REGULATION OF EXPRESSION OF THE M143 PROMOTER OF MOUSE CYTOMEGALOVIRUS

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DEDICATION

For my dad and mom, Balarami Reddy Eluru, Swarna Latha Eluru,
Thank you for your never-ending support and love.

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ABSTRACT

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The objective of this research is to examine the regulation of an essential gene (m143) in MCMV that has sequence and functional homology to HCMV genes IRS1/TRS1. All of these prevent the shutdown of protein synthesis that is an antiviral defense. Little is known about the regulation of these genes at either the transcriptional or posttranscriptional levels. Sequential deletion mutant analysis of the m143 promoter was done in the context of an SEAP reporter plasmid. The results indicated that viral proteins are required for activation and the presence of repressor-binding sites and activation-binding sites. EMSA shows that SP1 may be important for the activation. Two important viral transcriptional regulators IE1 and IE3, each alone weakly activates the m143 promoter, but together synergize to efficiently activate this promoter. The absence of repression with co-transfection experiments support repression by other viral proteins. Such studies may lead to novel treatments for cytomegalovirus infection.

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CHAPTER I

INTRODUCTION

Herpesviruses

Herpesviruses belong to the *Herpesviridae* family and come under the order of *Herpesvirales* (1). This order has been revised and now includes two new families along with *Herpesviridae*. New families include *Alloherpesviridae* that consists of bony fish and frog viruses, and *Malacoherpesviridae* that consists of oyster herpesvirus (OsHV1). Classification criteria for this family are based on the morphology of the virion, sequence similarity, serological relationships and biological characters (1, 2). The *Herpesviridae* is a large family and consists of more than 100 types of viruses that infect a wide range of hosts such as mammals, birds, fish and reptiles (1, 2). Within the family of *Herpesiridae*, non-human species viruses were renamed after the host genus rather than the subfamily, so that it would be easy to manage their rising number (1). Eight of the herpesviruses primarily infect humans; herpes simplex virus types 1 and 2, varicella-zoster virus, human cytomegalovirus (HCMV), Epstein-Barr virus, human herpesvirus 6, human herpesvirus 7, and Kaposi's sarcoma associated virus. Herpesviruses contain linear dsDNA surrounded by an icosahedral capsid that is enclosed by tegument proteins. All of this is encased by an envelope, which is composed of a lipid bilayer and glycoproteins. All herpesviruses share similar characteristics such as virion structure, remaining latent in their host and can reactivate from latency (1, 3). Both primary and reactivated

herpesvirus infections can either be asymptomatic or can result in diseases depending on the host's immune system (2, 4). The virus that is being addressed in this project is murine cytomegalovirus (MCMV), which is a model system for studying HCMV, which will be discussed later.

Classification. *Herpesviridae* are classified into three subfamilies based on their replication and host range: *alpha, beta and gamma herpesvirinae* (1, 3, 5). Alpha herpesviruses have a large number of hosts and are the least host species restricted, which means that human virus can be used to infect other organisms to study pathogenesis. Alpha herpesviruses can reproduce quickly in cell cultures and damage cells within a short period of time due to their short replication cycle. Alpha herpesviruses establish their latency in neuronal cells. Genera of alpha herpesviruses, with an example of each, include: *Simplexvirus* [herpes simplex virus type 1 (HSV-1)], *Varicellovirus* [varicella zoster virus, (VZV)], the avian *Mardivirus* [Turkey herpesvirus 1], and *lltovirus* [Marek desease herpesvirus 1(GaHV-1)] (3). Beta herpesviruses are more host specific than alpha herpesviruses and infection is limited to very closely related species. Beta herpesviruses spread slowly in cell cultures having a longer replication cycle than alpha herpesviruses. Beta herpesviruses also can cause host cell enlargement, for example HCMV infections hence the name cytomegalovirus due to cell enlargement. Beta herpesviruses establish their latency in a variety of cell types. Some of the examples of the beta herpesviruses include the genera of *Cytomegalovirus* [human cytomegalovirus, (HCMV)] *Murinemegalovirus* [mouse cytomegalovirus, (MCMV)], *Roseolovirus* [human

herpesvirus-6], and *Proboscivirus* [elephant endotheliotropic herpesviruses (EEHV)] (1) . Gamma herpesviruses also have a very limited host range. Gamma herpes viruses can replicate in lymphoblastoid cells and establish latency in lymphocytes. Examples of the gamma herpesviruses genera include *Lymphocryptovirus* [Epstein Barr virus (EBV)], *Rhadinovirus* [murine herpes virus-68 (MuHV4)], *Macavirus* [Porcine lymphotropic herpesvirus 1(SuHV3)] and *Percavirus* [Equine herpesvirus 2 (EHV2)] (1, 3).

Cytomegalovirus (CMV)

Cytomegalovirus causes cell enlargement, this was first called cytomegalia in 1921 (6). Cytopathic effect (CPE) can be noticed when cells are infected with CMV with characteristic nuclear and cytoplasmic inclusions and cell rounding in many cell types. HCMV infects 40%-90% of adults worldwide (7) and infects a large majority of the population almost 60% of adults in USA (8). CMV spreads through bodily secretions such as saliva, breast milk, urine and semen (9-11).

Transmission and pathogenesis of CMV. The seroprevalance of CMV infection is inversely related to socioeconomic conditions (11, 12). Transmission of this virus occurs in many ways such as transfer across the placenta from mother to fetus and via many body fluids such as mentioned above (9-11, 13). CMV infection can also occur through transplanted organs and blood transfusion (14). HCMV infection is generally asymptomatic but it can sometimes cause infectious mononucleosis in immune-competent individuals (7). HCMV causes serious problems in immuno-compromised individuals such as AIDS, cancer, and transplant patients either by reactivation from

latency or by primary infection as reviewed in (10, 15). Studies show that immuno-compromised patients can develop a multisystem disease that might be life threatening as reviewed by (16). For example, retinitis and pulmonary disease are some of the diseases that occur in HIV-AIDS patients (17). HCMV is one of the major causes of birth defects. In congenital infections this virus can cause severe damage to the central nervous system. The neonates will sometimes have permanent disabilities such as hearing loss or severe neurological disorders and also may have signs of enlarged liver and inflammation in the lung tissues (18). CMV has also been implicated in atherosclerosis (19-21). CMV can infect many types of cells such as: epithelial, endothelial, fibroblast cells, monocytes, macrophages, hepatocytes and neurons (22, 23). Understanding the important factors that are involved in CMV infection that can affect the pathogenesis, inflammation or immune response would help in developing additional therapeutic methods along with currently available antiviral drugs.

Antiviral therapy for CMV infections. There are some antiviral drugs available for treating CMV infections such as acyclovir, gancyclovir, valgancyclovir, cidofovir and foscarnet. Acyclovir is a 2'-deoxyguanosine analogue and is phosphorylated by host kinases to inhibit the viral DNA polymerase at multiple steps (24, 25). Acyclovir is very effective against several herpesviruses but only moderately effective against CMV. The related drug gancyclovir (GCV) is a nucleoside analogue of 2'-deoxyguanosine and it is phosphorylated into gancyclovir triphosphate by viral protein kinase, UL97 (26). Gancyclovir triphosphates incorporate by competing with dGTPs and inhibit the DNA

synthesis by cellular and viral DNA polymerases (27). This drug is commonly used for CMV retinitis, CMV pneumonitis, and CMV neurologic disease in AIDS patients. However mutations in either viral polymerase or UL97 kinase help CMV to develop resistance to gancyclovir (27). Valgancyclovir is a prodrug of ganciclovir, which was developed to supplant intravenous gancyclovir, because oral valgancyclovir achieves blood levels comparable with intravenous gancyclovir (28), in addition, CMV is less likely to develop resistance against valgancyclovir (28, 29). Valgancyclovir is used in solid organ transplant patients and HIV-associated CMV retinitis patients (30). Potential side effects of these drugs include nephrotoxicity and hematologic toxicity, notably neutropenia, and thrombocytopenia. It is recommended that the dose of these drugs should be reduced if toxicity occurs (31). Cidofovir is a nucleotide phosphate analogue, which is converted into an active diphosphoryl form of cidofovir diphosphate by host kinases, this will competitively inhibit viral DNA polymerase (25, 32). It is mainly used in the treatment of CMV retinitis in patients with AIDS (33). Foscarnet is used in patients who are failing GCV therapy due to dose limiting factors or viral resistance (34). Foscarnet is a pyrophosphonate analogue and it binds to pyrophosphate binding sites to block the cleavage of pyrophosphate from the terminal nucleoside triphosphate that is being added to the growing DNA chain and thus inhibit viral DNA polymerase activity (34). One of the common side effects of this drug is nephrotoxicity (34). All of these drugs have potential mutagenic effects. They have also been reported to inhibit spermatogenesis and hence new therapeutic targets are desirable (35).

Vaccine development. Currently, there is no effective therapy for pregnant women when they get primary infection due to CMV, because these drugs can't be given to pregnant women due to their potential mutagenicity (8, 35). It has been reported that congenital CMV diseases affect thousands of newborn children every year (8). CMV can cross the placenta during pregnancy and can be transferred to the fetus. This can cause neurological damage to the newborn. Hence it is desirable to develop a vaccine against CMV infections (36). However it is challenging to develop a vaccine against CMV due to its latency and potential reactivation and so live vaccines might be hazardous. An additional challenge is that CMV has developed multiple mechanisms to modify the immune response (37). Currently there are several vaccines that are being tested in humans. These include live attenuated vaccines and sub unit vaccines encoding glycoprotein gB (36, 37).

CMV Virion structure. All members of the *Herpesviridae* family share similar virion structures, which is used to classify the virus as a family member (1). The virions can vary in size ranging from 120-260 nm depending on the composition of the tegument and the morphology of the envelope. CMV is around 200 nm across. Virions consist of basically four components those include core, capsid, tegument and envelope shown in Figure 1 (38). The viral core contains a single linear double stranded DNA genome, which ranges in size from 120-230 kb depending on the virus, and is arranged around the protein core in the form of a torus (38). CMV has the largest genome of the herpesviruses. The CMV genome is predicted to encode approximately 200 genes (39,

40). A protein shell called the capsid surrounds this nucleic acid (dsDNA) and together this makes the nucleocapsid. Herpesvirus capsids are icosahedral-structured component contains 162 capsomeres and are 100 nm in size (41). Capsomeres are made with 5 capsid proteins, major capsid proteins (MCP), TR11, TR12, smallest capsid proteins (SCP) and PORT (10). The capsid is surrounded by an ordered structure called the tegument. Tegument contains several proteins and varies in thickness. The tegument proteins help the virus in virion assembly and establishing a productive infection in a new cell. During infection they interfere with host functions to overcome the host defense mechanisms, and help activate viral gene expression (42). The final component of the herpes virus virion is the envelope, which contains several viral glycoproteins; gB, gH, gL, gM, gN, gO and its lipid bilayer membrane is derived from the host cell endoplasmic reticulum (ER) membrane (41, 43).

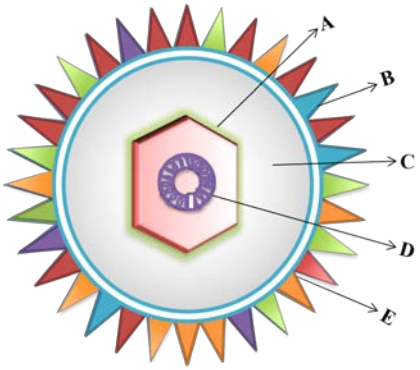


FIG. 1. CMV Virion Structure. The components shown are A, capsid; B, glycoproteins; C, tegument; D, DNA core; E, envelope.

CMV Replication. Replication starts with viral attachment to the cell surface and penetration into the cell (44). When the virus attaches to the cell, the virus interacts with proteoglycan complexes on the cell surface via the virus-encoded glycoprotein complex gM:gN. CMV then binds to more specific receptors, for example epithelial growth factor receptor (EGFR), through other virus-encoded glycoproteins such as gB (45-47). Other co-receptors to mediate entry must be present on cells that don't express EGFR. A recent study indicates that integrins can be involved in the attachment via highly conserved disintgrin-like domains in viral glycoproteins (48). The gB in the envelope of the virus facilitate the fusion with the plasma membrane, depositing the tegument and nucleocapsid in the cell cytoplasm. This fusion differs between cell types, fusion in endothelial cells requires endocytosis and low pH (49). After release into the cell, viral nucleocapsids migrate to the nucleus via microtubules. The viral genome gets into the nucleus through the nuclear pores, where the linear genome circularizes as an episome (10). Gene expression, replication of the CMV genome and assembly of new capsids take place in the nucleus. Replication times for HCMV is about 72 hours and for MCMV is about 24 hours in fibroblasts. These are the cells generally used in the laboratory. Most herpes virus's gene expression is temporally regulated (4). There are three phases of expression, first, the immediate-early (IE) phase during which necessary transcription factors start being made. Secondly, the early (E) phase during which proteins required for DNA replication is made. Thirdly, the late (L) phase during which structural components of the virions are made (10). As mentioned, early gene expression is required for viral

genome replication to take place. DNA synthesis occurs by a “rolling-circle” method. According to this model, the replication of a single circular DNA occurs in a unidirectional way, which results in several copies of the viral genome connected end to end, called concatemers (10). The DNA has to be cleaved for the genome to be packaged into capsids. Capsid proteins are transported into the nucleus and form capsomers surrounding scaffolding proteins. These are assembled into pro capsids. Cleavage of the genome is concurrent with entry into the capsid. Viral DNA gets into the capsid via portal proteins, the scaffolding proteins get out and portal gets closed (10). The details of this process are still not well understood. Tegument proteins are added in an ordered sequence and some are added in the nucleus, which facilitates nuclear egress, while some are added in the cytoplasm for final egress (10). The nucleocapsid egresses by budding. According to the “double envelope theory”, capsid attains an envelope by leaving the nucleoplasm through the inner nuclear membrane, which will then be lost by fusion with the outer nuclear membrane releasing the nucleocapsid into the cytoplasm (50). Then the final envelopment occurs at Golgi derived vesicles (10, 51, 52). The enveloped virus is then transported via cellular vesicular release mechanisms to the cell membrane. There the virion is released by vesicular fusion with the cell membrane (4, 10).

(i) Gene classes. The focus of this study is gene regulation, hence HCMV gene class are being discussed a little more here. CMV genes are temporally regulated and classified in to three kinetic classes depending on the time and sensitivity to different inhibitors. They are immediate early genes (IE), early genes (E) and late genes (L) (10,

53) . Immediate early genes are independent of any newly synthesized viral proteins and mRNA is made in the presence of protein synthesis inhibitors. The timing discussed here will refer to the kinetics of MCMV. During immediate early times, within the first 4 hours post infection (hpi), regulatory proteins are expressed including some which act as transactivators for expression of viral early and late proteins along with cellular proteins. IE1/IE2 are extensively studied immediate early transcriptional regulators in HCMV and IE1/IE3, are the homologues in MCMV (10, 54). IE1/IE3 are under the control of the major immediate early promoter (MIEP) and are splice variants. This gene region has five exons and two alternative polyadenylation signals. Exons 1, 2, 3 and 4 encode IE1 transcript and exons 1, 2, 3 and 5 encode IE2 transcript (IE3 of MCMV) (54, 55). However there are other transcriptional regulators that are expressed in all kinetic classes of gene expression. Expression of early genes requires the presence of functional IE products. During early times, starting around 4 hpi, proteins that are involved in DNA replication is expressed (10). Late genes are expressed after the onset of DNA replication starting around 12 hpi. Late proteins can be distinguished from early genes using DNA synthesis inhibitor such as phosphonoformic acid (PFA, foscarnet), which prevent viral DNA replication (10, 53). During late times structural proteins necessary for viral assembly get expressed (10).

Latency. When a herpes virus infects its hosts, this infection can either lead to a lytic infection in which the virus replicates and causes cell death or latent infection in which the virus stays dormant in the host. Latency is defined as the presence of viral

DNA but no infectious virus production (56). Histone modifications play an important role in the repression of gene expression. Acetylated H3 was found in HCMV lytic infections and dimethylated H3 causes repression of HCMV genes in latency (57). An additional mechanism is down regulation of immediate early gene expression. CCAAT displacement protein (CDP or CUX1), a cellular protein, binds to a CUX1 site in the major immediate early promoter (MIEP) and represses MIEP expression (58). Latency in CMV can be in several cell types. These include bone marrow and peripheral blood (59, 60). It has been shown that bone marrow precursors are a key site of latency for HCMV persistence and reactivation; peripheral blood monocytes circulate the virus, and differentiation of latently infected monocytes into macrophages leads to reactivation and productive infection (60). A study reported on latency in MCMV showed that during latency high copy numbers of DNA were present in lung tissue and could be reactivated (61).

Genome characteristics. MCMV contains a 230 kb genome that encodes for around 170 genes and HCMV contains a 229 kb genome that encodes for about 170 genes (39, 40). Herpesvirus's genomes consist of one or two unique sequences flanked by repeated sequences (62). The HCMV genome consists of two covalently linked segments: the unique long and unique short (U_L and U_S) regions, flanked by two sets of inverted repeats TRL/IRL and TRS/IRS (Figure 2). The HCMV open reading frames (ORFs) are numbered sequentially and designated by their location within the unique or repeated regions of the viral genome (39, 63). For example US22 is the 22nd ORF located

in the unique short (US) region. The genomes of other β−herpesviruses are linear and lack internal repeats. The MCMV genome consists of a single unique sequence with short direct repeats at either end (64). MCMV's ORF's are designated based on location within the genome. In addition, 'm' is for mouse. Homologs to HCMV ORFs are designated with upper case letters (M112/M113 for IE1/IE3 gene region), those with sequence similarity below a certain level (exact percentage is not clearly stated in the original paper) are designated with lower case letters (m22) (40).

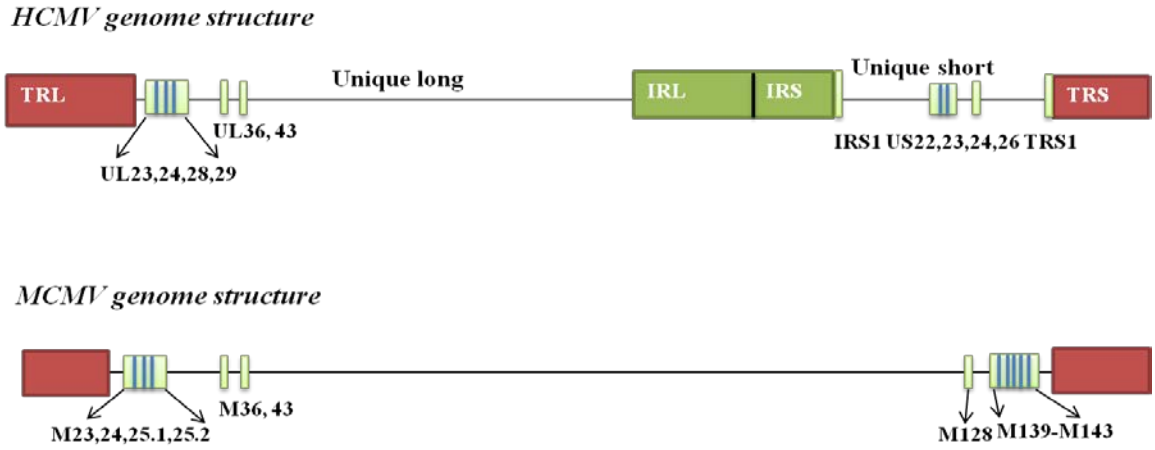


FIG. 2. HCMV and MCMV genome structures with relative locations of US22 gene family members. TRL; Terminal repeat long, TRS; Terminal repeat short, IRL; Internal repeat long, IRS; Internal repeat short.

MCMV as a model for HCMV. MCMV and HCMV share similarities in pathogenesis and genome organization. Both can cause diseases either by reactivation from latency or by primary infection and can establish a lifelong latency (10). Hence MCMV is useful to study conserved genes. There are several families of related open

reading frames among the conserved genes, resulting from gene duplication events. One of these gene families is the US22 gene family (10, 39, 40). US22 family members are characterized by the presence of at least one of four conserved sequence motifs and are specific to the beta herpesviruses. Motifs I and II are well defined and contain short stretches of hydrophobic and charged residues. Motifs III and IV are less well defined and contain stretches of nonpolar residues (39, 40). The US22 gene family consists of 12 members in each HCMV and MCMV(40). The closest homologue for each HCMV US22 family member is not another HCMV gene but the MCMV gene with similar location in the genome (Figure 2). This project addresses one of the US22 genes, m143, which is essential for viral replication. MCMV's m143 and its neighbor m142 are 3'–coterminal transcripts and are the closest homologues of IRS/TRS1 in HCMV, which inhibit the same antiviral response (65, 66). They also exhibit similarities in expression kinetics (67-69). When the cell is infected, cellular antiviral defense mechanisms are activated, such as the protein kinase R (PKR) pathway. PKR binds to dsRNA produced by the virus and becomes auto-phosphorylated, activating PKR. Activated PKR phosphorylates the translation initiation factor eIF2α leading to the shutdown of global protein synthesis. The protein m143 forms a complex with m142, which binds to dsRNA and PKR to inhibit the PKR antiviral pathway (70, 71). Because m143 is essential for virus replication, it is important to determine how this gene is regulated.

Promoter regulation

Gene expression can be regulated at various steps, such as polyadenylation (72), splicing (73), mRNA stability (74), chromatin remodeling (75), transcription initiation (76) etc. Regulation of transcription initiation is an essential step in determining whether a gene is expressed or not in a cell, which consequently results in the making of mRNA and protein. Transcriptional initiation is regulated by the binding of certain proteins to the sequences in a promoter. A eukaryotic promoter contains protein-binding sites for various transcription factors (TFs), which regulate the promoter (77). Hence, promoter analysis is important to identify the functional transcription binding sites. A TATA binding site is generally located 20-25 bases upstream of the transcriptional start site. This is called as core promoter where the RNA polymerase II binds with the help of other transcription factors. TATA binding protein (TBP) binds to TATA binding sequence. Transcription factor IID (TFIID) binds to TBP, this leads to the binding of RNA polymerase II along with five other transcription factors and forms a pre-transcription initiation complex (77). In promoters without the consensus TATA box, usually TBP binds to the promoter and TFIID complex requirements are similar to promoters with consensus TATA box (78).

Herpesviruses replicate inside the nucleus and they largely utilize the host cell's transcription machinery and DNA repair system for successful infection (3). Transcription is an important step for any genes expression. In herpesviruses cellular factors and chromatin remodeling complexes play an important role in lytic and latent

infections. Host cellular RNA polymerase II and transcription factors help the viral gene transcription. Viral activators and repressors are also involved in transcriptional regulation of viral as well as host genes (4, 79). MCMV gene regulation is not well-studied compare to HCMV, so most of what is discussed here is about HCMV.

Many cellular factors have been shown to be involved in regulating a variety of CMV promoters. Some of those factors activate and some repress the CMV promoters that are discussed here respectively. When the virus enters into cells, it activates several cellular transcription factors such as myc, NF-kB and mitogen activated protein kinases ERK1, ERK2 and p38 (79). In fibroblasts, extracellular-signal-regulated kinases (ERKs), members of the MAPK family are activated by the inhibition of an ERK-specific phosphatase upon HCMV infection (80). Several cellular factors are regulated by the MAPK pathway, mainly factors that are involved in HCMV's early gene regulation. Activated substrate of ERK kinase up regulates the activity of cAMP response-element-binding protein (CREB) (81). Previous studies on HCMV have shown in infected cells that ATF-CREB interacts with IE2 protein (the homologue of MCMV IE3) to trans activate the early UL112/UL113 promoter (82). ATF/CREB proteins up regulate the transcription of number of promoters when bound to cAMP responsive elements (CRE) upon the phosphorylation of cAMP dependent PKA upon infection with MCMV (83). Interferon gamma inducible protein 16, a cellular protein interacts with tegument protein pp65 (UL83) and activates the major immediate early promoter MIEP (84). Activator protein 1 (AP1) and necrotic factor kappa beta (NF-kβ) sites that are present in the major

immediate early enhancer were found to be important for the activation of MIEP in transfection assays (85). Mutational analysis of the consensus sequence for PEA3 that is present in late gene UL75 showed that a PEA3 like element is involved in its activation (86). According to previous studies, SP1 transcription factor contains a zinc finger protein motif, by which it binds directly to DNA and enhances gene transcription (87, 88). SP1 binds to GC-rich elements and is derived from a single gene product (89). It is known to undergo heavy posttranslational modifications (90). Several studies support that SP1 involves in the regulation of herpes virus gene expression and HCMV infection increases mRNA levels, proteins and SP1 binding activity (91-93). It has been shown that attachment of glycoproteins from HCMV to the cell surface up regulates the SP1 induction and IE2 of HCMV can physically interacts with SP1 to increase transactivation (91, 94). IE2 protein may be increasing this SP1 DNA binding by releasing the inactive SP1. This SP1 binds to the SP1 sites in p65 and p105/p50 promoters and upregulates NF-kB (93). It has been well documented that SP1 interacts with general transcription factors for basal transcription activity (95).

Some cellular factors can also repress CMV promoters. C/EBP interacts with NF-kB and represses NF-kB mediated activation of HCMV MIEP enhancer's activity (96). Association with transcription activators and chromatin modifiers may lead to YY1 mediated activation whereas association with other cellular repressors may lead to repression by YY1 (97). It has been documented that YY1 is involved in the regulation of HCMV gene expression and it represses HCMV IE gene expression in non-permissive

T2 cells (98). A study in MCMV shows that repressors bind to the viral promoters at the early stage of infection. The number of repressors bound to these promoters is decreased once the RNA polymerase and acetylated histones bind to these promoters to activate their expression. Viral repressor recruitment increased and occurs in a promoter specific manner during latency and YY1 and core binding factor 1 (CBF-1) act as repressors and play an important role during repression leading to latency in MCMV (99).

Viral proteins are involved in the regulation of viral promoters either directly or via cellular factors. Immediate early genes IE1 and IE2 (IE3 in MCMV) are spliced variants expressed from the MIEP. IE1 and IE2 proteins can activate many viral and cellular promoters and regulate temporal expression of HCMV genes (100). Work by Harel and Alwine (101), suggests that activated ERK play a role in regulating IE2 mediated transcription. It has been shown that IE2 mediated transactivation occurs via synergy between SP1 and IE2. This transactivation is evidently increased, when TBP is recruited by the promoters (102). A study shows that IE1 and IE2 regulate MIEP by interacting with NF-kB. Deletion of the MIEP enhancer or mutations in NF-kB sites in the MIEP abolished expression from the MIEP and viral replication (103). IE1 and IE3 in MCMV are homologs of IE1 and IE2 in HCMV, which are regulated in a similar mechanism discussed above in HCMV.

Since virus attachment, and entry of the tegument proteins up regulate signal transduction pathways that can promote different viral promoter's transcription (94, 104). Tegument proteins are known to regulate viral gene expression. Tegument protein pp71

accumulates at nuclear domains (ND10) upon HCMV infection. This occurs via Daxx binding to SUMO-modified PML and transports pp71 to ND10. All this happens before IE1 protein production. Accumulated pp71and interaction with hDaxx is important for efficient infection and to enhance IE transcription at ND10 (105-108). Attachment of HCMV (glycoproteins gB, gH) is sufficient to induce initial cellular signal transduction, which up regulates SP1 and NF-kB and help viral gene expression (94). Tegument protein pp71 plays a crucial role in activating immediate early gene in the beginning of lytic infection (109). In HCMV, pp71 activates US11 gene via IE proteins and ATF-CREB sites contribute to the activation of US11 (110) .

A wide variety of both viral and cellular factors can mediate viral promoter regulation. Very little is known about the promoter regulation of MCMV as compared to HCMV. The promoter studied in this project is for m143, which is essential for the virus. This promoter requires viral infection to be upregulated and immediate early genes (IE1/IE3) can activate this promoter (68). My studies on m143 have expanded the knowledge about gene regulation in MCMV and similarity to HCMV gene regulation.

CHAPTER II

MATERIALS AND METHODS

Primer Design

A series of 5' end primers were designed using the m143 promoter region sequence. These primers were designed to make a series of promoter deletion mutants differing by approximately 100 bases. A common 3' reverse primer was used. The designed primers are shown in the Table 1. The constructs are shown in Figure 3.

TABLE 1. Forward and reverse primer sequences designed to make deletion mutants from the full-length promoter sequence by deleting approximately 100 bases. These primers were used to amplify the deletion mutants.

Primer Name	**Primer Sequence (5'-3')**	**Annealing Temperature (^{0}C)**	**AnnealingTemperature used (^{0}C)**
-787 Forward	TACTCGCGTCCGCCGTATCAC	58.26	53.3
-728 Forward	GGCGGCATGAGACTACGGTG	57.93	55.4
-620 Forward	CAACACCACGACCCGCTACC	57.93	59.3
-511 Forward	TATCGCGACCGGGCTCGAAC	57.93	55.4
-403 Forward	TCGTGGTCGGCAGCGTGTTG	57.93	55.4
-315 Forward	GAAACGGCCGTCCTACCAGGTG	60.43	58.4
-205 Forward	CGGCGTATGCCGTCACGATG	57.93	55.4
Reverse	GGCAACCACCTCTCTGCTGTGG	60.43	-

Polymerase Chain Reaction. PCR was done to amplify the deletion mutants using the primers shown in Table 1. DNA from infected cells was used as a template. DNA from uninfected cells was used as a negative control. Total PCR reaction mixture was made up to 50 ul contained 25 ul of Amplitaq gold® 360 master mix (2X) (Applied Biosystems, Foster City, CA, USA), 2.0 ul of GC enhancer, 1.0 ul of forward primer (0.2 uM), 1.0 ul of reverse primer (0.2 uM), template DNA (1 ug) and the rest is PCR grade water to make it up to 50 ul. The Amplitaq gold system has an antibody that is blocking the active site of the polymerase, when heated at high temperatures this gets released and the DNA polymerase is active. This reaction was run with a hot start that was set as pre denaturation at 94 ^{0}C for 5 min. The program used was: denaturation at 94 ^{0}C for 1.0 min, annealing at primer specific temparature for 1.0 min, extension at 72 ^{0}C for 1.0 min, repeated for 34 cycles with a final extension at 72 ^{0}C for 5 min to improve complete double strand production. PCR products were run on 1% agarose gels and the products were purified using the QIAquick Gel Extraction Kit (Qiagen Sciences, Maryland, USA) following the protocol from the kit.

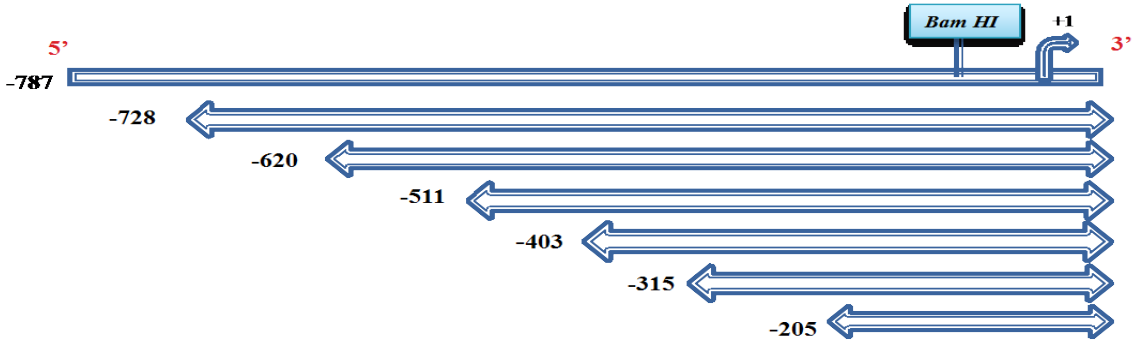


FIG. 3. Full length (-787) promoter and designed sequential deletions.

TA cloning

TA cloning was done with purified PCR products of the expected size. These were cloned into a PCR® 2.1-TOPO plasmid by topoisomerase following the company's protocol (Invitrogen™ by Life Technologies, Carlsbad, CA, USA) and used for transformation.

Bacterial transformation. Each TOPO TA cloning product was added to chemically competent *E. coli* (One shot TOP10 by Invitrogen), and heat shocked for 1.0 min and incubated 1.0 hour for recovery following manufacturer's protocol. Transformed *E.coli* was spread on LB Agar plates with kanamycin (*Kan)* antibiotic (50 ug/ml) and X-gal at 40 ug/ml. Kanamycin was used because the TOPO vector contains ampicillin and kanamycin antibiotic resistance, and kanamycin is less likely to have satellite colonies than ampicillin. X-gal was used for blue white colony selection. The plates were incubated overnight at 37 ^{0}C. White colonies and pale blue colonies were selected for further analysis. White colonies are the results of disruption of *lac Z* expression in the plasmid. Blue colonies result from *lac Z* expression from self-ligated plasmid. Pale blue colonies were chosen because some of our small constructs might not disrupt *lac Z* expression. Bacteria from white or pale blue colonies were grown overnight at 37 ^{0}C in 3.0 ml of liquid LB media with kanamycin (50 ug/ml). Plasmid DNA was isolated from the liquid cultures at around 16 hours using either the QAIGEN miniprep kit (QAIGEN, Sciences, Maryland, USA) following their protocol or using our lab made solutions. Miniprep protocol from our lab as follows: 1.5 ml of overnight culture was

transferred into a 1.5 ml eppendorf tube and centrifuged at 6000 g for 10 min. Supernatant was removed and the bacterial pellet was air dried. This pellet was resuspended in 100 ul of ice-cold GTE solution I (25 mM Tris, pH8.0, 50 mM glucose, 10 mM EDTA, 100 ug/ul RNAse A) by vortexing and pipetting up and down. This was incubated on ice for 5.0 min. Next 200 ul of fresh solution II (10% SDS and 1M NaOH) was added and mixed by inversion. This tube was incubated on ice for 3 min. then 150 ul of solution III (299.5 mM of KAcetate in 40 ml of water and adjusted pH with glacial acetic acid to 4.8, final volume was made up to 100 ml) was added, mixed by inversion and incubated on ice for 5.0 min. The tubes were centrifuged at 6000 g for 4 min to pellet the debris. Supernatant was transferred to a fresh 1.5 ml tube and 450 ul of phenol/chloroform was added in fume hood, mixed by vortexing and centrifuged at 6000 g for 2.0 min. The aqueous phase was transferred to a fresh tube, 900 ul of 100% ethanol was added, mixed and incubated for 2 min at room temperature. This was centrifuged for 4.0 min at 6000 g, to pellet the plasmid DNA, and supernatant was removed. The plasmid DNA pellet was washed with 70 % ethanol and centrifuged for 1.0 min at 6000 g. Supernatant was removed and plasmid DNA was air-dried. The plasmid DNA was resuspended in 50 ul of sterile water.

Restriction Digestion. For the confirmation and orientation of PCR products, the cloned deletion mutants were screened for insert using *BamHI* digestion. This enzyme was used because all the PCR products have a *BamHI* restriction site close the transcription start site. All the enzymes used in this project were purchased from New

England BioLabs (Ipswich, MA). Restriction digestion mixture with *BamHI* (10 U), 1X NEB buffer 4, 3.0 ul of plasmid DNA and water was incubated over night at 37 ^{0}C. Negative control tubes contained everything in the mixture but no restriction enzyme, which shows the activity of enzyme. This mixture was run on 1.2 % agarose gels to check the orientation.

Sequencing

After confirming the correct constructs, they were sequenced using M13 labeled primers on a LICOR 4300 sequencer to ensure that there are no mutations, mismatches or deletions. We used the sequitherm EXCEL™ II DNA sequencing kit for our sequencing. Sequencing reaction mixture was made with 2.25 ul of M13 800 IRDye labeled forward and M13 700 IRDye labeled reverse primers (1 pmol/µl) that have the complementary sequence in PCR2.1TOPO vector, 7.65 ul of template plasmid DNA (250 ng), 8.10 ul of 3.5 X sequitherm excel buffer II, 1.12 ul of sequitherm excel II DNA polymerase (5 U/ml). Four µl of this premix was added in each tube containing 2.0 µl of ddATP, ddTTTP, ddGTP, or ddCTP termination mix. This mixture was run on a thermocycler with pre- denaturation (95 ^{0}C for 5 min), denaturation (95 ^{0}C for 30 sec), annealing (50^{0}C for 15 sec), elongation and (70 ^{0}C for 1.0 min and repeated for 29 cycles), extension (72 ^{0}C for 5 min). The sequencing products were stored at -20 ^{0}C. Three µl of stop solution was added to all reaction products and the mixture was heated at 95 ^{0}C for 3 min before 1.7 µl of sample was loaded in each well of the sequencing gel. Sequencing gels were allowed to run for 8 hours to overnight depending on the product size. The primers used

for sequencing are M13 forward primer (5' G TAA AAC GAC GGC CAG 3') and M13 reverse primer (5' CAG GAA ACA GCT ATG AC 3'). The sequences obtained from sequencing were analyzed by BLAST from NCBI to check the sequences for similarity with the published MCMV sequences before sub cloning into a reporter plasmid.

Sub-cloning

In order to study the regulation of the m143 promoter we subcloned the constructs into a reporter plasmid. The Clontech SEAP reporter plasmid (PT3057-1, Clontech, Moutain Veiw, CA, USA) which uses a secreted embryonic alkaline phosphatase (SEAP) as a reporter molecule to monitor the promoter activity was used. The confirmed and sequenced constructs were digested using *EcoRI* restriction enzyme (10 U). This enzyme was used because the insert in the multiple cloning site (MCS) of PCR 2.1 TOPO plasmid is flanked by *EcoRI* sites and there is an *EcoRI* site upstream of the SEAP gene in the MCS. The SEAP plasmid was digested with *EcoRI* (10 U) and treated with Antarctic phosphatase (5 U) with 1X phosphatase buffer for 1 hour at 37 ^{0}C to avoid self-ligation by removing the phosphates at the ends of the linearized plasmid. Ligation of the gel purified insert and linearized reporter plasmid was set at a 3:1 molar ratio of insert to vector for 24 h at 4 ^{0}C using T4 ligase (5 U) with 1X ligase buffer. As previously described, ligation mix was transformed into chemically competent *E.coli*, spread on an ampicillin (Amp) (50 ug/ml) plate, and incubated overnight at 37 ^{0}C. Ampicillin was used because the SEAP reporter plasmid has ampicillin resistance. The colonies were analyzed for the orientation of insert in the vector using *BamHI* (10 U) digestion (Figure

4 A and B). Large-scale plasmid preparations were made using the Fast Ion Plasmid Maxi Kit (MIDSCI, IA, USA) following manufacturer's protocol. We were not successful in subcloning the full-length promoter into the reporter plasmid directly so we chose to subclone in two steps. The full-length (-787) construct in PCR 2.1 TOPO was digested using *KpnI* and *BglII* and this fragment was put into the -315 construct that was digested with *KpnI* and *BglII*. Some of the PCRs were not successful, so an alternate method was used, using restriction enzymes in the constructs that had been successfully sub cloned (Figure 4 C). The -511 construct was made with *Acc65 I* and *NruI* from the full-length construct. First the full-length construct was digested with *Acc65 I*, then the cohesive ends were filled with DNA Polymerase I, Large (Klenow) Fragment (1 U/ug was used) to make it blunt ended and gel purified. When this fragment was religated, it created a new restriction site for *SnaBI*. We used *Acc65I* because it is an isoschizomer of *KpnI* and produces a 4-base 5´ extension whereas *KpnI* produces a 4-base 3´extension. This allowed us to fill in the 5' overhangs to form blunt ends and avoid Klenow's possible 3'→5' exonuclease activity. This plasmid was then digested with *SnaBI* and *NruI* enzymes (5 U each), *NruI* is a blunt end cutter and cuts twice in the insert and *SnaBI*, also a blunt end cutter, cuts once at the newly generated site. After removing the *SnaBI-NruI* fragments, the plasmid was religated. The +4 construct was made by religating the *BglII* (5 U) digested plasmid from the -205 construct. To make the -124 construct, *BglII* digested +4 and a *BamHI-BglII* fragment (-124 to +4) was purified from the full-length construct (-787) and inserted into the *BglII* site of +4 construct.

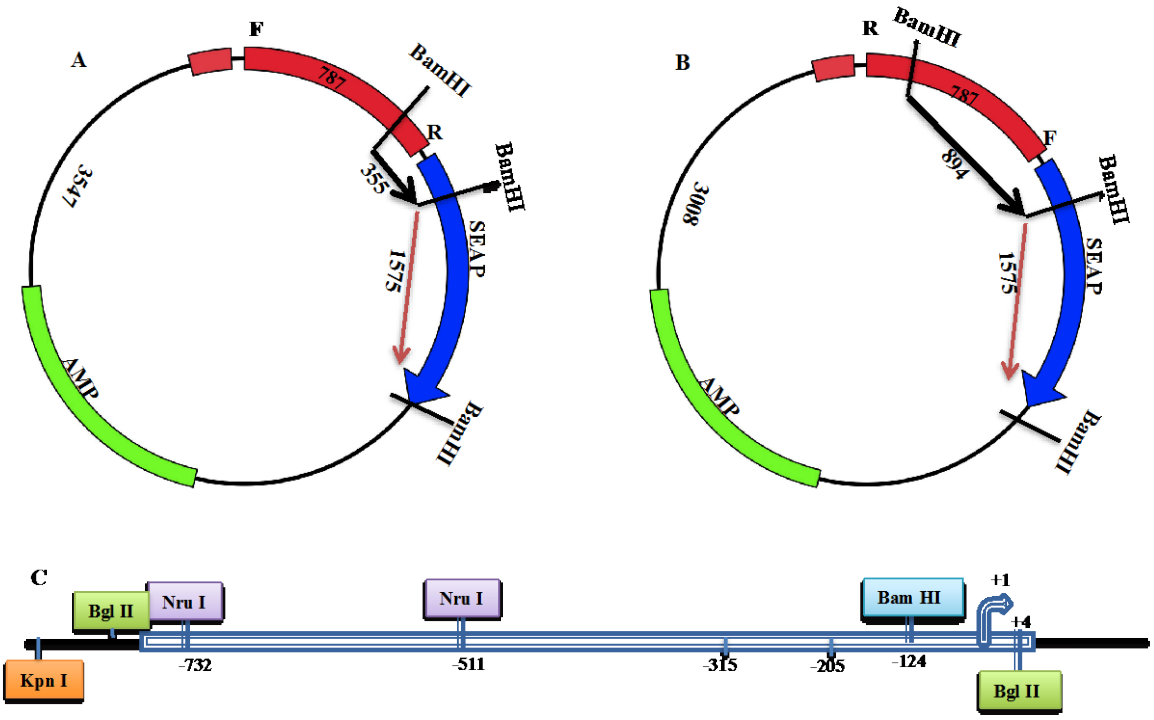


FIG. 4. **Analysis of insert orientation and restriction enzymes used: A**. correct orientation of the insert in pSEAP plasmid after subcloning the digested insert into pSEAP multiple cloning site. **B.** Incorrect orientation of the insert into pSEAP. **C.** Full-length construct with the enzymes used in making various constructs in SEAP reporter plasmid as described. Black lines indicate the MCS in the SEAP reporter plasmid. **F**: Forward primer, **R**: Reverse primer from PCR.

Cell cultures

Murine NIH3T3 fibroblasts cells (America Type Culture Collection (ATCC) CRL 1658, Rockville, MD) that are permissive for MCMV were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO® by Life Technologies, Carlsbad, CA, USA) that was supplemented with 10% bovine calf serum (BCS) (GIBCO® by Life

Technologies, Carlsbad, CA, USA). This is referred as DMEM complete media in later sections. These cells were grown in a CO_2 water-jacketed incubator (NUAIRE) at 37 ^{0}C with 5% CO_2. The cells were maintained on a strict 3T3 regimen, which means they were split every 3 days and seeded at 3 x10^5 cells in a 75 cm^2 growth surface area flask (MIDSCI, Valley Park, MO, USA). For splitting, cells were washed with PBS-EDTA (136.89 mM Nacl, 2.68 mM Kcl, 10.14 mM NA_2HPO_4, 1.76 mM KH_2PO_4, pH-7.4, 11.1 mM dextrose, 1.36 mM EDTA, 0.2 u filter sterilized) after removing the media. Then the cells were trypsinized using 1.0 ml of 0.5% trypsin in PBS-EDTA for 30-60 sec. Trypsinized cells were re-suspended in DMEM complete media to inactivate the trypsin. The cells were diluted 1:10 using 0.1% trypan blue dye to count the number of viable cells before seeding.

Virus Stocks

We used MCMV [Smith strain (ATCC VR 194)] for infections. NIH 3T3 fibroblast cells were seeded at 1x10^6 into a 150 cm^2 flask (MIDSCI, Valley Park, MO, USA). Twenty-four hours later these cells were infected at a multiplication of infection (MOI) of 0.001. Virus stock was harvested when there was 100% cytopathic effect (CPE) in the flask (around 5 days). To harvest the virus, the cells were frozen at -80 ^{0}C for 10 min and thawed. This will break open the cells and release the virus from inside the cells. These cells with media were collected into a tube and centrifuged at 425 g for 10 min to get rid of cell debris. The supernatant was collected into fresh tubes, aliquoted into

polypropylene cryogenic vials and stored at -80 ^{0}C. All virus stocks were then quantified by standard plaque assay.

UV Inactivated virus stock. After harvesting, the virus stock was divided in half into two sterile tubes, one half was poured into a sterile 60 mm petri dish and treated with UV radiation for 2 min in UV cross linker (FB-UVXL-1000, Fisher Biotech®), rocked to mix and irradiated for 2 more minutes. The other half was left untreated. Then these stocks were aliquoted and quantitated using standard plaque assay to confirm virus inactivation. In experiments using UV inactivated virus the parental stock was used for control infections.

Plaque Assay. NIH3T3 cells were seeded into 12 well plates (MIDSCI, Valley Park, MO, USA) at 1x 10^5 cells per well. Cells were infected 24 hours later. Tenfold serial dilutions of the virus stock were made in sterile tubes. One hundred ul of the virus inoculum was used in duplicate wells with each dilution. Cells were infected for 1 hour by rocking every 15 min. After removing the inoculum the cells were overlaid with 1X DMEM complete media with 0.5 % agar. Then the plates were incubated for 5 days. At day 5 cells were fixed using 10 % buffered formalin. Twenty-four hours later overlay and formalin were removed and plates were dried. Then the cells were stained with 1% crystal violet to facilitate counting of plaques. The average of 4 wells (2 dilutions) was used to get the virus titer of the stock.

Transfections

NIH3T3 cells were seeded at $6x10^5$ in a 25 cm^2 flask. Twenty-four hours later they were transfected with 9.0 ug of reporter plasmid using 18.0 ul of Transfectin™ lipid reagent (BIO-RAD, Hercules, CA, USA) at a 2:1 ratio of Transfectin to plasmid. Twenty-four hours post transfection the cells were counted and split into triplicate wells of a 12 well plate at equal cell densities. After an additional 24 hours, transfected cells were infected as described for the plaque assay at an MOI of 2 which would be sufficient to infect almost every cell, mock infected (media without virus) or treated with UV inactivated virus at a dilution comparable to the parental virus. Twenty fours post infection the cells were examined for CPE to confirm infection. Supernatants were collected to perform the SEAP assay and cells were harvested for DNA for normalization of transfection efficiencies.

Co-Transfections. NIH 3T3 fibroblast cells were transfected with 6.0 ug of reporter plasmid along with 6.0 ug of a plasmid that expresses both IE1 and IE3, or IE1 alone or IE3 alone using the same 2:1 ratio of Transfectin to plasmid and the cells were handled as for mock infection to avoid inconsistences of SEAP levels with other transfections. In some co-transfections the cells were treated with UV inactivated virus instead of media. Plasmids encoding MCMV immediate-early genes (IE3 and IE1/IE3) were kindly provided by Dr. Martin Messerle (University of Halle, Halle, Germany). The IE1-GFP tagged plasmid was generously provided by Dr. Qiyi Tang (Ponce School of Medicine, Ponce, Puerto Rico).

PFA treatments. Phosphonoformic acid (PFA, Foscarnet) is a drug that inhibits viral DNA replication as described in the antiviral therapy section. PFA treatment was done to inhibit late gene expression. After infecting the cells, DMEM complete media mixed with 50 ug/ul of PFA (Sigma-Aldrich, St Louise, MO, USA) was added for 24 hours. As mentioned above replicate wells received either normal media or media with PFA.

Normalization. DNA was isolated from cells using the MasterPure™ DNA purification kit (Epicentre® Biotechnologies, Madison, WI, USA) following the company's protocol. This DNA was used for quantitative PCR for the normalization of transfection efficiencies. Cellular gene primers for poly A binding protein (PABP) were used as a loading control to normalize equal amounts of cellular DNA (Table 2). Ampicillin primers (Amp) were used for the plasmid DNA (Table 2). Ampicillin primers could not be used to look for transfection efficiencies in co-transfected DNA samples because the IE1/IE3 plasmids also had *Amp* resistance. Hence we used primers for SEAP to see relative copies of reporter plasmid DNA present in co-transfected samples (Table 2). The qPCR reaction mixture contained: 12.5 ul of 2X iQ™ SYBER® green super mix (BIORAD, CA, USA), 1 µl of each forward and reverse primers (10 mM), 10.0 ng template DNA, and double distilled water. All the reactions were run in duplicate. PCR was done using the program conditions; 1 cycle of 3 min at 95 ^{0}C, followed by 35 cycles of 30 sec at 95 ^{0}C, 30 sec at 60 ^{0}C, and 30 sec at 72 ^{0}C which had previously been determined to work well with all the primers. Fluorescence was read after each cycle.

After 35 cycles, final incubation was carried out for 5 sec at 72 ^{0}C. Melting curves were done at starting temperature 65 ^{0}C, ending temperature 90 ^{0}C, with increments of 0.1 ^{0}C, and hold for 1sec. After completion of melting curves, there was a final extention at 72 ^{0}C for 1 min followed by holding at 10 ^{0}C. Quantitative PCR products were run on 1 % agarose gels to check for a single product of the correct size. For normalization, first the loading control was analyzed to ensure this was comparable between samples. Any differences in the loading control were used to correct for the plasmid of interest. Then we checked to see if there is any difference in the amount of plasmid of interest. If they were not the same then the fold difference was used to correct the SEAP levels. Cycle number varying in the loading control (PABP) means the DNA quantification is wrong. Cycle number varying in the plasmid of interest (AMP/SEAP) but not varied in the loading control means transfection efficiencies are different. This allowed us to identify whether any difference in SEAP levels is coming from different transfection efficiencies or the difference between the reporter constructs. The primers used in qPCR are shown in Table 2.

TABLE 2. qPCR primers used in the normalization of transfection efficiencies

Primer Name	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
PABP	GCAATGCTGGCCCAGTGATCATG	AAGGCCAGGGACGTCCTCACTGAC
Ampicillin	ATCGTTGTCAGAAGTAAGTTGG	GCCGCATATCACTATTCTCAG
SEAP	AGACCTTCATAGCGCACGTC	CATGTCTGCTCGAAGCGGC

SEAP Assay

Culture supernatants were collected and centrifuged at 10,621 g for 4 min to get rid of any cells and supernatant was transferred to fresh tubes. These samples were frozen at -20 ^{0}C until ready to do the assay. Initial studies indicated that freezing and thawing of the samples did not significantly affect SEAP activity. The assay was performed in triplicates. Samples were assayed using the Great EscAPe^{TM} Fluorescent SEAP assay (Clontech). In this assay 75.0 µl of 1X dilution buffer was added to 75.0 µl of supernatant in 0.5 ml PCR tubes (BIORAD, Hercules, CA, USA) and heated at 65 ^{0}C for 30 min in a PTC-200 Peltier thermal cycler (MJ Research) to inactivate cellular phosphatases. The samples were kept on ice for 2 min and cooled to room temperature, and transferred into triplicate wells of a micro plate-96 well plate with clear bottoms and black sides (MID-SCI, St Louise, MO, USA). Seventy five µl of assay buffer was mixed with 3.0 µl of substrate, [1 mM 4-methylumbelliferyl phosphate (MUP)], which was diluted in 1X dilution buffer and added to each sample well that had sample. The plates were incubated for 60 min in the dark at room temperature on a shaker. Fluorescence was detected using the Synergy HTS fluorometer at 360 nm transmittance, 340 nm absorbance and at sensitivity 60. SEAP activity in the reporter constructs was compared to pSEAP2-Basic (no promoter). We used a positive control plasmid under the control of SV40 promoter in our initial studies to check if the assay is working.

Statistical analysis

One-way ANOVA was used to determine statistical significance between treatments and student t-test was used to compare the individual constructs. P values of ≤ 0.05 were considered significant.

Site directed mutagenesis

Based on the results from the SEAP assay, we performed site directed mutagenesis of identified important DNA sequence regions.

Primer Designing. The most critical part in primer design is introducing a restriction site to destroy the consensus site and to simplify screening. The primers were designed that are complementary to each other so that they could anneal to the same sequence on the opposite strands of the plasmid. These primers were used in sequential PCR reactions. For the 1st round, one reaction was performed with the forward primer used to generate the -205 or the -315 construct and the reverse mutated TATA primer. A second reaction was done with forward mutated TATA primer and the common 3' reverse primer (Table 1). In the 2nd round PCR, the PCR products from the 1st round were used as template with -205 or -315 construct forward and common 3' reverse primer. The PCR was performed as described in the PCR section with an annealing temperature of 59.4 ^{0}C. The final PCR product of the expected size was gel purified, TA cloned and screened for the designed mutation via restriction digestion. The designed mutated bases are underlined, which makes a restriction site for *BsrGI*.

Original sequence: GATGGTGTAAAAGAGGGCCG

Mutated Forward primer: 5' GATGGTGTACACGAGGGCCG 3'

Mutated Reverse primer: 5'CGGCCCTCGTGTACACCATC 3'

Electrophoretic Mobility Shift Assay (EMSA)

The principle behind this assay is DNA runs slower when it is bound to a protein than DNA alone on acrylamide gels. In this study we used oligonucleotides designed based on the deletion analysis. These oligonucleotides were end labeled with LICOR 700 IRDye to allow detection in the gel. Nuclear extracts were prepared from mock infected or infected NIH3T3 cells as described in nuclear extraction preparation. The designed oligonucleotides are shown in the Table 3.

TABLE 3. Designed oligonucleotides for EMSA, consensus-binding sites are underlined.

Site Name	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
TATA box	GATGG**TGTAAAA**GAGGGCCG	CTACCACA**TTTTCTC**CCGGC
Core binding factor	TCTGTCTGGG**TGTGGTCA**CCCGGA	AGACAGACCC**ACACCAGT**GGGCCT
SP1	GCCGTCACGATGT**CCGCCCG**CGAGTTGTCG	CGGCAGTGCTACA**GGCGGGC**GCTCAACAGC

Nuclear Extraction preparation. Cells were seeded into 60 mm cell culture dishes at a density of 2 x 10^5 and 24 hours later infected with MCMV at an MOI of 2 or mock infected. Twenty-four hours post infection the cells were washed with PBS-EDTA, scraped from the surface of the dish in 1 ml of PBS-EDTA, transferred into a 1.5 ml eppendorf tube, and pelleted at 106 g for 10 min. The pellet was resuspended in 100 ul of

cytoplasmic extract (CE) buffer with detergent NP-40 (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 0.075% (v/v) NP40, 1mM DTT and 1:1000 diluted protease inhibitor [Protease inhibitor cocktail set III, CALBIOCHEM, La Jolla, CA] adjusted to pH 7.6). The samples were incubated on ice for 3 min and centrifuged at 239 g for 4 min. The cytoplasmic extract (supernatant) was collected into a new tube, glycerol was added to a final concentration of 20 % and stored at -80 ^{0}C. The nuclei were gently washed with 100 ul of CE buffer without NP-40 and centrifuged at 239 g for 4 min. Supernatant was removed and 50 ul of nuclear extract (NE) buffer (20 mM Tris Cl, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, protease inhibitor cocktail set III and 25 % (v/v) glycerol, adjusted to pH 8.0) was added to the nuclear pellet. After resuspending by vortexing, salt concentration was adjusted to 400 mM by adding an additional volume of 5.0 M NaCl. This extract was incubated on ice for 10 min with periodic vortexing. This extract was centrifuged at 10,621 g for 10 min to pellet any remaining nuclei. Supernatants were collected and stored at -80 ^{0}C.

Western blotting. In order to check for cytoplasmic protein contamination of nuclear extracts we performed western blot analysis. We used Bio-Rad's western mini PROTEAN 3 apparatus for running the gel. Approximately 40 ug of nuclear extracts were separated by SDS-PAGE on 10 % gels and transferred to nitrocellulose at 4 ^{0}C overnight. The mini wet trans blot system from Bio-Rad was used for transferring. The membranes were then incubated in blocking buffer [5 % milk in TBS (150 mM NaCl, 10 mM Tris pH 7.5)] for 1 hour, before primary antibodies were added at a dilution of 1:200

in blocking buffer. Primary antibodies used were mouse anti- α tubulin for cytoplasmic protein and rabbit anti-e1 to confirm viral infection. Then the membranes were washed 3 times in TBS 0.1% Tween 20 with 5 min incubation to get rid of primary antibody. Secondary antibodies were added at 1:15000 in blocking buffer. The secondary antibodies used were fluorescently labeled goat-anti rabbit and goat-anti mouse. The membranes were then washed again 3 times with TBS 0.1 % Tween 20. The membranes were detected on the Odyssey CLx infrared imaging system (LI-COR Biotechnology, Lincoln, NE).

(i) Antibodies. Mouse anti-α-tubulin (clone B-5-1-2) was purchased from Sigma (Sigma-Aldrich, St. Louis, MO). The generation and characterization of rabbit anti-e1 antibody were previously described. Secondary antibodies used goat-anti rabbit IRDye 800 CW and goat-anti mouse IRDye 680 RD from LICOR.

Protein Concentration Determination. We used the Bio-Rad DC ™ protein assay kit. We followed the Bio-Rad's microtitre plate protein assay protocol for determination of the concentration of protein in the extracts, using bovine serum albumin (BSA) as a standard. All BSA standard samples were performed in duplicates.

Oligo Preparation. Oligos were resuspended in 1X TE buffer (pH 7.5) to a final concentration of 20 pmol/µl. Five µl of forward IRDye 700 oligo and 5 µl of reverse IRDye 700 oligo were added into a new tube. These oligos were annealed by placing the oligo set in a 100 ^{0}C heat block for 5 min. then slowly cooled to room temperature. The annealed oligos were diluted 1: 200 with di H_2O and stored at 20 ^{0}C. This stock was used

as a working oligo stock.

Gel Preparation and Binding Reaction. Denatured polyacrylamide of gels 4% or 6% were prepared. The percent used depended on the oligo size. The binding reaction was set up which included, 100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5, 25 mM DTT/2.5%Tween® 20, NIH3T3 nuclear extract (5.0 μg) and 20 fmoles of annealed IRDye 700 primers. We used mock and infected nuclear extracts in binding reactions. There were three control samples in which one tube contained just the labeled probe, a second tube contained labeled probe, and nuclear extract (Mock or infected) and the third tube contained labeled probe, nuclear extract (Mock or infected), and non-specific unlabeled DNA [non-specific competitor (Mutated TATA primers) 1 μg/ul,]. If the non-specific unlabeled DNA cannot compete for binding that helps confirms specificity of the binding. Reaction mixtures were incubated for 30 min and 2.0 ul of 10X orange loading dye (LI-COR®) was added. Mixtures were loaded on the polyacrylamide gel and run using Tris borate EDTA buffer (TBE) at half the concentration used for DNA agarose gels until the dye front was 2 inches from the bottom of the gel. All of this was done in the dark at 4 ^{0}C because of the light sensitivity of the probe. Then the gel was detected on the Odyssey CLx infrared imaging system.

CHAPTER III

RESULTS

Identification of DNA sequences important for the m143 promoter. In order to identify what sequences are involved in the promoter regulation of m143, a series of nested deletion mutants of the m143 promoter were generated and tested for the ability to mediate expression of a secreted alkaline phosphatase in the pSEAP2-Basic plasmid. The presence of consensus transcription factor binding sites for factors previously found to be important for other herpesviruses promoters was noted. The regions were identified using transcription element search system (TESS) from the computational biology and informatics lab at University of Pennsylvania. These sites and the deletion mutants are shown in Figure 5 A and B respectively. The numbers indicate location relative to the transcriptional start site (+1).

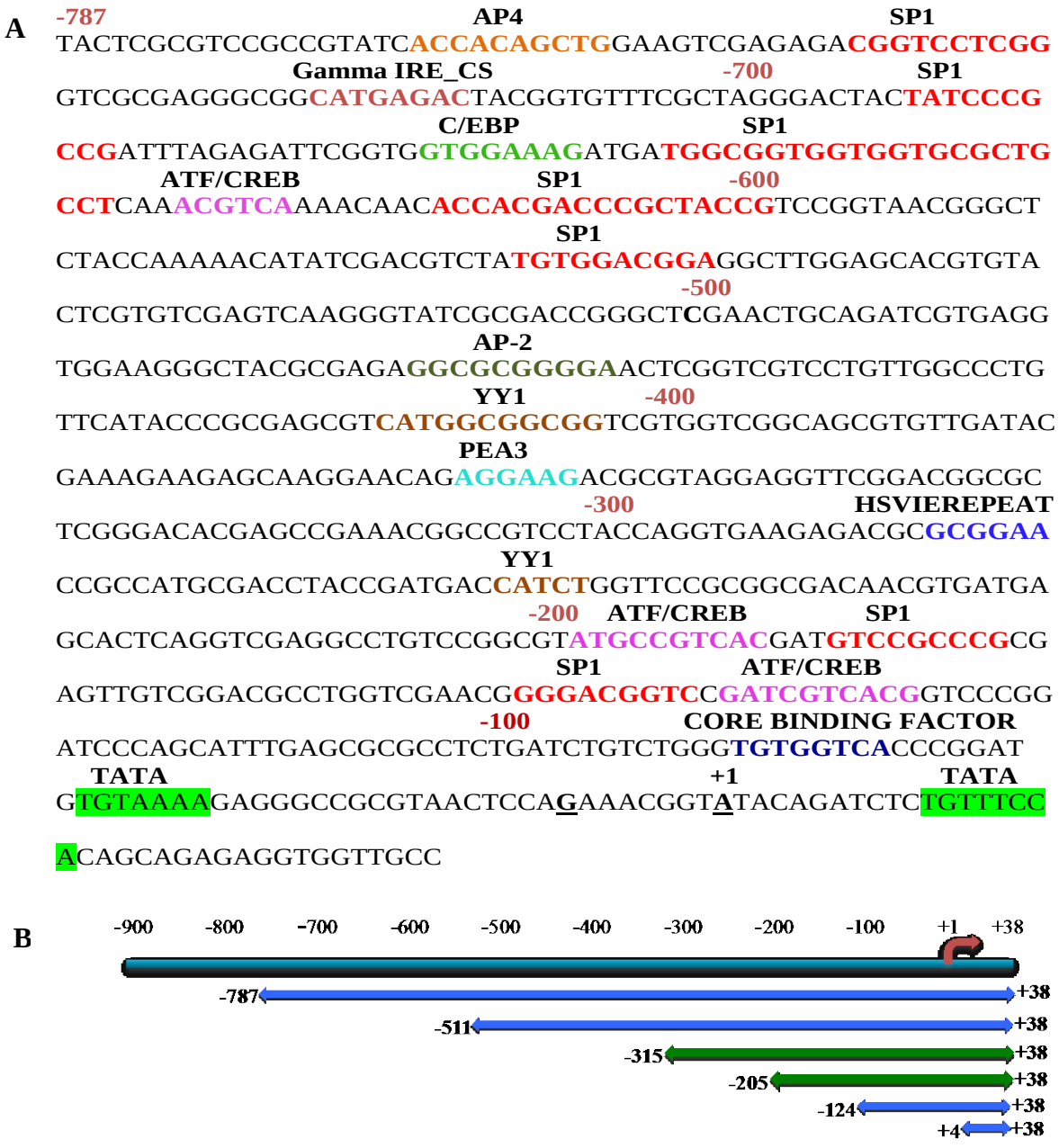


FIG. 5. **M143 promoter region and deletion mutants: A.** m143 promoter sequence used in this study. +1 indicates the transcriptional start site. The locations of consensus transcription factor binding sites are indicated. **B.** Map of deletion mutants used to analyze the functional binding sites involved in transcriptional regulation.

SEAP activity was assessed for mock and infected cells with each promoter reporter construct. In all cases, the phosphatase activity is expressed as fold increase over background. The background activity from cells transfected with the pSEAP2-Basic plasmid, which contains no promoter, was set at 1 in every experiment. Mock-infected SEAP levels for all the constructs were not significantly different than no promoter, indicating that cellular factors are not sufficient for detectable activity of the m143 promoter (Figure 6). SEAP levels from infected cells were significantly higher than uninfected indicating that viral factors are required for the activation from the m143 promoter. SEAP levels with the full-length promoter were 4 fold higher than -511, which means that there is at least one activator-binding domain within the deleted region. This sequence contains several potential important binding sites including one of each AP4, Gamma interferon response element, C/EBP, ATF/CREB and 5 SP1 sites. Deletion of the next 196 bases led to 3.5 fold increased SEAP levels, which indicates the removal of a repressor-binding site. This region contains one each of AP2, YY1 and PEA3 sites. SEAP levels between the -315 construct and the -205 construct were not significantly different from each other. The sites present in this region, HSV IE repeat and YY1 are unlikely to be important for regulation of the m143 promoter. Deletion of the region between -205 and -124 resulted in loss of all activity, which indicates that an activator-binding site or sites were removed. This region contains 2 ATF-CREB and 2 SP1 sites. SEAP levels of the +4 construct were the same as the background.

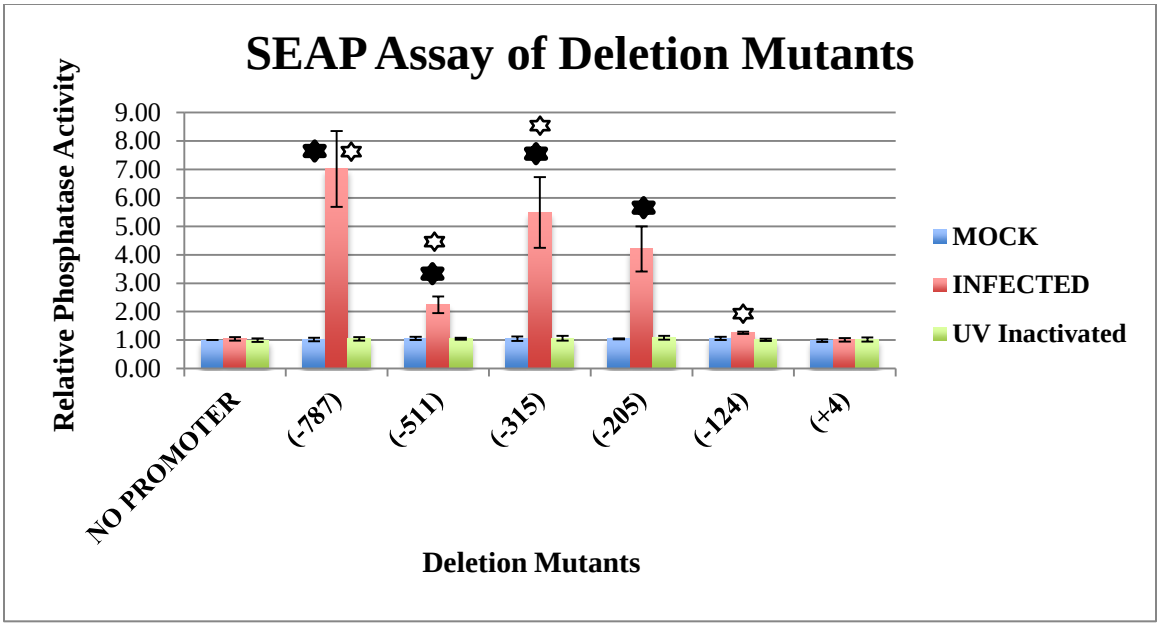


FIG. 6. **Viral infection is required for detectable activation from the m143 promoter:** NIH3T3 cells were transfected with the indicated promoter reporter constructs and were mock infected, infected at an MOI of 2 or treated with UV inactivated MCMV at the same dilution as the virus 48 hours post transfections. Supernatants were collected for SEAP assay 24 hours post infections. The level of activity is expressed relative to no promoter set at 1. n=3, error bars are std. error of means. Closed stars represent statistical significance between mock and infected. Open stars represent significant difference from the previous construct.

Newly synthesized proteins are required. It has been shown that the attachment of virus to the cell surface activates some cell signaling pathways (79). Using UV inactivated virus allowed us to address the importance of these changes in activation of the m143 promoter. Treatment with UV inactivated virus didn't show any significant difference from mock infection (Figure 6), which indicates that tegument proteins from the virus entry and attachment of virus to the cell surface are not sufficient to activate this promoter.

Immediate Early proteins (IE1/IE3) are sufficient for activation. Two important viral transcriptional regulators that are expressed rapidly after infection are splice variants (IE1and IE3) (54, 111). To investigate the importance of IE1and IE3 for activation of the m143 promoter, the reporter plasmids were co-transfected with a plasmid expressing both IE1 and IE3. The SEAP activity from co-transfections was significantly higher than background (no promoter) in all cases except the +4 construct as shown in Figure 7, SEAP activity in the full-length construct was 4 fold higher than the rest of the constructs, indicating that either IE1or IE3 are activating between -787 and -511 or other cellular factors may be enhancing the activation via this region. There was no significant difference in SEAP activity levels from the -511 to -124 constructs. There was a significant decrease in the +4 construct from the -124 construct, indicating either IE1, IE3 or both activate via this region. Unlike infection (Figure 6), repression between -511 and -315 was not seen in co transfections, indicating that other viral proteins likely mediate the repression of the m143 promoter via this region. The SEAP activity levels of the -124 construct in the presence of IE1/IE3 are double the SEAP activity levels from infections (Figure 6), indicating there may be other viral proteins repressing in this region as well. There are two putative TATA boxes present one at -33 and another at +11. The +11 one was removed in the +4 deletion mutant. Decrease in the SEAP levels between the -124 construct and the +4 construct support that TATA sequence present at -33 is more likely to be functional.

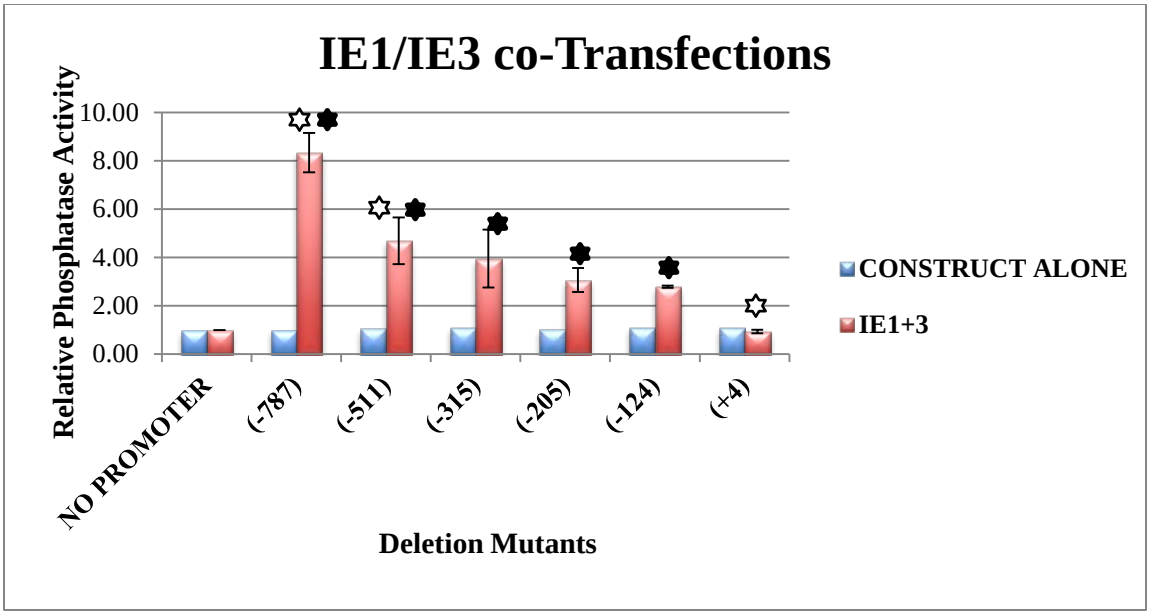


FIG. 7. **Immediate early proteins IE1/IE3 are sufficient to activate the promoter:** NIH3T3 cells were co-transfected with a plasmid that expresses both IE1 and IE3, and treated as mock infection. Supernatants were collected for SEAP assay 48 hours post transfections. The level of activity is expressed relative to no promoter set at 1. n=3, Error bars are std. error of means. Closed stars represent statistical significant difference from no promoter. Open stars represent significant difference from the previous construct.

IE1 alone can activate m143 promoter. Since cytomegalovirus promoters may vary in the importance of IE1 and IE3 for regulation we next examined the ability of IE1 alone to activate the m143 promoter (Figure 8). Cells were co transfected with a plasmid that expressed IE1 alone along with the m143 promoter constructs. SEAP levels of all the constructs with IE1 co-transfection were above background except the +4 construct, indicating that the region required for activation by IE1 is in this minimal promoter. SEAP levels of IE1 co-transfections were much lower than the SEAP levels of IE1/IE3

co-transfections (Figure 7) in all the constructs, indicating that IE1 alone only weakly activates the m143 promoter. IE1 likely cooperate with IE3 to activate this promoter.

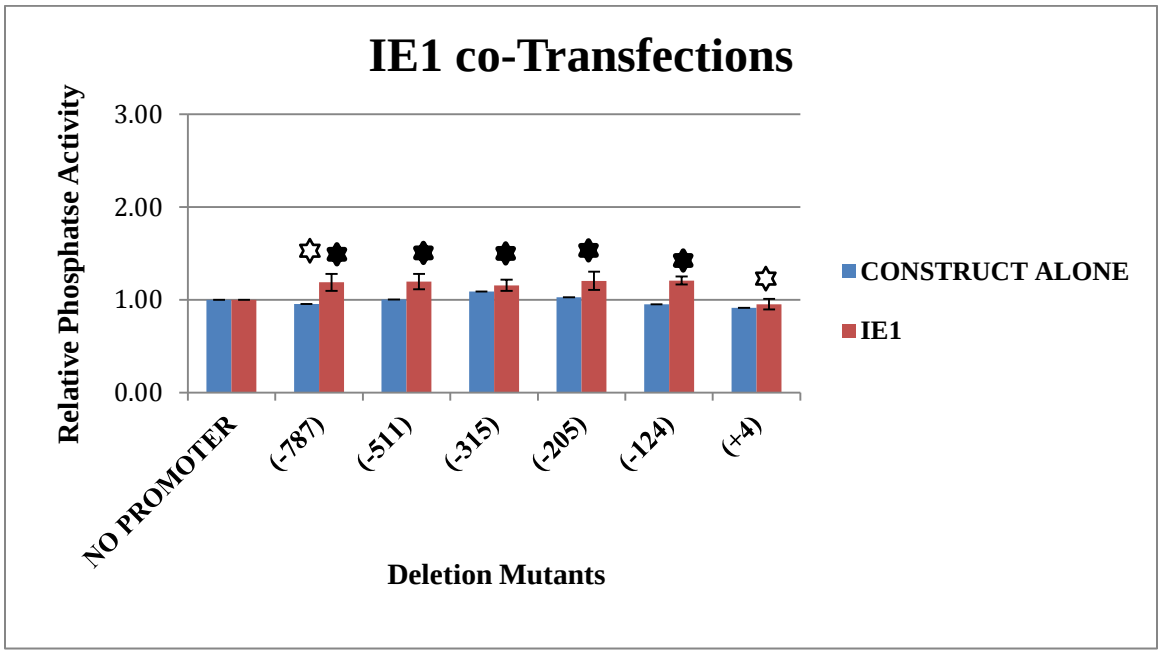


FIG. 8. **IE1 alone can activate the m143 promoter:** NIH3T3 cells were co-transfected with a plasmid that expresses IE1. Supernatants were collected for SEAP assay 48 hours post transfections. The level of activity is expressed relative to no promoter set at 1. n=3, Error bars are std. error of means. Closed stars represent statistical significance between treatments. Open stars represent significant difference from previous construct.

IE3 alone can activate the m143 promoter. SEAP levels of all the constructs with IE3 co-transfection were not significantly different from the background SEAP levels except full-length promoter (Figure 9), indicating that IE3 alone can activate the m143 promoter weakly. Although IE1 and IE3 alone can activate weakly, IE1 and IE3 appear to work synergistically to activate the m143 promoter.

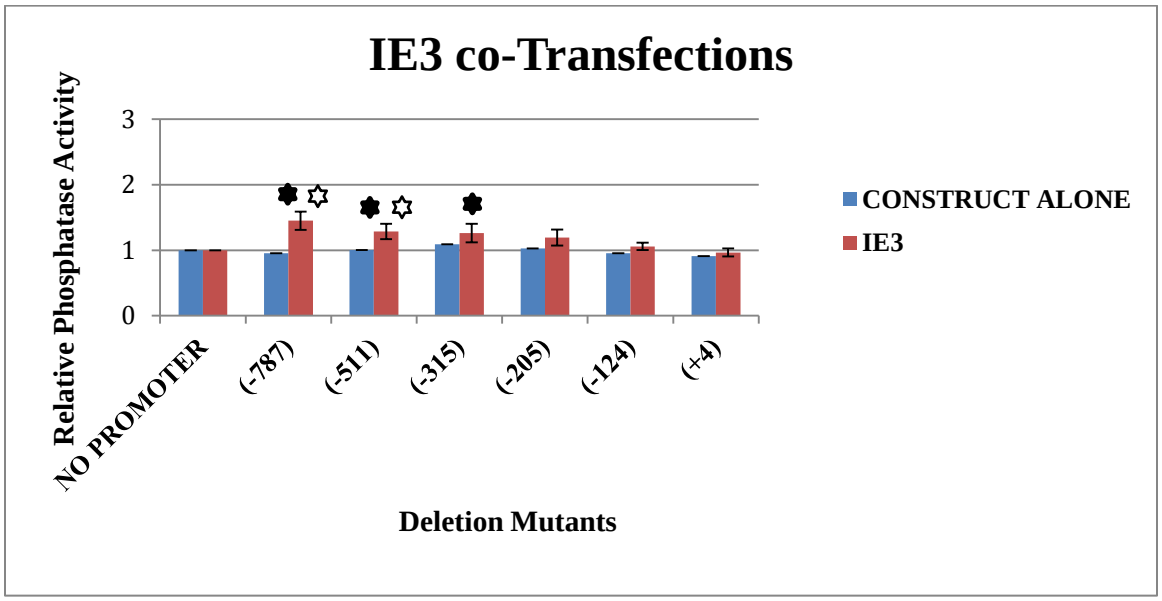


FIG. 9. **IE3 alone can activate the m143 promoter:** NIH3T3 cells were co-transfected with IE3 plasmid. Supernatants were collected for SEAP assay 48 hr post transfections. The level of activity is expressed relative to no promoter set at 1. n=3, Error bars are std. error of means.

Late proteins are not involved in the regulation. Since the data indicates that other viral proteins are also involved in regulation of the m143 promoter, we did treatment with phosphonoformic acid (PFA, foscarnet) to determine whether late proteins were involved (Figure 10). PFA is a drug that inhibits viral DNA replication thus preventing late gene expression. SEAP levels in the presence of the inhibitor were not significantly different from infected without drug in all cases, indicating that late viral proteins are not involved in the regulation of m143 promoter. Thus the viral regulators are likely to be early proteins.

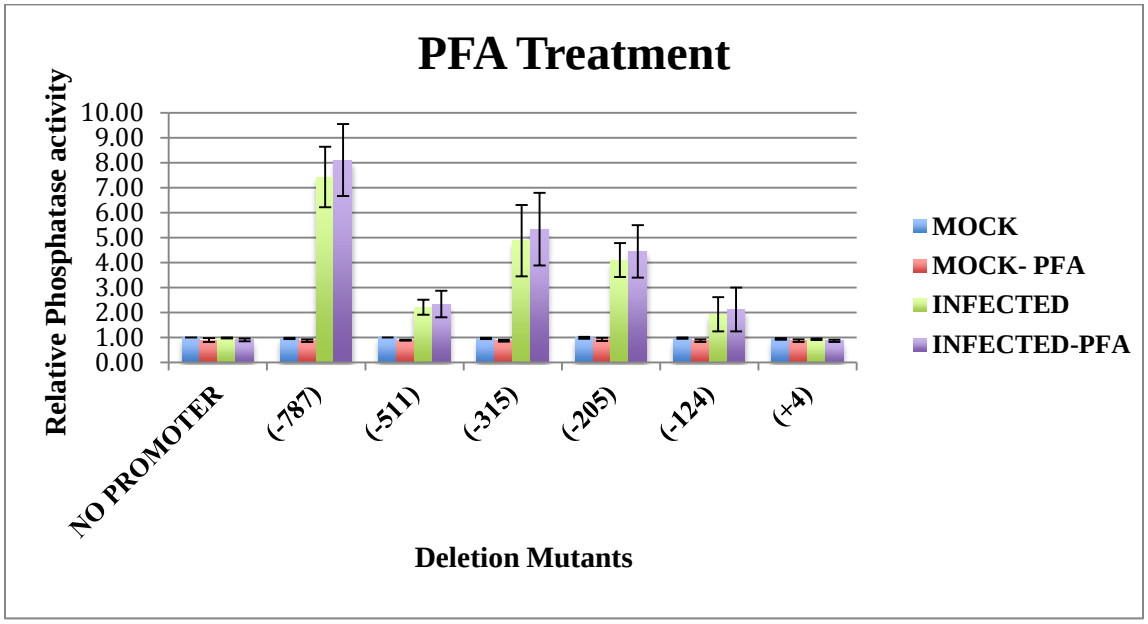


FIG. 10. **Late viral proteins are not involved in regulation of the m143 promoter:** NIH3T3 cells were transfected and treated as in Figure 6 except 6 ul/ml of PFA was present throughout the infection in the indicated samples. Supernatants were collected for SEAP assay 24 hours post infections. The data shown above are the mean and the standard error of the mean of three individual experiments.

Viral factors are involved in m143 promoter regulation. In order to see if tegument or capsid proteins are involved in the repression of m143, we did co-transfections with IE1/IE3 and treated with UV inactivated virus (Figure 11). There was no significant difference in all the constructs between IE1/IE3 co-transfections without UV inactivated MCMV and with UV inactivated MCMV, indicating that tegument proteins and capsid proteins together are not involved in the repression of the m143 promoter.

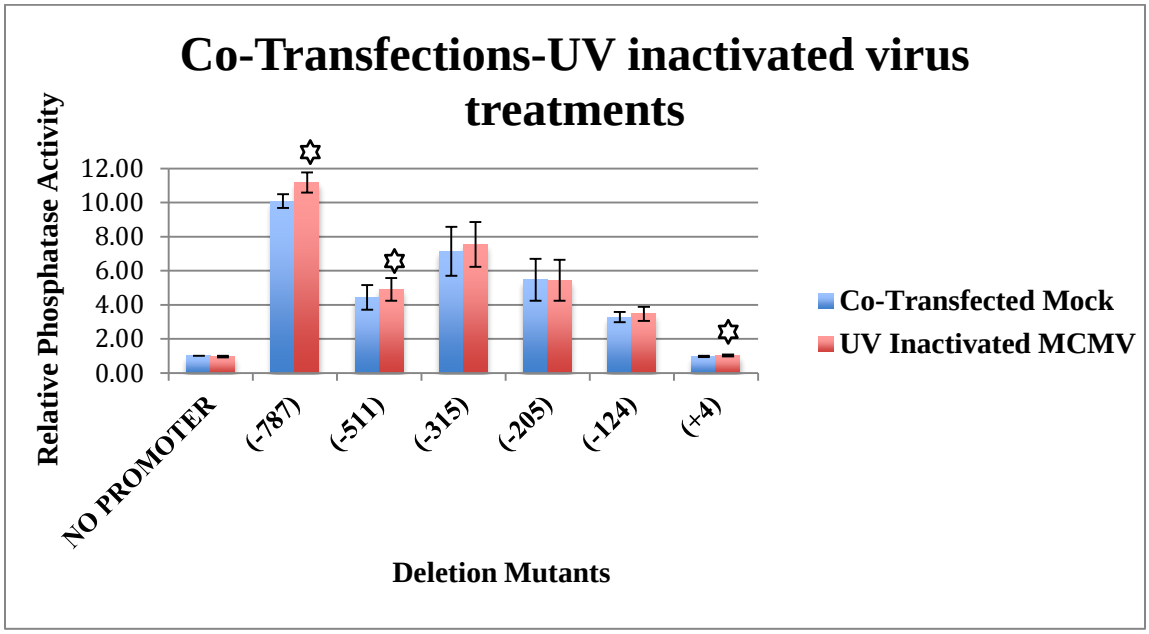


FIG. 11. **Viral proteins enhance/repress m143 promoter activation:** NIH3T3 cells were co-transfected as in Figure 7, except that replicate wells were treated with UV inactivated virus as in Figure 6. Supernatants were collected for SEAP assay 48 hours post transfections. The data shown above are the mean and the standard error of the mean of three individual experiments. Open stars represent significant difference from

Mutational analysis of the putative TATA box

To determine if the putative TATA box in the -124 construct is important, Several PCR attempts were done using the mutated primers of TATA box and the primers for the -315 or the -205 construct as described in the methods section. Plasmid DNA of the -315 or the -205 construct was used as template. After TA cloning of the gel purified PCR product into PCR2.1 TOPO, clones were screened for the mutated insert using *BsrGI*. We didn't get the mutated TATA insert. So the template was changed to DNA from infected cells, because we used the same template in our initial PCR while making deletion mutants. This time the PCR product was TA cloned into pGEM-T easy vector system I (Promega, Madison, WI). The clones were again screened for the introduced *BsrGI* restriction enzyme site. But we were not successful in getting the mutated plasmid. It may be because one of the mutated fragments from the mutation to the common 3' reverse primer is only 70 bases long and might have been confused with primer dimers. Probably conditions need to be worked out more or a different method may need to be used.

Identification of transcription binding factors that are involved in the regulation of the m143 promoter

Electrophoretic mobility shift assay was performed to identify if any proteins are binding to specific SP1 and TATA binding sites in the m143 promoter. A SP1 site was chosen because of activation from m143 promoter wherever this site is present in the sequence. Nuclear extracts from mock infected and infected cells were prepared as

described in the methods section. These extracts were tested for cytoplasmic contamination by western blotting (Figure 12). With anti tubulin, there was only a slight contamination with cytoplasmic proteins in the nuclear extracts. After quantifying the nuclear extracts they were used for EMSA as described.

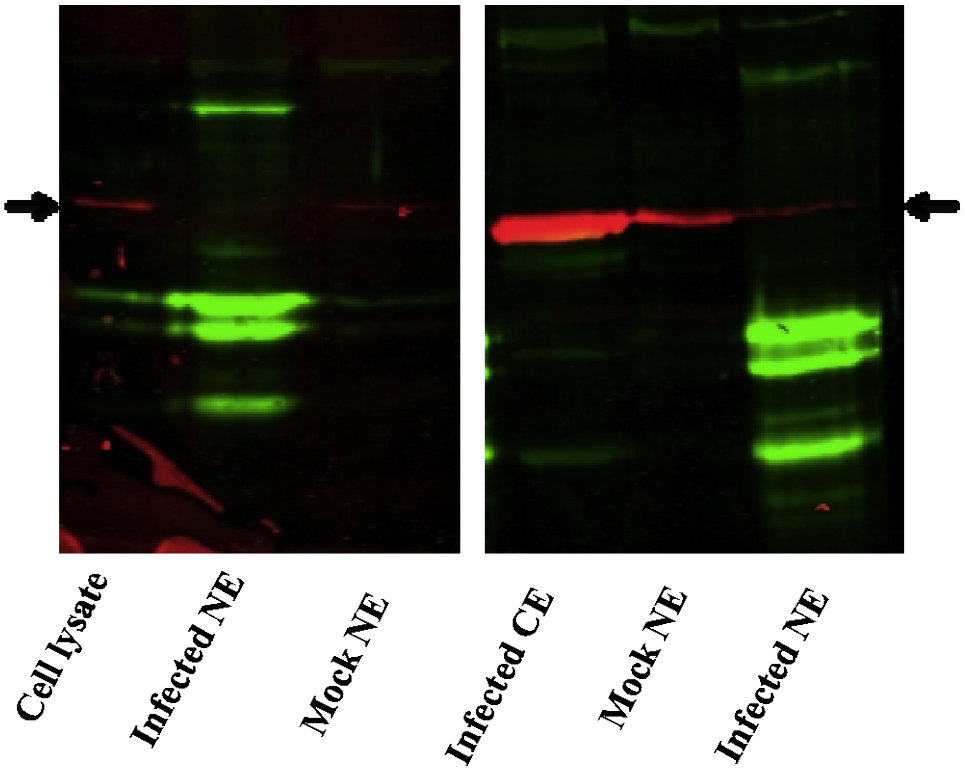


FIG. 12. **Western blot:** western blots for cytoplasmic protein contamination check in infected and mock nuclear extracts (NE). CE: cytoplasmic extracts. The red indicates tubulin and green indicates viral nuclear protein e1.

To examine the reproducibility of binding patterns, we used three different nuclear extracts. The patterns were similar for all three extracts, which indicates that the binding is reproducible. The labeled SP1 probe alone seen in the first well is a control for migration and so the shift of the probe with bound protein can be differentiated on the gel (Figure 13). To see if the binding is specific, we used a non-specific unlabeled DNA competitor both with mock and infected nuclear extracts. Addition of nonspecific competitor helps to reduce the concentration of nonspecific proteins and can indirectly enhance the binding of specific proteins to the labeled probe as seen for the band indicated by the arrow. Mock nuclear extracts were used to see if the proteins or complexes that bind to the probe are cellular or viral proteins addressed in more detail in the discussion. Additional bands on the gel show nonspecific binding because binding is reduced by the non-specific competitor.

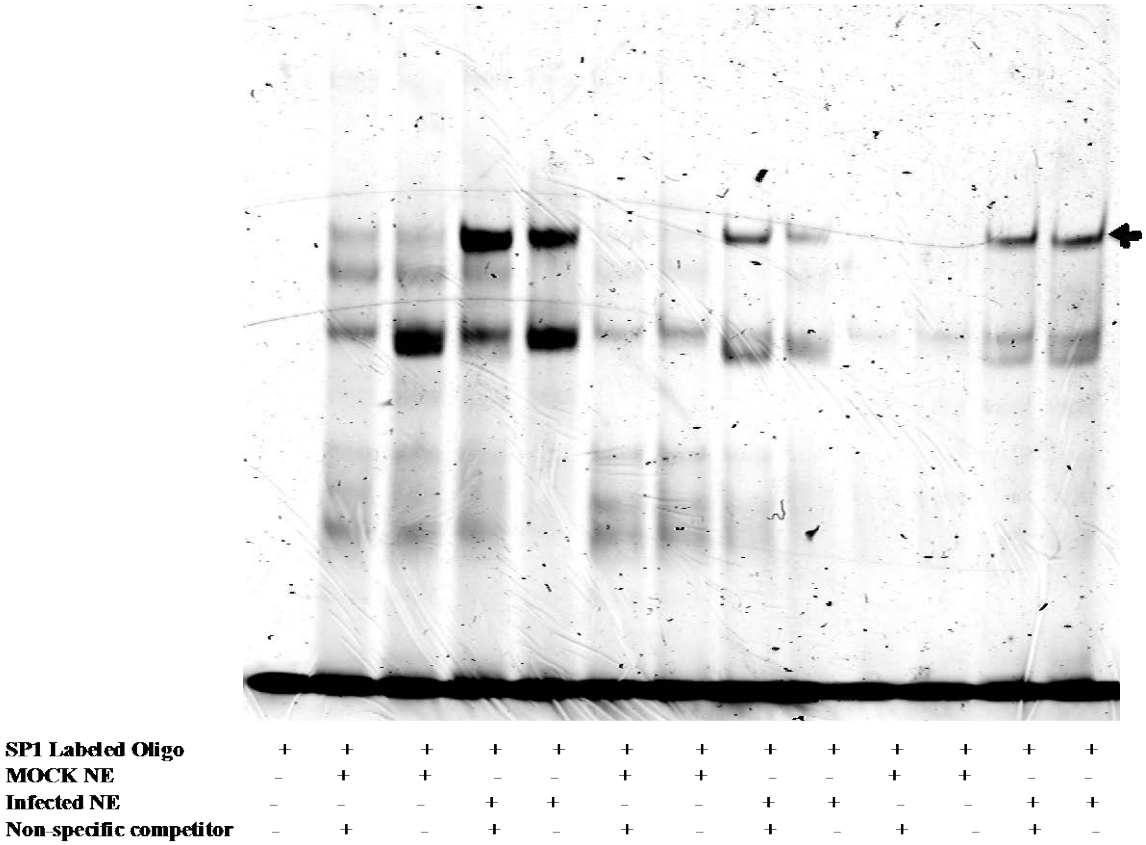
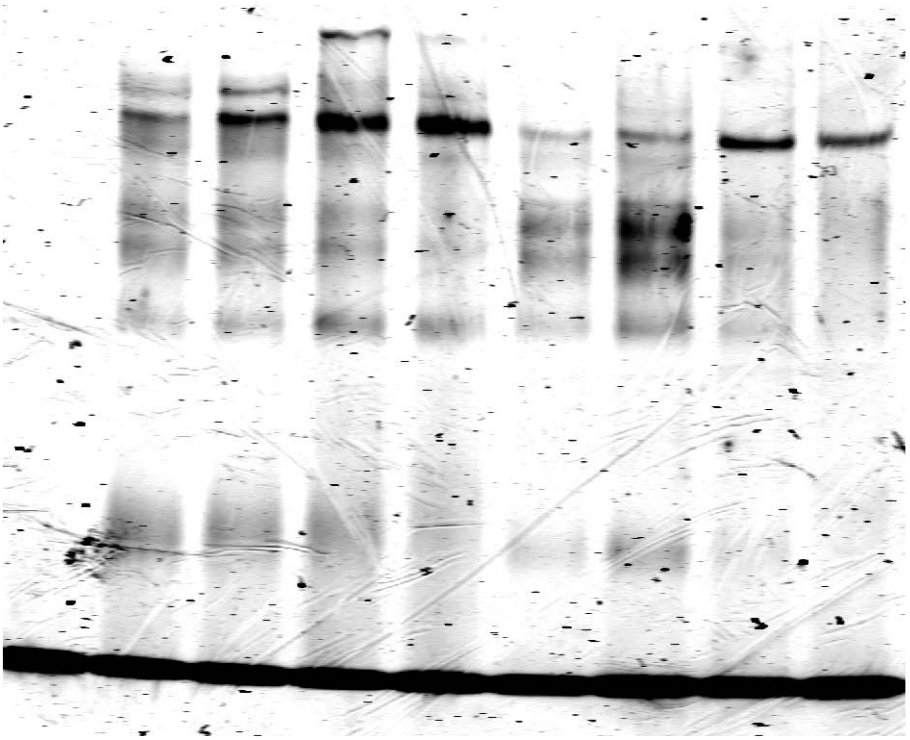


FIG. 13. **EMSA for SP1 binding:** EMSA was done for the SP1 binding site present at -187 to -178 with three different nuclear extracts (NE)**.** Conditions used are described in materials and methods. The band indicated with an arrow is likely a specific interaction.

We wanted to see if anything binds to the putative TATA sequence of the m143 promoter. TATA labeled oligo was used to see if any proteins from nuclear extracts are binding to this sequence. Figure 14 shows the binding of multiple complexes to the labeled oligo in mock and infected nuclear extracts. Since the results with the competitor

used were inconsistent, the specificity is unclear. The conditions may need to be worked out further.



TATA Labeled Oligo	+	+	+	+	+	+	+	+	+
MOCK NE	-	+	+	-	-	+	+	-	-
Infected NE	-	-	-	+	+	-	-	+	+
Non-specific competitor	-	+	-	+	-	+	-	+	-

FIG. 14. **EMSA for TATA binding:** EMSA was done for TATA binding site with two different nuclear extracts (NE)**.** Conditions used are described in materials and methods.

CHAPTER IV

DISCUSSION

Identification of DNA sequences important for the m143 promoter

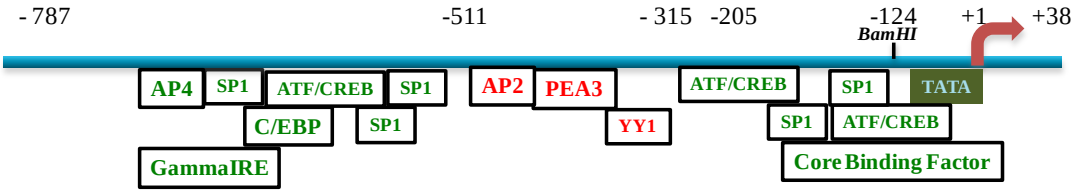


FIG. 15. **Consensus transcription binding sites:** The locations of consensus transcription factor binding sites on the m143 promoter. Green color font sites indicate cellular activator sites and red color indicates repressor sites.

M143 is an essential gene of MCMV and is required for inhibition of the PKR mediated antiviral pathway (70, 71). Because it is essential for the virus, understanding m143 gene regulation, especially by viral factors, could help in developing new antiviral therapeutics. Previously it has been shown that expression from the m143 promoter is up regulated upon viral infection by Hanson et al (68). In their work they used luciferase as a reporter, which is more sensitive than the SEAP reporter system and saw detectable activation in mock infections. Despite this difference, our system is very useful since the normal environment would be infected cells. This is a continuation of the same work to identify the regulatory elements of the m143 promoter. Deletion analysis of a promoter

is one of the common methods used for identification of important regulatory regions of that promoter. All of the regions identified as important for activation contain consensus binding sequences for SP1 and ATF-CREB suggesting that these may be important for activation (Figure 15). As discussed in the promoter regulation section, SP1 and ATF-CREB are upregulated upon HCMV infection and are important in regulating HCMV gene expression (83, 91, 93). The significant increase in SEAP levels after the deletion of the region between -315 and -511 supports the presence of a repressor-binding site. This region contains 3 sites PEA3, AP2 and YY1 (Figure 15). YY1 is known to activate and repress transcription depending on associated factors such as location of its binding relative to transcription start site and post translational modifications (112). Some previous work indicates that YY1 can repress the HCMV MIEP enhancer (98), so it is reasonable to hypothesize that YY1 may be binding directly to the recognition sequence in m143 promoter or may be recruiting additional repressors to form a co-repressor complex to repress the m143 promoter. HCMV attachment stimulates the production and activity of several cellular transcription factors (79, 113). When the virus is UV inactivated it becomes a non-infectious viral particle, which means that viral DNA is not capable of replication and gene expression. When it is used for treatment it can attach, enter and release viral structural proteins and DNA in to the cell, these events alone can trigger various initial signaling pathways as described in promoter regulation (79, 114). We have investigated if treatment with UV inactivated MCMV affected the regulation of the m143 promoter. Treatment with UV inactivated MCMV resulted in no detectable

changes in m143 promoter regulation either alone or after activation by IE1/IE3 suggesting that viral attachment, entry and tegument proteins do not have any effect on m143 promoter which agrees with previous luciferase data (68). Some immediate early proteins of CMV are known to regulate various viral and cellular promoters. Previously discussed splice variants, IE1/IE3 are the known transcriptional regulators of MCMV. IE1 can activate the shared promoter, the MIEP and IE3 can repress this promoter (54, 115). We wanted to see if these immediate early proteins have any effect on m143 promoter's activation, Co-transfections with a plasmid that expresses both IE1/IE3 showed these are sufficient to activate the m143 promoter. Even though IE1 and IE3 alone are able to activate the m143 promoter, when IE1/IE3 are present together, the SEAP activity levels of m143 promoter increases significantly which indicates that they are acting synergistically. An explanation for this behavior could be, that IE1/IE3 form a complex and bind to the promoter either directly or via other transcriptional factors. Since there is no repression seen in co-transfections with IE1/IE3, the repression between -511 and -315 and -124 and +4 constructs may be coming from a viral protein other than IE1/IE3. No significant difference was seen with IE1/IE3 co-transfections and treated with UV inactivated virus, indicating that no combined effect of tegument and IE1/IE3 on the m143 promoter regulation. Since proteins other than immediate early proteins are involved in the regulation of the m143 promoter, we did MCMV infection followed by PFA treatment to prevent late gene synthesis and the results suggests that late proteins are not important in regulating the m143 promoter. The results from UV treatment and PFA

treatments suggest that the repressors are viral proteins, which are not tegument or late proteins. Recent studies show a viral repressor in HCMV, UL34, the UL34 gene expresses two forms of proteins, an early and a late. UL34 has 14 binding sites in the HCMV genome and has been shown to repress at least two HCMV promoters, an immediate early gene (US3) and an early gene (US9) (116). UL34 has a homologue in MCMV, m34, which could be binding to m143 promoter and repressing its gene expression (40). Although the DNA sequence bound by UL 34 was not found in the m143 promoter, it is unknown if m34 binds to the same sequence.

Identification of transcription binding factors that are involved in the regulation of the m143 promoter

After we found important regulatory regions of the m143 promoter, we wanted to see if SP1 and TATA binding factors are involved in the regulation of m143 promoter. The SP1 cellular transcription factor was suspected to have an important role in the regulation of the m143 promoter because of the activation in the constructs with this site present, in addition previous work indicates that HCMV increases SP1 levels and activity (93). Electrophoretic mobility shift assay was performed to examine binding of protein complexes to SP1 site that is present at -187 to -178 in the m143 promoter, this site was chosen because of the low number of consensus sites in this region. Our gel shift results indicate binding with multiple protein complexes. Proteins or complexes from nuclear extracts bound to the labeled SP1 oligo and at least one was specific binding because addition of non-specific unlabelled competitor did not inhibit the binding, if anything

binding was enhanced. Faint bands of the same size were observed in the mock nuclear extract, which indicates that MCMV infection may be up regulating this binding or increasing nuclear localization of the required proteins. The multiple bands observed in the gel shift could be explained the capacity of SP1 to bind to multiple cellular and viral proteins. Probe bound SP1 may be binding to different proteins and resulting into multiple specific shifted bands. These results would be consistent with an upregulation in SP1 protein or activity as reported for HCMV, supporting similar mechanisms in MCMV. Several bands were seen with TATA oligo, which indicates the binding of proteins, but it is not consistent in all extracts. So no final conclusions could be drawn.

The results of this study indicate that multiple viral and cellular factors are involved in regulating the m143 promoter. The major immediate early proteins IE1 and IE3 are required. Cellular SP1 may also be important and may be upregulated. Finally, It appears that other viral proteins repress the m143 promoter. Identification of the repressive mechanisms for this essential gene could lead to new ways to control cytomegalovirus infection and disease.

REFERENCES

1. **Davison, A. J., R. Eberle, B. Ehlers, G. S. Hayward, D. J. McGeoch, A. C. Minson, P. E. Pellett, B. Roizman, M. J. Studdert, and E. Thiry.** 2009. The order herpesvirales. Arch. Virol. **154:**171-177.

2. **Roizman, B., L. Carmichael, F. Deinhardt, A. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf.** 1981. Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses. Intervirology. **16:**201.

3. **Pellett, P.E., and Roizman, B.** 2007. The Family *Herpesviridae,* A Brief Introduction, p. 2479-2500, *In* D.M. Knipe and P.M. Howley (Ed), Fields Virology, 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

4.**Boehmer, P., and A. Nimonkar.** 2003. Herpes virus replication. IUBMB Life. **55:**13-22.

5. **Davison, A. J., B. L. Trus, N. Cheng, A. C. Steven, M. S. Watson, C. Cunningham, R. Le Deuff, and T. Renault.** 2005. A novel class of herpesvirus with bivalve hosts. J. Gen. Virol. **86:**41-53.

6. **Goodpasture, E. W., and F. B. Talbot.** 1921. Concerning the nature of" protozoan-like" cells in certain lesions of infancy. Arch. Pediatr. Adolesc. Med. **21:**415.

7. **Taylor, G. H.** 2003. Cytomegalovirus. Am. Fam. Physician. **67:**519-524.

8. **Colugnati, F. A. B., S. A. S. Staras, S. C. Dollard, and M. J. Cannon.** 2007. Incidence of cytomegalovirus infection among the general population and pregnant women in the United States. BMC Infectious Diseases. **7:**71.

9. **Brown, H. L., and M. P. Abernathy.** 1998. Cytomegalovirus infection. Semin. Perinatol. **22:**260-266.

10. **Mocarski E S jr., Robert Shank Thomas,** 2007. Cytomegaloviruses, *In* D.M. Knipe and P.M. Howley (Ed), Fields Virology, 5th ed, Lippincott Williams & Wilkins, Philadelphia, PA.

11. **Mustakangas, P., S. Sarna, P. Ämmälä, M. Muttilainen, P. Koskela, and M. Koskiniemi.** 2000. Human cytomegalovirus seroprevalence in three socioeconomically different urban areas during the first trimester: a population-based cohort study. Int. J. Epidemiol. **29:**587-591.

12. **Wong, A., K. Tan, C. Tee, and G. Yeo.** 2000. Seroprevalence of cytomegalovirus, toxoplasma and parvovirus in pregnancy. Singapore Med. J. **41:**151-155.

13. **Revello, M. G., and G. Gerna.** 2002. Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant. Clin. Microbiol. Rev. **15:**680-715.

14. **Pass, R. F.** 1985. Epidemiology and transmission of cytomegalovirus. J. Infect. Dis. **152:**243-248.

15. **Krmpotic, A., I. Bubic, B. Polic, P. Lucin, and S. Jonjic.** 2003. Pathogenesis of murine cytomegalovirus infection. Microb. Infect. **5:**1263-1277.

16. **Griffiths, P. D.** 2002. The treatment of cytomegalovirus infection. J. Antimicrob. Chemother. **49:**243-253.

17. **Whitley, R. J., M. A. Jacobson, D. N. Friedberg, G. N. Holland, D. A. Jabs, D. T. Dieterich, W. D. Hardy, M. A. Polis, T. A. Deutsch, and J. Feinberg.** 1998. Guidelines for the treatment of cytomegalovirus diseases in patients with AIDS in the era of potent antiretroviral therapy: recommendations of an international panel. Arch. Intern. Med. **158:**957.

18. **Bale, J. F.,Jr, J. A. Blackman, and Y. Sato.** 1990. Outcome in children with symptomatic congenital cytomegalovirus infection. J. Child Neurol. **5:**131-136.

19. **Lemström, K., P. Koskinen, L. Krogerus, M. Daemen, C. Bruggeman, and P. Häyry.** 1995. Cytomegalovirus antigen expression, endothelial cell proliferation, and intimal thickening in rat cardiac allografts after cytomegalovirus infection. Circulation. **92:**2594-2604.

20. **Streblow, D. N., S. L. Orloff, and J. A. Nelson.** 2001. Do pathogens accelerate atherosclerosis? J. Nutr. **131:**2798S-2804S.

21. **Horváth, R., J. Černý, J. Benedík Jr, J. Hökl, I. Jelínková, and J. Benedík.** 2000. The possible role of human cytomegalovirus (HCMV) in the origin of atherosclerosis. Journal of Clinical Virology. **16:**17-24.

22. **Sinzger, C., A. Grefte, B. Plachter, A. S. H. Gouw, and G. Jahn.** 1995. Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissues. J. Gen. Virol. **76:**741-750.

23. **van den Pol, A. N., E. Mocarski, N. Saederup, J. Vieira, and T. J. Meier.** 1999. Cytomegalovirus cell tropism, replication, and gene transfer in brain. The Journal of Neuroscience. **19:**10948-10965.

24. **Biron, K. K.** 2006. Antiviral drugs for cytomegalovirus diseases. Antiviral Res. **71:**154-163.

25. **De Clercq, E.** 2003. New inhibitors of human cytomegalovirus (HCMV) on the horizon. J. Antimicrob. Chemother. **51:**1079-1083.

26. **Biron, K. K.** 2006. Antiviral drugs for cytomegalovirus diseases. Antiviral Res. **71:**154-163.

27. **McGavin, J. K., and K. L. Goa.** 2001. Ganciclovir. Drugs. **61:**1153-1183.

28. **Cvetkovic, R. S., and K. Wellington.** 2005. Valganciclovir. Drugs. **65:**859-878.

29. **Chou, S., N. S. Lurain, K. D. Thompson, R. C. Miner, and W. L. Drew.** 2003. Viral DNA polymerase mutations associated with drug resistance in human cytomegalovirus. J. Infect. Dis. **188:**32-39.

30. **Martin, D. F., J. Sierra-Madero, S. Walmsley, R. A. Wolitz, K. Macey, P. Georgiou, C. A. Robinson, and M. J. Stempien.** 2002. A controlled trial of valganciclovir as induction therapy for cytomegalovirus retinitis. N. Engl. J. Med. **346:**1119-1126.

31. **Hardy, W. D.** 1991. Combined ganciclovir and recombinant human granulocyte-macrophage colony-stimulating factor in the treatment of cytomegalovirus retinitis in AIDS patients. JAIDS J. Acquired Immune Defic. Syndromes. **4:**S22-28.

32. **Xiong, X., J. L. Smith, and M. S. Chen.** 1997. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. Antimicrob. Agents Chemother. **41:**594-599.

33. **Safrin, S., J. Cherrington, and H. S. Jaffe.** 1997. Clinical uses of cidofovir. Rev. Med. Virol. **7:**145-156.

34. **Jacobson, M., J. O'Donnell, and J. Mills.** 1989. Foscarnet treatment of cytomegalovirus retinitis in patients with the acquired immunodeficiency syndrome. Antimicrob. Agents Chemother. **33:**736-741.

35. **Biron, K. K.** 2006. Antiviral drugs for cytomegalovirus diseases. Antiviral Res. **71:**154-163.

36. **Modlin, J. F., A. M. Arvin, P. Fast, M. Myers, S. Plotkin, and R. Rabinovich.** 2004. Vaccine development to prevent cytomegalovirus disease: report from the National Vaccine Advisory Committee. Clinical Infectious Diseases. **39:**233-239.

37. **Griffiths, P., S. Plotkin, E. Mocarski, R. Pass, M. Schleiss, P. Krause, and S. Bialek.** 2013. Desirability and feasibility of a vaccine against cytomegalovirus. Vaccine. **31:**B197-B203.

38. **McGeoch, D. J., F. J. Rixon, and A. J. Davison.** 2006. Topics in herpesvirus genomics and evolution. Virus Res. **117:**90-104.

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

40. **Rawlinson, W. D., H. E. Farrell, and B. G. Barrell.** 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. J. Virol. **70:**8833-8849.

41. **Rixon, F.** 1993. Structure and assembly of herpesviruses, p. 135-144. *In* Anonymous Seminars in Virology. Elsevier.

42. **Boyle, K. A., R. L. Pietropaolo, and T. Compton.** 1999. Engagement of the cellular receptor for glycoprotein B of human cytomegalovirus activates the interferon-responsive pathway. Mol. Cell. Biol. **19:**3607-3613.

43. **Britt, W. J., and M. Mach.** 2008. Human cytomegalovirus glycoproteins. Intervirology. **39:**401-412.

44. **Compton, T., D. M. Nowlin, and N. R. Cooper.** 1993. Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. Virology. **193:**834-841.

45. **Boyle, K. A., and T. Compton.** 1998. Receptor-binding properties of a soluble form of human cytomegalovirus glycoprotein B. J. Virol. **72:**1826-1833.

46. **Wille, P. T., T. W. Wisner, B. Ryckman, and D. C. Johnson.** 2013. Human Cytomegalovirus (HCMV) Glycoprotein gB Promotes Virus Entry In Trans Acting as the Viral Fusion Protein Rather than as a Receptor-Binding Protein. mBio. **4:**.

47. **Wang, X., S. M. Huong, M. L. Chiu, N. Raab-Traub, and E. S. Huang.** 2003. Epidermal growth factor receptor is a cellular receptor for human cytomegalovirus. Nature. **424:**456-461.

48. **Feire, A. L., H. Koss, and T. Compton.** 2004. Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain. Proc. Natl. Acad. Sci. U. S. A. **101:**15470-15475.

49. **Ryckman, B. J., M. A. Jarvis, D. D. Drummond, J. A. Nelson, and D. C. Johnson.** 2006. Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. J. Virol. **80:**710-722.

50. **Mettenleiter, T. C.** 2004. Budding events in herpesvirus morphogenesis. Virus Res. **106:**167-180.

51. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. J. Virol. **76:**1537-1547.

52. **Radsak, K., M. Eickmann, T. Mockenhaupt, E. Bogner, H. Kern, A. Eis-Hübinger, and M. Reschke.** 1996. Retrieval of human cytomegalovirus glycoprotein B from the infected cell surface for virus envelopment. Arch. Virol. **141:**557-572.

53. **Wathen, M. W., and M. F. Stinski.** 1982. Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. J. Virol. **41:**462-477.

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

55. **Stenberg, R. M., P. Witte, and M. Stinski.** 1985. Multiple spliced and unspliced transcripts from human cytomegalovirus immediate-early region 2 and evidence for a common initiation site within immediate-early region 1. J. Virol. **56:**665-675.

56. **Steiner, I., and P. G. Kennedy.** 1995. Herpes simplex virus latent infection in the nervous system. J. Neurovirol. **1:**19-29.

57. **Ioudinkova, E., M. C. Arcangeletti, A. Rynditch, F. De Conto, F. Motta, S. Covan, F. Pinardi, S. V. Razin, and C. Chezzi.** 2006. Control of human cytomegalovirus gene expression by differential histone modifications during lytic and latent infection of a monocytic cell line. Gene. **384:**120-128.

58. **Stern, J. L., J. Z. Cao, J. Xu, E. S. Mocarski, and B. Slobedman.** 2008. Repression of human cytomegalovirus major immediate early gene expression by the cellular transcription factor CCAAT displacement protein. Virology. **378:**214-225.

59. **Pollock, J. L., R. M. Presti, S. Paetzold, and H. W. Virgin IVth.** 1997. Latent murine cytomegalovirus infection in macrophages. Virology. **227:**168-179.

60. **Minton, E., C. Tysoe, J. H. Sinclair, and J. Sissons.** 1994. Human cytomegalovirus infection of the monocyte/macrophage lineage in bone marrow. J. Virol. **68:**4017-4021.

61. **Balthesen, M., M. Messerle, and M. Reddehase.** 1993. Lungs are a major organ site of cytomegalovirus latency and recurrence. J. Virol. **67:**5360-5366.

62. **Weir, J. P.** 1998. Genomic organization and evolution of the human herpesviruses. Virus Genes. **16:**85-93.

63. **Dunn, W., C. Chou, H. Li, R. Hai, D. Patterson, V. Stolc, H. Zhu, and F. Liu.** 2003. Functional profiling of a human cytomegalovirus genome. Proceedings of the National Academy of Sciences. **100:**14223-14228.

64. **Ebeling, A., G. M. Keil, E. Knust, and U. H. Koszinowski.** 1983. Molecular cloning and physical mapping of murine cytomegalovirus DNA. J. Virol. **47:**421-433.

65. **Cassady, K. A.** 2005. Human cytomegalovirus TRS1 and IRS1 gene products block the double-stranded-RNA-activated host protein shutoff response induced by herpes simplex virus type 1 infection. J. Virol. **79:**8707-8715.

66. **Marshall, E. E., C. J. Bierle, W. Brune, and A. P. Geballe.** 2009. Essential role for either TRS1 or IRS1 in human cytomegalovirus replication. J. Virol. **83:**4112-4120.

67. **Romanowski, M. J., and T. Shenk.** 1997. Characterization of the human cytomegalovirus irs1 and trs1 genes: a second immediate-early transcription unit within irs1 whose product antagonizes transcriptional activation. J. Virol. **71:**1485-1496.

68. **Hanson, L. K., B. L. Dalton, L. F. Cageao, R. E. Brock, J. S. Slater, J. A. Kerry, and A. E. Campbell.** 2005. Characterization and regulation of essential murine cytomegalovirus genes m142 and m143. Virology. **334:**166-177. doi: 10.1016/j.virol.2005.01.046.

69. **Hanson, L. K., B. L. Dalton, Z. Karabekian, H. E. Farrell, W. D. Rawlinson, R. M. Stenberg, and A. E. Campbell.** 1999. Transcriptional analysis of the murine cytomegalovirus HindIII-I region: identification of a novel immediate-early gene region. Virology. **260:**156-164. doi: 10.1006/viro.1999.9796.

70. **Child, S. J., L. K. Hanson, C. E. Brown, D. M. Janzen, and A. P. Geballe.** 2006. Double-stranded RNA binding by a heterodimeric complex of murine cytomegalovirus m142 and m143 proteins. J. Virol. **80:**10173-10180. doi: 10.1128/JVI.00905-06.

71. **Budt, M., L. Niederstadt, R. S. Valchanova, S. Jonjić, and W. Brune.** 2009. Specific inhibition of the PKR-mediated antiviral response by the murine cytomegalovirus proteins m142 and m143. J. Virol. **83:**1260-1270.

72. **Wahle, E., and W. Keller.** 1996. The biochemistry of polyadenylation. Trends Biochem. Sci. **21:**247-250.

73. **Purcell, D., and M. A. Martin.** 1993. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. J. Virol. **67:**6365-6378.

74. **Mitchell, P., and D. Tollervey.** 2000. mRNA stability in eukaryotes. Curr. Opin. Genet. Dev. **10:**193-198.

75. **Kingston, R. E., C. A. Bunker, and A. N. Imbalzano.** 1996. Repression and activation by multiprotein complexes that alter chromatin structure. Genes Dev. **10:**905-920.

76. **Carey, M., and S. T. Smale.** 2000. Transcriptional regulation in eukaryotes. Cold Spring Harbor Laboratory Press Cold Spring Harbor:.

77. **Lemon, B., and R. Tjian.** 2000. Orchestrated response: a symphony of transcription factors for gene control. Genes Dev. **14:**2551-2569.

78. **Pugh, B. F., and R. Tjian.** 1991. Transcription from a TATA-less promoter requires a multisubunit TFIID complex. Genes Dev. **5:**1935-1945.

79. **Fortunato, E. A., A. K. McElroy, V. Sanchez, and D. H. Spector.** 2000. Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. Trends Microbiol. **8:**111-119.

80. **Rodems, S. M., and D. H. Spector.** 1998. Extracellular signal-regulated kinase activity is sustained early during human cytomegalovirus infection. J. Virol. **72:**9173-9180.

81. **Xing, J., D. D. Ginty, and M. E. Greenberg.** 1996. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science. **273:**959-963.

82. **Lang, D., S. Gebert, H. Arlt, and T. Stamminger.** 1995. Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB. J. Virol. **69:**6030-6037.

83. **Englander EW, W. S.** 1992 Nov 11;. DNA damage response of cloned DNA beta-polymerase promoter is blocked in mutant cell lines deficient in protein kinase A. Nucleic Acids Res. **20(21):**5527-31.

84. **Cristea, I. M., N. J. Moorman, S. S. Terhune, C. D. Cuevas, E. S. O'Keefe, M. P. Rout, B. T. Chait, and T. Shenk.** 2010. Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein. J. Virol. **84:**7803-7814.

85. **Isern, E., M. Gustems, M. Messerle, E. Borst, P. Ghazal, and A. Angulo.** 2011. The Activator Protein 1 Binding Motifs within the Human Cytomegalovirus Major Immediate-Early Enhancer Are Functionally Redundant and Act in a Cooperative Manner with the NF-κB Sites during Acute Infection. J. Virol. **85:**1732-1746.

86. **McWatters, B. J., R. M. Stenberg, and J. A. Kerry.** 2002. Characterization of the human cytomegalovirus UL75 (glycoprotein H) late gene promoter. Virology. **303:**309-316.

87. **Tan, N. Y., V. C. Midgley, M. M. Kavurma, F. S. Santiago, X. Luo, R. Peden, R. G. Fahmy, M. C. Berndt, M. P. Molloy, and L. M. Khachigian.** 2008. Angiotensin II–inducible platelet-derived growth factor-D transcription requires specific Ser/Thr residues in the second zinc finger region of Sp1. Circ. Res. **102:**e38-e51.

88. **Narayan, V. A., R. W. Kriwacki, and J. P. Caradonna.** 1997. Structures of Zinc Finger Domains from Transcription Factor Sp1 insights into sequence-specific protein-DNA recognition. J. Biol. Chem. **272:**7801-7809.

89. **Dynan, W. S., and R. Tjian.** 1983. The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. Cell. **35:**79-87.

90. **Pugh, B. F., and R. Tjian.** 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell. **61:**1187-1197.

91. **Wu, J., J. O'Neill, and M. S. Barbosa.** 1998. Transcription Factor Sp1 Mediates Cell-Specifictrans-Activation of the Human Cytomegalovirus DNA Polymerase Gene Promoter by Immediate-Early Protein IE86 in Glioblastoma U373MG Cells. J. Virol. **72:**236-244.

92. **Everett, R., D. Baty, and P. Chambon.** 1983. The repeated GC-rich motifs upstream from the TATA box are important elements of the SV40 early promoter. Nucleic Acids Res. **11:**2447-2464.

93. **Yurochko, A. D., M. W. Mayo, E. E. Poma, A. Baldwin, and E. Huang.** 1997. Induction of the transcription factor Sp1 during human cytomegalovirus infection mediates upregulation of the p65 and p105/p50 NF-kappaB promoters. J. Virol. **71:**4638-4648.

94. **Yurochko, A. D., E. Hwang, L. Rasmussen, S. Keay, L. Pereira, and E. Huang.** 1997. The human cytomegalovirus UL55 (gB) and UL75 (gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-kappaB during infection. J. Virol. **71:**5051-5059.

95. **Emili, A., J. Greenblatt, and C. J. Ingles.** 1994. Species-specific interaction of the glutamine-rich activation domains of Sp1 with the TATA box-binding protein. Mol. Cell. Biol. **14:**1582-1593.

96. **Prösch, S., A. Heine, H. Volk, and D. H. Krüger.** 2001. CCAAT/enhancer-binding proteins α and β negatively influence the capacity of tumor necrosis factor α to up-regulate the human cytomegalovirus IE1/2 enhancer/promoter by nuclear factor κB during monocyte differentiation. J. Biol. Chem. **276:**40712-40720.

97. **Bushmeyer, S., K. Park, and M. L. Atchison.** 1995. Characterization of functional domains within the multifunctional transcription factor, YY1. J. Biol. Chem. **270:**30213-30220.

98. **Liu, R., J. Baillie, J. P. Sissons, and J. H. Sinclair.** 1994. The transcription factor YY1 binds to negative regulatory elements in the human cytomegalovirus major immediate early enhancer/promoter and mediates repression in nonpermissive cells. Nucleic Acids Res. **22:**2453-2459.

99. **Liu, X., S. Yan, M. Abecassis, and M. Hummel.** 2010. Biphasic recruitment of transcriptional repressors to the murine cytomegalovirus major immediate-early promoter during the course of infection in vivo. J. Virol. **84:**3631-3643.

100. **Hagemeier, C., S. M. Walker, P. Sissons, and J. H. Sinclair.** 1992. The 72K IE1 and 80K IE2 proteins of human cytomegalovirus independently trans-activate the c-fos, c-myc and hsp70 promoters via basal promoter elements. J. Gen. Virol. **73:**2385.

101. **Harel, N. Y., and J. C. Alwine.** 1998. Phosphorylation of the human cytomegalovirus 86-kilodalton immediate-early protein IE2. J. Virol. **72:**5481-5492.

102. **Kim, J., Y. Hong, K. Jeang, and S. Kim.** 2000. Transactivation activity of the human cytomegalovirus IE2 protein occurs at steps subsequent to TATA box-binding protein recruitment. J. Gen. Virol. **81:**37-46.

103. **Gustems, M., E. Borst, C. A. Benedict, C. Pérez, M. Messerle, P. Ghazal, and A. Angulo.** 2006. Regulation of the transcription and replication cycle of human cytomegalovirus is insensitive to genetic elimination of the cognate NF-κB binding sites in the enhancer. J. Virol. **80:**9899-9904.

104. **Liu, B., and M. F. Stinski.** 1992. Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements. J. Virol. **66:**4434-4444.

105. **Ishov, A. M., O. V. Vladimirova, and G. G. Maul.** 2002. Daxx-mediated accumulation of human cytomegalovirus tegument protein pp71 at ND10 facilitates initiation of viral infection at these nuclear domains. J. Virol. **76:**7705-7712.

106. **Cantrell, S. R., and W. A. Bresnahan.** 2005. Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication. J. Virol. **79:**7792-7802.

107. **Saffert, R. T., and R. F. Kalejta.** 2006. Inactivating a cellular intrinsic immune defense mediated by Daxx is the mechanism through which the human cytomegalovirus pp71 protein stimulates viral immediate-early gene expression. J. Virol. **80:**3863-3871.

108. **Woodhall, D. L., I. J. Groves, M. B. Reeves, G. Wilkinson, and J. H. Sinclair.** 2006. Human Daxx-mediated repression of human cytomegalovirus gene expression correlates with a repressive chromatin structure around the major immediate early promoter. J. Biol. Chem. **281:**37652-37660.

109. **Spaete, R. R., and E. S. Mocarski.** 1985. Regulation of cytomegalovirus gene expression: alpha and beta promoters are trans activated by viral functions in permissive human fibroblasts. J. Virol. **56:**135-143.

110. **Chau, N. H., C. D. Vanson, and J. A. Kerry.** 1999. Transcriptional regulation of the human cytomegalovirus US11 early gene. J. Virol. **73:**863-870.

111. **Keil, G. M., A. Ebeling-Keil, and U. H. Koszinowski.** 1987. Immediate-early genes of murine cytomegalovirus: location, transcripts, and translation products. J. Virol. **61:**526-533.

112. **Gordon, S., G. Akopyan, H. Garban, and B. Bonavida.** 2005. Transcription factor YY1: structure, function, and therapeutic implications in cancer biology. Oncogene. **25:**1125-1142.

113. **Evers, D. L., X. Wang, and E. Huang.** 2004. Cellular stress and signal transduction responses to human cytomegalovirus infection. Microb. Infect. **6:**1084-1093.

114. **Browne, E. P., B. Wing, D. Coleman, and T. Shenk.** 2001. Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. J. Virol. **75:**12319-12330.

115. **Stenberg, R., J. Fortney, S. Barlow, B. Magrane, J. Nelson, and P. Ghazal.** 1990. Promoter-specific trans activation and repression by human cytomegalovirus immediate-early proteins involves common and unique protein domains. J. Virol. **64:**1556-1565.

116. **Liu, Z., and B. J. Biegalke.** 2013. Human Cytomegalovirus UL34 Binds to Multiple Sites within the Viral Genome. J. Virol. **87:**3587-3591.

APPENDIX

Abbreviations

Cytomegalovirus	CMV
Human cytomegalovirus	HCMV
Murine cytomegalovirus	MCMV
Cytopathic effect	CPE
Gancyclovir	GCV
Endoplasmic reticulum	ER
Epithelial growth factor receptor	EGFR
Immediate-early	IE
Early	E
Late	L
Hours post infection	hpi
Major immediate early promoter	MIEP
Phosphonoformic acid	PFA
CCAAT displacement protein	CDP
Open reading frames	ORFs
Unique short	US
Unique long	UL
Protein kinase R	PKR
Transcription factors	TFs
TATA binding protein	TBP

Transcription factor IID	TFIID
Extracellular-signal-regulated kinases	ERKs
cAMP response-element-binding protein	CREB
Necrotic factor kappa beta	NF-kβ
Nuclear domains	ND10
Kanamycin	Kan
Ampicillin	Amp
Secreted embryonic alkaline phosphatase	SEAP
Dulbecco's modified Eagle's medium	DMEM
Bovine calf serum	BCS
Multiplication of infection	MOI
Cytopathic effect	CPE
Poly A binding protein	PABP
Electrophoretic Mobility Shift Assay	EMSA
Nuclear extract	NE
Cytoplasmic extract	CE
Bovine serum albumin	BSA
Tris borate EDTA buffer	TBE
Transcription element search system	TESS