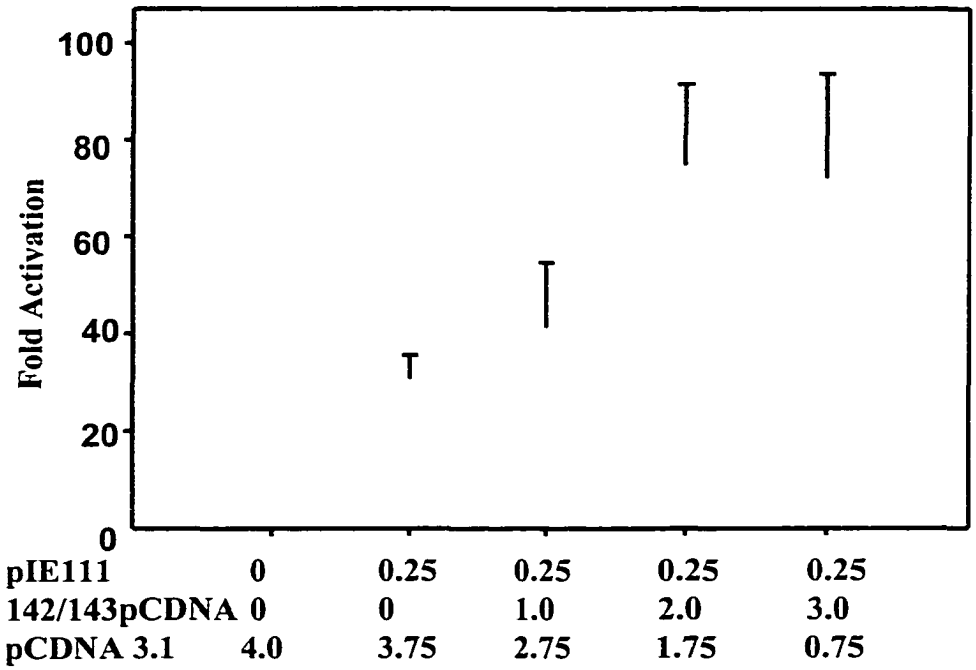

FIG. 27. Cooperation between the proteins pm142/pm143 and IE1/3 in activating the early e1 promoter. A total of 1.0 μg of the reporter plasmid e1CAT was transfected into NIH 3T3 fibroblasts with the empty vector pCDNA3.1 (lane 1) or with plasmids pIE111 (IE1 and IE3) and m142/143pCDNA. The amount of pIE111 DNA was kept constant at 0.25 μg per sample (lane 2-5) and 142/143pCDNA was titrated in at 1 μg (lane 3), 2 μg (lane 4) or 3 μg (lane 5). The empty vector pCDNA3.1 (Invitrogen) was used to maintain a constant amount of plasmid DNA per transfection. Experiments were conducted as described in the Methods. The transfections were done in duplicate and each experiment was repeated three times. Error bars represent standard deviations.

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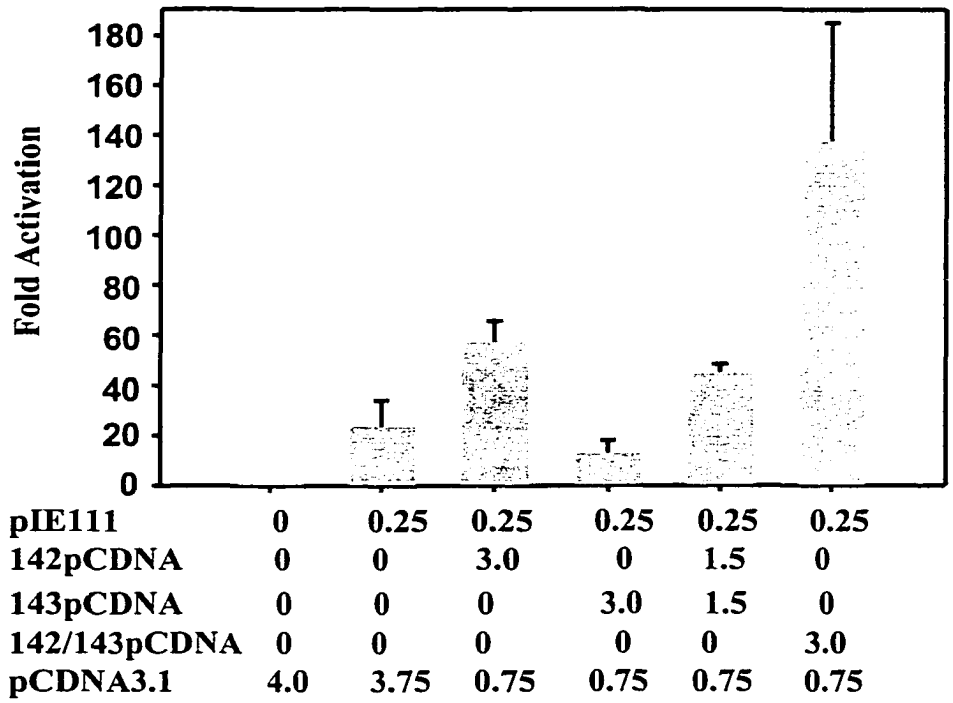

FIG. 28. Both pm142 and pm143 are required for maximal cooperation with IE1/IE3 proteins in the activation of the early e1 promoter. A total of 1.0 μg of the reporter plasmid e1CAT was transfected into NIH 3T3 fibroblasts with the empty vector pCDNA3.1 (lane 1) or with plasmids pIE111 (IE1 and IE3) (lanes 2-6), 142pCDNA (lane 3), 143pCDNA (lane 4), 142pCDNA and 143pCDNA (lane 5) or m142/143pCDNA (lane 6). The numbers denote the amount of DNA added in μg. The empty vector pCDNA3.1 (Invitrogen) was used to maintain a constant amount of plasmid DNA per transfection. Experiments were conducted as described in the Methods. The transfections were done in duplicate and each experiment was repeated three times. Error bars represent standard deviations.

Both pm142 and pm143 are required for the cooperative activity with IE1 and IE3 in the transactivation of the e1 promoter. We went on to determine if pm142, pm143 or both might cooperate with IE1 and IE3 in activating the e1 promoter. In order to do this we transfected the e1-CAT construct and pIE111 along with 142pCDNA, 143pCDNA, or both 142pCDNA and 143pCDNA as shown in figure 28. Unfortunately we do not have

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data from cells transfected with e1-CAT and 142pCDNA or 143pCDNA alone. Therefore, we can only speculate that proteins expressed from 142pCDNA and 143pCDNA fail to transactivate the e1 promoter, like those expressed from 142/143pCDNA. We found that in the presence of pm142 alone, the activity mediated by IE1 and IE3 doubles. The protein pm143 however, does not assist IE1/IE3 in the activation of the e1 promoter. When 142pCDNA and 143pCDNA were transfected together, transactivation by IE1/IE3 was increased but to levels that were not significant. However, pm142 and pm143 expressed from 142/143pCDNA was found to cooperate with IE1 and IE3, allowing transactivation of the MIEP to reach 140 fold above basal levels. Therefore the co-expression of the plasmids 142pCDNA and 143pCDNA did not restore cooperation with IE1 and IE3 to the levels seen with 142/143pCDNA. As mentioned previously, this can be explained by squelching since there is more pm142 and pm143 expressed from 142pCDNA and143pCDNA than from 142/143pCDNA under these conditions (Fig. 29). Also, since the m142 and m143 proteins are overexpressed from 142pCDNA and 143pCDNA, there could be improper localization of the m142 and m143 proteins. Also, an alternate protein may be made from the 142/143pCDNA vector, which may contribute to the activation of this promoter. From these data we can conclude that both pm142 and pm143 act cooperatively with the immediate early proteins IE1 and IE3, since the proteins expressed from 142/143pCDNA dramatically increased the activation seen with IE1 and IE3.

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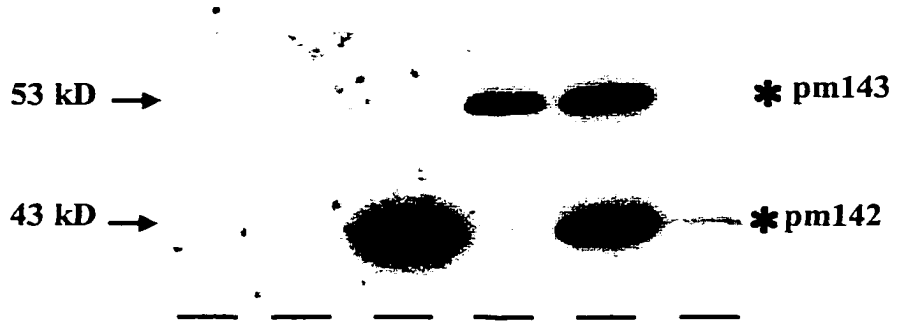

pIE111	**0**	**0.25**	**0.25**	**0.25**	**0.25**	**0.25**
142pCDNA	**0**	**0**	**3.0**	**0**	**1.5**	**0**
143pCDNA	**0**	**0**	**0**	**3.0**	**1.5**	**0**
142/143pCDNA	**0**	**0**	**0**	**0**	**0**	**3.0**
pCDNA3.1	**4.0**	**3.75**	**0.75**	**0.75**	**0.75**	**0.75**

FIG. 29. Elevated expression of pm142 and pm143 may have caused squelching to occur in the e1 promoter analysis. NIH 3T3 cells were transfected with the indicated plasmids. The numbers refer to μg of DNA. Cell lysates were harvested 48 hours post transfection and analyzed by Western blot analysis using a pm142 and pm143 antibody mixture. Asterisks denote low levels of protein expression.

Summary of transactivation results. Proteins pm142 and pm143 were able to transactivate the MCMV MIEPE in a dose dependent manner to at least 3 fold above basal levels, and both proteins were necessary for transactivation to occur. These proteins were also able to transactivate the HCMV MIEPE. However, pm142 and pm143 proteins exhibited noncooperative effects with IE1/IE3 in the activation of the MCMV MIEPE. Collectively pm142 and pm143 failed to activate the early e1 promoter. However when in combination with IE1 and IE3, cooperative activity by pm142 and pm143 was observed in the activation of the e1 promoter. Although pm142 alone assisted IE1 and IE3 to some degree, both pm142 and pm143 were required for optimal activity.

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CHAPTER V

DISCUSSION

Kinetics of m142 and m143 RNA Expression

MCMV genes m142 and m143 are required for replication in NIH 3T3 fibroblast cells. Therefore, these genes are essential and play a very important role in the biology of MCMV. The gene m142 includes MCMV sequences 199444-200748, and the gene m143 includes MCMV sequences 200923-202593. The m142 transcript is 1.8 kb in size and the 5' end of the transcript has been mapped to MCMV sequences 200847 or 200848. The m143 transcript is 3.8 kb in size and the 5' end of the transcript starts at MCMV sequences 202694 or 202686 (Fig. 5, 6). Both m142 and m143 are transcribed at immediate early times, and their steady state levels increase and remain abundant at early and late times. In addition to the m142 and m143 transcripts, several unexpected transcripts were expressed from the m142-m143 gene region at early and late times. These unexpected transcripts are 1.6 kb and 2.2 kb in size (Fig. 8). The 1.6 kb transcript may result from splicing of the 1.8 kb m142 transcript. However, nuclease mapping studies would have to be done to confirm this. The 1.6 kb band could also be an early transcript containing another potential ORF within m142, designated m142b. Alternatively, the 1.6 kb transcript may result from a shorter poly (A) tail of the 1.8 kb transcript. A similar type of regulation has been observed for the HCMV ie1 transcript (Stamminger, 1991). The 2.2 kb transcript most likely contains ORF m142c, however the mapping of the 5' end of the 2.2 kb transcript has not yet been done (Fig. 6). Therefore, more extensive mapping is needed in this region to confirm the origin of the 1.6 kb and 2.2 kb RNAs and to determine if splicing occurs in this region.

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The expression pattern of m142 and m143 is different from the expression of the other MCMV immediate early genes. The immediate early genes ie1 and ie3 are expressed at abundant levels at IE times. However, they are not expressed at early and late times of viral infection. The expression kinetics of m142 and m143 RNAs are quite similar to HCMV immediate early genes IRS1/TRS1, which are required for the activation of early genes involved in HCMV viral replication. IRS1 and TRS1 transcripts are present at immediate early, early and late times. Interestingly, m142 and m143 also have homology to IRS1/TRS1. This suggests that, unlike ie1 and ie3, m142 and m143 are similar to IRS1/TRS1 in that they likely have important functions at early and late times of viral infection. In our studies, we have shown that m142 and m143 do in fact have functional similarity to IRS1/TRS1, by cooperating with IE1/IE3 in the activation of an early gene.

Characterization of pm142 and pm143

The m142 and m143 protein products (designated pm142 and pm143, respectively) were characterized according to size, kinetics and location within an MCMV infected cell. According to the DNA sequence, the expected size for the m142 protein product was 48 kD, and the expected size for the m143 protein product was 64 kD. However these proteins proved to be smaller than expected. The m142 protein corresponds to a 43 kD protein product and the m143 protein corresponds to a 53 kD protein product (Fig. 10). These protein products may be smaller than expected due to proteolytic processing of the proteins. However, since there was no variation in the sizes of the m142 and m143 proteins as the infection progressed, this is unlikely (Fig. 12).

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Splicing of the m142 transcript could also be taking place since the m142 transcript shifts from 1.8 kb to 1.6 kb as the viral infection progresses, possibly resulting in a shorter ORF. There is also precedence for proteins with a high proportion of basic or charged amino acids to migrate faster or slower than their actual molecular weight on a polyacrylamide gel (Takano et al., 1988). Since both pm142 and pm143 have a high content of charged residues, they could be running faster on the polyacrylamide gel. Because the sizes of pm142 and pm143 are smaller than expected, it is likely that no major posttranslational processing like phosphorylation or glycosylation is taking place although m143 has two potential N-glycosylation sites.

The proteins pm142 and pm143 were expressed differently from the MCMV IE1 and IE3 proteins. The IE1 and IE3 proteins are present by 2 hours post infection, and then gradually decrease. These proteins (IE1 and IE3) are not detectable at early and late times. The pm142 and pm143 proteins, however, were detectable at 3 hours post infection and increased as the viral infection progressed (Fig. 12). Interestingly, the kinetics of pm142 and pm143 expression (like their RNA) was similar to IRS1/TRS1. IRS1/TRS1 are expressed at 2 hours post infection and remain throughout the entire HCMV replication cycle at relatively high levels (Romanowski and Shenk, 1997). However unlike IRS1/TRS1, pm142 and pm143 are not expressed to detectable levels under conditions in which only the immediate early proteins are expressed. Therefore, the m142 and m143 protein products cannot be designated as immediate early proteins. However, since Western blot analyses are not very sensitive assays, it is possible that pm142 and pm143 are present at IE times, but not to levels that are not detectable by this

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method. To address this, we could perform ^{35}S labeling of infected cells followed by immunoprecipitations using pm142 and pm143 antisera.

It was surprising to find that the m142 and m143 genes, which are immediate early genes, expressed their protein products at early times (Fig. 13). Since this occurs, there is likely control of m142 and m143 at the level of translation. This control can be mediated by sequences at the 5' untranslated region (UTR) or the 3' UTR of the m142 and m143 transcript. For example, the HCMV UL99 gene is regulated by leader sequences (Kerry et al., 1997), having secondary structure that interferes with the scanning of the ribosomal preinitiation complex (Gray and Wickens, 1998). Other CMV genes can be regulated at the level of translation by short ORFs. The CMV UL4 gene contains a 22 codon upstream ORF, whose protein product represses downstream translation by blocking translational termination at its own stop codon, thus causing ribosomes to stall on the mRNA (Alderete et al., 1999).

It is possible that m142 and m143 are regulated by secondary structure at the 5' UTR or short ORFs. The former is not likely because algorithmic analyses predict the 5' UTR of m142 and m143 to be moderately stable, and therefore secondary structure at the 5'UTR should not inhibit migration of the ribosomal subunit. The stem loop structure of the m142 5' UTR was calculated to have an optimal standard free energy value (ΔG) of about -32 Kcal/mole. The stem loop structure of the m143 5' UTR was calculated to have an optimal standard free energy value (ΔG) of about –30 Kcal/mol. Stem loop structures that inhibit translation usually have an optimal standard free energy value of

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-50 Kcal/mol or lower (Kozak, 1989). However, this does not rule out the involvement of other cellular or viral proteins that may bind to sequences within the 5' UTR and hinder the progression of the preinitiation complex.

It is possible that translation of m142 and m143 mRNA is regulated by a short open reading frames contained in the m142 5' UTR (Fig. 6). The protein product of this ORF could be involved in the repression of m142 or m143 protein expression. However, analysis of the 5' end revealed no ORF in this region. It is also possible that sequences at the 3' UTR of m142 and m143 can regulate translation by changing poly (A) tail length or by making contacts with the proteins involved in translational initiation (Gray and Wickens, 1998).

Since the m142 and m143 proteins were expressed during early times, they likely have important functions during this time in the viral replication cycle. Therefore, these genes could be involved in 1) viral pathogenesis or host immune evasion, 2) viral DNA replication or the regulation of genes involved in viral DNA replication or 3) the regulation of the major immediate early promoter and enhancer (MIEPE) by functioning similarly to the virion tegument proteins that have transactivating activity. We investigated the role pm142 and pm143 play in the regulation of the MIEPE and the regulation of an early gene involved in viral DNA replication. Our results show that pm142 and pm142 regulate the MIEPE and the promoter of at least one early gene involved in MCMV DNA replication.

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Intracellular Localization of pm142 and pm143

Most viral regulatory proteins are nuclear proteins that bind promoter/enhancer sequences or interact with cellular transcription factors. However, viral proteins located outside the nucleus can also regulate gene expression. For example, the UL37 protein is an integral membrane glycoprotein that traffics through the endoplasmic reticulum and Golgi apparatus. It is believed that UL37 protein product regulates viral gene expression via cell signaling (Zhang et al., 1996). However recent studies show that the acidic domain of UL37 plays a key role in transactivating viral promoters (Colberg-Poley et al., 1998). Some investigators speculate that the acidic domain of membrane bound UL37 may be released by proteolytic cleavage, translocate to the nucleus, and regulate gene expression in the nucleus (Colberg-Poley et al., 1998). Other viral regulatory proteins have been shown to shuttle between the nucleus and the cytoplasm, and perform different functions depending on their location. For example, the Tax protein of human T-cell leukemia virus type 1 shuttles between the nucleus and the cytoplasm (Burton et al., 2000). While in the nucleus, Tax activates the promoters of viral and cellular genes. While in the cytoplasm, Tax indirectly regulates NFkappaB. Tax does this by interacting with the cytoplasmic inhibitor of NFkappaB, thus allowing NFkappaB to translocate to the nucleus to regulate gene expression (Li and Gaynor, 1999). So although most proteins involved in gene regulation are nuclear proteins, we cannot assume that proteins that localize to the cytoplasm are not involved in the regulation of gene expression.

The cell fractionation studies have shown that both pm142 and pm143 localized to the nucleus and cytoplasm at 3, 4, and 24 hours post infection (Fig. 14A, 16A). In the immunofluorescence studies m142 and m143 localized predominantly to the cytoplasm at

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24 hours post infection, with low levels of pm142 and pm143 nuclear protein (Fig. 14A, 14B). The difference in the immunofluorescence and cell fractionation results can be explained by the inherent differences between these two assays. In the cell fractionation experiments, the cell lysates were analyzed by Western blot under denaturing conditions, disrupting any protein-protein interactions. In the immunofluorescence studies, the cells were fixed in paraformaldehyde. Although this fixative preserves the architecture of the cell, it may cross-link amino groups on adjacent molecules stabilizing protein-protein and protein-nucleic acid interactions. If these interactions are taking place in the cell, it is possible that the antigenic epitopes of pm142 and pm143 may be masked. It is also possible that pm142 and pm143 are not present in the nucleus at a density that is detectable in this assay, or nuclear shuttling may be involved.

In the cell fractionation assays, it also appeared that pm142 and pm142 were present in both the nucleus and the cytoplasm at relatively similar amounts (Fig. 15A, 16A). In the immunofluorescence assays however, most of the pm142 and pm143 appeared to localize to the cytoplasm (Fig. 14A, 14B). The nuclear and cytoplasmic fractions from the cell fractionation experiments were analyzed by Western blot. Since the Western blot method has a narrow quantitative range, we cannot assume that pm142 and pm143 localize to the cytoplasm and the nucleus at equal amounts in an infected cell. In order to determine the relative amount of pm142 or pm143 in the nucleus and the cytoplasm, the cell fractionation experiments can be done using lysates from radiolabeled MCMV infected cells, followed by immunoprecipitation of the nuclear and cytoplasmic fractions with pm142 or pm143 antisera. The immunoprecipitated samples then can be run on an acrylamide gel under denaturing conditions, the gel dried, and analyzed on a

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phosphorimager to quantitate the amount of pm142 or pm143 in the nuclear versus cytoplasmic fraction. These results would also indicate if pm142 and pm143 associate with other cellular or viral proteins while in the nucleus or the cytoplasm. It is also possible that pm142 and pm143 may associate with each other.

MCMV US22 family members located adjacent to m142 and m143 in the MCMV genome form protein-protein interactions (Karabekian, unpublished data). It is likely that pm142 and pm143 also associate, and therefore co-localize in a MCMV infected cell. The pm142 and pm143 proteins are expressed similarly, localize to the same cellular compartments, and are both needed to enhance expression of several MCMV genes. In order to determine if pm142 and pm143 associate with each other, immunoprecipitation experiments can be done on MCMV infected cells using pm142 or pm143 antisera. The immunoprecipitated samples then can be analyzed by Western blot using pm142 and pm143 antisera. Also, immunofluorecence studies can be done using confocal laser scanning microscopy to determine if these proteins co-localize. However, both pm142 and pm143 antisera were generated in rabbits. Therefore if in the colocalization studies we use our pm142 and pm143 antisera as the primary antibody, followed by anti-rabbit secondary antibodies, pm142 will not be distinguished from pm143. Antisera against pm142 or pm143 must be generated in another animal like the mouse. This will allow a goat anti-mouse secondary antibody conjugated to a fluorescent molecule like Texas red to be used to localize pm142, while a goat anti-rabbit secondary antibody conjugated to FITC can be used to localize pm143. Alternatively, either pm142 or pm143 antisera can be directly conjugated to a fluorescent molecule like Texas red. This will enable the

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pm142 and pm143 proteins to be distinguished by the confocal microscope since FITC and Texas red fluoresce at different wavelengths of light.

As stated previously, pm142 and pm143 were present in the nucleus and cytoplasm at early times (3 and 4 hours post infection). Both pm142 and pm143 were also present in the nucleus and cytoplasm at late times (24 hours post infection). Interestingly, HCMV IRS1 and TRS1 are present in the nucleus and the cytoplasm at immediate early and early times of viral infection, however they localize predominantly to the cytoplasm at late times of viral infection (Romanowski and Shenk, 1997). During a viral infection, HCMV IRS1 and TRS1 are packaged in the virion and transactivate the HCMV MIEPE following viral entry into the host cell. These proteins are also involved in the regulation of the early replication fork proteins. Since pm142 and pm143 have homology to IRS1/TRS1 and localize to the nucleus, we went on to determine the transactivating capability of these proteins by testing their ability to transactivate the MIEP and an early promoter involved in viral DNA replication.

Transactivation of the Major Immediate Early Promoter and Enhancer by pm142 and pm143

The pm142 and pm143 proteins transactivated the MCMV major immediate early enhancer and promoter (MIEPE) 3 fold above basal levels (Fig. 20). Since pm142 and pm143 are early proteins this could occur if 1) pm142 and pm143 are packaged in the virion and transactivate the MIEPE after virion attachment and penetration, or if 2) the pm142 and pm143 proteins transactivate the MIEPE at early times in order to keep IE1 and IE3 protein available for early gene expression. Since IE1 and IE3 protein are not

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expressed in the presence of phosphonoacetic acid (at early times), the former possibility is more likely.

HCMV IRS1 and TRS1 are packaged in the virion and they transactivate the HCMV MIEP following viral attachment and penetration. Interestingly m142 and m143 have homology to IRS1/TRS1, and are regulated similarly to IRS1/TRS1. The proteins pm142 and pm143 are also nuclear proteins. Therefore, pm142 and pm143 could act as virion regulatory proteins in a manner similar to IRS1/TRS1. According to our immunofluorescence analysis, pm142 and pm143 are also found predominantly in the cytoplasm at late times, like IRS1/TRS1. This might be important in the packaging of these proteins in the virion. To date, no MCMV virion components have been identified with transactivating function. Our laboratory is currently investigating the ability of pm142 and pm143 to be packaged in the virion.

In order to do this, we will be purifying virion particles from glycerol gradients. These purified virions will be subjected to Western blot analysis using pm142 and pm143 antisera. If pm142 and pm143 do localize to the virion, we will continue our studies by determining if pm142 and pm143 localize to the surface of the viral envelope or reside within the virion. In order to do this, protease digestion assays will be done using trypsin. Trypsin treatment of the virion will degrade proteins outside the viral envelope. Following trypsin treatment, Western blot analyses will be done using pm142 and pm143 antisera. If pm142 and pm143 are located within the virion particle, this will suggest that they may be tegument proteins. If pm142 and pm143 are tegument proteins, they likely transactivate the MCMV major immediate early enhancer and promoter (MIEPE) immediately following viral entry into the host cell.

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Alternatively, it is possible that there may be some low level expression of IE1 and IE3 protein at early and late times. In a time course experiment, IE1 and IE3 are expressed most abundantly at 2 hours post infection. However, these proteins remain throughout the course of the viral infection at low levels. The IE1 and IE3 proteins however are not detectable under conditions in which only early genes are expressed. This suggests that the IE1 and IE3 protein is relatively stable. Therefore, it is not likely that pm142 and pm143 transactivate the MIEPE at early times.

In order to determine if pm142 and pm143 transactivate the MIEPE, we varied the amount of 142/143pCDNA transfected with the MIEP-reporter construct (Fig. 20). When 1 μg of 142/143pCDNA effector plasmid was cotransfected with 1μg of target plasmid MIEP-CAT (E:T ratio of 1:1), no transactivation above basal levels was observed. Transactivation was observed (3-4 fold above basal levels) when 2 μg or 3 μg of 142/143pCDNA effector plasmid was cotransfected with 1 μg of target plasmid MIEP-CAT (E:T ratio of 2:1 or 3:1). The effector to target ratio of 1:1 is what likely occurs during a natural infection, not 2:1 or 3:1 ratio, since there is one copy of the ie1/ie3 gene locus per m142/m143 gene locus in an infected cell. Therefore, it is possible that these findings were not biologically relevant and do not occur during a normal infection. On the other hand, if pm142 and pm143 are packaged in the virion, it is likely that a target ratio of 1:1 would not be taking place during a natural infection. Depending on how much pm142/pm143 is packaged in the tegument, ratios of at least 2:1 or 3:1 could be reached, thus making these findings more indicative of what takes place during a natural infection.

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We also found that pm142 and pm143 have additive effects with IE1 and IE3 in the activation of the MIEP (Fig. 25). These results were not surprising because the other immediate early proteins (IE1 and IE3) have independent effects on the activation of the MIEP and when these proteins are introduced collectively, their effect on the MIEP is additive (Fig. 3). The potential mechanism by which these proteins could transactivate the MIEP will be discussed later.

Failure of the 142pCDNA and 143pCDNA expression vectors to reproduce the results found with 142/143pCDNA. Proteins expressed from 142/143pCDNA (which expresses pm142 and pm143) were able to transactivate the MIEP. However when the m142 expression vector (142pCDNA) and the m143 expression vector (143pCDNA) were cotransfected, they failed to reproduce the results found with 142/143pCDNA (Fig. 21). We believe that the phenomenon of squelching may account for the discrepancies in our transfection experiments. There is precedence that squelching occurs when transcriptional transactivators are overexpressed in transfection assays. For example, when the acidic domain of the HSV-1 regulatory protein VP16 (virion protein 16) was overexpressed in transfection assays, the transactivating ability of this protein was inhibited (Pfitzner et al., 1993). More specifically, the overexpressed VP16 protein interacted with and sequestered a basal transcription factor that was required for transcription. Other overexpressed transcriptional transactivators interact with and squelch common transcriptional transactivators like SP1 (Pfitzner et al., 1993).

High levels of pm142 and pm143 protein were expressed from the plasmids 142pCDNA and 143pCDNA, respectively when compared to proteins expressed from

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142/143pCDNA (Fig. 22). This was because the expression from 142pCDNA and 143pCDNA was driven by the strong HCMV enhancer located directly upstream of the m142 and m143 genes. A low level of expression of pm142 and pm143 from the 142/143pCDNA expression vector, was likely driven by the m142 and m143 native promoter, which is relatively weak. It is likely that the high levels of pm142 and pm143 expressed from 142pCDNA and 143pCDNA, respectively cause transcriptional interference by sequestering a transcription factor, coactivator or protein within the basal transcription factor complex which is present in limiting quantities. In the absence of this component, expression from the reporter plasmid is inhibited. We can determine if squelching is taking place with these constructs by transfecting cells with the 142/143pCDNA and MIEP-CAT. If the levels of CAT activity decrease when 142pCDNA or 143pCDNA is titrated in, this would suggest that squelching is taking place.

It is also possible that because of the high level of protein expression from 142pCDNA and 143pCDNA, pm142 and pm143 do not localize to the correct cellular compartment. If pm142 and pm143 do not localize properly, they will not be able to function properly. It is also possible that an alternate transcript may be expressed from 142/143pCDNA that is absent in 142pCDNA and 143pCDNA, which may be important in transactivation. In addition to the m142 and m143 transcripts, an additional transcript is expressed from this region (designated m142c). Sequences from m142c are contained within 142/143pCDNA. If a proteins expressed from m142c are involved in transactivation, 142pCDNA and 143pCDNA combined would not be able to reproduce the results found with 142/143pCDNA. Lastly, a negative feedback loop mechanism

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may be involved, such that if the level of pm142 and pm143 surpasses a certain threshold, the activity of these proteins will be shut off.

In the future, expression vectors containing m142 or m143 under the control of its native promoter will be made. These constructs will be tested in transient assays in a similar fashion as 142pCDNA and 143pCDNA. The constructs containing m142 or m143 under the control of its native promoter will allow the function of pm142 or pm143 to be determined while maintaining protein levels that are biologically relevant.

Transactivation of the e1 promoter by pm142 and pm143

We determined if pm142 and pm143 were able to transactivate an early gene that is involved in viral DNA replication. We tested the ability of pm142 and pm143 to transactivate the e1 promoter (M112-113). The e1 gene is homologous to the HCMV UL112-113, which is required for HCMV ori-lyt dependent DNA replication. We found that the pm142 and pm143 proteins were not able to transactivate the e1 promoter alone (Fig. 26). However in the presence of IE1 and IE3, cooperative activity was observed (Fig. 27). In the presence of 1 μg of 142/143pCDNA, transactivation by IE1/IE3 increased from 30 fold to 50 fold above basal levels. When the amount of 142/143pCDNA was increased to 3 μg, transactivation by IE1/IE3 increased to at least 100 fold above basal levels. This was the first demonstration that other MCMV viral proteins assist in IE1/IE3 mediated transactivation of the e1 promoter (M112-112) or any other promoter.

HCMV IRS1/TRS1 acts synergistically with HCMV IE1 and IE2 to transactivate the UL112-113 promoter. Once more in these studies, pm142 and pm143 are similar to

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IRS1/TRS1. The m142 and m143 gene products and IE1/IE3, act cooperatively to activate the e1 promoter (M112-113), which is involved in viral DNA replication. The pm142 and pm143 proteins may help to activate the expression of the other replication fork proteins as well. Like in HCMV, MCMV regulatory proteins are probably more efficient at activating viral replication fork proteins when present collectively.

As stated previously, transactivation by IE1/IE3 was increased in the presence of proteins expressed from 142/143pCDNA (Fig. 27). However, when the IE1 and IE3 expression vector was cotransfected with 142pCDNA and 143pCDNA, cooperative activity did not occur (Fig. 28). This is likely due to squelching, since very high levels of pm142 and pm143 were expressed from 142pCDNA and 143pCDNA under these conditions (Fig. 29). It is also possible that improper protein localization may occur due to the high protein levels expressed from 142pCDNA and 143pCDNA, or a negative feedback control mechanism is being used. In the future, these experiments will be repeated using constructs expressing the m142 or m143 protein under the control of its native promoter. The m142 and m143 native promoters are not as strong as the HCMV MIEP, and therefore the squelching phenomenon will probably be prevented.

The cooperative results with IE1/IE3 and m142/m143 can be interpreted in different ways, since the effector plasmid (pIE111 which expresses IE1 and IE3) used in the experiment is under the control of the MIEP, and the IE3 protein represses expression from this promoter (Messerle, 1992). The pm142 and pm143 proteins have also been shown to regulate this promoter. Furthermore, the m142 and m143 expression vectors contain the HCMV MIEP that may be regulated by MCMV IE1/IE3 proteins and pm142 and pm143 as well. Therefore, it is difficult to know for sure if the cooperation occurs

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because pm142 and pm143 are increasing the expression of IE1 and IE3 by activating the MIEPE, or if the effect of the pm142 and pm143 proteins is more directly related to the e1 promoter. In the future, these experiments will be repeated with the effector plasmids cloned under the control of a heterologous promoter like the SV40 promoter.

Potential Mechanism of pm142 and pm143 Gene Regulation

There are several potential mechanisms by which pm142 and pm143 could regulate the MIEPE (M122-123) and the e1 (M112-1123) promoters. These proteins could 1) activate the expression of a cellular transcription factor, 2) bind directly to viral promoter or enhancer regions 3) interact with transcription factors or components of the basal transcription factor complex to facilitate transcription, or 4) bind to an inhibitor of a transcription factor.

The pm142 and pm143 proteins could activate the expression of a cellular factor that is important in the transactivation of viral promoters. The MCMV MIEPE and the M112/113 promoter have very complex promoter/enhancer regions containing several cellular transcription factor binding sites. In HCMV, IE1 and IE2 proteins can regulate the expression of cellular genes involved in cell growth or cell cycle control. HCMV IE1 and IE2 can transactivate the c-fos, c-myc and hsp70 promoters, thus allowing the conditions within the cell to become more favorable for a permissive viral infection (Hagemeier et al., 1999). A similar scenario could be taking place with the pm142 and pm143. The pm142 and pm143 proteins transactivate the promoter of a cellular factor like c-fos, which then binds to viral promoter/enhancer regions to activate viral gene

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expression. The pm142 and pm143 could also activate cellular factors by other mechanisms such as effects on phosphorylation state of these proteins.

Proteins pm142 and pm143 could directly bind DNA promoter/enhancer regions to regulate cellular and viral genes. The HCMV immediate early protein IE2, directly binds to DNA to autoregulate its own expression. HCMV IE2 binds to a region in the promoter referred to as the crs (cis repression sequence) (Lang and Stamminger, 1993) and thereby interferes with the formation of the preinitiation complex (Wu et al., 1993). HCMV IE2 also binds to elements within the promoter of the HCMV early UL112-113 gene. It is possible that pm142 and pm143 could bind to cellular and viral promoters directly, to activate viral gene expression.

It is also possible that pm142 and pm143 could interact with components of the basal transcription factor complex or other transcription factors to facilitate the transcription of the cellular and viral genes. The HCMV IE2 protein has been shown to interact with several cellular transcription factors. The HCMV IE2 protein can bind to cellular transcription factors E2F-1, SP-1, TEF-1 and CTF-1 (Furnari et al, 1993; Lukac et al., 1994). HCMV IE2 can also interact with the TATA binding protein, which is a component of the basal transcription factor complex TFIID (Jupp et al., 1993). Therefore pm142 and pm143 could regulate cellular and viral promoters by protein/protein interactions with a transcription factor or component of the basal transcription factor complex.

I believe that pm142 and pm143 regulate viral promoters by protein/protein interactions with a transcription factor or component of the basal transcription factor complex. The interactions between these proteins could enhance the function of TFIID,

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by making TFIID more efficient or promoting the establishment of the preinitiation complex. Since squelching likely occurs when pm142 and pm143 are over expressed, this suggests that these proteins are able to complex with other factors that are important in the transactivation of the MIEPE and the e1 promoter. Also, pm142 and pm143 were not able to transactivate the e1 promoter alone, but were able to cooperate with IE1 and IE3. This suggests that protein-protein interactions are taking place.

To elucidate between the possible mechanisms in which pm142 and pm143 may regulate gene expression, several experiments can be done. In order to determine if pm142/pm143 binds to a certain viral or cellular promoter, Dnase1 footprinting can be done. In this case, an end labeled probe containing a viral or cellular promoter sequence is incubated with pm142/pm143 (generated by vitro transcription/translation) to allow the formation of protein-DNA complexes. The protein-DNA complex is then exposed to Dnase1 and electrophoresed. If pm142/pm143 binds to the DNA, the DNA will be protected in this region. The exact sequence of protein binding can also be determined with this method.

Electrophoretic mobility shift assays (EMSA) can also be used to determine if pm142 and pm143 bind to DNA directly. In this case, promoter sequences are labeled and used as probe. This probe is then incubated with in vitro translated pm142 and pm143. If the pm142 and/or pm143 protein interacts with the labeled probe, there will be a retardation in the migration of the labeled probe upon electrophoresis compared to control samples. If there is evidence that pm142 and pm143 bind to the promoter sequence, more elaborate EMSA experiments can be done using 1) pm142 or pm143 antibody to confirm the presence of pm142 or pm143 in the protein-DNA complex by

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supershift 2) specific or nonspecific DNA competitors to determine the binding affinities of the proteins or 3) mutated probes to determine to what sequences pm142 and pm143 bind.

In order to determine if pm142 or pm143 interact with other viral or cellular transcriptional transactivators, MCMV infected cell lysates can be immunoprecipitated with pm142 or pm143 antibody, and co-precipitating proteins analyzed by Western blot using an antibody against the potential interacting protein. If there is evidence of an interaction, more extensive studies can be done using glutathione -S-transferase (GST) fusion proteins. The m142 and m143 genes can be cloned in frame to GST to generate GST-m142 and GST-m143. GST-m142 or GST-m143 will then be immobilized on glutathione-agarose beads, and incubated with the labeled in vitro translated potentially interacting protein. The complexes will be run on an acrylamide gel, the gel dried, and exposed to xray film. If a band is detected, the labeled protein is likely complexing with pm142 and pm143.

Conclusions

We have characterized the expression of the m142 and m143 genes and their products, which are essential for MCMV viral DNA replication. These findings were significant because m142 and m143 are novel immediate early genes and prior to these studies only three MCMV IE genes had been characterized. It was shown the pm142 and pm143 act cooperative with IE1 and IE3 to transactivate an early promoter. This was the first demonstration of a MCMV protein assisting IE1/IE3 mediated transaction. However, since m142 and m143 are essential for viral replication in fibroblasts, they

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likely have other functions that deem them necessary for viral replication. Therefore, more study needs to be done on the m142-m143 genes to elucidate other roles these proteins may play in viral infection.

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CHAPTER VI

FUTURE DIRECTIONS

Since pm142 and pm143 are able to transactivate the same genes as MCMV IE1 and IE3, pm142/pm143 probably have an additional function that deems them essential for viral replication. In order to determine what this function is, fibroblast cell lines stably transfected with m142 and m143 genes will be made. Next mutant viruses, deleted of m142 and m143 will be generated using these stably transfected cell lines to complement mutant virus growth. After the m142-m143 mutant viruses have been generated, they will be grown on normal fibroblast cells under condition in which IE, E or L genes are expressed. These cells will then be harvested and total RNA will be isolated. The RNA will be converted to cDNA. The cDNA will be labeled and used to probe microarrays containing MCMV genes. The results of the microarray analysis will determine where in the viral replication cycle m142 and m143 are necessary; IE, E or L times. These studies will give further insight as to the function of these genes. Similar experiments will be done after growing the m142-m143 mutant viruses on different cell lines, like macrophages. This will allow us to determine if these genes are important in a specific cell type that is involved in the pathogenesis of the virus.

In addition, promoter deletion analyses and gel shift analysis will be done to determine to which promoter sequences pm142 and pm143 may bind. In parallel, single-site and deletion mutations will be made within the m142 and m143 gene to determine which motifs are important in promoter activation. These mutants can also be tested with respect to DNA binding and protein-protein interactions.

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