REGULATION OF EXPRESSION FROM THE M142 PROMOTER OF MOUSE CYTOMEGALOVIRUS

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DEDICATION

For my father, Pramod Pandhe, mother Nutan Pandhe and my sisters, Supriya and Vrushali, thank you for your never-ending support, patience, and love.

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ABSTRACT

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Human cytomegalovirus (HCMV) is a common cause of infection with serious illness in immune-compromised people. HCMV can only infect humans, limiting the studies done with this virus. Animal viruses, such as mouse cytomegalovirus (MCMV) are useful models for HCMV. Despite extensive use as a model, very little is known about gene regulation in MCMV compared to HCMV. Hence, the goal of this research was to expand the knowledge of promoter regulation in MCMV. We examined regulation of an essential MCMV gene, m142. The m142 gene's closest homologs are HCMV IRS1 and TRS1 which have similar expression and function. Previous analysis showed that m142 is an immediate early gene, meaning cellular factors are sufficient for activation, although viral infection increased expression. However, neither the sequences required nor the regulatory factors were identified. Using a series of sequential deletion mutants of the m142 promoter controlling a secreted alkaline phosphatase (SEAP) reporter, we have identified important regulatory regions and some probable regulators. We found that a sequence between -901 and -875 nucleotides upstream of the transcription start sites was required for activation in the absence of viral infection. Further analysis by

EMSA indicated involvement of a consensus Elk-1 site and the binding of Elk-1 protein in this region. Two important viral transcriptional regulators are IE1 and IE3. Co-transfection with plasmids expressing these proteins showed that IE1 and IE3 separately activated via distinct regions of the promoter, but also co-operated. Comparison of the co-transfections with infection indicated that additional viral factors likely regulate the m142 promoter via sequences between -713 to -579. We were able to narrow down the potential viral genes involved by inhibition of a subset of viral proteins, known as late genes, which were not required for this regulation. Finally, deletion of sequences between -875 and -713 resulted in an increase in promoter activity. As this was detected not only in the context of viral infection, but also when either IE1 or IE3 were co-transfected, a cellular regulator is likely involved in this repression. In summary, we have found that like immediate early promoters of HCMV, m142 is a long promoter with complex regulatory mechanisms.

ABBREVIATIONS

AC - Assembly complex

APs - Assembly proteins

ATCC - American Type Culture Collection

ATF - Activating transcription factor

ATRX - ATP linked helicase

BLAST - basic local alignment tool

CBF-1 - Core binding factor 1

CE - Cytoplasmic extracts

ChiP - Chromatin Immuno-precipitation

CMV – Cytomegalovirus

CREB - c-AMP response element binding protein

crs - cis-repression sequence

DMEM - Dulbecco's Modified Eagle Medium

E – Early

EBV - Epstein Barr virus

eIF-2α - Eukaryotic initiation factor 2 alpha

EMSA – Electrophoretic mobility shift assay

ER - Endoplasmic reticulum

F – Forward

HAT - Histone acetyletransferase

HCMV - Human cytomegalovirus

HMTs - Histone methyl transfereases

Hpi – Hours post infection

IE - Immediate early

IFI16 - Inducible protein 16

IRL - Internal repeat long

IRS - Internal repeat short

IRS1 - Internal repeat short 1

KSHV - Kaposi’s sarcoma-assocated herpesvirus

L – Late

mC-BP - Minor capsid binding protein

MCMV - Mouse cytomegalovirus

MCP - Major capsid protein

mCP - Minor capsid protein

MHC - Major histocompatibilty complex

MIEP - Major immediate early promoter

MOI - Multiplicity of infection

NB - Nuclear bodies

nBLAST - Nucleotide Basic Local Alignment Tool

ND10 - Nuclear domain 10

NE - Nuclear extract

NEP - Non-infectious enveloped particles

NF-κB - Nuclear factor-kappa B

ORFs - Open reading frames

OriLyt - Origin of lytic replication

PFA - Phosphonoformic Acid

PFU - Plaque forming units

PKR - Protein kinase R

PML - Promyelocytic leukemia protein

PODs - PML oncogenic domains

PORT - Portal protein

qPCR - Quantitative PCR

R – Reverse

SCID - Severe combined immunodeficiency disease

SCP - Smallest capsid protein

SDS-PAGE – Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

SEAP - Secreted Alkaline Phosphatase

Sp - Specificity protein

SRE - Serum Response Element

SSB - Single stranded DNA binding protein

TAF - TBP associated factor

TBP - TATA binding protein

TCF - Ternary complex factors

TESS - Transcription element search system

TRL - Terminal repeat long

TRS - Terminal repeat short

TRS1 - Terminal repeat short 1

U_L - Unique long

U_S - Unique short

VZV - Varicella zoster virus

YY1 - Yin Yang 1

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CHAPTER I

INTRODUCTION

CYTOMEGALOVIRUS CLASSIFICATION

Cytomegalovirus (CMV) belongs to the order *Herpesvirales* and family *Herpesviridae* (1). The family *Herpesviridae* contains viruses infecting higher vertebrates (mammals, reptiles, and birds). *Herpesviridae* have similar virion structures, linear dsDNA, and the capacity to undergo latency and reactivation. Infected individuals can be symptomatic or asymptomatic during primary infection or during reactivation depending on the immune system of the host (2-4). This family is divided into three sub-families: *alphaherpesvrinae* (α), *betaherpesvrinae* (β), and *gammaherpesvrinae* (γ) (5). Classification into these sub-families is based on viral host range, replication rate and replication kinetics, genetic similarity, and sites of latency (1). The *alphaherpesvirinae* are the least host species specific of the *Herpesviridae*; hence non-human hosts can be used to study human alphaherpesviruses (6, 7). Alpha herpesviruses undergo a quick replication cycle and they can establish latency in neuronal cells (3, 8, 9). The sub-family *gammaherpesvrinae* includes viruses which replicate in lymphoblastoid cells, can establish latency in lymphocytes, and also have very limited host range (10, 11). Members of the sub-family *betaherpesvirinae* undergo a slow replication cycle compared

to alpha herpesviruses and they are very species specific (12). Cytomegaloviruses, the topic of this study, belong to the *betaherpesvirinae*.

CMV can undergo lytic or latent infection. During lytic infection, infectious viral particles are produced. During latent infection viral DNA is maintained episomally with little transcription, and infectious viral particles are not produced (13). The sites where CMV establishes latency are not related to the sites of primary infection (14). Active viral infection is categorized into three types: 1) Primary infection when a host is infected for the first time; 2) endogenous infection when virus reactivates from latency; and 3) exogenous infection when a new strain infects a previously infected individual (13, 15, 16).

There are cytomegaloviruses specific to various species. Human cytomegalovirus (HCMV) was first isolated and cultured in 1956 (17, 18). Mouse cytomegalovirus (MCMV), was first isolated by Margaret Smith in 1954 from mouse salivary gland tissue (19).

MCMV AS A MODEL FOR HCMV

Because of the species specificity of HCMV, HCMV research is limited to tissue culture study and severe combined immunodeficiency disease (SCID) mice engrafted with human tissue (20). SCID mice with human tissue grafts do not represent the natural course of viral pathogenesis and disease progression (21). HCMV and MCMV genomes show similar sequences. The overall DNA sequence identity is 42.5% but the amino acid homology in the central more conserved region of some 78 genes with FastA scores over

100 (22). The less conserved regions are also less conserved within a virus, within HCMV strains the sequence conservation in the less conserved genes can be 49% (23). The genomes are also largely co-linear with many genes with similar locations exhibiting functional homologies (6, 7). Both HCMV and MCMV can cause similar diseases (such as hepatitis, colitis, retinitis, encephalitis) either by primary infection or reactivation from latency (24). Thus, MCMV is a good model to study many aspects of cytomegalovirus infection and develop new therapies (25, 26).

ROUTES OF INFECTION

The spread of CMV occurs via different bodily fluids such as saliva, blood, urine, semen, cervicovaginal secretions, amniotic fluid, and breast milk (27). Some of the major factors contributing towards CMV infection are poor hygienic conditions in lower socio-economic groups, crowded living conditions, sexual activity, and close contact with children less than 2 years old (28-30). The rate of seroconversion in a normal population is 2.2% whereas the rate for day care workers is around 11% (31, 32). Many genital infections result in inflammation providing optimum conditions for entry of other infectious agents, including CMV, during intercourse (33). Youth, pregnancy, and multiple sexual partners cause increased cervical shedding of HCMV compared to urinary shedding in infected individuals (34, 35).

PATHOLOGIES

Immuno-competent Individuals

For the immune-competent individuals, the chances of developing CMV-related pathologies upon infection are low (36, 37). Immuno-competent individuals occasionally show symptoms of mononucleosis (36, 37). Mononucleosis symptoms include fever, extreme fatigue, mild inflammation of pharynx, cough, nausea, diarrhea, mild liver enzyme elevation, lymphocytosis, and headache (36, 37). There is also clinical correlation between cytomegalovirus and atherosclerosis and some cases of glioblastoma (38-40).

In Immuno-compromised Individuals

Organ Transplant Recipients and AIDS Patients

Individuals with compromised immune systems, such as organ transplant recipients receiving immune-suppression treatment, and AIDS patients are at a high risk for CMV related diseases (such as retinitis, colitis, hepatitis) and the diseases are generally similar (41, 42). Because CMV can infect many different cell-types in the host, complications caused by HCMV in immune-compromised patients are varied and include: leukopenia (decreased number of white blood cells), pneumonitis, gastrointestinal problems, graft atherosclerosis (development of plaques inside grafted arteries), encephalitis, hepatitis, and retinitis (39, 43-47). CMV negative individuals receiving transplanted tissue from a CMV positive donor are at high risk for CMV

disease (48, 49). A CMV infected individual is also at risk either if the donor tissue is infected with a different strain of CMV or because the immune suppressive therapy leads to lack of control of reactivated virus (41, 42). Findings from several clinical studies indicate about 70% of renal transplant and 90% of lung transplant patients are infected with multiple CMV strains (50, 51).

Neonates

The neonate can get infected either due to a primary infection or reactivation of latent infection of the mother. In 30-40% cases of 1° maternal infection, fetal infections will develop (31, 52). The chances of reactivation of latent HCMV in pregnant women are 10-30%, but only in 0.15 to 2% of cases does the fetus get infected (31, 52). Intrauterine infection can be spread through blood from mother to fetus. Another route is via infection of the basal plate of the placenta that results in spreading of virus to the fetus (53, 54). As viral genital shedding is tremendously increased during pregnancy, the baby may also be infected as he/she passes through the birth canal (34, 35). Post pregnancy, virus tents to activate in many tissues, such as mammary glands, and virus spread can also occur through breast feeding (55, 56). The transfer of virus via such routes can cause disease in premature infant, however the risk of disease is low (54).

The signs of neonatal infection may include seizures, chorioretinitis (inflammation of the choroid- a thin vascular coat of the eye with retinitis), other ocular abnormalities, hypotonia (poor muscle tone), poor suckling, elevated cerebrospinal fluid protein, hypoxia, psycho-motor retardation, and/or hearing loss (57-60). Newborns

infected *in utero* or during delivery can have complications later even if they are not symptomatic at the time of birth (61). It has been proposed that routine HCMV screenings for newborns should be introduced so that appropriate therapy can be used as early as possible (61). Not all complications are caused by direct infection of fetal cells or organs. HCMV infection of placental tissue can cause changes in the placenta leading to insufficient oxygen and nutrient supply to the fetus (62, 63).

HCMV DIAGNOSIS

Some of the commonly used methods for CMV detection in adults are quantitative nucleic acid testing, CMV culture, histopathology, detection of IgM antibodies against CMV, antigenemia assay where CMV antigens are detected, and screening of CMV specific proliferation of T-cells (64-67). In hematopoietic stem cell transplant patients, CMV viral load is tested in whole blood and plasma (68). In pregnant women, Amniotic fluid cultures used to detect the presence of HCMV DNA via PCR are sensitive and specific, but they cannot predict the damage to the fetus (69). Thus, amniotic fluid testing is usually used to confirm HCMV infection after non-invasive procedures, such as ultrasound or MRI, detect possible CMV related abnormalities because these are non-invasive, cost effective tests with high success rates (32, 70). Urine, blood, dried blood spot, saliva, or cerebrospinal fluid of a newborn can be collected during the first 3 weeks after birth and CMV PCR is performed (71-73).

CURRENT TREATMENT METHODS FOR HCMV

Current available drugs for the treatment of HCMV are ganciclovir and valganciclovir, with cidofovir, foscarnet, and fomivirsen having more limited use (74). Ganciclovir is an analog of the nucleoside guanosine but needs to be administered intravenously if high efficiency is desired (75, 76). Valganciclovir is converted to ganciclovir but can be given orally (77). Ganciclovir gets phosphorylated to ganciclovir monophosphate by a viral phosphokinase, the product of the UL97 gene in HCMV (78). Phosphorylation to the triphosphate form is by cellular kinases. The viral DNA polymerase is 10 times more likely to incorporate this guanosine analog than the cellular enzyme (78). Incorporation of ganciclovir results in termination of the elongation in DNA replication (79). Cidofovir is a nucleotide analog of cytidine and does not require activation by a viral kinase (80). Because cidofovir is not activated by the viral kinase, it can be used to treat infections with strains resistant to ganciclovir due to kinase mutations, but has higher toxicity (81). As these drugs can incorporate into cellular DNA; they have potential teratogenic effects, hence are not recommended for pregnant women (82, 83). Other side effects of these drugs include neutropenia (low count of neutrophils), kidney and bone marrow toxicity, as well as selection of drug resistant virus mutants (Some of the mutations detected in gancyclovir resistant viral strains: A594V, L595S, N510S, A590T, A591V, A591D, C592G, L595W, L595T, E596G, E596D, N597I, G598V, K599M, C603Y, A606D, and V665I) (82-85). Foscarnet mimics pyrophosphate and inhibits pyrophosphate binding to the viral DNA polymerase. The

observed toxicity by foscarnet is more than by other replication inhibiting drugs; hence foscarnet is mostly used for the treatment of viral strains resistant to other drugs (86, 87). Fomivirsen has a very different mode of action. It is a modified oligonucleotide which is complementary to essential CMV mRNA transcripts and inhibits via an antisense mechanism. Due to stability issues, it is used exclusively for local treatment of CMV retinitis by injection into the eye (88, 89).

VIRAL STRUCTURE

Herpesviruses contain linear dsDNA genomes ranging from 124-230 kb, wrapped around core proteins (4). From inside out, the herpesvirus particle is composed of core proteins, DNA, viral capsid, tegument proteins, and viral envelope. Core proteins are responsible for the compaction of DNA (3, 4). The general structure of a herpesvirus is shown in Figure 1 (2-4).

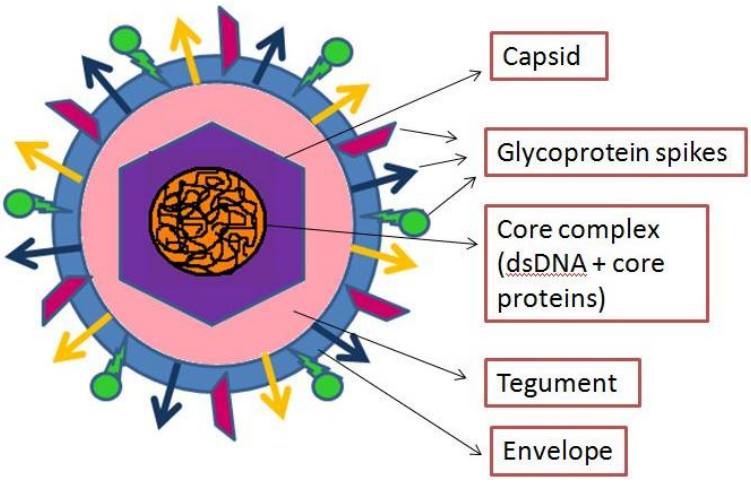


Figure 1. General structure of herpesviruses. The dsDNA-core protein complex is surrounded by an icosahedral viral capsid surrounded by protein tegument and an envelope lipid bilayer containing virally encoded glycoproteins (2-4).

Viral Capsid

All herpesviruses have similar capsid structures and the diameter of the icosahedral capsid is around 130 nm (6, 7). The CMV capsid is composed of four major components: major capsid protein (MCP), minor capsid protein (mCP), minor capsid binding protein (mC-BP), and smallest capsid protein (SCP) (90-92). These are assembled into 12 pentons forming vertices, 150 hexons forming faces and edges, and 320 triplexes forming structures for interconnection of pentons and hexons shown in Figure 2 (6, 93). The pentons are composed of 5 copies of MCP, while each hexon is composed of 6 copies of MCP and 6 copies of SCP. The triplex structures are composed of 2 copies of mCP and a mC-BP (94). One specialized penton consists of 12 molecules of portal protein (PORT). These PORT proteins form a channel for the entry and exit of viral DNA (95). The capsid is assembled with the aid of a set of viral scaffolding assembly proteins (96).

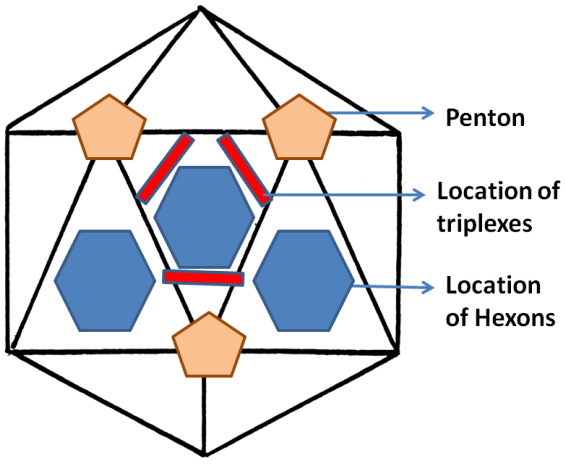


Figure 2. Schematic representation of the location of capsid components. The organization of pentons, hexons, and triplexes is shown.

During viral infection, three types of capsids are observed in the nucleus: "A" capsids which appear empty; "B" capsids which are believed to have scaffolding proteins; and "C" capsids which contain viral DNA (97, 98). The C capsid undergoes primary envelopment at the inner nuclear membrane and enters the cytoplasm (99). The process of maturation is debated, whether A and B capsids are both steps in the process or A capsids are a dead-end. Scanning electron microscope images are not able to explain the sequence of steps during DNA packaging (100).

HCMV Tegument Proteins

A distinctive characteristic of herpesviruses is the presence of the tegument composed of multiple sub-layers of proteins. The tegument consists of specific viral proteins synthesized during infection and enters the host cell along with the viral capsid (101). So far 14 tegument proteins of HCMV have been studied extensively to understand their functions (102). During initial stages of infection, tegument proteins are responsible for changing host cell metabolism, regulating expression of some viral genes, and disrupting host cell defenses (101, 103-105). During later infection stages; tegument proteins act as chaperones, transport newly synthesized viral proteins into the nucleus, control DNA packaging, and help maturation and egress of virus (105-107). Capsid structure is essential for the organization and arrangement of the tegument proteins on the outside of the capsid (2-4). Tegument proteins are divided into two types based of their location relative to the capsid. The inner tegument proteins form an orderly packed,

densely compact layer on the virion capsid while loosely attached tegument proteins present between this and the viral envelope are known as outer tegument proteins (94). Tegument layers do not only contain viral tegument proteins, but also cellular proteins but it is unknown whether they have a functional significance (108).

Envelope

The viral envelope is derived from a host membrane and contains viral glycoproteins. Within this phospholipid bilayer the capsid could be located symmetrically or asymmetrically within the envelope (94). As well as enveloped nucleocapsids, there are non-infectious enveloped particles (NEP) are produced. These include particles with defective genomes and dense bodies which contain tegument proteins without viral capsid (109). The strain of virus, number of passages in cell culture, multiplicity of infection (MOI) used, type of infected cells, and *in vivo* or *in vitro* infection play important roles in the relative proportion of the different types of particles (110). Defective viral particles formed during the infection process are useful for viral infection and spread because it saturates immune surveillance and reduces the immune control over viral spread.

HCMV Glycoproteins

Viral envelope glycoproteins play important roles during entry, cell to cell spread, and virion maturation. Six glycoproteins were originally identified from laboratory strains of HCMV: gB, gN, gO, gH, gM, and gL. These glycoproteins, plus additional

UL128-131 (missing from the lab strains), are involved in the process of entry into cells (90, 91, 111). A homodimeric complex of gB is called gCI, the complex of gM and gN complex is gCII, and the gH/gL/gO complex is named gCIII. Glycoprotein complex gCI, gCII, and gCIII are highly conserved among herpesviruses (91, 112). The gM/gN complexes are highly abundant followed by gB and gH/gL/gO complexes (112). Glycoproteins are essential for entry into the host cells (113). If the ORF of gB, gH, gL, or gM is disrupted, viral particle cannot infect (111, 114, 115).

VIRAL INFECTION CYCLE

Attachment and Entry

Attachment of virus to the cell surface depends on multiple factors determining the probability of entry of virus in a cell. Cellular receptors such as integrins play vital roles in the entry of CMV. Integrins are expressed on the cell surface in non-covalently linked heterodimers containing α and β subunits (116). During CMV infection, integrins perform multiple functions such as receptors for viral entry, signal transduction, and changes in cytoskeletal structure helpful for transport of viral capsid towards the nucleus (117, 118). The gB homodimer binds beta 1 integrins via disintegrin-like domain (118). Other cellular receptors used by CMV in different cell types are epidermal growth factor receptors (EGFR), heparin sulfate glycosaminoglycans (GAGs) and platelet derived growth factor receptors alpha (PDGFR-α) (111, 119-121).

In HCMV, gH/gL/gO complexes are important for entry into fibroblast cells whereas gH/gL/UL128-131 complexes are important for entry into endothelial, epithelial, monocytes, and macrophage cells (111, 114, 115). After initial attachment gB undergoes conformational changes causing fusion of viral envelope with the cell membrane (122). Along with fusion with the plasma membrane, cytomegalovirus can be endocytosed or macropinocytosed and fuse with vesicular membranes (Step 1, Figure 3) (123, 124).

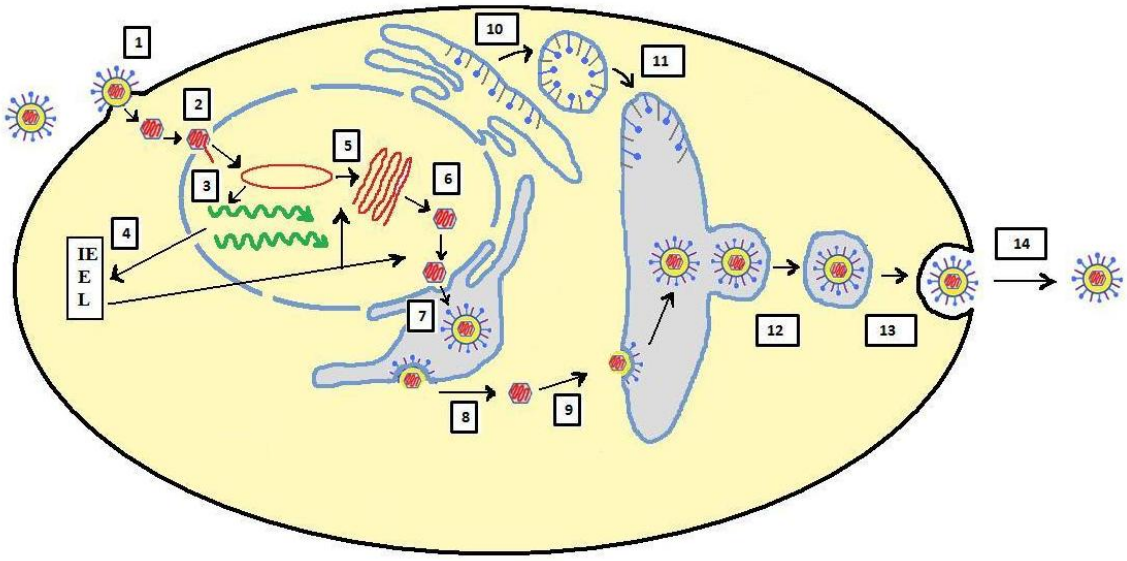


Figure 3. Overview of herpesvirus replication cycle showing all the major stages from the entry of infectious particle to the release of mature virus. Stages: 1 - Attachment and entry, 2 - entry of viral DNA into the nucleus, 3 and 4 - viral gene expression, 5 - genome replication, 6 - nucleocapsid assembly, 7- budding though the inner nuclear leaflet into perinuclear space, 8- Release from outer nuclear membrane into the cytoplasm, 9 – Envelopment at Golgi complex, 10 – budding of transport vesicle containing viral glycoproteins from ER, 11 – fusion of transport vesicle with Golgi complex, 12 and 13 – envelopment and release of enveloped viral particle from Golgi complex and fusion with plasma membrane, 14 – release of enveloped viral particle.

The reducing environment in the cytoplasm is one of the factors responsible for detachment of viral tegument proteins from the viral capsid. Tegument proteins are

targeted to the nucleus (125). With the help of the microtubule network, capsid is transported to the nuclear pore. Only viral DNA and tegument proteins enter the nucleus and they are transported separately (Step 2, Figure 3) (126, 127).

HCMV AND MCMV GENOMES

CMV encodes around 170 genes in an approximately 230 kb genome (22, 128). A characteristic of herpesvirus genomes is the presence of repetitive sequences flanking one or two unique sequences which are important for encapsidation of the genomes (128, 129). The HCMV genome consist of two unique segments: the unique long (U_L) and unique short (U_S). The U_L segment is flanked by repetitive sequences or direct repeats known as terminal repeat long (TRL) and Internal repeat long (IRL) whereas the U_S segment is flanked by repetitive sequences internal repeat short (IRS) and terminal repeat short (TRS) (130). In the MCMV genome internal repeats are absent. CMV open reading frames (ORFs) are named based on then location in the genome. Thus for HCMV the 22nd ORF located in the unique short region is US22. The homologous gene in MCMV is m139, the 139th ORF in the MCMV genome (131, 132).

Gene Classes

Herpesvirus genes are classified into three groups depending on the time of expression and sensitivity to different inhibitors. These three classes are immediate early (IE) or α, early (E) or β, and late (L) or γ (133). Transcription of IE genes is independent of prior viral protein synthesis after viral entry in the target cell, and protein synthesis

inhibitors are used to identify IE genes (134, 135). Early gene expression requires IE proteins (135). Viral DNA replication is a prerequisite for L gene expression; hence viral DNA synthesis inhibitors such as foscarnet are used to differentiate between E and L genes (133).

HCMV Gene Expression/Transcription

Viral gene transcription takes place in specialized compartments present inside an infected cell nucleus. These structures are known as nuclear bodies (NB), nuclear domain 10 (ND10) bodies or promyelocytic leukemia protein (PML) oncogenic domains (PODs), as the cellular PML protein is a key in organizing these domains (136). In an uninfected cell ND-10s are important for gene regulation, post-translational modification, controlling cell growth, cell differentiation, and apoptosis. Upon infection, CMV DNA is targeted to ND-10s and transcription starts there (137, 138). Viral proteins modify levels and localization of ND-10s proteins, leading to enhanced viral transcription. For example viral tegument protein pp71 accumulates in ND10 and initiates viral infection (139).

Genome Replication

In an infected cell, viral genome replication occurs independently from cellular DNA synthesis (140). Herpesviruses form a large replication compartment, which is a globular amorphous structure containing viral DNA and required replication machinery (141, 142). The CMV origin of lytic replication (OriLyt) spans around a 2.4 kb region containing various transcription factor binding sites, pyrimidine rich sequences, direct,

and inverted repeat sequences, and some conserved motifs of unknown function (143). Although the rate of replication varies by cell type, in fibroblasts maximum viral DNA synthesis was detected by 72 hours post infection (hpi) for HCMV and by 12 hpi for MCMV (26). Six proteins essential for viral DNA replication and conserved among all herpes viruses are DNA polymerase, polymerase processivity factor, single stranded DNA binding protein (SSB) which binds to single-stranded regions of DNA to prevent premature annealing, helicase, primase, and primase associated factor (5, 26). Viral DNA is synthesized in a concatemeric form and then cleaved in to individual units as it is packaged into capsid (144).

VIRAL DNA PACKAGING

Three essential steps before the initiation of viral DNA packaging are viral DNA synthesis, synthesis of late proteins, and viral capsid assembly (145). Capsid assembly takes place inside the nucleus (Step 6, Figure 3). Multiple studies have been done in the area of viral DNA packaging, but assembly and egress are still not well understood. During the process of maturation a procapsid is formed containing scaffolding or assembly proteins (APs) (100). These APs are required for proper capsid formation, often contain nuclear localization signals which are important for the transport of capsid components into the nucleus, form complexes to bring together the capsid components, and must be cleaved to dissociate from the capsid (146-148). The dsDNA enters and APs leave the procapsid structure leading to development of C capsid or matured capsid. There is debate about the possible route used by scaffolding proteins for the exit from the

procapsid structure (100). Specific sequences in the viral DNA are recognized by proteins in the PORT complex for loading of the genomes and cleavage of the concatomeric DNA into genome lengths (149).

MATURATION AND RELEASE

The CMV capsids undergo primary envelopment at the inner nuclear membrane (Step 7, Figure 3). The process of tegumentation occurs in nucleus and cytoplasm via protein-protein interactions between tegument proteins and capsid proteins (94, 150) and it is likely that different layers of tegument proteins are important for primary and secondary envelopment. Virus induced specialized membrane structural domains play an important role in the process of envelopment (151, 152). The Primary envelope is lost via fusion with the outer leaflet (Step 8, Figure 3). In an infected cell Golgi complex, trans-Golgi network, and early endosomes are arranged together in a circular form inside the cytoplasm. This structure, known as the assembly complex (AC), is induced due to viral infection and promotes effective viral envelopment and release (153). The viral glycoproteins are synthesized in ER and transported to the Golgi complex membrane (Steps 10 and 11, Figure 3) (154). These proteins get incorporated in the viral envelope during final envelopment, the process of budding into the trans-Golgi network (Step 11, Figure 3) (145, 155). Golgi derived secretary vesicles fuse with the plasma membrane and releases the matured viral particles (Step 12-14, Figure 3) (154). This process eventually leads to cell death (26).

CMV LATENCY AND REACTIVATION

Not all cells infected by CMV undergo lytic viral replication. Virus can also enter into a latent infection state where viral DNA is present but no infectious viral particles are produced (156, 157). Smooth muscle cells, endothelial cells, epithelial cells, fibroblast, macrophages, and dendritic cells are permissive to HCMV infection leading to production of infectious virus, but bone marrow progenitor cells and monocytes promote latent CMV infection (158-160). Latently infected cells show an episome associated with dimethylated H3 causing repression of CMV genes whereas during lytic expression, CMV DNA is associated with acetylated H3 (161).

Viral tegument protein pp71 plays an important role in reactivation from latency. Pp71 interacts with and degrades Daxx in a proteasome dependent pathway to reverse HDAC mediated viral gene repression (162-164). The differentiation stage of a cell is also important for reactivation of HCMV. IE genes are more prone to activation in differentiated cells as compared to undifferentiated or precursor cells (165, 166).

ACTIVITY OF m142/m143 IN MCMV AND IRS1/TRS2 IN HCMV

The focus of this study is the m142 gene which belongs to the US22 gene family. This gene family was named for the HCMV gene which was the first studied and is based upon the presence of at least one of 4 conserved hydrophobic motifs (167, 168). The m142 gene product and its binding partner m143 are the structural and functional homolog of IRS1 and TRS1 of HCMV (169, 170). All of these proteins bind protein

kinase R (PKR) and dsRNA to prevent PKR activation (170-172). PKR binds dsRNA inducing autophosphorylation, converting PKR into an active form (171). Activated PKR phoshorylates eukaryotic initiation factor 2 alpha (eIF-2α), a regulatory component of eIF2. Phosphorylation of eIF-2α prevents binding of the initiator tRNA to the small ribosomal subunit and protein synthesis. As a result, cells eventually undergo apoptosis.

TRANSCRIPTION REGULATION

Gene Regulation of HCMV

HCMV DNA localizes to the nucleus and utilizes mostly cellular and some viral proteins for transcription. In HCMV, genes expressed under IE conditions include IE1, IE2, internal repeat short 1 (IRS1) and terminal repeat short 1 (TRS1), US3, and UL36-38 (173, 174). As IE genes are transcribed in the presence of protein synthesis inhibitors, cellular transcription factors are major regulators. However, viral factors can also play various roles in the regulation of these promoters. The best studied of these promoters is the major immediate early promoter (MIEP), which is used in many plasmids as "the CMV promoter". Since m142 is an IE transcript, we are interested in how the regulation compares to other IE genes.

Major Immediate Early Promoter

Immediate early genes ie1 and ie2 are controlled by a single promoter known as the MIEP; this promoter is highly active in many cell types and has a very strong enhancer (175). IE1 and IE2 proteins are produced by differential splicing (176). IE1

and IE2 mRNAs share exons 1, 2, and 3 as shown in Figure 4 (26, 177). IE1 is a 72 kDa protein also known as IE72 or ppUL123, whereas IE2 is an 86 kDa protein and is also known as IE86 or ppUL122a (178). IE1 and IE2 are responsible for activation of expression from different early and late genes of HCMV including auto-regulation of the MIEP.

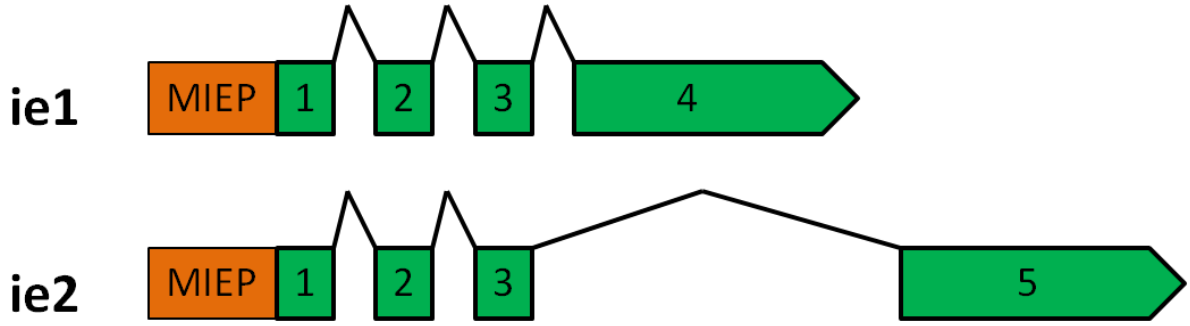


Figure 4. Differential splicing of ie1 and ie2 mRNA in HCMV for the production of IE1 and IE2 proteins. IE1 and IE2 expression is regulated by the MIEP. Exon 1, 2, and 3 are shared by IE1 and IE2, whereas exon 4 is present in IE1 and exon 5 is present in IE2.

In MCMV, the MIEP controls the expression of IE1, and IE3 in a similar manner. However, unlike HCMV, an additional abundant transcript, IE2, is encoded on the opposite side of the MCMV MIEP. The names were given before the sequences and origins were known, thus IE1 and IE3 of MCMV are organizational and functional homologs of IE1 and IE2 of HCMV and the splicing pattern is comparable (179).

MIEP Regulation

In addition to cellular transcription factors, various CMV tegument proteins, and IE1 and IE3 are important in regulating expression from the MIEP of HCMV. Some

important cellular regulators of the HCMV MIEP include AP-1, NF- kappa B, and Sp1 family members (180-182).

AP-1 is a dimer made up of Jun, Fos, or activating transcription factor (ATF) subunits that bind to the AP-1 target binding site (183). Two AP-1 binding sites are present in the HCMV MIEP enhancer region and the HCMV MIEP contains 4 consensus binding sites for nuclear factor-kappa B (NF-κB). Deletion of NF-κB sites alone impair virus replication in cells actively going through cell cycle (184) and for replication in quiescent cells (180). Deletion of the AP1 sites alone has little effect, but knock out in combination with NF-κB shows greater impairment than either alone (181).

The specificity protein (SP) is a major family of transcription regulators. There are four proteins present in this family: SP1, SP2, SP3, and SP4. The SP family proteins have a conserved DNA binding domain and an activation domain. Expression of SP4 is limited to brain cells whereas SP1, SP2, and SP3 are expressed in all cell types (185). HCMV infection causes activation of SP1 and SP3 expression. Two specific GC rich consensus binding sites are present in the HCMV MIEP. SP1 and SP3 binding are responsible for enhancing expression in a synergistic or co-operative manner. Mutation of one of the sites is insignificant but mutation of both target sites significantly hampers activation by SP1 and SP3 (186).

PUL83 or pp63 is a highly abundant tegument protein of HCMV which promotes activation of the MIEP (187). Cellular interferon gamma inducible protein 16 (IFI16) was found to interact with and support the activation process by pUL83, which recruits

IFI16 to the target binding site of the MIEP (188). That viral tegument proteins and cellular proteins can co-operatively mediated IE gene activation is found to be universal among all members of the herpesvirus family (26). The type of cellular proteins involved may differ in different families but the overall process is the same.

Expression from the MIEP is an effect of competition between activators and repressors. The cellular transcriptional regulator ATP linked helicase (ATRX also known as X-linked nuclear protein or X-linked helicase II) suppresses gene expression from the MIEP, by recruiting hDaxx and causing chromatin modifications. This hDaxx mediated repression is prevented by viral tegument protein pp71 (UL-82) (162-164, 189).

Activation by IE1

The IE1 protein is one of the major activator of the MIEP (190). In addition, IE1 has a more general mechanism of promoting transcription. Binding of IE1 to HDAC-2 causes sequestration of HDAC2 leading to reduce deacetylation of histones at viral promoters and active viral gene expression. IE1 is also responsible for interacting with PML and Daxx so that these two proteins will be unavailable for repression of viral transcription (191). Proteins IE1 and IE2 are involved in disruption of PODs at early times (137).

IE2 Mediated Auto-repression

IE2 acts as an activator for CMV E and L genes, but it also represses its own promoter (192, 193). Binding of IE2 to the cis-repression sequence (crs), located

between the TATA box and the transcriptional start site is important for this auto-repression. IE2 recruits HDAC2 (192), which causes deacetylation of histones bound to the MIEP. These deacetylated histones undergo further methylation by histone methyl transfereases (HMTs) which are also recruited by IE2 (193). Thus IE2 and IE1 have opposing roles in the deacetylation process (194). IE2 also binds to two other crs like elements in the MIEP, the -240 and -180 boxes. This interaction has the same repressive effect as that of binding with crs (195).

Other IE Gene Regulation

US3 is another IE gene whose expression is controlled by a complex set of protein. US3 is a membrane protein responsible for interacting with class I major histocompatibilty complex (MHC) proteins and retaining them in the endoplasmic reticulum (ER). This prevents the presentation of HCMV antigens to cytotoxic T cells and activation of cell mediated immunity is controlled (196). Regulation of US3 has several similarities to the MIEP. Tegument protein pp71 (UL82), NF-kB, and IE1 up-regulate US3 gene expression (197). However, for US3 IE2 is an activator, unlike its repressor function for the MIEP IE2. In addition, IRS1, and TRS1 promote activation of theUS3 promoter while the IE gene product pUL37ex1/UL38 and E gene product pUL84 and pUL34 repress (197).

Activation of Other Genes via IE1 and IE2

IE1 activates expression from the promoter of DNA polymerase alpha via interaction with the CCAAT box (198). IE1 and IE2 can both promote activation of a variety of promoters via basal promoter elements (199, 200). This is likely due to TATA binding protein (TBP) associated factor (TAF)-like activity of both of these proteins (201-203). In addition, IE1 can inhibit cellular repressors. The cellular protein p107 interacts with the E2F family of transcription factors and prevents cell cycle progression. One of the important triggers for inhibition via p107 is infection with DNA viruses. During HCMV infection, IE1 alleviates p107 mediated repression of E2F target genes (204).

IE2 mediates activation of various viral promoters via interaction with cellular transcription factors as well including c-AMP response element binding protein (CREB) (205, 206) and SP1 (207). Some sequences in the IE2 protein important for regulation by IE2 have been identified. Autoregulation or transactivation by IE2 is inhibited by mutation in a region between amino acids 450 and 544 (208) while phosphorylation of IE2 between amino acids 266 and 275 inhibits its interaction with TBP and cause a decrease in activation capacity (177).

MCMV GENE REGULATION

Gene regulation of MCMV has not been extensively studied. The MCMV MIEP is the major exception, and found to be similar to the HCMV MIEP (209, 210). MCMV IE1 has a similar function to HCMV IE1 of preventing HDAC mediated repression of

gene expression (211). MCMV IE3 also represses the MIEP like the homologous IE2 of HCMV (179). *In vivo* studies have supported additional repressors of the MIEP of MCMV such as YY1 and core binding factor 1 (CBF-1) which were bound to specific sites during latency but not lytic infection (212). MCMV IE1 and IE3 also function as activators of viral E and L genes, like HCMV IE1 and IE2 (26).

IMPORTANT CELLULAR TRANSACTIVATORS

SP1

SP1 is a cellular zinc-finger protein which acts as a transcription regulator for various cellular and viral promoters. It has an N-terminal transcriptional activator domain and a C-terminal conserved functional domain (185). Depending on other factors associated with SP1, it can act as an activator or a repressor of the target promoter. HCMV has been shown to cause increased expression of SP1 and increased DNA binding activity by SP1 in infected cells (213). SP1 activation and activation by SP1 follows a positive activation loop in case of HCMV. The process of viral attachment and entry with target cell is enough to initiate signals leading to SP1 and NF-kβ activation (214, 215). The SP1 protein can activate CMV promoters via consensus SP1 binding sites or via inverted repeat sequences which do not contain consensus site in cooperation with the CMV IE2 protein (216, 217).

Elk-1

Elk-1 is a transcription factor responsible for regulating expression of various genes via the serum response element (SRE) consensus site. Elk-1 belongs to the Ets family of ternary complex factors (TCF) subfamily of ETS domain transcription factors (218, 219). Elk-1 either interacts with the target sequence in a complex with other ETS-domain complex factors or with second transcription factor SRF by direct protein-protein interaction (219, 220).

The activator function mediated by binding of Elk-1 to the target promoters could be mediated by CBP or p300 recruited by Elk-1. These two proteins have intrinsic histone acetyletransferase (HAT) activity, resulting in reliving repression (218). In the primate cytomegalovirus immediate early promoter, Elk-1 along with serum response factor enhanced basal expression from the IE promoter. This Elk-1 mediated effect was observed in monocytes and T-lymphocytes (221).

YY1

The transcription factor Yin Yang 1 (YY1) has been reported to activate or repress different herpesvirus promoters. In Kaposi's sarcoma-assocated herpesvirus (KSHV), YY1 activated expression from the promoter of viral ORF50 (222). Similarly, in varicella zoster herpesvirus (VZV) deletion of an YY1 binding site in the promoter region of ORF28/29 causes significant inhibition of viral replication in melanoma cells (223). In MCMV, YY1 binding to a 21 bp repeat element of the MEIP resulted in repression of expression (212). Similarly in Epstein Barr virus (EBV), an YY1 binding

sites between -206 to -277 is essential for YY1 binding and YY1 mediated repression of a viral gene (224).

AP2

Proteins from AP2 family are involved in various cellular processes such as growth, differentiation, programmed cell death, mutations, and regulation of gene expression (225). There are five isoforms of AP2 (α, β, γ, δ, and ε) (226). The cellular protein AP2 contains a dimerization domain and a DNA binding domain. The dimerization domain consists of two alpha helices separated by a span region (227). The DNA binding sequence is located in the C-terminal region of the protein. In addition to the DNA binding domain a proline rich N-terminal activation domain is highly essential for the tranactivator function of AP2 (228). The IE2 protein of HCMV interacts with various cellular transcription factors such as SP1, AP1, and AP2. These complexes perform transactivator functions (229).

The MCMV m142 Promoter

The m142 is an immediate early promoter and it may behave similar to other IE promoters (such as MIEP) of MCMV or HCMV. Not much is known about the m142 promoter regulation. Initial characterization of the m142 promoter of MCMV showed that it had activity in the absences of viral infection and could be activated by IE1 and IE2 (230). Our research focuses on understanding the regulation of expression from the promoter of the essential gene m142.

The specific aims of this study were: 1) Identification of DNA sequences important for m142 promoter regulation; 2) Identification of cellular and viral proteins involved in regulation of the m142 promoter; and 3) Analysis of the activity of cellular and viral regulators in the context of viral infection.

CHAPTER II

MATERIALS AND METHODS

TISSUE CULTURE

NIH/3T3 mouse fibroblast cells [American Type Culture Collection (ATCC) CRL-1658, Rockville, MD] were used for all experiments. NIH/3T3 cells were maintained on a strict schedule by splitting every three days and seeding 3×10^5 cells in a 75 cm^2 flask. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM GIBCO® cell culture, Grand Island, NY) supplemented with 10% bovine calf serum (BCS, Atlanta Biologicals, Flowery Branch, GA). For further reference this media will be referred to as complete media.

VIRUS

The virus used for all studies was MCMV Smith strain (ATCC VR 194). Virus stocks were prepared in NIH/3T3 cells. Virus titers were measured by standard plaque assay by Campbell el al.

INFECTION OF CELLS

Cells were seeded 24 hours prior to infection. Virus stock was diluted in complete media to get an MOI of 2 PFU/cell unless otherwise indicated for the experiment. Volumes were as indicated for specific experiments. Cells were incubated

at 37°C with virus for 1 h with rocking after every 15 min. After the incubation period, the media was replaced with complete media. Mock infected cells were treated identically except that media without virus was used.

In some experiments phosphonoformic acid (PFA = foscarnet, Sigma-Aldrich, St. Louis, MO) was added to inhibit viral replication, which results in the inhibition of late gene expression (Snoeck et al. 1988, Manischewitz el al. 1990). When the media was changed after the incubation period, PFA was added to a final concentration of 300µg/ml. Replica plates without PFA were used as control for the experiments. Supernatants from PFA treated and untreated cells were collected and plaque assays were carried out to confirm that the PFA had inhibited production of virus. Supernatants from cells treated with PFA had no detectable virus by plaque assay (data not shown). In addition, viral DNA was harvested as described under Generation of Infected Cell DNA and quantitated by qPCR for the m142 promoter DNA as described in the Quantitative PCR (qPCR) section using the forward (5'- TCGAGGTCCACGACGG-3') and reverse (5'-GGAACACGGGTGGAGAAG-3') primer pair. The cellular poly A binding protein (PABP) [forward primer 5'-GCAATGCTGGCCCAGTGATCATG-3' and reverse primer 5'-AAGGCCAGGGACGTCCTCACTGAC-3'] was used as a loading control for the PCR. Quantitative PCR was performed using a DNA Engine Opticon 2 (MJ Research, Waltham, MA) as described in the qPCR section, using the same conditions which were optimal for both primer pairs. UV inactivated virus was prepared by treating one half of a newly prepared viral stock in a 100 mm tissue culture plate at 10 ml/plate

for 5 min under optimal crosslink setting (120 mJ/cm^2) in an UV crosslinker-FB-UVXL-1000 (Thermo Fisher Scientific, Waltham, MA). The matched un-treated and UV treated viral stocks were then aliquoted and stored at -80°C for further use. Plaque assay was carried out on both UV treated and untreated viral stock to confirm inactivation of the virus. During infection an equal volume of UV treated virus was used as that of the matched untreated virus stock to get the same number of viral particles per ml.

GENERATION OF INFECTED CELL DNA

Sixty mm tissue culture plates were seeded with 5x10^5 NIH/3T3 cells and 24 hours later cells were mock infected or infected with MCMV at an MOI of 2 PFU/cell. Cells were harvested 24 to 48 hours after infection. Total cellular and viral DNA was isolated using the MasterPure DNA extraction kit (Epicentre, Madison, WI) as per manufactures' instructions.

CONSTRUCTION OF m142 REPORTER PLASMIDS

Primer Design

A series of nested, approximately 100 bps deletion mutants of the m142 promoter were designed for PCR amplification. Ten forward (F) and two reverse (R) primers were designed using the primer basic local alignment tool (BLAST) primer designing tool. The primers and the expected products are listed in Table 1. Not all the primer pairs produced expected products, so we turned to restriction enzyme digestion to generate these desired promoter constructs.

Table 1. Primers designed to create nested deletions of the m142 promoter

Promoter constructs produced by PCR amplification are indicated in bold letters.

Primer sequence (F-Forward, R-Reverse)	Number of bases deleted compared to full length	Deletion constructs relative to transcription start site
GGAGCTCCGTCGCATG (F1)	**Full Length**	**-901**
ACCAGGAGCTGTGGTTCC (F2)	95	-806
CGTGGATCTCACGTCCC (F3)	**188**	**-712**
TGACGGAGGAGAACAGCTC (F4)	284	-617
GAGTCGGCGGACAAGG (F5)	397	-504
GACACGGATCAGTCTCCG (F6)	474	-427
GAGGGAGATTGTCACGAATG (F7)	600	-301
GTCGACGCCGAACTCC (F8)	677	-224
TCGAGGTCCACGACGG (F9)	**792**	**-109**
GCCATCGGTATTCGGAGTGT (F10)	902	+4
GGAACACGGGTGGAGAAG (R1)	-	+74
AACACGGGTGGAGAAGG (R2)	-	+72

Generation of Promoter Constructs by PCR

Individual reactions were carried out at annealing temperatures based on the predicted melting temperature of F and R primer as shown in Table 2. PCR reactions were done in parallel with DNA from infected and uninfected cells to ensure that

products were actually derived from viral rather than cellular DNA. PCR was conducted using the Amplitaq Gold PCR master mix (Applied Biosciences, Life technologies, Grand Island, NY) using a PTC-200 Peltier Thermal Cycler (MJ Research, Ramsey, MN). The PCR conditions were 1 cycle of 10 min at 94°C, followed by 34 cycles of 1 min at 94°C, 1 min at respective standardized annealing temperatures of the primer pairs as mentioned in Table 2, and 1 min at 72°C, and finished with 72°C incubation. PCR products were analyzed by agarose gel electrophoresis based on the expected product size. Bands of the expected size from infected cells were purified using the QIAquick gel extraction kit according to manufacturer's instruction (Qiagen, Valencia, CA).

Table 2. Standardized annealing temperatures used to amplify PCR products

Mutant	Annealing temperature
-901 (FL)	47.60
-806	47.60
-713	52.60
-504	49.00
-109	48.30

TA Cloning

Eluted PCR products were cloned into the PCR® 2.1-TOPO plasmid by topoisomerase (TA) cloning according to manufacturer's instructions (Invitrogen, Grand

Island, NY). The resulting plasmids were transformed into chemically competent *E. coli* (One shot DH5α-TOPO® 10, Invitrogen, Grand Island, NY).

Sequencing

Sequencing of constructs cloned into PCR 2.1 was carried out using M13 reverse and M13 forward sequencing primers flanking the TA insertion site using the EXCEL™ II DNA sequencing kit-LC (Epicentre, Madison, WI). Sequencing reactions were run on a PTC-200 Peltier Thermal Cycler (MJ Research, Ramsey, MN) with conditions of 1 cycle of 5 min at 95°C to denature the template, followed by 29 cycles of 30 sec at 95°C, 15 sec at 50°, 1 min at 70°C, and finished with a 72°C incubation for 10 min. Products were stored at -20°C or directly run on a 4000L automated sequencer (LICOR Biosciences, Lincoln, NE) according to manufacturer's directions. Just prior to loading 3 µl of stop solution was added in every reaction, samples were heated at 95°C for 3 min and 1.7 µl of sample was loaded in every well. The sequencing gels were allowed to run for 8 h to overnight. Sequencing results were analyzed with the nucleotide basic local alignment tool (nBLAST). Constructs obtained without any mutation were used for cloning into a reporter plasmid.

Generation of Constructs After PCR

Constructs -901 and -713 were initially constructed in the PXP luciferase reporter plasmid (Nordeen el al., 1988). The pCR™ 2.1 plasmids (Invitrogen, Grand Island, NY) containing the confirmed promoter sequences were double digested with HindIII and

EcoRV. The PXP vector was double digested with HindIII and SmaI. All restriction enzymes were purchased from New England Biolabs (NEB) and used according to manufacturer's recommendations (NEB, Ipswich, MA). The -109 construct was made by double digested from the PCR 2.1 plasmid with BamHI and EcoRV while the PXP plasmid was double digested with BglII and EcoRV. The digestion reactions were separated on 1% agarose gels, the desired bands (insert) were excised and purified with the QIAquick gel extraction kit according to manufacturer's instructions (Qiagen, Valencia, CA). A vector: insert molar ratio of 1:3 was used to increase the probability of successful insertion and samples were incubated at 4°C, overnight. The ligation mixture was transformed into chemically competent *E. coli* according to manufacturer's instructions (One shot DH5α-TOPO® 10, Invitrogen, Grand Island, NY).

The luciferase detection system available was not able to detect luciferase produced by the m142 promoter or enough luciferase was not produced for the detection. Hence, we decided to shift to a fluorescence-based assay compatible with our equipment based on a secreted alkaline phosphatase (SEAP) reporter plasmid pSEAP2 (Clontech, Mountain View, CA). Subcloning from the PXP2 plasmids into pSEAP2 was done by digestion of both plasmids with EcoRI, and purification, ligation, and transformation were performed as described previously.

Generation of Promoter Constructs by Restriction Digestion

Construct -875 was prepared from the -901 construct in pSEAP2 by digesting with BglII and relegation. Construct -579 was prepared by double digestion of the -901

full length promoter in PCR2.1 with BmgBI and EcoRV and insertion into the PXP plasmid digested with SmaI. Similarly, the -222 construct was prepared from the -901 PCR 2.1 construct by double digest with SalI and BamHI and cloned into PXP digested with SalI and BglII. Both of these promoters were put into the pSEAP2 plasmid using double digestion with HindIII and EcoRI.

To generate the -377 construct the -712 PCR 2.1 plasmid was double digested with HaeIII and BglII and the resulting fragment was cloned into PXP digested with SmaI and BglII. This promoter was put into the pSEAP2 plasmid using double digestion with HindIII and EcoRI. Construct -9 was prepared from construct -109 in the pSEAP2 vector by digestion with XhoI followed by re-ligation.

Some PCR products had errors upon sequencing and restriction digestion was used to replace the erroneous sequences. In the -806 promoter all sequences samples had at least 1 mutation in the region between -777 and +74. The un-mutated region between -806 and -777 was digested from a PCR2.1 -806 plasmid using MluI and cloned into the pSEAP2 -901 plasmid which had also been digested with Mlu 1. Correct orientation of the inserted fragment was detected by double digestion with HindIII and EcoRI. Constructs prepared by PCR and restriction digestion method are shown in Figure 5.

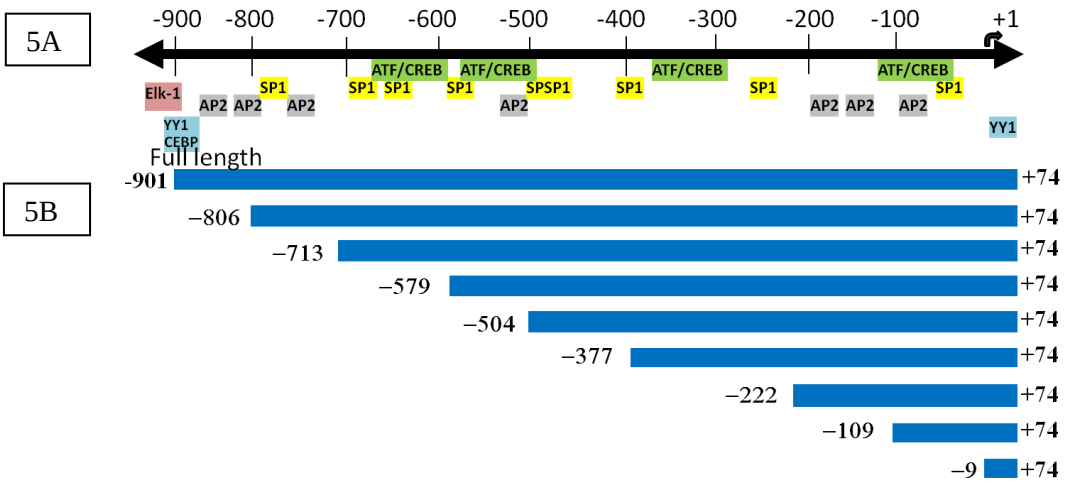


Figure 5. Schematic map of putative transcription factor binding sites in the m142 promoter and relative sizes of deletion constructs used in this study. (A) Potential transcription factor binding sites in the m142 promoter. Transcription factor binding sites found important for the regulation of other herpesvirus promoter are shown. **(B) Map of the m142 promoter constructs generated by PCR and restriction digestion.** Generation of the promoter constructs is explain in detail in the materials and methods section.

CHARACTERIZATION OF PROMOTER CONSTRUCTS

Transient Transfection

T25 flasks were seeded with 6×10^5 cells in a final volume of 4 ml of complete media. After 24 h, transfection was carried out with Transfectin™ (BioRad, Hercules, CA). DNA: Transfectin was used at a ratio of 1µg DNA: 2 µl Transfectin (230). The day after transfection, cells were observed for viability and equal numbers of cells from each transfection were seeded in 60 mm dishes or replicate wells of a 12 well plate. The number of wells seeded from every sample differs according to experiment and treatments. The remaining cells were used for DNA isolation according to the protocol in

the DNA isolation section. The next day cells were infected with MCMV at a MOI of 2 PFU/cell. Reporter plasmid with no promoter was used as a control. Twenty four hours later supernatant was harvested for quantitation of secreted alkaline phosphatase and DNA was harvested for qPCR. The same protocol was followed during co-transfection experiment except 6 μg of each plasmid (reporter plasmid and plasmid expressing protein of interest) were transfected instead of 9 μg of single plasmid. This was done because previous experiments had shown that these ratios worked well (230).

Assay for Production of Secreted Alkaline Phosphatase (SEAP)

Three independent transfection experiments were performed for each assay. Supernatants from transient transfections were collected and processed for the secreted alkaline phosphatase (SEAP) assay according to manufactures' instructions (Clontech, Mountain View, CA) and each supernatant was analyzed in triplicates to control for pipetting variability. Briefly, samples were centrifuged at 5000g for 10 min at 4°C to pellet cell debris. Supernatants were transferred to another tube, mixed with an equal volume of 1X dilution buffer, and heated at 65°C for 35 min to denature cellular AP. SEAP produced by the reporter plasmid is heat stable, hence can withstand 65°C. Replicate samples were taken from each supernatant tube. Heat treated samples (50 μl) were added into 96 well plates with clear bottoms and black sides (MidSci, St Louis, MO). A mixture of 75 μl of assay buffer and 3 μl of 1mM 4-methylumbelliferyl phosphate (MUP), a substrate for SEAP, diluted in 1X dilution buffer was added in each well of 96 well plate containing heat treated supernatants. The 1X dilution buffer, 1mM

MUP, and mixture of MUP and assay buffers were prepared fresh each time. Plates were incubated on a shaker at RT for 1 hr then read in a Synergy-HT microplate reader (BioTek, Winooski, VT) at excitation 360 nm, transmission 460 nm, and sensitivity 60 at RT.

Quantitative PCR

DNA was isolated from cells using the MasterPure DNA extraction kit (Epicentre, Madison, WI) as per manufacturers' instruction. DNA was quantitated using the nanophotometer™ P-class spectrophotometer (Implen, Westlake Village, CA). Quantitative PCR was carried out in the DNA Engine Opticon 2 (MJ Research, Waltham, MA). In order to normalize for the efficiency of transfection, parallel PCR reactions for cellular DNA and plasmid were performed. Primers for a cellular gene, Poly A binding protein (PABP) (forward 5'-GCAATGCTGGCCCAGTGATCATG-3' and reverse 5'-AAGGCCAGGGACGTCCTCACTGAC-3'), were used as loading control for PCR. The ampicillin primer pair (Forward 5'-ATCGTTGTCAGAAGTAAGTTGG-3' and reverse 5'-GCCGCATATATCACTATTCTCAG-3') was used to detect reporter plasmid in case of infected samples except in the case of co-transfections. Because the plasmids expressing viral proteins IE1 and IE3 were ampicillin resistant, in these cases the pSEAP2 plasmid was detected with the F9 and R1 primer pair, to ensure that evaluation was of the reporter plasmid. The PCR conditions used for all three primer pairs were 1 cycle of 3 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C. The plate was read after each cycle 72°C. After 35 cycles a final incubation

was carried out for 5 seconds at 72°C. Melt curve analysis was performed with starting temperature 65°C, ending temperature 90°C, with increment of 0.1°C, and holding for 1 sec. After completing of the melt curve samples were held at 72°C for 1 min followed by 10°C for maximum 10 hrs.

SODIUM DODECYL SULFATE – POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Total cell extracts were prepared from mock and infected cells. Cells were washed twice with cold PBS (Nacl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM). Western lysis buffer (50 mM Tris, 1% SDS, pH 7.5,) at 300 ul for a 30 mm plate was added to the cells and cell lysates were collected by scraping. Lysates were collected in 1.5 ml tubes and stored at -80°C. Protein concentrations were measured using the DC protein assay (Bio-Rad, Hercules, CA) according to manufacturer's instructions. The resolving (5%), separating (10%) SDS-PAGE gels, and samples were prepared according to Mini-PROTEAN® 3 Cell Instruction Manual (BioRad, Hercules, CA). The SDS-PAGE electrophoresis was performed in a cold room for 2-3 hours at constant voltage of 200 V. A Mini Trans-blot® Electrophoretic Transfer Cell (Biorad, Hercules, CA) was used for the transfer of proteins to 0.22 mm pore size nitrocellulose membrane (MidSci) at 25 V constant voltage overnight at 4 C. After transfer the membranes were air dried prior to blocking and detection.

Membranes were blocked with 5% non-fat dry milk solution prepared in Tris-buffered saline (50mM Tris, 150 mM NaCl, pH 7.6) for 1 h at RT. After blocking, the membrane was probed with 1° antibody for 1h at RT. The 1° antibodies and the suppliers used were as follows: Mouse monoclonal Sp1 at 1:200 dilution (1C6:sc-420), rabbit polyclonal Sp2 at 1:200 dilution (K-20: sc-643), and mouse monoclonal Sp3 at 1:200 dilution (F-7: sc-28305) (Santa Cruz Biotechnology, Dallas, TX), mouse anti-actin (A5316; Sigma) at 1:5000 dilution, rabbit anti-tubulin at 1:5000 dilution (T5192; Sigma), rabbit anti-m143 at 1:5000 dilution (230).

After 1° antibodies, the blots were subjected to three 10 min washes with TBS-Tween 20 (TBS with 0.05% Tween 20 added). Membrane was probed with 2° goat anti-mouse IRDye® 680 RD antibody (LI-COR Biosciences, Lincoln, NE) or goat anti-rabbit IRDye® 800 CW antibody (LI-COR Biosciences) for 1 hour at room temperature followed by three washes with TBS-Tween. Secondary antibodies were used at 1:15,000 dilutions and incubated for 1 hour at room temperature. Detection was carried out on an Odyssey CLx (LI-COR Biosciences) at the wavelengths to match the conjugates.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extract (NE) and cytoplasmic extracts (CE) were prepared from mock, infected, and transfected cells 24 hours after infection or transfection according to the protocol published by Baldwin et al. (Baldwin et al., 1996). Protein concentrations were determined as described previously and samples were stored at -80°C. Single stranded

oligonucleotides labeled with IRDye® 700 at the 5' end of the probe (Integrated DNA Technologies, Coralville, IA) were diluted in 1X TE to 20 pmole/µl final concentration. Forward and reverse labeled probes were mixed at a 1:1 concentration and heated at 100°C for 3 min. Oligonucleotide mixtures were cooled slowly to anneal. The stock was diluted in water to obtain the desired working concentration of 0.1 pmole/µl according to manufactures' instructions (Integrated DNA Technologies, Coralville, IA). Annealed probes were used at a concentration of 0.1 picomole per sample for EMSA. Depending upon the results of optimization experiments, one to six µg of nuclear extract was incubated with 0.4 µg/sample of poly dI:dC a non-specific DNA competitor dissolved in TE (10 mM Tris, 1mM EDTA, pH7.5), DNA binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5), 25mM DTT/2.5% Tween 20 for the electromobility shift assay (LI-COR Biosciences, Lincoln, NE). Nuclear extracts were mixed with poly dI:dC in DNA binding buffer and incubated for a 10 min before addition of 0.1 pMole of labeled probe followed by incubation for 30 min (according to preliminary standardization experiments) in the dark at room temperature. For competition experiments, unlabeled double stranded competitor was added before labeled probe and samples were incubated for 10 min followed by 30 min incubation with labeled probe. Although both forward and reverse sequences were generated for the primers, only forward sequences will be indicated here. Forward sequences for labeled probes used for EMSA were:

-901 to -879 (5'-GGAGCTCCGTCGCATGGGACTGCTGAAG-3');

-757 to -733 (5’-CGACGTGGCACCCCCCGGCGAGGC-3’);

-459 to -428 (5’-CCGCCGCCTCCGTCGCCGGAGGCGGGGCGCT-3’);

-409 to -386 (5’-GGCGGCGGTGGCGAGGCGCCGCG-3’);

and -37 to -13 (5’-CACTTGGGCGATAAAACGCCGATC-3’).

Mutant unlabeled competitors used for competition assay were:

-901 to -879 YY1 mutant (5’-GGAGCTCCGTCG**TTG**GGGACTGCTGAAG-3’);

-901 to -879 Elk-1 mutant (5’-**TGC**GCTCCGTCGCATGGGACTGCTGAAG-3’);

and AT rich mutant -37 to -13 (5’-CACTTGGGCGAT**CG**AACGCCGATC-3’).

Consensus unlabeled competitors used were:

KSHV consensus TATA 5’-GCAGAGCA**TATAA**AATGAGGTAGGA-3’

and AP2 consensus competitor 5’-GATCGAACTGA**CCGCCCG**CGGCCC GT-3’.

For supershift assays, specific antibodies or isotype matched control antibodies were used. Nuclear extracts were incubated with antibody on ice for 1 hour then poly dI:dC, binding buffer, and probe were added and mixtures were incubated at room temperature for an additional 30 min. Mouse monoclonal SP1 antibody (1C6:sc-420), rabbit polyclonal Sp2 antibody (K-20: sc-643) (Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal TFAP2B antibody (NBP1-89063, Novus Biologicals, Littleton, CO), mouse monoclonal AP2 antibody (NB600-202, Novus Biologicals, Littleton, CO), rabbit

polyclonal YY1 antibody (ab12132, Abcam, Cambridge, MA), and rabbit monoclonal Elk-1 antibody (ab32106, Abcam, Cambridge, MA) were used at 1ug/sample. Orange loading dye (LI-COR Biosciences, Lincoln, NE) was added to the reaction mixture and samples were loaded onto non-denaturing 5% acrylamide gels prepared and run according to the LI-COR instruction manual (LI-COR Biosciences, Lincoln, NE). Electrophoresis was carried out in a cold room at a constant voltage of 200V for 3 hours or based on migration of the orange dye which runs at approximately 200bps. The gel was scanned at 700 nm on the Odyssey Clx (LI-COR Biosciences, Lincoln, NE). The images were obtained and bands were quantitated using Image Studio™ Software (LI-COR Biosciences, Lincoln, NE).

MASS SPECTROMETRY

Specific bands from EMSA gels were cut out based upon measured distance as they were not detectable by Coomassie staining, samples were prepared according to instructions given by the Proteomics Core, UT Southwestern Medical Center at Dallas, and sent for mass spectrometry analysis (Proteomics Core, UT Southwestern Medical Center at Dallas). The short reverse-phase LC-MS/MS method was used to analyze bands. Proteins were identified from samples using an in-house data analysis pipeline (CPFP) with quantitation performed using the Normalized Spectral Index method (SINQ) by UTSW proteomics core staff. The Excel summary of identifications and access to full results online was provided. The mass-spectrometer collects MS/MS fragment spectra, which are generated from peptides. Multiple spectra can be acquired for the same

peptide, and different variants of the same peptide may be seen (with PTMs, or ionized in different charge states). Protein identifications are inferred from the identified peptides. Protein inference is performed across samples. The results contained lists the proteins identified across all samples, and provides spectral counts and spectral index values / ratios that allowed us to compare between samples. For this assay, negative controls from nuclear extracts with no DNA probe were run to determine what protein complexes might be present at these sizes in a non-denaturing gel.

CHROMATIN IMMUNOPRECIPITATION (ChiP) ASSAY

Chromatin immune-precipitations were performed based on the methods of (Xu el at., 2004). Briefly, mock and infected cells were infected at an MOI of 2 PFU/cell and harvested 24 hours after the infection. Cells were crosslinked with 1% formaldehyde for 15 min at room temperature and $1x10^7$ cells were resuspended in 750 µl of lysis buffer (50 mM Tris HCl [ph7.4], 150 mM NaCl, 1mM EDTA, 0.5%NP-40, 2 mM MgCl, 0.2 mM EDTA, and 10 µl of protease inhibitor cocktail/ml). Cell lysates were sonicated at 50% amplitude for a total of 10 minutes of sonication in cycles of 15 seconds ON and 30 seconds OFF using the Q800R Sonicator (Qsonica, Newtown, CT). Ten µl samples were run on a 1% agarose gel to check the size of fragmented DNA. Approximately 200 base pairs size single product was generated. Samples were used for immunoprecipitation or stored at -80°C. Immunoprecipitation was carried out with 0.5 µg of control or specific antibody followed by DNA elution and phenol:chloroform extraction according to the X-ChiP protocol (Abcam). For preliminary standardization experiments 0.5 µg of Elk-1

antibody and isotype matched control antibody were used for precipitation. PCR was carried out with forward primer 5'- GGAGCTCCGTCGCATG-3' reverse primer 5'-GGGACGTGAGATCCACG-3' to amplify a region between -901 to -695 containing the Elk-1 binding site from the m142 promoter. The primer pair (Forward 5'-GCAACGTGACCTTTAAAGCCTACTTTCCC-3' reverse 5'-TCAGACCGAAGACTGCGACGGTAC-3') amplifying the MIEP region of MCMV was used as a positive control. The previously described PABP primers, which map near the poly A tail, were used as a negative control. The qPCR for all three primer pairs were conducted under the conditions given in the PCR section.

STATISTICAL ANALYSIS

To examine differences in SEAP and intensity of shifted bands in EMSA, a Kruskal-Wallis analysis was conducted on SEAP values across each of the 10 constructs and shifted EMSA bands. To explore significant post hoc comparisons, Mann-Whitney *U* tests were conducted for each of the pair wise comparisons. These non-parametric analyses were used as they are considered appropriate alternatives to ANOVA for small sample sizes when standard errors of the mean are not equal across samples (231-234).

CHAPTER III

RESULTS

IDENTIFICATION OF DNA SEQUENCES IMPORTANT FOR THE m142 PROMOTER ACTIVITY IN UNINFECTED AND INFECTED CELLS

Initial analysis of the regulation of m142 showed that the m142 promoter can activate expression from a reporter gene, but did not identify the DNA sequences required for regulation (230). In order to identify important regulatory sequences, a series of nested deletion mutants of the m142 promoter were generated using restriction digestion and PCR. The series of deletion mutants are shown in Figure 6. The numbers indicate the location relative to the transcription start site (+1) (235).

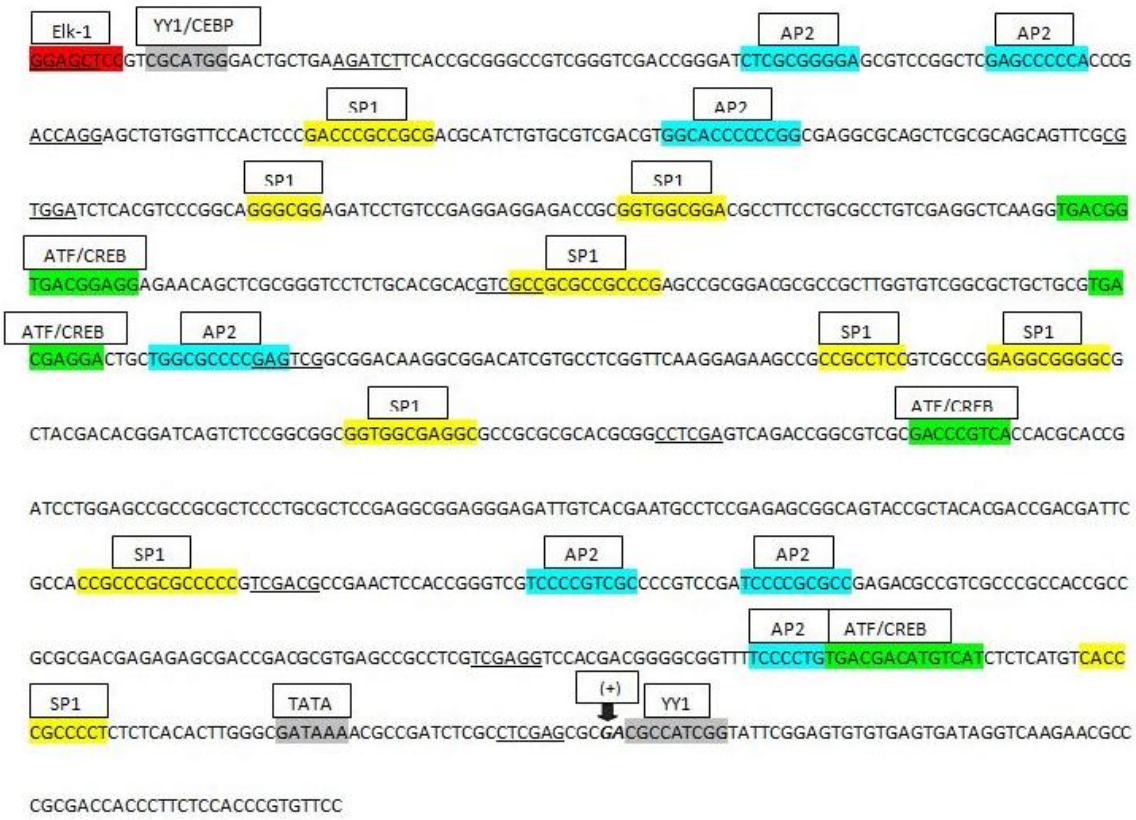


Figure 6. The m142 promoter sequence showing the exact location of transcription factor binding sites. The exact locations of transcription factor binding sites as previous shown in Figure 5A are highlighted here. The beginning of every deletion construct is represented by underlining the first 6 bases of the particular construct. Different consensus binding site are indicated by color: Red = Elk-1, Grey = YY1/CEBP, Blue = AP2, Yellow = SP1, Green = ATF/CREB.

The promoter constructs were cloned into reporter plasmid pSEAP2-Basic (Clontech laboratories, Mountain View, CA) controlling expression of the secreted alkaline phosphatase (SEAP) reporter gene (236). Replicates of transfected cells were treated with media (mock) or virus at a multiplicity of infection (MOI) of 2 plaque forming units/cell (PFU/cell) to identify regions required for regulation by cellular factors and viral factors respectively. For all experiments the relative SEAP activity was determined, normalized to the activity from mock infected cells transfected with pSEAP2-Basic plasmid (referred to as no promoter). The results of the deletion mutant analysis are shown in Figure 7.

In the absence of virus infection, only the full length -901 construct showed significant expression above basal level. Thus sequences between -901 and -806 are required for activation of the m142 promoter via cellular factors.

In the context of viral infection additional regions important for regulation of expression from the m142 promoter were identified. Deletion of -806 to -713 resulted in a significant increase in SEAP expression, indicating the likely presence of repressor binding site(s). The additional deletion of -713 to -579 resulted in a significant decrease in expression, consistent with the presence of possible activator binding site(s). Similarly, a 4 fold reduction in activity upon deletion of -504 to -377 indicates the presence of additional activator site(s). Complete loss of detectable activation was seen upon deletion of the region between -109 to -9 indicating that the minimal promoter sequences required for activation in the presence of viral infection are in this region.

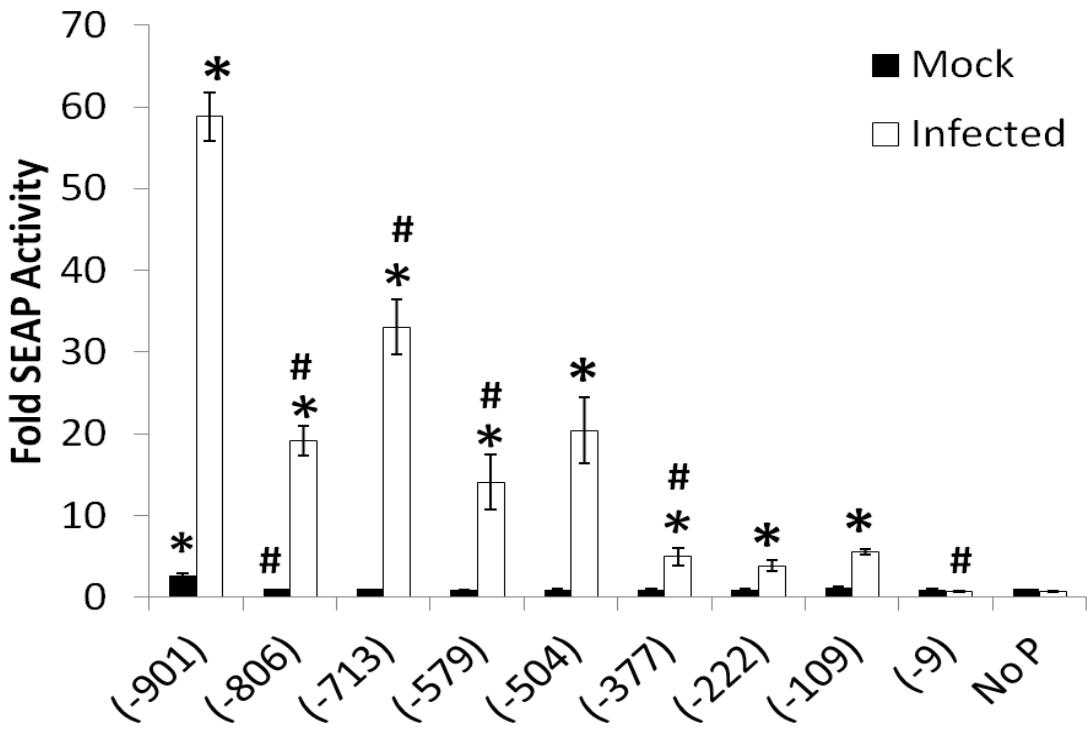


Figure 7. Reporter expression analysis of m142 deletion promoter constructs in mock and infected cells. NIH/3T3 cells were transfected with SEAP reporter plasmids containing the indicated promoter constructs and split into replica wells the next day. Twenty four hours later, replicate samples were infected with MCMV at an MOI of 2 PFU/cell or mock infected with media alone. Twenty four hours after infection supernatants were harvested for analysis of SEAP activity and cell-associated DNA was extracted and used to normalize for transfection efficiency. The numbers indicate the 5' end of the promoter relative to the transcriptional start site, No P = no promoter. The mean SEAP activity of mock sample with no promoter was set to one and fold increases are indicated. The black bars represent mock infected cells. White bars represent infected cells. n = 3 independent transfections. To examine differences in SEAP due to deletions, a Kruskal-Wallis analysis was conducted, * = significantly different expression as compared to mock no promoter ($p \leq 0.05$). To explore significant post hoc comparisons, Mann-Whitney *U* tests were conducted for each of the pair-wise comparisons. # = significantly different expression compared to the previous construct under the same conditions ($p \leq 0.05$).

To identify consensus sites present in the m142 promoter region, we analyzed the promoter sequence using the transcription element search system (TESS) (237) from the computational biology and informatics lab at University of Pennsylvania. A large number of consensus factor binding sites were detected in the m142 promoter. We decided to focus on transcriptional factor binding sites previously reported important in other herpesviruses. These sites are shown in Figure 6. As MCMV belongs to family herpesviridae, sites found important in other herpesviruses might be important for m142 promoter regulation as well.

The original study of m142 promoter regulation found an approximately 120 fold activity of the m142 promoter over no promoter in mock infected cells, with an approximately 3 fold increase in activity upon infection using the luciferase reporter plasmid pxp2 (230). As our results were distinctly different, with modest activation in the absence of infection and a 30 fold increase upon infection, we wanted to determine possible causes of this discrepancy. The m142 promoter used by Hanson et al. 2005 contained an extra 21 bases after the transcription initiation site (+74 to + 95) which were not present in our full length construct. Sequences within the transcribed region can regulate transcription (238, 239), so these 21 bases could be important. Alternatively, the pxp2 plasmid was originally generated from an SV40 promoter-containing construct, and could have cryptic promoter elements (240).

In order to distinguish between these possibilities we constructed a new -901 and -802 constructs with the 21 bps added. As shown in Figure 8, the addition of these bases did not significantly affect SEAP expression. Thus, it is more likely that the pxp2 reporter plasmid contains cryptic promoter element(s) which are absent from the more extensively tested SEAP expression plasmid used in the current studies.

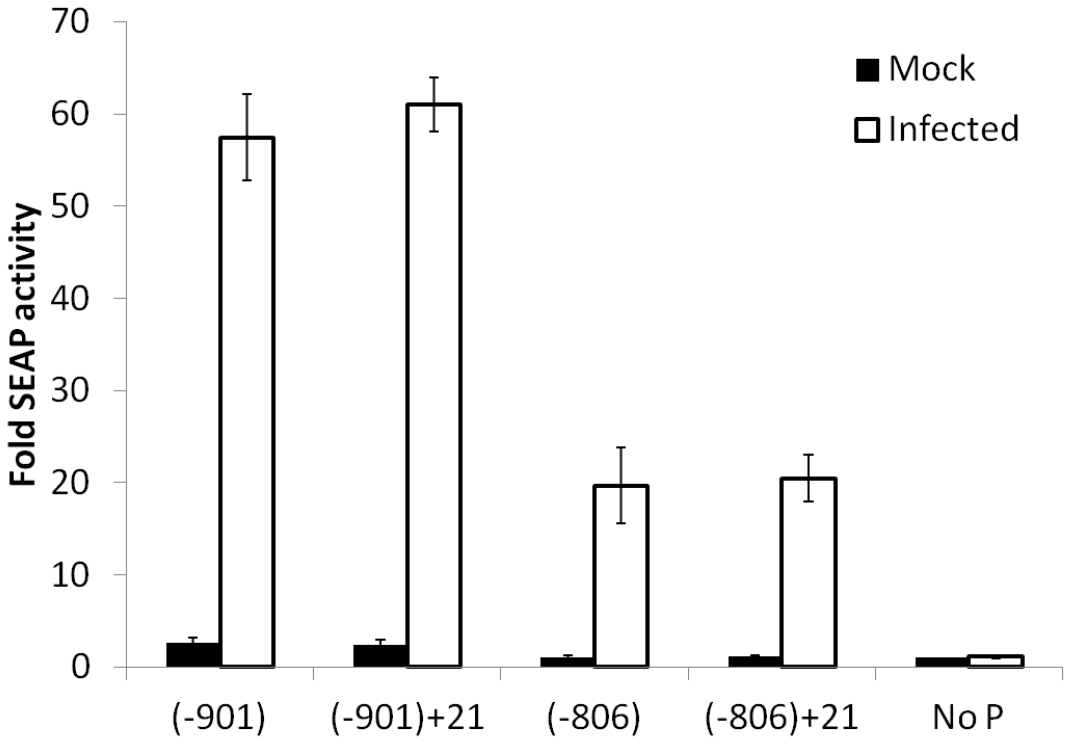


Figure 8. The region between +74 to +95 from the transcription start site is not involved in the transcription regulation of the m142 promoter. SEAP analysis was performed as described for Figure 7. The black bars represent fold SEAP activity in mock infected cells, white bars represent fold SEAP activity from infected cells. No P = no promoter. n = 3 independent transfections. ($p \leq 0.05$).

Cellular Activators Regulate the m142 Promoter via the Region Between -901 to -875

As seen in Figure 7, the region between -901 to -806 is important for activation of the m142 promoter in the absence of virus. As there are several consensus transcriptional regulatory binding sites in this region, we made use of a BglII site at -875 to narrow down the sequences that are important for this activation. Deletion of region -901 to -875 was sufficient to reduce detectable activation to background level in mock infected cells (Figure 9). In the context of infection, deletion of -901 to -875 resulted in a slight, but statistically significantly lower activity than the -806 construct. The slight difference between the -875 and -806 may indicate the presence of a repressor binding site.

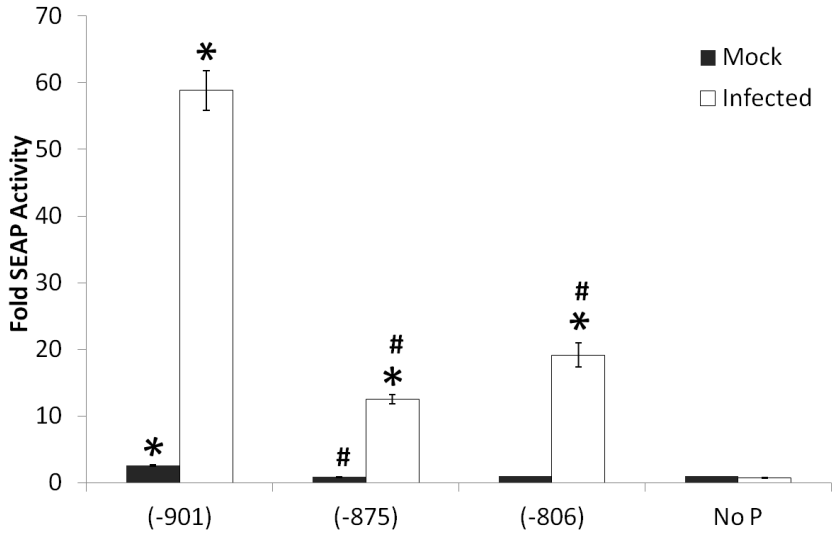


Figure 9. The region between -901 and -875 from the transcriptional start site is required for activation of the m142 promoter in uninfected cells. Analysis was as in Fig. 7 using the indicated plasmids. The black bars represent fold SEAP activity of mock infected cells and white bars represent fold SEAP activity of cells infected with MCMV. No P = no promoter. * = significantly different expression compared to mock ($p \leq 0.05$).

= significantly different expression compared to the next larger construct under the same conditions ($p \leq 0.05$). n = 3 independent transfections.

Identification of Sequences Involved in Protein Binding to the -901 to -875 Regulatory Region

As the region between -901 to -875 is required for detectable activation of the m142 promoter in uninfected fibroblasts, we set out to identify cellular factors binding to this region. Three consensus transcription factor binding sites are located in this region: an Elk-1 binding site, and overlapping YY1 and CEBP binding sites (Figure 10A). A labeled probe (-901 to -879) was designed and used for EMSA with nuclear extracts from mock and infected cells (Figure 10A). Two shifted bands were consistently detected, however the relative intensities varied (Figure 10B). With mock infected nuclear extracts the slower migrating band (designated band A) was more intense, while with infected samples the faster migrating band (designated band B) was stronger. In order to determine which sequences are important for the binding; we performed competition assays with unlabeled competitors: a non-mutated competitor, or with mutations in the YY1/CEBP sites (Figure 10B and 11) or the Elk-1 site (Figure 12 and 13).

The YY1/CEBP mutant competitor exhibited competition similar to the non-mutated competitor (Figure 10B), however, the competitor with a mutated Elk-1 site competed poorly for binding to the faster migrating band B (Figure 12). This supports that the Elk-1 site is important for regulation via this region, while the YY1 and CEBP

sites are probably not. However, as neither mutation resulted in failure to compete with band A, the sequences mutated are not required for binding of that complex.

A

Elk-1 YY1/CEBP

5’-GGAGCTCCGTCGCATGGGACTGCTGAAG-3’ Specific probe
5’-GGAGCTCCGTCG**TTG**GGGACTGCTGAAG-3’ YY1 mutant
5’-**TGC**GCTCCGTCGCATGGGACTGCTGAAG-3’ Elk-1 Mutant

B

NE/M(6 µg)	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-
NE/I (6 µg)	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
Specific Competitor	-	0	10X	100X	1000 X	-	-	-	0	10X	100 X	1000 X	-	-	-
Competitor with mutated YY1/CEBP site	-	-	-	-	-	10X	100 X	1000 X	-	-	-	-	10X	100 X	1000 X

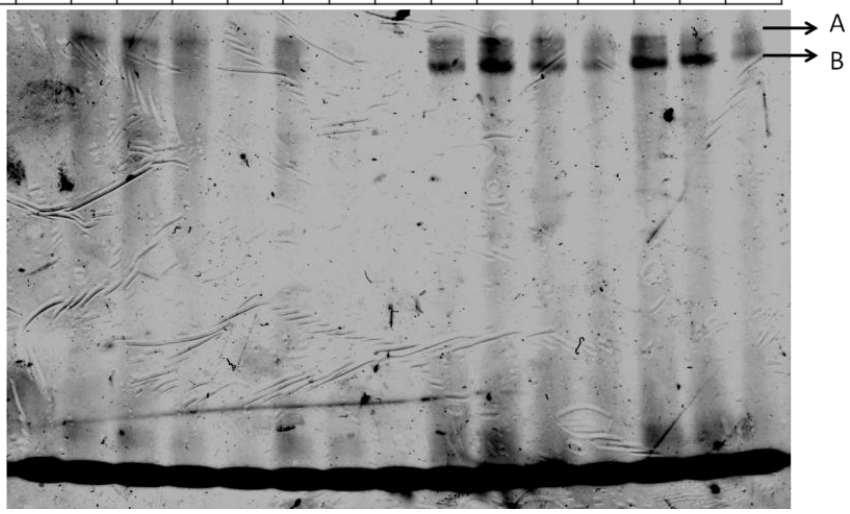


Figure 10. Mutations in the YY1/CEBP do not alter completion of binding to the -901 to -875 region of the m142 promoter. (A) Sequence used for EMSA. The sequences of probe and competitors used for EMSA are given. Consensus transcription factor binding sites are highlighted. The mutations which were introduced in the competitors are underlined. **(B) Mutation of the YY1 and CEBP sites failed to affect binding.** Six µg of nuclear extracts from mock (M) or infected (I) cells were mixed with the labeled probe and subjected to EMSA. Increasing concentrations of unlabeled specific or mutant competitor were added as indicated. NE – Nuclear Extract.

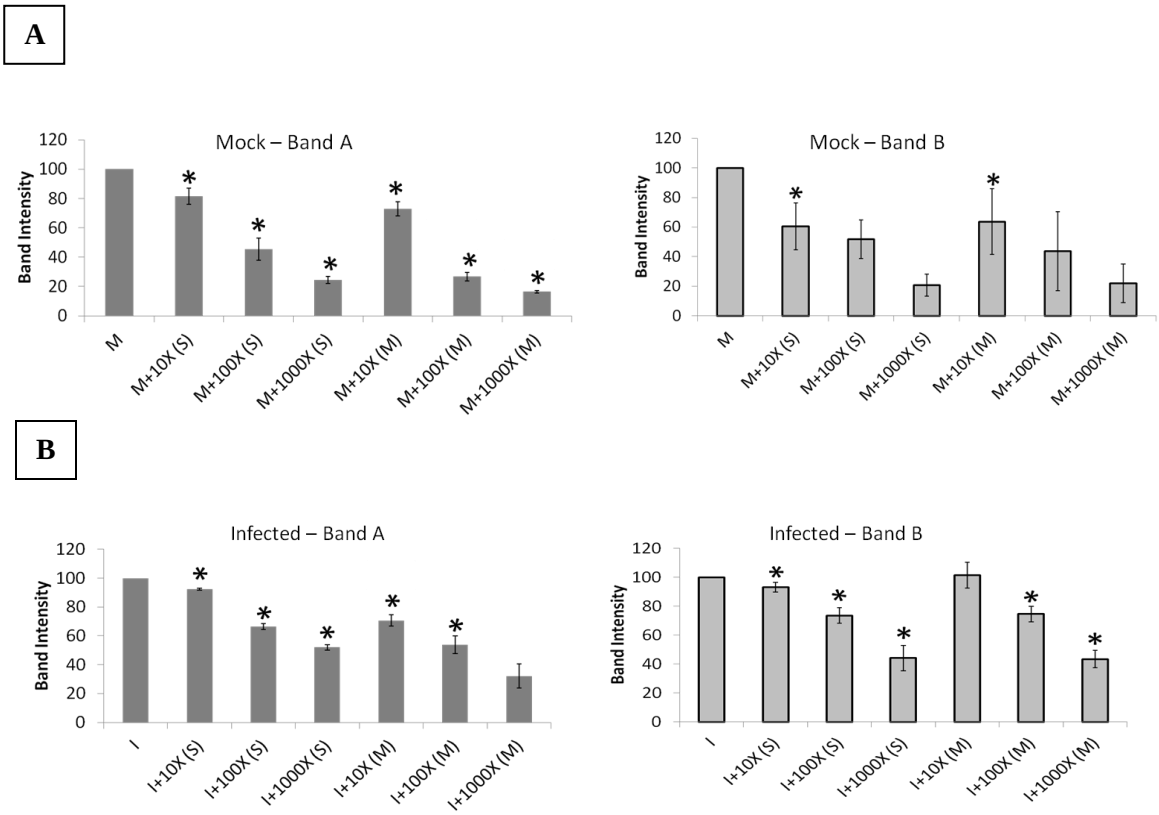


Figure 11. Densitometric analysis of bands in Figure 10B. Analysis was performed on a LiCor Odyssey CLx. Densitometric analysis was performed on EMSAs from 3 separate experiments and mean and standard error of the mean are shown. * = significantly different from previous lower concentration of unlabeled probe by Kruskal-Wallis test and Mann-Whitney pairwise comparisons, ($p \leq 0.05$). **(A) Densitometric analysis of bands from mock samples. (B) Densitometric analysis of bands from infected samples.** S = Unlabeled specific competitor and M = Unlabeled mutant competitor.

NE/M(6 µg)	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-
NE/I (6 µg)	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
Specific Competitor	-	0	10X	100X	1000 X	-	-	-	0	10X	100 X	1000 X	-	-	-
Competitor with mutated Elk-1 site	-	-	-	-	-	10X	100 X	1000 X	-	-	-	-	10X	100 X	1000 X

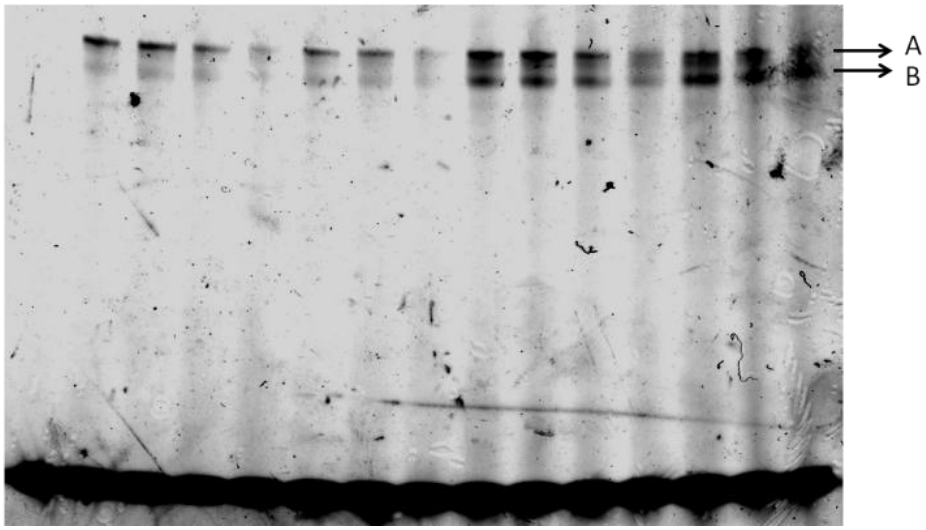


Figure 12. Mutation of the Elk-1 site impairs competition for binding the -901 to -875 region of the m142 promoter. Experiment was performed as in 10B using the Elk-1 mutant competitor. S = Unlabeled specific competitor and M = Unlabeled mutant competitor.

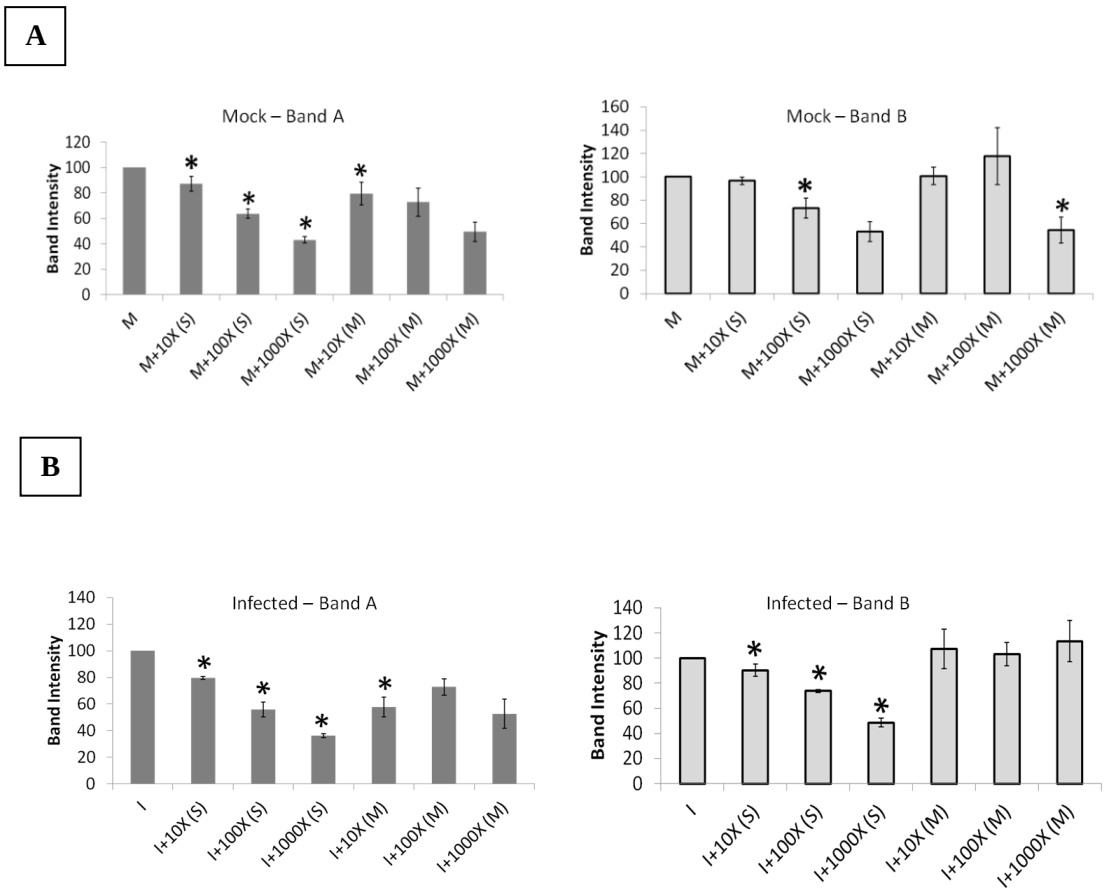


Figure 13. Densitometric analysis of bands in Figure 12. Analysis was performed on a LiCor Odyssey CLx. Densitometric analysis was performed on EMSAs from 3 separate experiments and mean and standard error of the mean are shown. * = significantly different from previous lower concentration of unlabeled probe by Kruskal-Wallis test and Mann-Whitney pairwise comparisons, ($p \leq 0.05$). **(A) Densitometric analysis of bands from mock samples. (B) Densitometric analysis of bands from infected samples.** S = Unlabeled specific competitor and M = Unlabeled mutant competitor.

A mutation in the Elk-1 consensus site, in the GGA triplet (241) lead to a loss of competition, we hypothesized that band B contains Elk-1 protein. In order to test this hypothesis, we performed a supershift assay with anti-Elk-1 antibody. As seen in Figure 14, addition of Elk-1 specific antibody resulted in decreased in intensity of band B with increased intensity of band A, which would be consistent with a supershift of band B to a size similar to band A. A similar effect was not seen with the control antibody, indicating that this is specific to anti-Elk-1 antibody. Taken together, the results of the competition and supershift analysis support that Elk-1 is a component of a complex which binds to this important activator region and may be involved in regulation of the m142 promoter. Attempts to confirm the association of Elk-1 with this promoter region in the context of the viral DNA by chromatin immunoprecipitation (ChIP) analysis were unsuccessful as the viral DNA non-specifically co-precipitated with the beads which was not true for the cellular controls (data not shown).

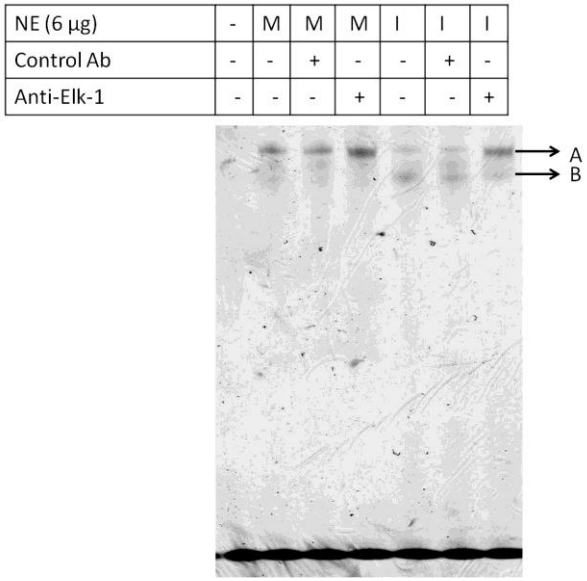


Figure 14. Supershift with anti-Elk-1 antibody in the -901 to -879 region of the m142 promoter. EMSAs were performed as in Figure 10B except that 3 µg of nuclear extract was used and where indicated the samples were pre-incubated for an hour with 1 µg of the indicated antibody.

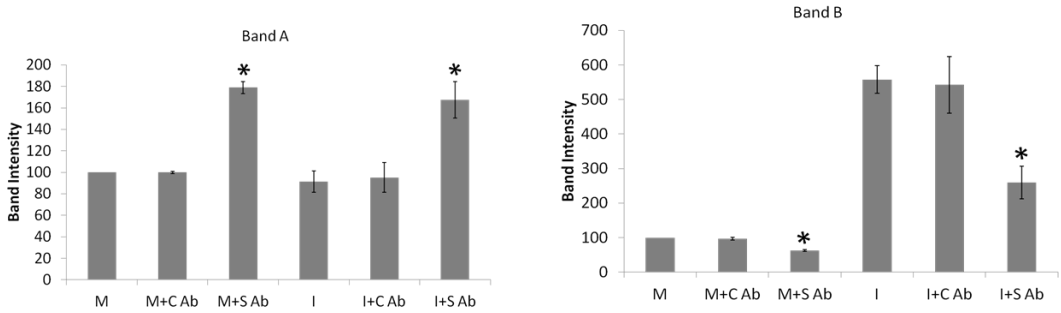


Figure 15. Densitometric analysis of bands in Figure 14. Densitometric analysis was performed on EMSAs from 3 separate experiments and mean and standard error of the mean are shown. * = significantly different from no antibody control by Kruskal-Wallis test and Mann-Whitney pairwise comparisons. ($p \leq 0.05$). M = Mock, I = Infected, C Ab = Control antibody, S Ab = Specific antibody.

Mass Spectrometric Analysis of Proteins Associated with the -901 to -875 Region of the m142 Promoter

In order to obtain more information about what is binding to this important regulatory region, we conducted mass spectrometry analysis of proteins present in band A and B of both mock and infected samples. The bands were cut out from the EMSA gel and samples submitted to the Protein Chemistry Technology Core of UT Southwestern Medical Center, although we were warned that as the bands could not be detected by Coomassie staining sensitivity might be an issue. No cellular transcription factors were detected in this analysis, which suggests mass spectrometry is not sensitive enough to detect Elk-1 or other important regulators under the conditions used. Interestingly, though most histones were detected in both bands, histone H2B type 1-F/J/L and histone 1.2 were not detected in infected sample band A but they were detected in mock samples. For band B, the same histones were detected in both mock and infected samples indicating that proteins binding in band A and band B may be interacting with different regions of the probe. In addition to cellular proteins, there were also viral proteins which were identified in the mass spectrometry analysis.

Table 3 shows a list of viral proteins detected in bands A and B of infected samples and not detected in the control. Brief information about the function of each identified viral protein is given in the second column.

Table 3. MCMV proteins associated with band A and B from the -901 to -875 probe

Viral protein	Function	In band
M36 protein	Prevent apoptosis (IE transcript) (242, 243)	A + B
M27 protein	Inhibit induction of IFN [antiviral responses] (E transcript) (244, 245)	A
M45 protein	Inhibits RIP activation and suppresses cell death (E protein) (246-248)	A
M142 protein	Prevent PKR mediated inhibition of protein synthesis (IE transcript) (249, 250)	A + B
M20 protein	Unknown function	A
M57 protein	Single stranded DNA binding protein (22)	A + B
M32 protein	Viral tegument protein which prevents CDK block of IE gene expression (251)	A
M140 protein	Capsid assembly (E transcript) (250, 252)	A + B
Immediate-early protein 1	Prevent repression via cellular repressors (IE transcript) (253)	A + B
M28 protein	Tegument protein, promotes IE gene expression (L transcript based on HCMV homolog) (254)	A + B
M80 protein	Protease (255)	A
M98 protein	Cleave DNA, required for DNA replication (256)	A
M102 protein	Primase Associated factor (E transcript) (257, 258)	A + B
M44 protein	Processivity subunit of DNA polymerase (E transcript) (22, 259)	A
M94 protein	Viral envelopment (L transcript) (260, 261)	B

THE ROLE OF VIRAL TRANSCRIPTIONAL REGULATORS IE1 AND IE3 IN REGULATION OF EXPRESSION FROM THE m142 PROMOTER

Virus infection is responsible for strong activation from the m142 promoter of MCMV (Figure 8). In previous studies using the pxp2 luciferase plasmid, activation of the full length m142 promoter was mediated by both viral transcription regulators, IE1 and IE3 (230). However, as our evidence supports the possibility of cryptic promoter sequences confounding the results of those studies with pxp2, and that study did not address what promoter sequences might be involved in the activation, we analyzed activation via IE1, IE3, or IE1 and IE3 together. IE1 and IE3 are splice variants, so can be expressed in combination from a plasmid containing the viral genomic sequence, or individually from plasmids from which either exon 4 (needed for IE1) or exon 5 (needed for IE3) have been removed (179).

All promoter constructs that were activated by viral infection were activated by IE1 and IE3 when expressed in combination, and the general pattern of activation was similar to infection (Figure 16). Thus IE1 and/or IE3 are important for activating the minimal promoter, and nothing else from the virus is absolutely required for this activation.

Examination of the activation by IE1 or IE3 alone showed us that they activate primarily though different regions. IE1 alone (Figure 17) weakly activated the minimal promoter and all larger constructs. IE3 alone could activate the full length promoter and

all constructs until the region between -377 to -222 was deleted, which abolished activation by IE3 (Figure 18). The IE3 may be involved in activation via region -377 to -222. The activation via IE1 or IE3 alone is minimal as compared to IE1 and IE3 co-transfection (Figure 16-18) indicating that IE1 and IE3 also work co-operatively or synergistically.

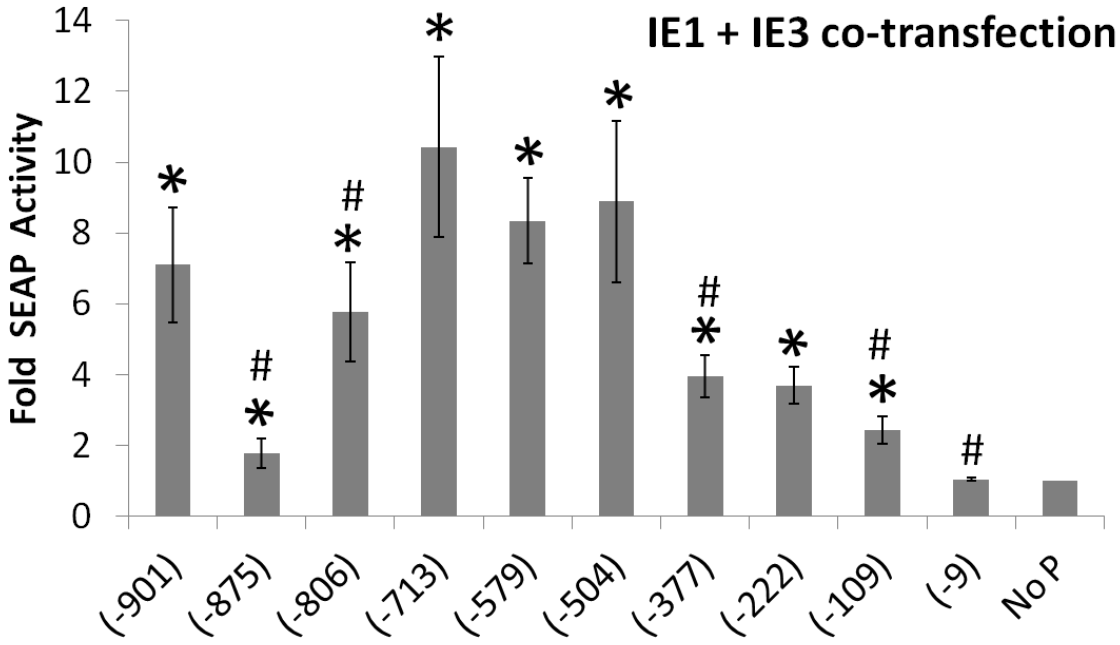


Figure 16. The viral immediate early proteins IE1 and IE3 activate deletion mutants of the m142 promoter in the absence of other viral proteins. Assays were performed as in Figure 7 for mock infection, except that cells were co-transfected with the reporter constructs and plasmids containing the entire IE gene region, to express both IE1 and IE3, or which express only IE1 or IE3. The ratio of reporter and IE plasmid was 1:1 in all experiments. * = significantly different expression compared to no promoter ($p \leq 0.05$). # = significantly different expression compared to the next larger construct. ($p \leq 0.05$). No P = no promoter. n = 3 independent co-transfections.

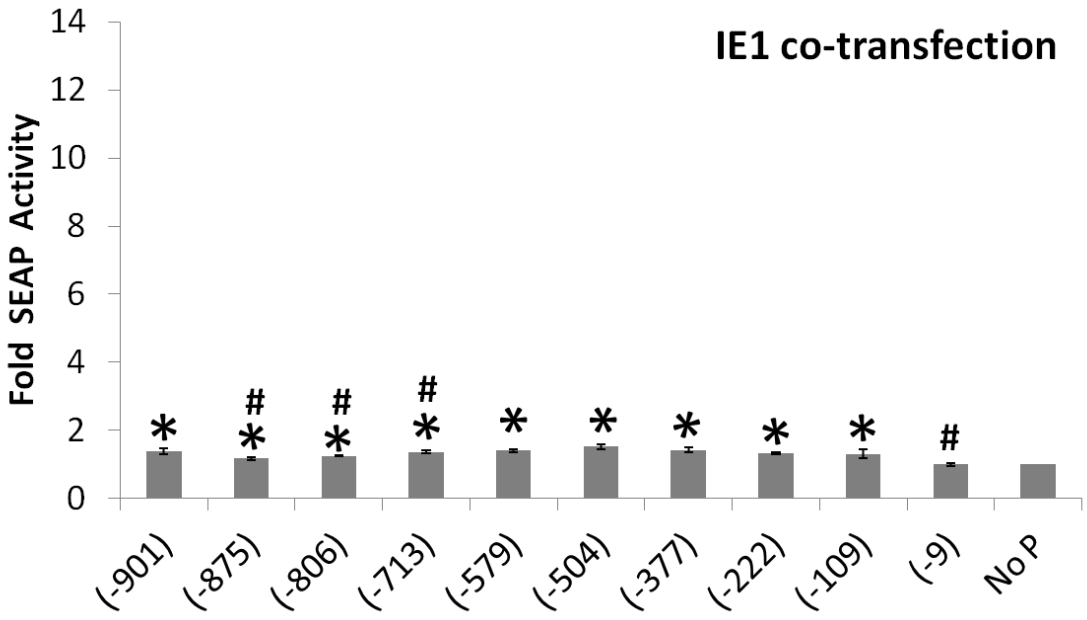


Figure 17. IE1 alone activates all m142 constructs containing the -109 minimal promoter. Assay was performed as in Figure 16. * = significantly different expression compared to no promoter ($p \leq 0.05$). # = significantly different expression compared to the next larger construct. ($p \leq 0.05$). No P = no promoter. n = 3 independent co-transfections.

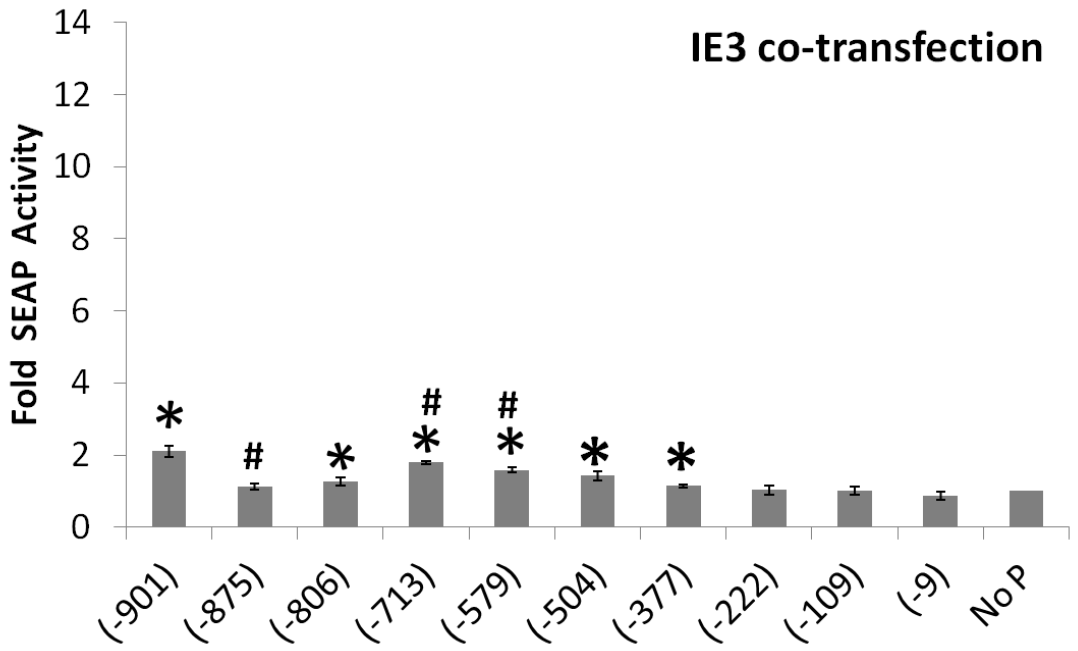


Figure 18. IE3 alone activates the m142 promoter via a different region than IE1 alone. Assay was performed as in Figure 16. * = significantly different expression compared to no promoter ($p \leq 0.05$). # = significantly different expression compared to the next larger construct. ($p \leq 0.05$). No P = no promoter. n = 3 independent co-transfections.

IE1 has been reported to sometimes have TATA Binding protein (TBP) associated factor (TAF) like activity (262) and the minimal promoter region between -109 to -9 is required for activation by IE1. This region between -109 and -9 has no consensus TATA box, but does contains an AT rich sequence located 23 bps upstream of the transcription start site (Figure 6) which might function as binding site for TBP (263). Gel shift analysis was performed to detect binding of cellular and/or viral proteins to a labeled probe containing the AT rich region (Figure 19B). A single shifted band (band 2, Figure 19B) was detected in mock sample whereas three shifted bands were detected in

infected sample (lane 9, Fig. 19B) although band 2 was very faint and inconsistent. Competition analysis with competitor containing a Kaposis sarcoma associated herpesvirus (KSHV) TATA box containing sequence (264) compared to cold consensus was performed (Figure 19B). Both competitors competed with labeled probe for binding with proteins in band C of mock and A and C of infected sample in a dose dependent manner. This supports that the region -37 to -13 may be important for binding of the basal transcriptional components.

A

5’-CACTTGGGCGATAAAACGCCGATC-3’ Probe sequence
5’-GCAGAGCATATAAAATGAGGTAGGA-3’ KSHV TATA competitor

B

NE/M(6 µg)	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-
NE/I (6 µg)	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
Specific Competitor	-	0	10X	100X	1000 X	-	-	-	0	10X	100 X	1000 X	-	-	-
KSHV TATA box competitor	-	-	-	-	-	10X	100 X	1000 X	-	-	-	-	10X	100 X	1000 X

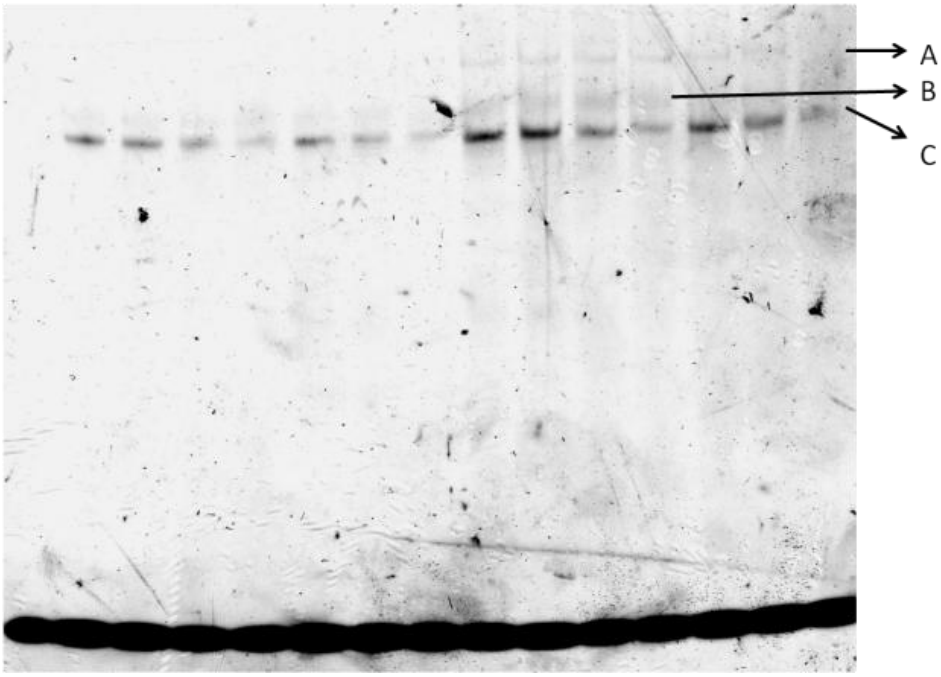
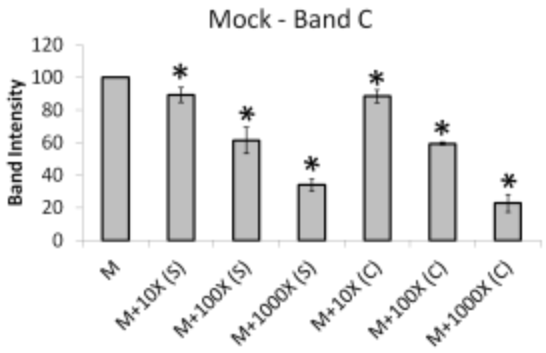


Figure 19. Binding to the AT rich region in the minimal promoter (-37 to -13) is competed by a consensus TATA box. Samples were prepared as explained in Figure 10. (**A) Sequence of probe.** The AT rich region is indicated in grey. (**B) Competition assay for -37 to -13 labeled probe with specific competitor or a consensus TATA-box containing competitor.**

A



B

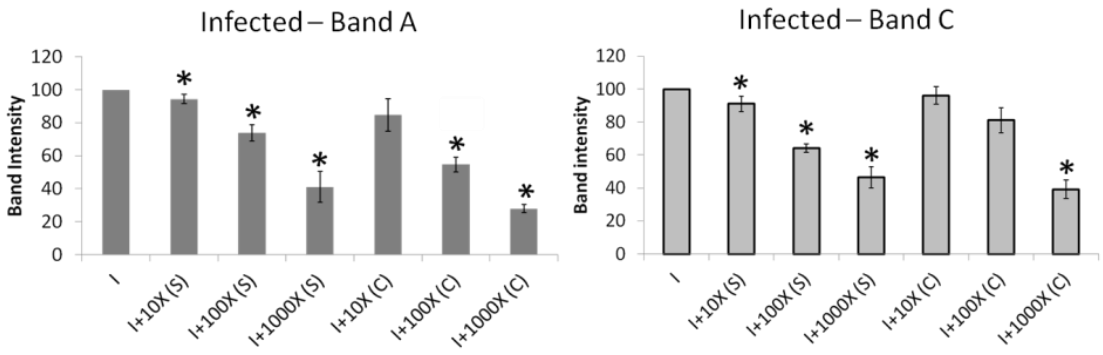


Figure 20. Densitometric analysis of bands from Figure 19B. * = significantly different from previous lower concentration of unlabeled probe. n = 3. S = Specific competitor, C = Consensus competitor, ($p \leq 0.05$).

To evaluate the important of the AT rich region of for binding another competition assay was performed using a mutant AT rich competitor (Figure 21). Mutant probe competed for binding with labeled probe in mock samples in a dose dependent manner (Figure 21B and 22A), however in infected samples, competition was less efficient, no longer being dose dependent (Figure 21B and 22B). The loss of competition at higher concentrations of competitor in the context of viral infection suggests utilization of the AT rich region in the context of viral infection.

A

5’- CACTTGGGCGATAAAACGCCGATC-3’ Probe sequence
5’-CACTTGGGCGAT**CG**AACGCCGATC-3’ Mutated competitor sequence

B

NE/M(6 µg)	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-
NE/I (6 µg)	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
Specific Competitor	-	0	10X	100X	1000 X	-	-	-	0	10X	100 X	1000 X	-	-	-
Competitor with mutant AT rich site	-	-	-	-	-	10X	100 X	1000 X	-	-	-	-	10X	100 X	1000 X

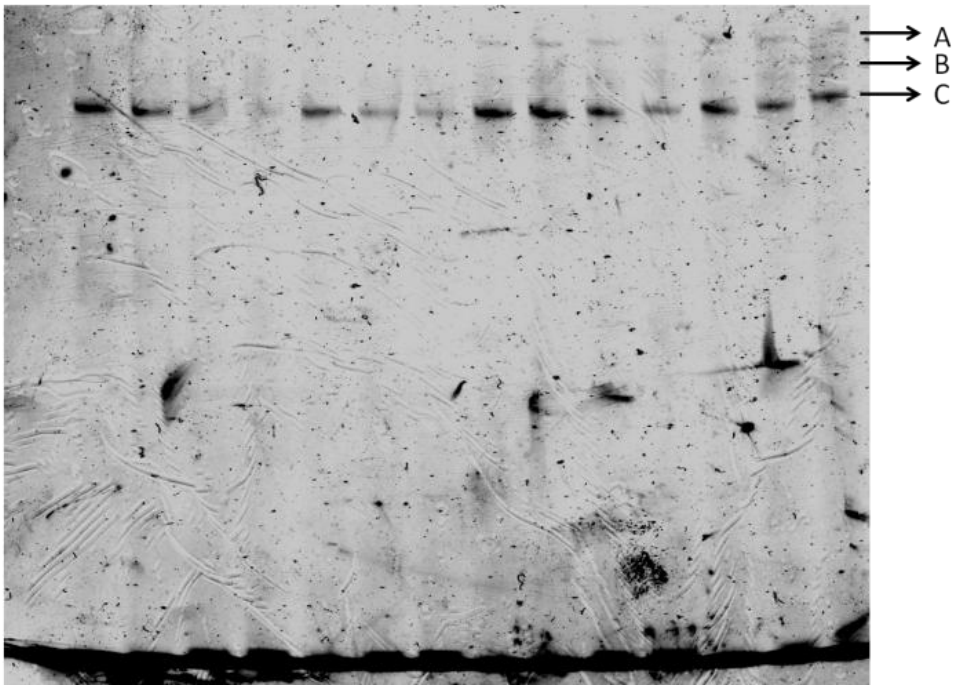


Figure 21. Analysis of importance of the AT rich sequence of -37 to -13 probe using unlabeled mutated competitor. (**A) Sequences of probe and competitor. (B) EMSA with wild-type and mutant competitor.** Samples were prepared and analyzed as in Figure 10 using the indicated probes and competitors.

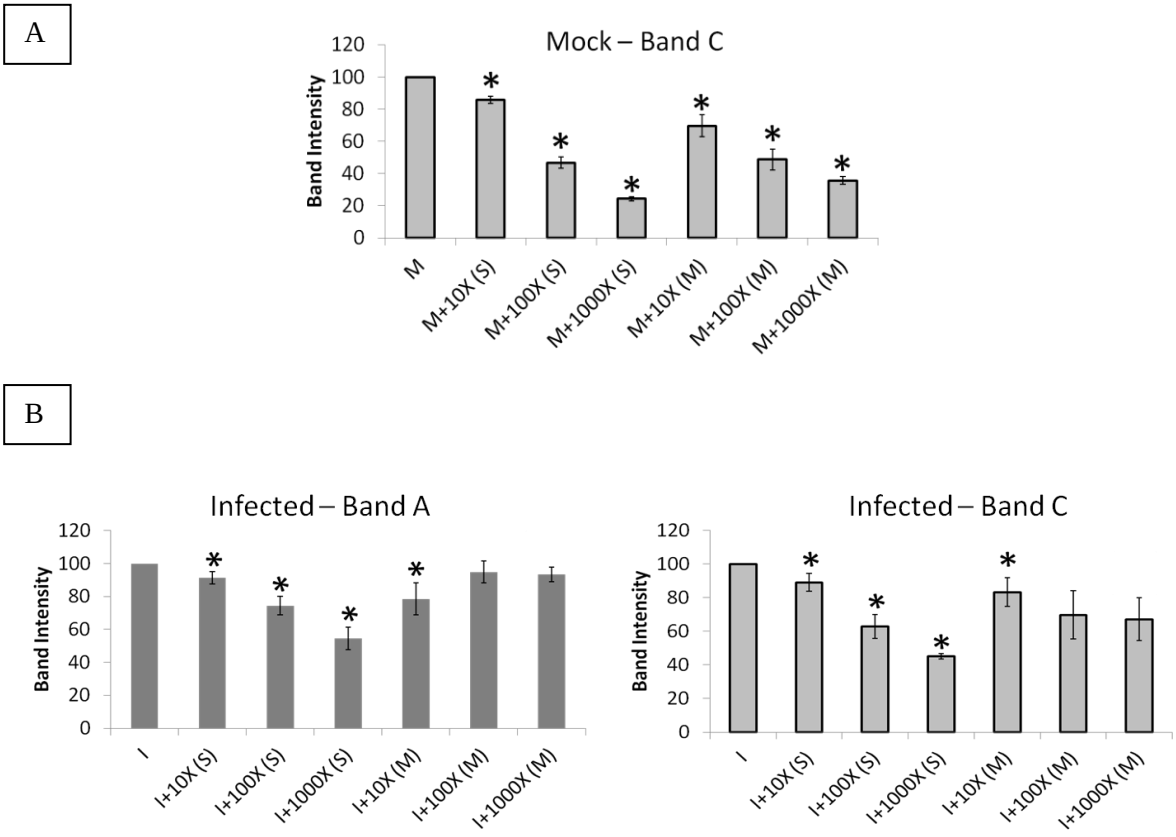


Figure 22. Densitometric analysis of bands in Figure 21B. n = 3. * = significantly different from previous lower concentration of unlabeled probe. S = Specific competitor, M = Mutant competitor. ($p \leq 0.05$).

Failure of Anti-TBP Antibody to Supershift

Since the competition by the consensus TATA competitor indicated that basal transcriptional components interacted with the -37 to -13 probe, we performed a supershift assay with anti-TBP antibody to see if it was present (Figure 23). No supershift was detected indicating that either TBP is not part of the complexes, or the complex is such that the sites recognized by this antibody are not accessible.

NE (3 µg)	-	M	M	I	I
-37 to -13 Probe	+	+	+	+	+
Control IgG1	-	+	-	+	-
Anti-TBP	-	-	+	-	+

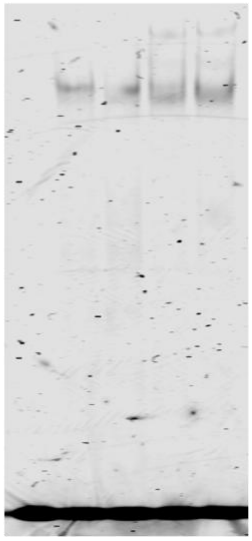


Figure 23. Supershift was not detected with anti-TBP antibody for the AT rich regin of the minimal promoter -37 to -13. Samples were prepared as in Figure 14. The amount of nuclear extract (3 µg) used was standardized during preliminary experiments.

SP1 Binding Sites in the m142 Promoter

The region between -504 and -377 contains activator binding site(s). This region contains three consensus SP1 binding sites (Figure 6). As the HCMV homolog of IE3 can co-operate with SP1 in promoter activation (265), and the combination of IE1 and IE3 could activate via this region it is possible that these sites are important for this regulation. We designed two labeled probes -459 to -428 and -409 to -386 containing two and one SP1 binding sites respectively.

The mock or infected NEs were analyzed with EMSA to detect binding with these probes (Figure 24). For both probes a single band C was detected after incubation with extracts from mock infected cells. The migration was slightly faster for the smaller probe -409 to -386 (23 bps) (lane 2, Figure 24) than for the larger probe -459 to -428 (31 bps) (lane 5, Figure 24), so it is possible that these are similar complexes and difference in migration is due to the difference in the size of probe. For both probes, incubation with infected cell lysates resulted in 2 additional slower migrating bands (A and B), and a much faster migrating band D. The fact that the same pattern was seen lends more support to the possibility that these are similar complexes.

NE	-	M	I	-	M	I
-459 to -428 probe	+	+	+	-	-	-
-409 to -386 probe	-	-	-	+	+	+

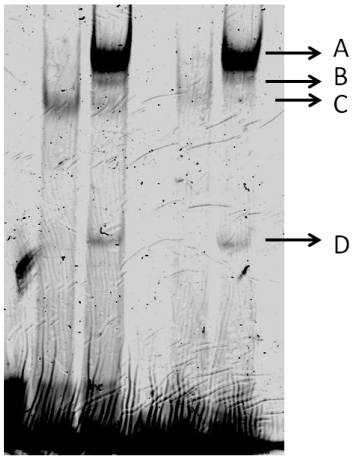


Figure 24. Gel shift analysis of probes containing SP1 consensus binding sites from a major activator binding region (-504 to -377) of the m142 promoter. Samples were prepared as described in Figure 10B except that labeled probes -459 to -428 and -409 to -386 were used.

There were dramatic changes in binding in the context of viral infection to both of these probes. Cellular proteins SP1, SP2, and SP3 can bind to the same consensus DNA binding site (185, 266) so we evaluated steady state levels of SP1, SP2, and SP3 in mock and infected NIH/3T3 cells. All three proteins were detected in both mock and infected cell lysates (Figure 25).

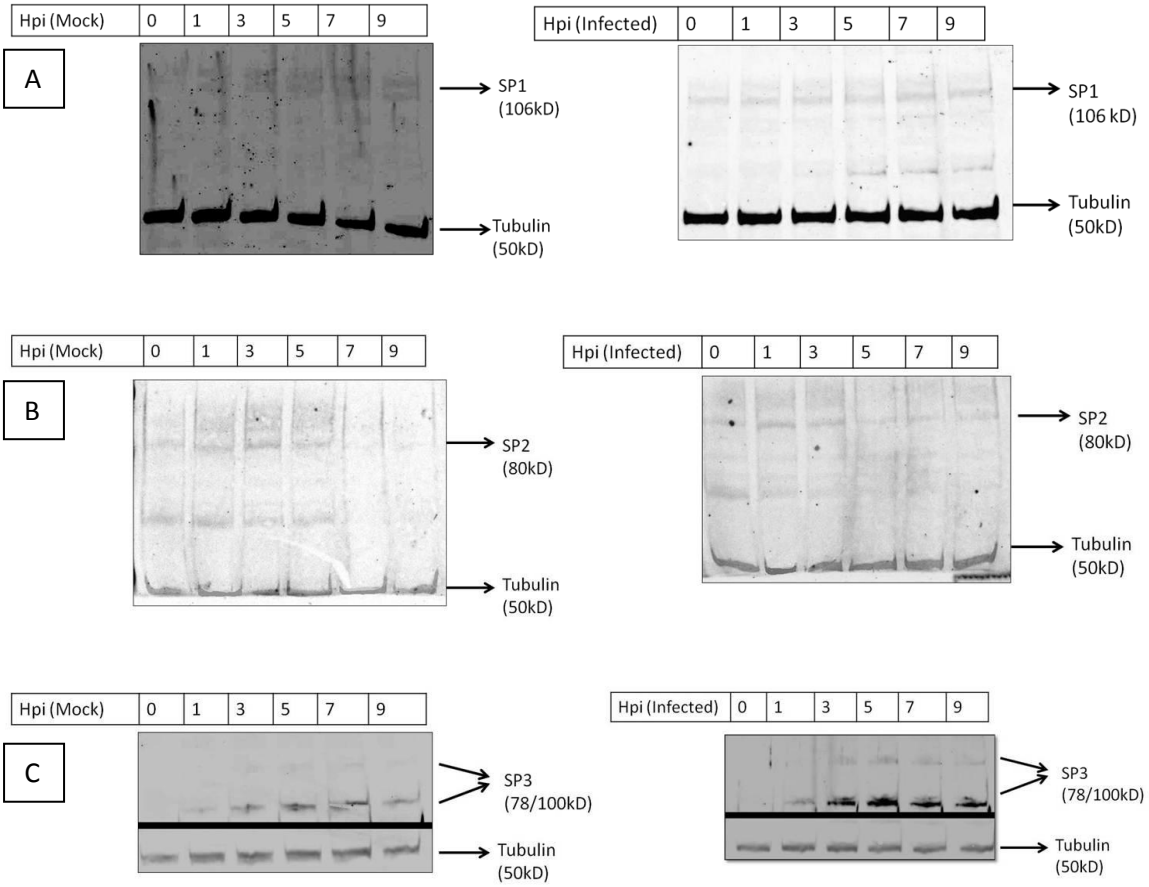


Figure 25. Cellular transcription factors SP1, SP2, and SP3 were expressed in mock and infected NIH/3T3 cells. $1x10^6$ cells were infected with virus at MOI of 2 PFU/cell and whole cell extracts were harvested at the indicated times post infection. Mock = uninfected cells. Sixty µg of total protein per sample was subjected to SDS-PAGE on a 10% gel before transfer to nitrocellulose membrane, and detection. **(A) SP1 expression.**

Blots were probed with mouse anti-SP1 and mouse anti-tubulin followed by goat anti-mouse (IRDye® 700) before scanning on the Odyssey CLx. **(B) SP2 expression.** Blots were probed with rabbit anti-SP2 and mouse anti-tubulin followed by goat anti-rabbit (IRDye® 800) and goat anti-mouse (IRDye® 700). **(C) SP3 expression.** Blots were probed with mouse anti-SP3 and mouse anti-tubulin followed by goat anti-mouse (IRDye® 700).

As all three, SP1, SP2, and SP3 were expressed in our cells, supershift experiments were conducted with SP1, SP2, and SP3 antibody to evaluate the presence of these proteins in the shifted bands. Under the conditions used, supershift was not detected with any antibody for either probe (Figure 26-29). SP1, SP2, and SP2 may not be part of any of the complexes binding to these probes. Alternatively, although these antibodies have previously been used successfully for supershift, other components in these specific complexes potentially could block the antibody binding sites.

NE	-	M	I	M	I	M	I	M	I
-409 to -386 probe	+	+	+	+	+	+	+	+	+
Control IgG1 Ab	-	+	+	-	-	-	-	-	-
Anti-SP1 Ab	-	-	-	+	+	-	-	-	-
Anti-SP2 Ab	-	-	-	-	-	+	+	-	-

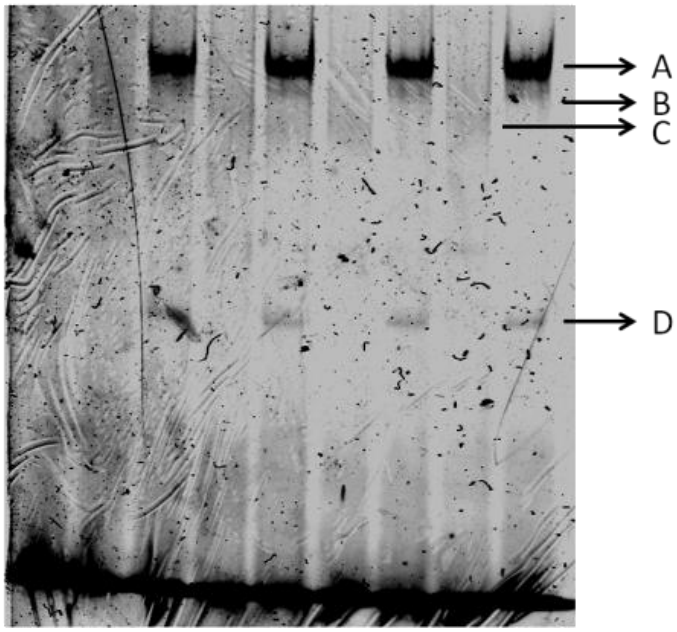


Figure 26. Supershift was not detected with SP1 and SP2 antibody for -409 to -386 labeled probe containing consensus SP1 sites. Samples were prepared as in Figure 14.

NE	-	M	I	M	I	M	I	M	I
-459 to -428 Probe	+	+	+	+	+	+	+	+	+
Control Ab	-	+	+	-	-	-	-	-	-
Anti-SP1 Ab	-	-	-	+	+	-	-	-	-
Anti-SP2 Ab	-	-	-	-	-	+	+	-	-

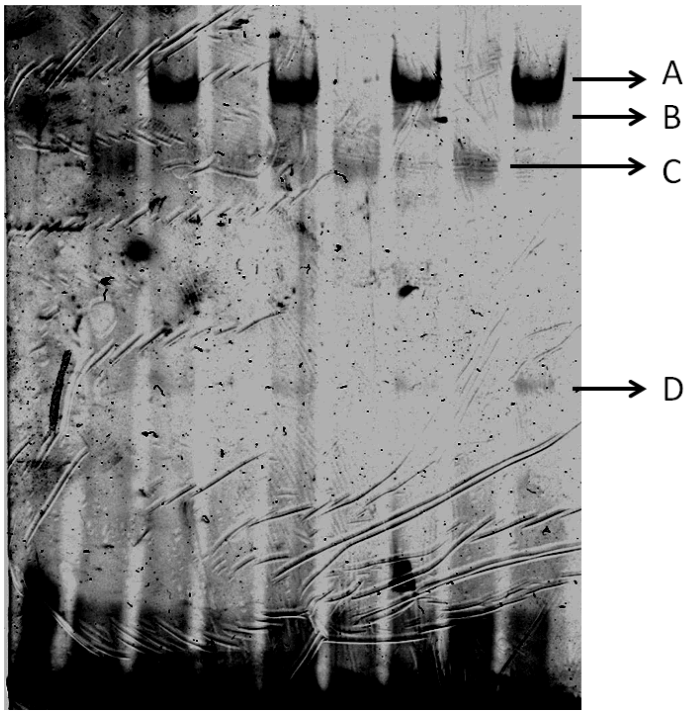


Figure 27. Supershift was not detected with SP1 and SP2 antibody for -459 to -428 labeled probe containing consensus SP1 sites. Samples were prepared as in Figure 14.

NE	-	M	I	M	I	M	I
-459 to -428 Probe	+	+	+	+	+	+	+
Control Ab	-	+	+	-	-	-	-
Anti-SP3 Ab	-	-	-	+	+	-	-

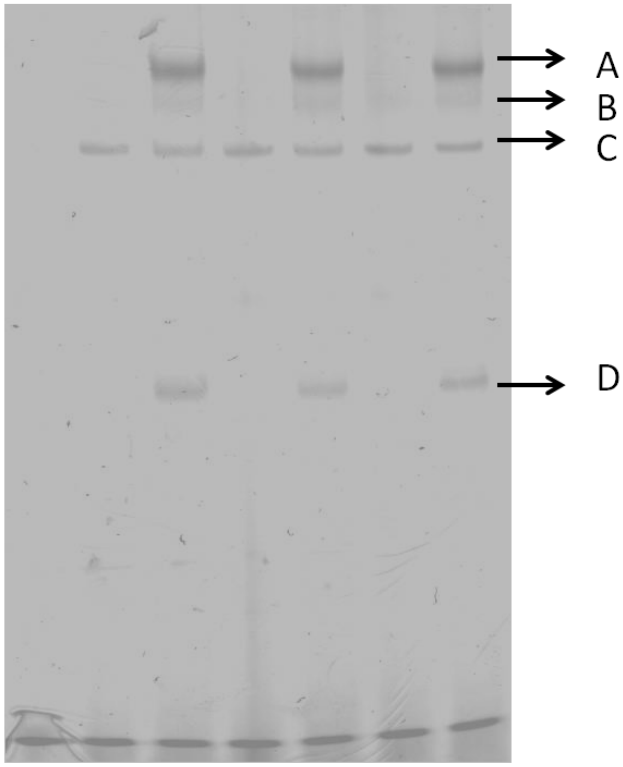


Figure 28. Supershift was not detected with SP3 antibody for -459 to -428 labeled probe containing consensus Sp1 sites. Samples were prepared as in Figure 14.

NE	-	M	I	M	I	M	I
-409 to -386 probe	+	+	+	+	+	+	+
Control Ab	-	+	+	-	-	-	-
Anti-SP3 Ab	-	-	-	+	+	-	-

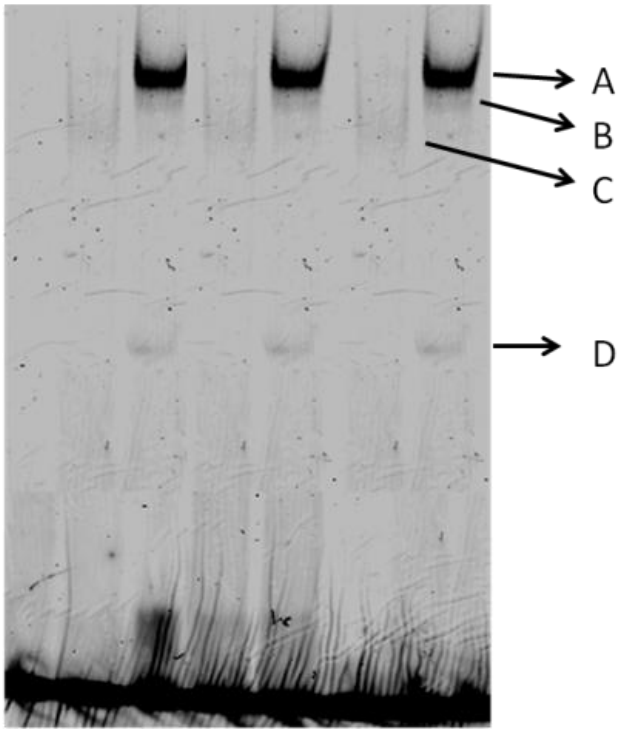


Figure 29. Supershift was not detected with SP1 and SP2 antibody for the -409 to -386 labeled probe containing consensus Sp1 sites. Samples were prepared as in Figure 14.

OTHERS FACTORS REGULATING THE m142 PROMOTER

Late Viral Proteins Are Not Involved in Regulation of Expression from the m142 Promoter

As viral infection induced resulted in an approximately 60 fold activation of the m142 promoter over background, while the IE1 and IE3 co-transfection resulted in only an approximately 7 fold increase (Figure 7 and 16 respectively), it suggests that viral proteins other than IE1 and IE3 could be involved. We analyzed the effect of the absence of late viral proteins on expression from the m142 promoter deletion constructs to determine the importance of late proteins in the regulation. Phosphonoformic acid prevents viral DNA replication which is required for the expression of late genes (267). No significant difference in expression from the m142 promoter constructs was detected indicating that late proteins are not required for activation or repression of the m142 promoter.

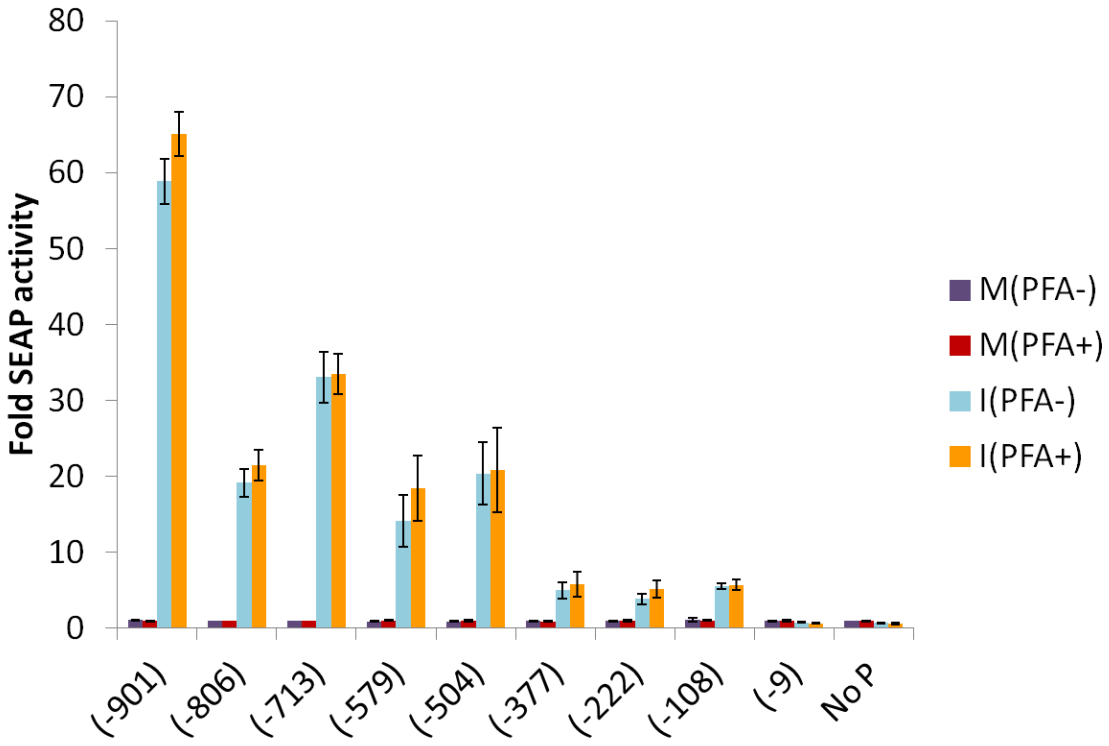


Figure 30. Late gene products are not required for activation or repression of the m142 promoter. NIH/3T3 cells were transfected with the SEAP reporter plasmids containing the indicated promoters and infected as in Figure 7 except that replica plates received either phosphonoformic acid (PFA) to a final concentration of 300 µg/ml or complete media as a control. Twenty four hours after infection supernatants were harvested and analyzed as in Figure 7. No P = no promoter. Blue bars = mock infected cells. Brown bars = mock with PFA. Green bars = infected. Purple bars = infected with PFA. Samples were tested in triplicate and the mean and standard error of 3 separate experiments is shown. Differences were analyzed as for Figure 7 and no significant differences between PFA treated and control treated samples were detected. ($p \leq 0.05$).

Viral Attachment and Entry Is Not Sufficient to Activate the m142 Promoter

We were interested in finding out the effect of virus attachment and entry on the activation of the m142 promoter. During the process of viral attachment various intracellular pathways are activated leading to altered cellular transcription factor levels and viral tegument proteins also can affect transcription (103, 268-272). To see whether

viral tegument proteins or altered gene expression due to virus binding were involved, in the activation of the m142 promoter we used UV inactivated virus.

For initial testing, we selected the -713 construct because this had the highest level of activation without the confounding factor of basal activation seen with the full-length promoter. Cells transfected with the -713 construct and no promoter control were mock infected, infected at an MOI of 2, or treated with an equal amount of UV inactivated virus from the same virus preparation as used for infection. There was no significant difference in the expression from mock infected cells and cells treated with UV inactivated virus (Figure 31). Hence, virus attachment, entry, and viral tegument proteins from the virus were not sufficient to activate the m142 promoter.

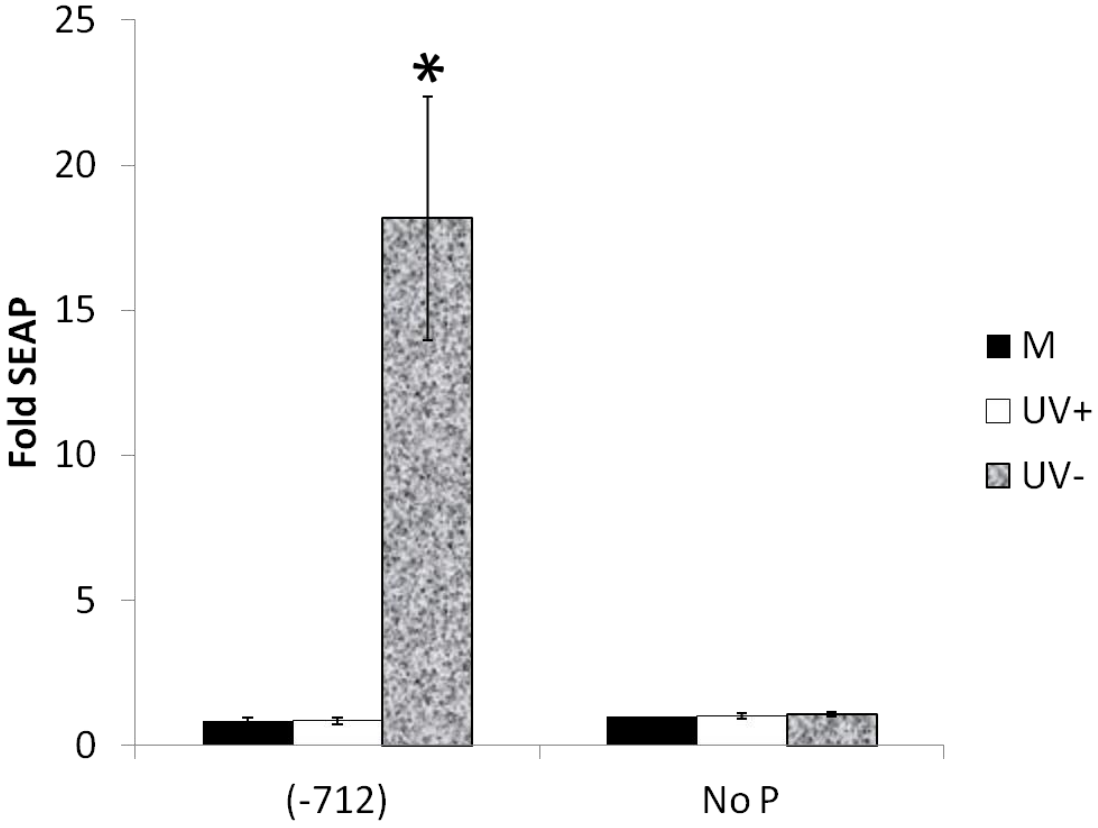


Figure 31. Viral attachment and entry is not sufficient to activate the m142 promoter over background. Cells were transfected as in Figure 7 with plasmid -713 and either infected with MCMV at an MOI of 2 PFU/cell or treated with an equivalent amount of UV inactivated virus from the same viral preparation. No P = no promoter. Cells were harvested and analysis was performed as in Figure 7. n = 3.

Role of AP2 Site in m142 Promoter Regulation

From the deletion analysis, the region between -806 to -713 was involved in apparent repression of the m142 promoter. This region contains one potential SP1 binding site and a potential AP2 binding site. AP2 is a known repressor as well as activator of various viral and cellular promoters (273-275). In addition, the region between -875 and -806 also has AP2 sites and the difference in activity between these

constructs again supported possible repressor binding activity. Hence we analyzed binding of proteins from mock and infected NEs to the region containing the consensus AP2 site, -757 to -733. There was no detectable binding to this probe with nuclear extracts from mock infected cells, while two shifted bands were detected with infected NEs (Figure 32B, lanes 2 and 6). Addition of a competitor containing a consensus AP2 site, but otherwise unrelated in sequence resulted in a dose-dependent reduction in binding in the very strong band A of infected NE but a similar dose dependent effect was not detected in band B (Figure 32B). This data supports that the AP2 sequence may be involved in binding of proteins from infected NEs.

A

5’-CGACGT**GGCACCCCCCGG**CGAGGC-3’ Probe sequence
5’-GATCGAACTGA**CCGCCCG**CGGCCC GT-3’ AP2 Consensus competitor

B

NE (6ug)	-	M	M	M	M	I	I	I	I
-757 to -733 Probe	+	+	+	+	+	+	+	+	+
AP2 Unlabeled Consensus Competitor	-	0	10X	50X	100 X	0	10X	50X	100 X

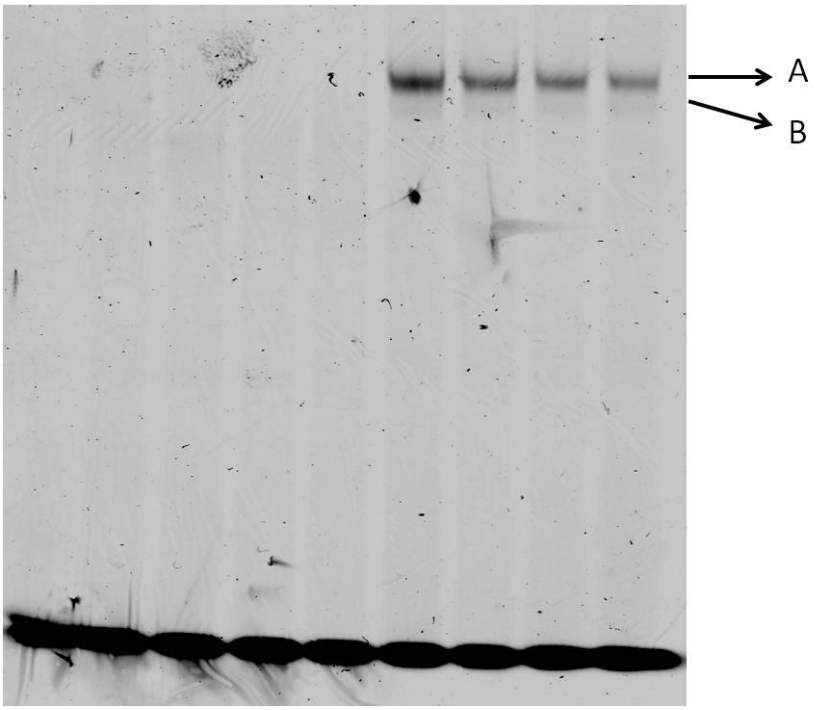


Figure 32. AP2 consensus competitor competes for binding the m142 -757 to -733 probe containing a consensus AP2 site. Samples were prepared and analyzed as in Figure 10. **(A) Sequence of probe and competitor.** The consensus AP2 sites are highlighted. **(B) Competition assay with increasing concentrations of unlabeled AP2 consensus competitor.**

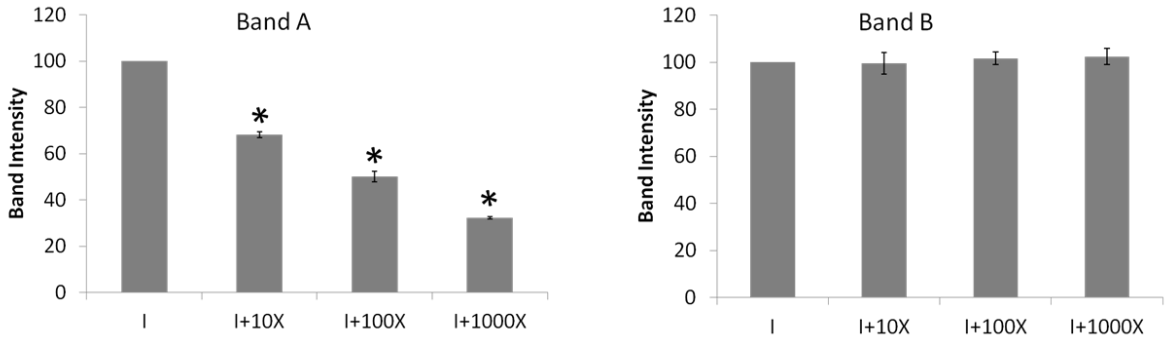


Figure 33. Densitometric analysis of bands in Figure 32B. Samples were analyzed as described in Figure 11.

Because the addition of specific competitor containing an AP2 consensus binding site resulted in a dose dependent decrease in binding, we hypothesized that AP2 is a part of the complex binding to the m142 -757 to -733 region. There are five isoforms of AP2 (α, β, γ, δ, and ε). They all can bind to the AP2 binding site. In Figure 34, supershift analysis was performed with rabbit polyclonal TFAP2B antibody. This antibody was chosen because it had previously been shown that TFAP2B is expressed in NIH/3T3 fibroblasts (company website). No evidence for supershift was detected under the conditions used for this assay. It is possible that other AP2 isoforms are part of the complex.

NE	-	M	M	M	I	I	I
-757 to -733 Probe	+	+	+	+	+	+	+
Control Ab	-	-	+	-	-	+	-
Anti-AP2 Ab	-	-	-	+	-	-	+

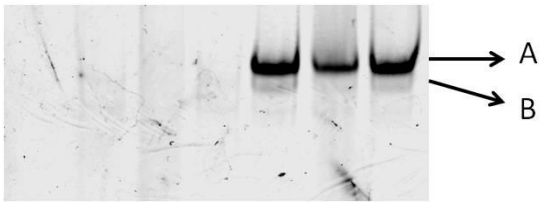


Figure 34. Rabbit anti- TFAP2B antibody failed to supershift the band obtained with -757 to -733 probe. Six µg of NE was used and samples were prepared as in Figure 14 except the labeled probe -757 to -733 was added.

CHAPTER IV

DISCUSSION

The m142 gene is an essential gene of MCMV. The m142 protein is required for inhibition of PKR mediated shutdown of protein synthesis during viral infection (171, 276). The structural and functional homologues of the m142 protein in HCMV are IRS1 and TRS1. Similar PKR mediated inhibition of protein synthesis is inhibited by these proteins in HCMV (171). Because the m142 protein is essential for MCMV, understanding factors, especially viral proteins, regulating expression from the m142 promoter could be helpful for developing new antiviral therapies. Not much is known about other IE promoters of either HCMV or MCMV except MIEP. The fact that they are IE suggest that there might be some similarities. Understanding m142 promoter regulation could provide information to predict the promoter regulation of IRS1 and TRS1 of HCMV. MCMV m142 and HCMV IRS1/TRS1 have similar kinetics of expression and are also lower abundance IE proteins than the genes controlled by MIEP. The lower abundance suggests that may be there are differences in regulation between the MIEP and IE promoters. The HCMV MIEP is one of the most frequently used promoters in plasmids for transient transfection, but the high level of expression is sometimes a problem, hence other viral IE promoters which mediate lower levels of expression could be potentially useful. It is important to know the size of the promoter, important

regulatory sequences, and regulatory proteins in order to select any promoter to express target genes.

The initial study done by Hanson el al showed the m142 promoter could activate luciferase reporter gene expression (230). The activation could be detected in both the absence and presence of viral infection, indicating that the m142 promoter likely contained significant sequences for regulation as an immediate early gene and would be useful for identifying important regulatory sequences/regions. In this study we have built on work done by Hanson et al to identify sequences involved in the regulation both in the presence and absence of viral infection. As the average promoter size is around 300-500 bp (277-279), but many herpesvirus IE promoters are longer (280, 281), the m142 promoter (around 1000 bps) is not an exception to this characteristic observed in other herpesvirus IE promoters.

We were able to identify five major regulatory regions as shown in Figure 25. One of these, -901 to -875, was required for detectable promoter activity in the absence of viral infection. This region contains three consensus binding sites: Elk-1 and YY1 overlapping with CEBP and our results of EMSA with mutant competitors and supershift analysis indicated that the Elk-1 site was likely to be important. When supershift was performed with anti-Elk-1 antibody on the MIEP of primate cytomegalovirus, supershift was detected in some but not all bands (221). As an activator function of Elk-1 was detected for the MIEP of HCMV (282). Elk-1 has been shown to activate the MIEP of

HCMV, a primate CMV, and now we have shown that it can activate a "minor" immediate early promoter m142, suggesting that Elk-1 may have a more general role in IE promoters than just the MIEP. In addition, supershift analysis with anti-Elk-1 antibody for the primate MIEP had similar results to ours, in which some bands were supershifted and others were not indicating that Elk-1 may be present in some of the complexes associated with these promoters. We detected biding from both mock and infected samples to the probe containing the Elk-1 site, but the two major bands were detected with differing relative intensity in infected samples as compared to mock samples. Possible explanations for detecting different intensity bands and different competition in mock and infected samples could be: 1) Viral proteins up-regulate the expression of cellular proteins resulting in changes in intensities of shifted bands; 2) viral proteins are responsible for increased recruitment of cellular proteins into the nucleus; 3) possibly viral proteins replace cellular components in infected cells, resulting in similar size shifted bands but with a different composition. All together, these results indicate that Elk-1 is likely to be important for regulation of the m142 promoter including under immediate early conditions, before other viral gene products have been expressed, as well as in the context of viral protein expression.

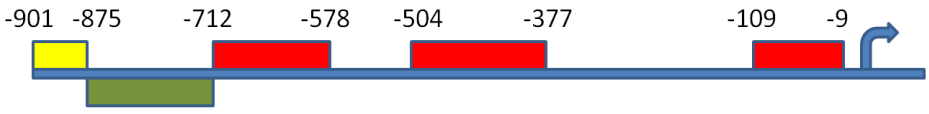


Figure 35. Location of identified regulatory regions of the m142 promoter binding with cellular and viral proteins. Yellow: region required for activation in the absence of

viral infection however could also be activated via viral proteins, Red: region required for activation in the presence of viral infection, Green: region containing repressor binding site(s).

We attempted to analyze the composition of the shifted bands by mass spectrometry. Although there were some interesting results, with some viral proteins detected, and some possible differences in histones detected in these bands, these studies were unsuccessful for the purpose of identifying transcription factors binding the region of interest from the m142 promoter, as no transcription factors were detected (Table 3). This is likely due to the low sensitivity which is one of the drawbacks of using mass spectrometry. Attempts to confirm the association of Elk-1 with the m142 promoter in the virus with ChiP assays were unsuccessful as the viral DNA (but not cellular) was non-specifically precipitated with the beads.

There were three additional regions whose deletion resulted in decreased activity in the context of viral infection, shown in red in the Figure 25. These were -713 to -578, -504 to -377, and the minimal functional promoter -109 to -9. All three of these regions contain SP1 consensus binding sites, but do not share other consensus sequences. According to previous studies done with HCMV, SP1sites are important in activation of several viral promoters (182, 283); hence we decided to look at the importance of SP1 binding site in the context of the m142 promoter by EMSA. Probes for two regions containing these SP1 sites (-459 to -428 and -409 to -386) were bound by what appeared to be similar complexes, with more bands binding from infected cell nuclear extracts, based upon gel migration pattern. Although our western blot analysis indicated that SP1,

SP2, and SP3 are all expressed in NIH/3T3 fibroblasts, super-shift assays were negative for all three antibodies. However, as similar shifted banding patterns were not seen with other probes, and the sequences of these probes are not the same outside the consensus SP1 sites, our results support that SP1 sites may be important for regulation. It is possible that other things are binding, or that viral proteins interacting with SP proteins are blocking access for the antibodies which has been previously reported for IE3 (265).

Comparison of the results of promoter activation by viral infection and the viral immediate early transcriptional regulators IE1 and IE3 supports the importance of viral components in the regulation via all three of these regions. IE1 alone was sufficient to activate all constructs until deletion of the minimal promoter from -109 to -9, while IE3 was not. When IE1 and IE3 were both present the activation was higher than seen with IE1 alone, suggesting that IE3 may be co-operating with IE1 to promote expression of m142 via the minimal promoter region. IE1 and IE2 of HCMV are known to function as TAFs (201). Our results support that in MCMV, IE1 and IE3 likely perform a similar function to promote basal transcription. Finally, viral infection activated m142 expression of the -109 construct to a higher level than co-transfection with IE1/3. This could indicate that other viral proteins are contributing to the activation. Alternatively, in co-transfections not all cells which received the promoter constructs necessarily also took up the IE expression plasmid. We and others have seen evidence of this (Julie Kerry, personal communication). Since we use an MOI of 2 PFU/cell, there should be few

transfected cells which were not infected, so the lower activation in co-transfection may just reflect these differences.

The region between -504 to -377 was found important for the activation of the m142 promoter during viral infection and during co-transfection with IE1 and IE3, but similar effect was detected during transfection with IE1 alone and IE3 alone. Hence, IE1 and IE3 must be required for the activation of the m142 promoter via region -504 to -377. Intense binding and additional bands were detected in infected samples as compared to mock samples in gel shift assays. Cellular proteins could also be involved in binding with this region along with IE1 and IE3. Increased intensity of bands in infected samples indicates the possible binding of other viral factors with this region.

Deletion of the region between -713 and -579 resulted in a drop from 30 to 20 fold activation over background in the context of viral infection, but there was no significant difference during co-transfection with the plasmid co-expressing IE1 and IE3 or upon treatment with UV inactivated virus. This suggests that the factors needed to efficiently activate via this region were not present without infection. As late gene expression was not required, it is likely that IE proteins other than IE1/3 or early proteins are involved.

The regions between -806 and -713, and possibly the region between -875 and -806 appear to contain repressor binding site(s) as deletion results in a significant increase in promoter activity. As the increase after deleting these regions was also seen in the co-

transfection experiments, it is likely that cellular factors are involved. Several pieces of evidence support that the repression is mediated via the AP2 sites: both have consensus AP2 binding sites and the dominant shifted band could be competed with an unlabeled competitor containing an otherwise unrelated consensus AP2 site, supporting that the AP2 site is important for binding, and it is known that AP2 isoforms can repress promoter activity (273-275). However, as the shifted bands were only detected with infected nuclear extracts, it is possible that a viral protein is binding via the AP2 consensus binding site. It is also possible that the AP2 binding is not related to the repressive activity. If this binding is related to the repression, it is encouraging that it was only detected in the context of virus infection. If the repression is being mediated by cellular factors interference with viral replication via this mechanism is unlikely to work without serious side effects. However, if the repression is mediated, even in part, by viral components this could lead to ways to interfere with expression of this essential viral protein.

In summary, the m142 promoter covers a large region and regulation involves multiple sequences important for activation or repression of promoter activity. The fact that sequences around 900 bases upstream of the transcriptional start site is discouraging for the possibility of utilizing this immediate early promoter for plasmids when lower levels of expression than provided by the HCMV MIEP ("the CMV promoter") are desired. However the apparent presence of repressor binding sequences between this region and other regions involved in activation holds the possibility that deletion of these

repressor regions might yield a smaller useful promoter. Our results support similarities in regulation with other immediate early promoters of cytomegaloviruses, and indicate that any possible future studies on the promoters for IRS1 and TRS1 likely should include at least 1000 bp upstream from the start of the open reading frame. We identified Elk-1 as a likely cellular factor involved in activating the m142 promoter and that IE1 is sufficient to activate via the minimal promoter. IE3 alone can also activate the m142 promoter, but via a different region, and the two proteins appear to co-operate through at least 2 regions of the promoter. Additional viral proteins may also be required for optimal expression via sequences between -713 and -579. There is evidence for repressor binding to regions between -806 and -713, and possibly also between -875 and -806. As this region is between the sequences required for activity in the absence of virus and the transcriptional start site, perhaps they can affect the ability of the DNA to take on an appropriate conformation to bring the activating factors into proximity with each other.

A summary of these results is shown in Figure 26.

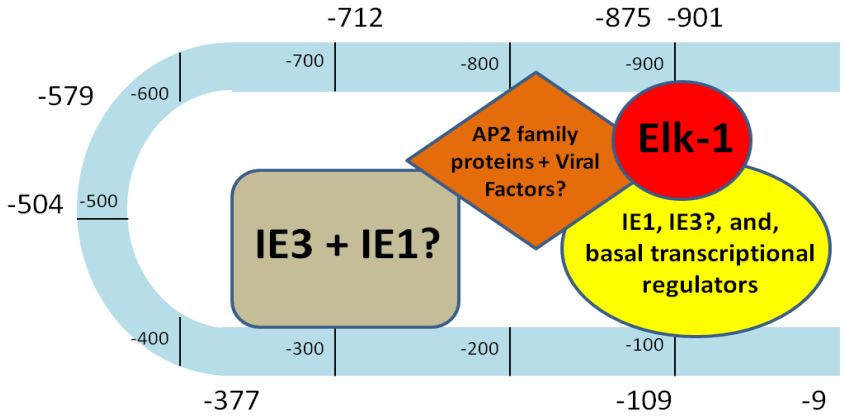


Figure 36. Summary and proposed model of identified regulatory proteins binding to the m142 promoter. A predicted model for the regulation of expression from the m142 promoter.

The mock infection represents the situation where only cellular factors are present. The samples collected 24 hpi represent a condition when both cellular and all viral proteins have been made. The conditions immediately after the entry of virus into the cell will be similar to mock infection. A single shifted band was detected binding to the probe containing an AT rich region from the minimal functional promoter in mock. The competition with a consensus TATA sequence suggests that this AT region may interact with basal transcription factors, but the failure to supershift indicates that TBP may not be a component of this complex. Thus the model is that in mock cells basal transcription factors are recruited to the AT rich region of the promoter, but on their own this is not enough to activate m142 expression, sequences between -901 and -879 are

needed to have detectable activation in the absence of any viral products. The results of the competition and supershift analysis support that the cellular transcription factor Elk-1 binds to the region between -901 to -879. This could promote activation via protein-protein interactions with the basal transcription factors bound to the minimal promoter region possibly via DNA looping. This could be the first step during the process of m142 transcription activation.

As the infection progresses, other cellular and viral proteins take part in the process of m142 transcription regulation. For the AT rich probe from the basal promoter region, two additional slower migrating bands were detected in infected samples indicating the binding of viral proteins, cellular proteins up-regulated upon viral infection, or cellular proteins re-localized upon viral infection in addition to the basal transcriptional factors. As this region was found to be important during IE1 and IE3 co-transfection, and IE1 co-transfection alone then viral proteins interacting with this region could be IE1and possibly IE3. They could interact with the AT rich region directly or via basal transcription factors. Our results are consistent with previous reports where IE1 and IE3 showed TAF like activity (201-203). This now allows activation without requiring upstream regulatory regions.

The region between -806 to -713 was found important for repression. This repression was detected during infection and co-transfection assays. The repression could be mediated by cellular factors or IE1 and IE3, however the most likely candidate

is cellular factor(s). This region contains AP2 binding sites. According to our model, cellular factors from the AP2 family of transcription factors could bind to the region between -806 to -713, only during infection. This effect might be due to either direct involvement of viral proteins or involvement of cellular proteins activated by viral proteins. In infected cells this complex may interfere with the activation of the m142 promoter; however the strong activation appears to mask the effect of repression at E and L time. This phenomenon ties in with previous findings about m142 transcription. Even though the m142 transcript start appearing at IE time point, The transcript steady state peaks at 3 hpi, with a modest decrease afterwards which would be compatible with the model of an induced repressor (250). We propose that inhibitor might interfere with protein-protein interactions and prevent DNA looping by inhibiting interaction of upstream regulators with transcriptional regulators binding to the minimal promoter region. Creating a new construct where this repressor binding region is deleted or just the AP2 site is mutated will give a better understanding of the role of this repressor and the AP2 site.

The region between -377 to -504 is promotes activation of the m142 promoter in the presence of IE1, IE3, or infection. This region contains three consensus SP1 sites. A single shifted band was detected with mock samples and in infected samples; more bands were detected in EMSA binding to probes containing these SP1 sites. We are hypothesizing that the single complex is common within mock and infected samples whereas the other three complexes involve additional cellular/viral proteins or consist of

entirely different proteins. Viral proteins IE1 and IE3 might be directly interacting or work indirectly. It is more likely that IE3 is interacting because IE3 is known to be able to activate via SP1 sites (265). We hypothesize that proteins bound to this region do not interact with the components of complexes bound to the other regulatory regions in uninfected cells but that the different complexes in infected or IE1/IE3 expressing cells now make interactions with other complexes formed at the minimal promoter enhancing transcription enhancing expression even in the absence of the upstream Elk-1 site

In some regions, the EMSA bands from infected samples are stronger than bands from mock samples and for probes from the minimal promoter region or Sp1 or AP2 consensus sites additional shifted bands were detected after infection. Some of the reasons for this could be: viral proteins might be interacting with these regions; increased binding of cellular proteins in the presence of viral proteins; induction of additional cellular factors upon viral infection; or viral proteins and cellular proteins are responsible for stabilizing the binding complexes. Purified proteins could be used to understand the sequence of binding of various proteins to important regulatory regions of this promoter.

The sequence of binding of cellular and viral proteins to the m142 promoter is still unknown. In infected cells the majority of proteins present after viral entry are cellular proteins, hence cellular proteins would likely be the first proteins to interact with this promoter. Identifying the steps and order of attachment of various proteins to the promoter will shed light on the process of viral infection. It will help us better

understand the regulation of essential promoter m142 of the MCMV. In the long term, this information might be helpful to make predictions about HCMV homologs of MCMV m142, IRS1 and TRS1 promoter activation.

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