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# Role of the Human Cytomegalovirus IE2 Protein on Transcriptional Regulation of Viral Promoters

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# ROLE OF THE HUMAN CYTOMEGALOVIRUS IE2 PROTEIN ON TRANSCRIPTIONAL REGULATION OF VIRAL PROMOTERS

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## ABSTRACT

ROLE OF THE HUMAN CYTOMEGALOVIRUS IE2 PROTEIN IN TRANSCRIPTIONAL REGULATION OF VIRAL PROMOTERS

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Human cytomegalovirus (HCMV) is a ubiquitous pathogen able to cause severe mortality and morbidity in immuno-compromised individuals. Successful infection by HCMV is dependent on expression of viral genes essential for replication. Immediate early (IE) gene products are the first subset of viral genes to be expressed during infection and function as key transcriptional regulators. IE2 is one the most predominantly expressed IE proteins and is essential for HCMV infection. IE2 transactivates several viral promoters, including those of the essential viral DNA polymerase (UL54) and UL112-113 gene regions. IE2 is also able to autoregulate is own expression and repress expression of the major IE gene products. This study aims to investigate the role of IE2 and cellular proteins in regulation of viral promoters in order to gain a better understanding of early events required for HCMV replication. Through the use of both transient assays and complementing assays in recombinant HCMV clones, we show that single amino acid mutations in the C-terminus of IE2 impair both IE2-mediated transactivation of early gene promoters and autorepression of the major IE promoter. These mutations in IE2 also result in nonviable recombinant viruses, emphasizing the importance of IE2 in HCMV replication. GST pulldown assays demonstrate that mutagenesis of Tyrosine 544 in IE2 reduces IE2 interactions with

TATA binding protein (TBP) when compared to the wildtype IE2 protein. Using ChIP assays, we demonstrate that wildtype IE2 is recruited to the UL54 promoter in transiently transfected cells. However, mutagenesis in the IE2 protein at Proline 535 and Tyrosine 544 significantly decrease recruitment of IE2 to the UL54 promoter. Interestingly, these functional defects in mutated IE2 protein had no effect on RNA polymerase II recruitment, suggesting that IE2 may function in transcriptional regulation after formation of the transcriptional pre-initiation complex. In this dissertation, we have further characterized regions and functional properties of IE2 essential for UL54 activation and HCMV replication. Our studies are significant in understanding the regulation of viral genes essential for replication of HCMV and in the development of novel HCMV therapies.

This thesis is dedicated to my husband, Kevin, and our son, Devin.

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"...and burning with curiosity, she ran across the field after it, and was just in time to see it pop down a large rabbit-hole under the hedge. In another moment down went Alice, never once considering how in the world she was to get out again."

Lewis Carroll, *Alice's Adventures in Wonderland*

# LIST OF ABBREVIATIONS

| | |
|---|---|
| aa | Amino Acid(s) |
| Ala = A | Alanine |
| ATF1 | Activating transcription factor 1 |
| BAC | Bacterial Artificial Chromosome |
| bp | Basepair |
| BSA | Bovine serum albumin |
| CRS | *Cis* repression sequence |
| cDNA | Complementary DNA |
| ChIP | Chromatin Immunoprecipitation |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| DEPC | Diethyl pyrocarbonate |
| *E. coli* | Escherichia coli |
| EDTA | Ethylenediamine-tetraacetic acid |
| g | Gram |
| *g* | Relative centrifugal force |
| G6PD | Glucose-6-phosphate dehydrogenase |
| Glu = E | Glutamic Acid |
| GST | Glutathione S-transferase |
| HCMV | Human cytomegalovirus |
| HEPES | N-(2-Hydroxyethyl) piperazine-N'-2-ethansulphuric acid |
| HHV | Human Herpesvirus |

| | |
|---|---|
| hr | hour |
| HSV | Herpes Simplex Virus |
| IE | Immediate early |
| Ile = I | Isoleucine |
| IPTG | Isopropyl-beta-D-thio-galactopyranoside |
| kan | Kanamycin gene |
| kb | Kilobase(s) |
| kDa | Kilodalton |
| L | Liter |
| LB | Luria Broth |
| M | Molar |
| mA | Milliamps |
| mg | Milligrams |
| ml | Milliliter |
| µl | Microliter |
| min | Minute |
| MIE | Major Immediate Early |
| mM | Millimolar |
| ng | Nanogram |
| nt | Nucleotide |
| OD | Optical density |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| pfu | Plaque forming units |
| Pro = P | Proline |

| | |
|---|---|
| RNase | Ribonuclease |
| RNA | Ribonucleic acid |
| SDS | Sodium dodecyl sulfate |
| Sp1 | Specificity protein-1 |
| TBE | Tris/borate/EDTA |
| TBS | Tris-buffered saline |
| TBS-T | TBS with Tween-20 |
| TE | Tris-EDTA buffer |
| TEMED | N,N,N',N'-Tetramethylethylendi-amine |
| Thr = T | Threonine |
| Tyr = Y | Tryrosine |
| UL | Unique Long |
| US | Unique Short |
| UV | Ultraviolet light |
| V | Volt |
| Val = V | Valine |
| WT | wildtype |
| X-gal | 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside |
| Zeo | Zeocin gene |

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# CHAPTER I

## INTRODUCTION

### The family *Herpesviridae*

The Herpesviridae family is generally categorized into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. All members of the Herpesviridae family possess the ability to establish persistent and latent infections. Alphaherpesviruses are neurotropic viruses, such as herpes simplex virus (HSV)-1, HSV-2, and varicella-zoster virus, which are characterized by a relatively rapid replication cycle and broad host cell specificity (115). The betaherpesviruses, including human cytomegalovirus (HCMV), human herpesvirus (HHV)-6 and HHV-7, are characterized by a slow life cycle and species-restricted host range (115). Gammaherpesviruses, including Epstein-Barr virus and Kaposi's sarcoma herpesvirus, are distinguished by slow replication and infection of lymphoid cells (115). Members of the herpesviruses represent an opportunistic group of viruses which are significant human pathogens.

### Human Cytomegalovirus

#### General Properties

HCMV is the most well characterized member of the *Betaherpesvirinae* subfamily. It is distinguishable from other herpesvirus due to its high species-specificity and slow replication period and growth in cultured cells (64). HCMV is a ubiquitous and opportunistic pathogen, easily controlled by a functional immune system in immuno-

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This dissertation follows the format of Journal of Virology.

competent individuals. In normal, immuno-competent populations, HCMV infections are usually asymptomatic, although infection may cause a brief mononucleosis-like condition in some individuals (64). However, in conditions of immune immaturity or suppression, such as neonates, transplant recipients, and AIDS patients, HCMV is a major cause of disease and mortality (64). Epithelial cells in the upper alimentary, respiratory and genito-urinary tracts are thought to be the primary sites of viral replication following direct contact (110). After infection, macrophage and endothelial-cell associated virus is important in the transmission of virus to other host tissues, such as the heart, lung, liver, and spleen. HCMV can replicate in several cell types including human fibroblasts, epithelial cells, endothelial cells, vascular smooth muscle and macrophages (126).

**Clinical Significance of HCMV**

In the United States, HCMV infection occurs at a rate of 1.6 infections per 100 susceptible individuals between 12-49 years of age (25). This rate of infection increases significantly in non-Hispanic Black, Mexican American, and low income populations (25). The virus persists throughout an individual's lifetime, and can reactivate in immunosuppressed periods to cause viral disease. HCMV is transmitted through several mechanisms: (1) horizontally, through direct contact with bodily fluids such as urine, semen or saliva, (2) vertically, or *in utero*, from an infected mother to her unborn child and (3) through organ transplants or medical transfusions of blood, platelets, and white blood cells (67, 104).

Infections of most immuno-competent individuals are subclinical and adverse symptoms are limited. However, HCMV infection causes severe disease and mortality in

individuals with compromised immune systems such as patients with AIDS, patients being treated with chemotherapy and patients undergoing immuno-suppressive therapies for tissue and solid organ transplantation (33, 67, 94, 104). Significant clinical symptoms include retinitis, gastrointestinal infections, pneumonia and a strong association with organ rejection (31). Furthermore, HCMV is known to be the major viral cause of birth defects leading to mental retardation and hearing loss (36, 87, 104). Current treatments for HCMV include ganciclovir and foscarnet, which target the viral DNA polymerase (85). However, since 1991 a number of HCMV strains resistant to ganciclovir and foscarnet have been isolated from patients and well characterized, demonstrating diminished efficacy of these treatments (130). Thus, novel therapeutic strategies are needed to effectively treat HCMV infection.

**Virion Structure and Morphology**

The virion of HCMV consists of a double stranded, DNA genome (235 kb) contained within an icosahedral capsid, a tegument region surrounding the viral capsid, and a lipid bilayer envelope studded with various virus-encoded glycoproteins (Fig. 1) (94). The capsid is approximately 100-nm in diameter and mature virions range from 150 and 200 nm (64). The HCMV capsid is composed of five structural proteins: the major capsid protein (MCP; pUL86), minor capsid protein (mCP; pUL85), minor capsid binding protein (mC-BP; pUL46), the smallest capsid protein (SCP; pUL48-49) and portions of the assembly protein (pUL80) (13, 38-40, 51, 123). A tegument or matrix region, is located between the capsid and the envelope (64). The tegument is composed of approximately 20 proteins with pp65 (ppUL83), pp28 (ppUL83), pp71 (ppUL82) and

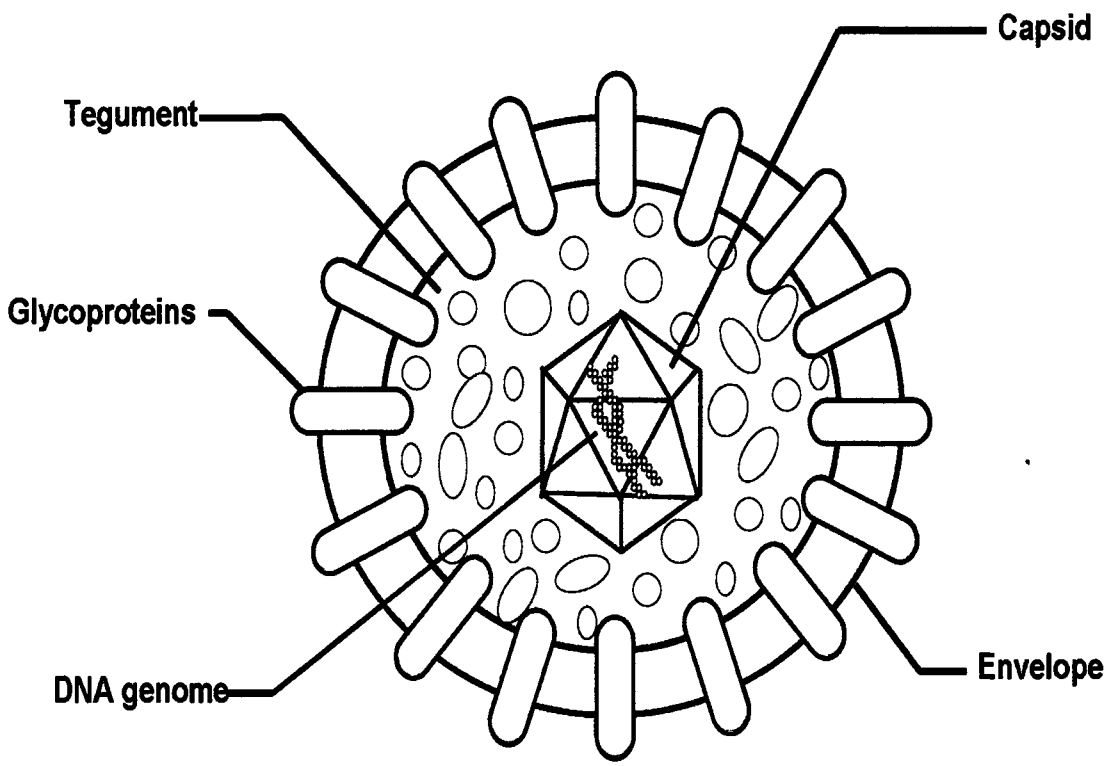


FIG. 1. Schematic diagram of the HCMV virion structure. Mature HCMV virions contain a large double stranded, DNA genome surrounded by an icosahedral capsid. The entire virion is surrounded by a lipid bilayer studded with glycoproteins. Between the capsid and the lipid bilayer is a region called the tegument.

pp150 (ppUL32) being the most abundant tegument proteins (6, 118). In addition, a more recent study by Varnum, *et al.* identified a number of cellular and viral proteins located in the HCMV virion through proteomic approaches (141). Approximately 59 viral structural proteins and over 70 host cellular proteins, including enzymes and chaperone proteins, have been identified suggesting a possible role of these proteins in the initiation of viral infection (141).

**Genome Organization**

The 235-kb double-stranded, linear DNA genome of HCMV is separated into 2 covalently linked segments, referred to as the unique long (UL) and unique short (US) region (Fig. 2) (19). Each segment is flanked by internal (IRL, IRS) and terminal repeat (TRL, TRS) sequences. Gene loci are identified according to their position in the genome (TRL, UL, IRL, IRS, US, and TRL) (94).

**Lytic (Productive) Infection.**

HCMV replicates in a number of susceptible cell types, including epithelial cells, endothelial cells, macrophages, smooth muscle tissues and fibroblasts (Fig. 3) (94). Viral entry involves attachment of virus to the cell surface through glycoprotein gM and gN interactions with heparin sulphate proteoglycan complexes on the cell surface (94). The viral glycoprotein, gB, has also been found to interact with the epithelial growth factor receptor (EGFR) to initiate viral entry (145). However, several EGFR-negative cell lines are permissive to HCMV infection, suggesting other receptors are involved in attachment/penetration of the virion (52). Following attachment of the

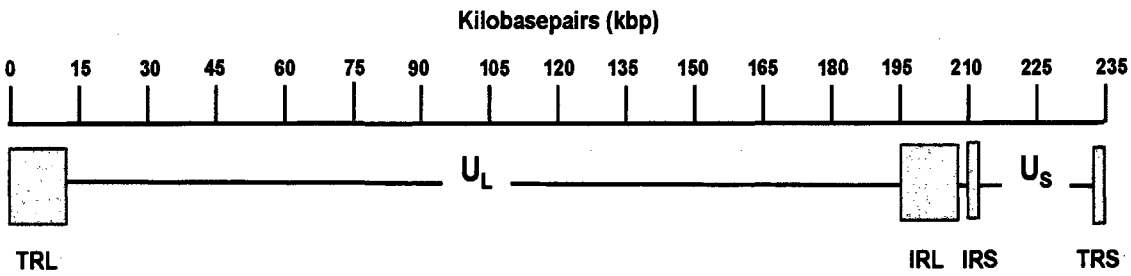


FIG. 2. Genomic Organization of HCMV. The HCMV genome contains two unique segments, unique long and unique short (solid lines), flanked by inverted repeat sequences (open rectangles). Gene loci are named according to their position along the genome.

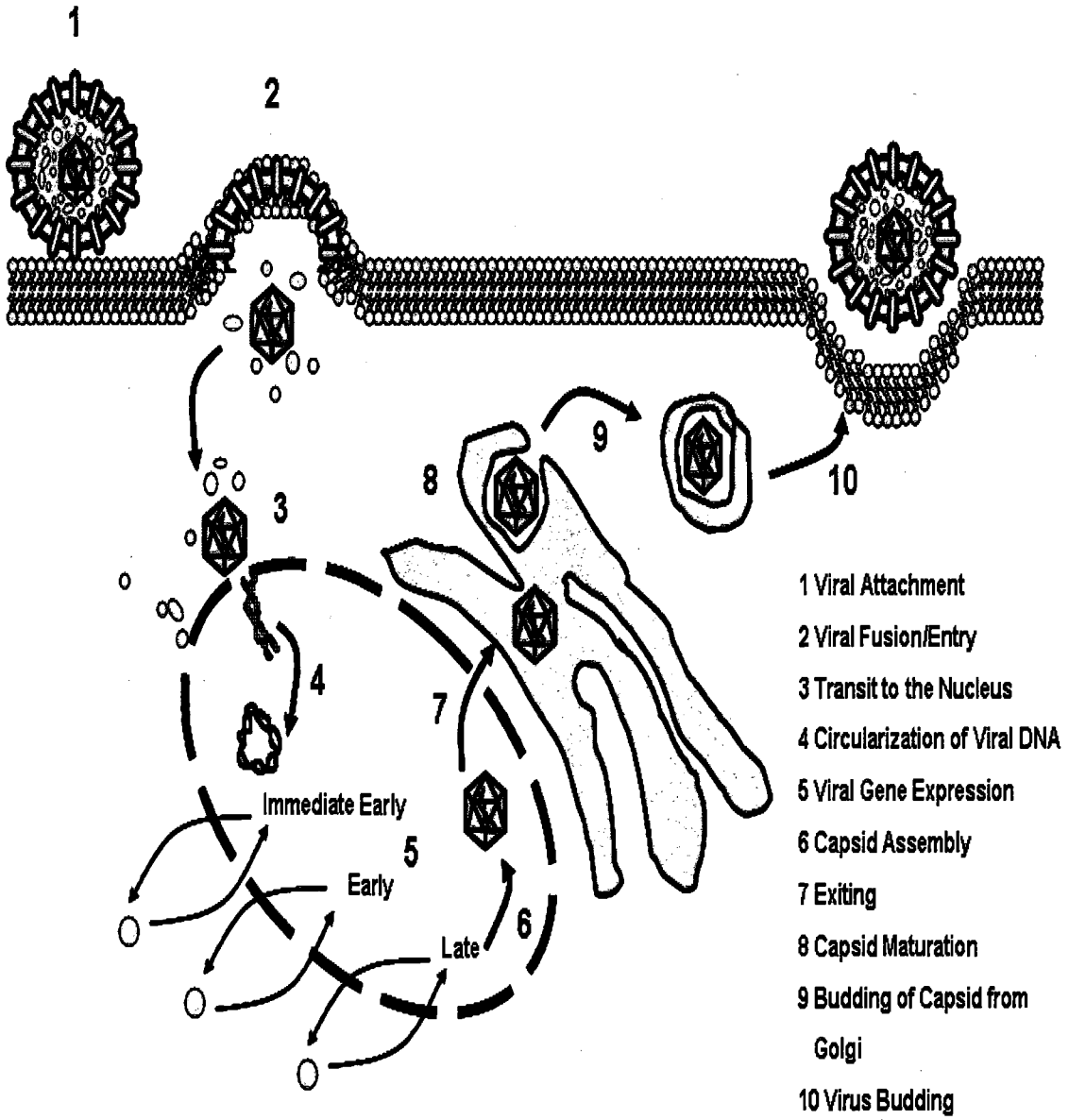


FIG. 3. The HCMV replication cycle. Please refer to text for additional information on HCMV replication.

viral particle to the cell surface, fusion occurs between the cell membrane and the viral envelope. The gH/gL/gO heterotrimeric complex is required for membrane fusion (49). The virus particle is then released into the cytoplasm and within minutes, transits to the nucleus. Transport through the nuclear pore allows the viral DNA to be deposited into the nucleus and to be transcribed into mRNA by the cellular DNA dependent RNA polymerase II (31).

A number of cellular signaling pathways are activated by binding and/or fusion of the HCMV virion to the cell membranes (32). Several pathways thought to be important for successful virus replication and immune evasion are activated, including modulation of calcium levels, activation of phospholipases C and A2, and release of arachidonic acid and subsequent metabolites (32). Activation of these pathways occur in the absence of viral expression, as demonstrated through the use of UV-inactivated virus, suggesting these actions are triggered by structural proteins involved in viral attachment and/or penetration. Viral attachment also triggers activation of cellular factors such as SP1, myc, c-fos/c-jun, NF-κB, ERK1/2, p38, and phosphatidylinositol 3-kinase (PI3-kinase) which are thought to prepare the cell for virus infection (8, 10, 56, 66, 157). In addition, prior to *de novo* protein synthesis, attachment and penetration of HCMV is able to trigger immediate effects on the host cell through the actions of tegument proteins and glycoproteins (33). Specifically, pp71, a tegument protein, has been shown to enhance activation of cellular promoters containing CREB/ATF and AP-1 regulatory elements (75).

Similar to other herpesvirus, the expression of HCMV genes are temporally regulated within the nucleus of the host cell in the following order: immediate early (IE),

early and late gene expression (64). The first subset of viral genes to be expressed are the IE gene products (64). The major IE (MIE) proteins, including IE1 and IE2, are the most abundantly expressed proteins during the IE phase (16, 94). Unlike early and late genes, IE genes do not require *de novo* cellular or viral protein synthesis, as determined by cyclohexamide treatment, and can be expressed within several hours following infection (64). As mentioned previously, IE promoters are primarily dependent on cellular proteins already present in the cell for activation (32).

A large majority of IE proteins facilitate the activation of HCMV early genes (104, 148). Early genes are typically involved in the replication of the viral genome and may code for structural proteins (94). Examples of early viral genes required for replication include the UL54 gene, which encodes the virus DNA polymerase, and UL112-113 gene products, which encode a number of phosphoproteins required for replication (94).

Late gene products are produced after initiation of DNA replication and encode proteins primarily involved in virion assembly, maturation, and spread (94). In addition to early gene activation, several of the IE proteins are involved in activation of late genes. For example, IE2 regulates expression of pp28, a tegument protein (60).

HCMV DNA replication begins approximately 24 hours post-infection (hpi) and peaks at 48-72 hours in human fibroblasts (88). Viral DNA replication occurs through a rolling circle mechanism resulting in concatemeric molecules (88). DNA is then cleaved and packaged into capsids as linear, double stranded DNA (88). After packaging, current research suggests that HCMV intranuclear capsids bud through the nuclear membrane, losing their primary envelope, and acquire a final envelope and tegument in cytoplasmic

compartments (93). Following tegumentation, the viral particle undergoes a second envelopment in the *trans*-golgi assembly compartment where it will obtain additional viral glycoproteins (13, 93). Finally, HCMV particles are released from the cell and enter into the intercellular space in order to infect nearby cells (93).

## HCMV Transcriptional Regulation

Transcriptional regulation of cellular and viral genes is a highly controlled and regulated process (Fig. 4). During virus infection, HCMV "hijacks" the cellular transcription machinery to enable transcription of viral genes (32). Transcription of cellular genes involves the assembly of a transcription pre-initiation complex at the transcription start site (+1) of the designated promoter (15). The TATA box, located at position -25 to -30, is recognized by the TATA-binding protein (TBP), a component of the TFIID complex. The TFIID complex consists of TBP and TBP-associated factors (TAFs). TFIIA and TFIIB are then recruited to the pre-initiation complex to assist in TFIID binding. Next, TFIIF and RNA polymerase II bind to the pre-initiation complex. TFIIE and TFIIH are recruited to allow transcriptional initiation and RNA polymerase II phosphorylation (15).

General transcription factors, such as SP1, CREB, and/or AP1 aid in recruitment of the TBP and RNA polymerase II protein complex to the promoter (47, 73). Transcription factors also facilitate recruitment of two types of enzymes, ATP-dependent chromatin remodeling enzymes and histone-modifying enzymes which function to control accessibility of the transcriptional machinery to the promoter (96, 139, 140).

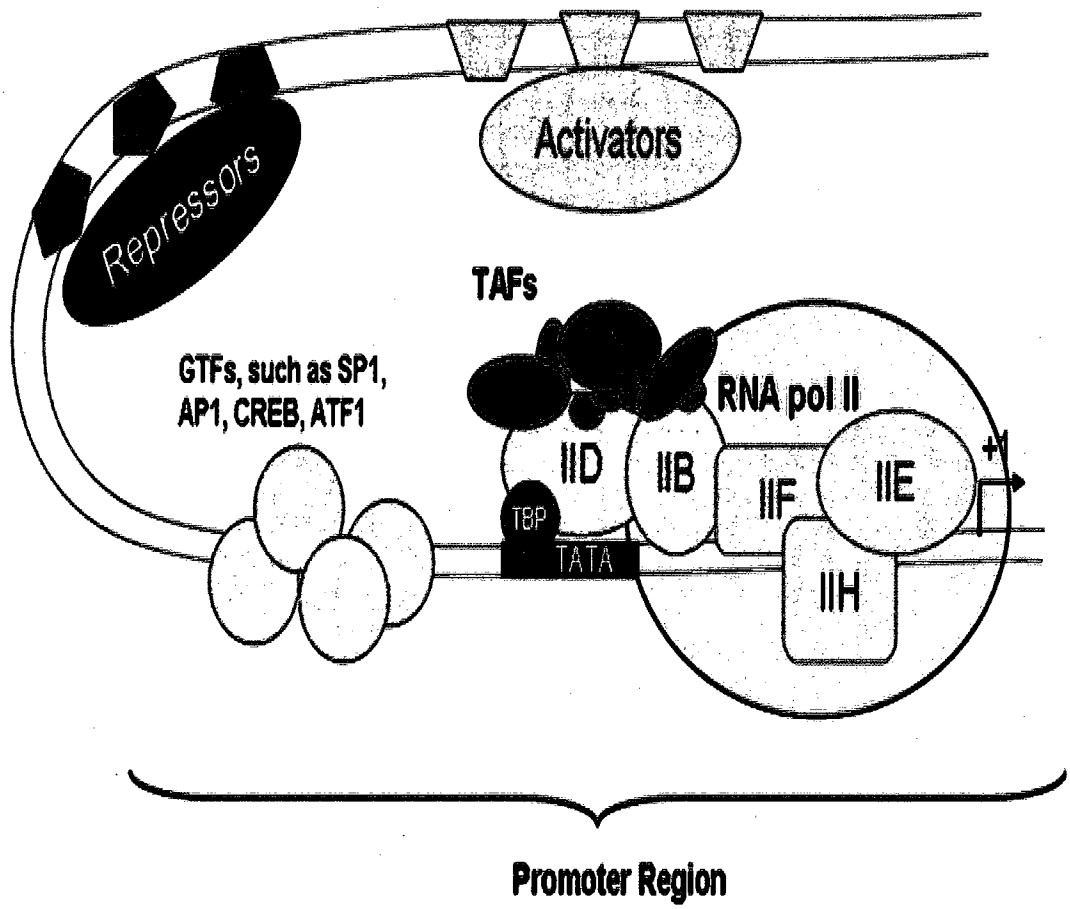


FIG. 4. Assembly of the Basal Transcription Machinery in Eukaryotic Cells. Initiation of transcription in eukaryotic cells involves a complex interplay of cellular factors recruited to individual promoters. Please refer to the text for additional information on transcriptional initiation.

ATP-dependent chromatin remodelers, such as SWI/SNF and Gcn5, disrupt histone-DNA contacts of the nucleosome (96, 139, 140). Histone modifying enzymes, such as P/CAF or p300/CBP, posttranscriptionally modify the N- and C-terminal tails of histone proteins for transcriptional regulation (7, 96, 139, 140).

Most regulatory proteins involved in eukaryotic gene transcription are termed activator or repressor proteins and possess a modular structure comprised of distinct functional domains including, but not limited to, a DNA binding and a transcriptional regulatory domain (11, 34). The DNA binding domain is usually specific for individual promoter sequences (34). The transcriptional regulatory domain facilitates transcriptional activation or repression through properties such as recruitment of co-regulators or interactions with basal transcription machinery (23, 24, 34).

Coactivators are proteins that do not bind directly to DNA, but function in transcriptional activation of RNA polymerase II promoters. Coactivators can regulate transcription by one or more of the following mechanisms (1) serving as a scaffolding factor at the promoter between transcription factors and members of the basal transcription machinery, (2) interacting with the basal transcription machinery, and/or (3) recruiting or functioning as histone modifiers (42, 55, 95, 100, 101, 150).

HCMV infection leads to an increase in the RNA and DNA synthesis within an infected cell, including an increase in expression of cellular transcription factors (32, 129). The IE2 protein is then able to interact with several cellular proteins involved in transcriptional regulation. IE2 has been shown to interact with members of the basal transcription machinery, including TBP, TFIID, and TBP-associated factors (TAFs) (43, 44, 57, 58). IE2 also interacts with a number of cellular transcription factors such as SP1,

AP1, and CREB (68, 119, 120, 146). In addition, IE2 has been found to interact with chromatin remodeling proteins such as P/CAF, p300/CBP, and HDAC3 (14, 98). Based on these properties of transcriptional regulation, IE2 appears to function as a coactivator to stimulate activation of several viral promoters. In this dissertation, we will further investigate the functions of IE2 at viral promoters and identify properties involved in regulation of transcription.

## Properties of the IE2 Protein

### IE2 Gene Expression

The majority of IE transcription occurs from the UL122/123 gene region, termed the major IE gene region (Fig. 5) (19). The major IE gene region encodes several splice variants during virus infection (135, 136, 138). The IE1 72 kDa and IE2 86 kDa proteins are the most abundantly expressed gene products from this locus and play a significant role in transcriptional regulation of viral genes (16, 94). These gene products share 85 amino acids in the N-terminus (135, 136, 138). In addition, the MIE promoter drives expression of a number of minor transcripts with sequences overlapping IE1 and IE2 which are currently not well-characterized (54, 63, 111, 125, 133). Regulation of the MIE promoter is controlled by both cellular and viral factors, as described later in this dissertation.

### Regulation of Viral Gene Transcription

IE2 is a potent transactivator of viral and cellular genes and is essential for viral replication (45, 74, 86, 107, 109, 134). This protein activates a number of HCMV early

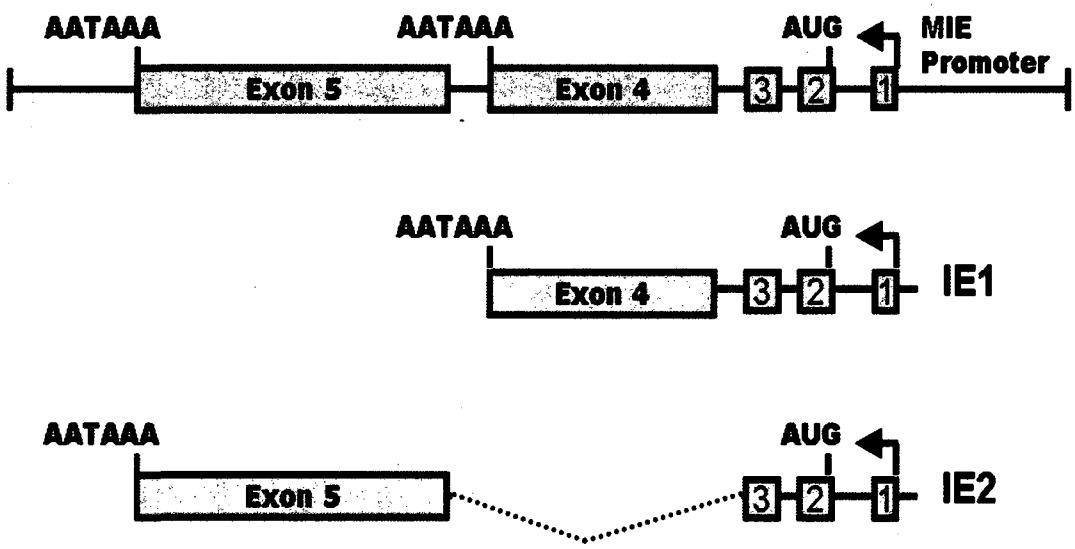


FIG. 5. Genomic Organization of the MIE Gene Region (UL122-123). The major IE gene region (UL122-123) is the most extensively transcribed region during the immediate early phase of viral gene expression. The major IE promoter encodes a number of splice variants (not all variants are shown). The IE1 and IE2 gene products are the most predominant UL122-123 gene products expressed. Figure adapted from (79).

promoters that are required for viral replication, including the UL112/113 (encoding four phosphoproteins required for replication) and UL54 (encoding the viral DNA polymerase) gene promoters. IE2 also activates a variety of heterologous cellular and viral promoters, regulating expression of the cyclin E, c-fos, hsp70 genes and the human immunodeficiency type I (HIV-1) long terminal repeat (28, 94, 120, 121, 128, 132, 156).

Regulation of viral and cellular genes is thought to be mediated by direct binding of IE2 to DNA and/or protein interactions with cellular and viral transcription factors (33, 128). Activation of TATA-containing promoters, such as the c-fos and HIV LTR promoter, is thought to be mediated by IE2 interactions with members of the basal transcription machinery, TFIIB and TBP (43, 44, 57, 58). IE2 is also able to interact with cellular transcription factors to regulate viral gene transactivation, including SP1, CREB, AP1 (68, 119, 120, 146). In addition, IE2 has been found to interact with histone modifiers such as P/CAF and HDAC3, suggesting IE2 is involved in chromatin modification to facilitate transcriptional activation (15, 95, 155). IE2 can also regulate early gene transcription by direct binding to DNA (2, 18, 48, 121, 122). For example, expression of UL112-113 gene products is regulated by an ATF/CREB binding site and a nonessential IE2 binding site, identified by deletion mutagenesis and gel mobility shift analysis (120, 121). Deletion of this IE2 binding site in UL112-113 within a HCMV recombinant virus causes a reduction of IE2-mediated transactivation of this promoter during infection (114).

**Structure-Function Analysis**

As a result of the essential role of IE2 in viral replication, numerous studies have been performed to identify functional domains of the IE2 protein and assess the biological significance of these regions (Fig. 6). Two distinct regions of the N- and C-terminus of IE2 are involved in transactivation of early genes (amino acids 1-98 and 195-570) (46, 84, 108, 122, 127, 134, 154). In addition to a transactivation function, the C-terminus of IE2 also plays a role in autoregulation (20, 48, 69, 74, 83, 107) and binding to cellular transcription factors and members of the basal transcription machinery (17, 22, 58, 127). Due to this large overlap of function domains, it is difficult to determine specific regions of IE2 important for a single function. Mutational analyses of IE2 performed by Asmar, *et al.* identified a region in IE2 between amino acids 450 and 544 that plays a major role in IE2 transcriptional regulatory properties during transient transfections, referred to as the "core" region (3). Mutations in the core region of transfected fibroblasts disrupted DNA binding of IE2 to the MIE promoter, autoregulation of the MIE promoter and transactivation of UL112-113 promoter (3). Research in our laboratory has shown that insertion of 4 amino acids at amino acid 349 or 540 result in a 6-fold or greater decrease in activation of the UL54 promoter in transient transfections (134). These insertions also resulted in a 10-fold increase of MIEP activation (134). Based on these results, it has been suggested that the IE2 regions involved in transcriptional activation and repression overlap. However, these large scale mutations in IE2 most likely disrupt one or more functional properties of IE2, emphasizing the need to examine these properties using single amino acid mutations.

More recent studies involving less intrusive mutagenesis of IE2 have confirmed

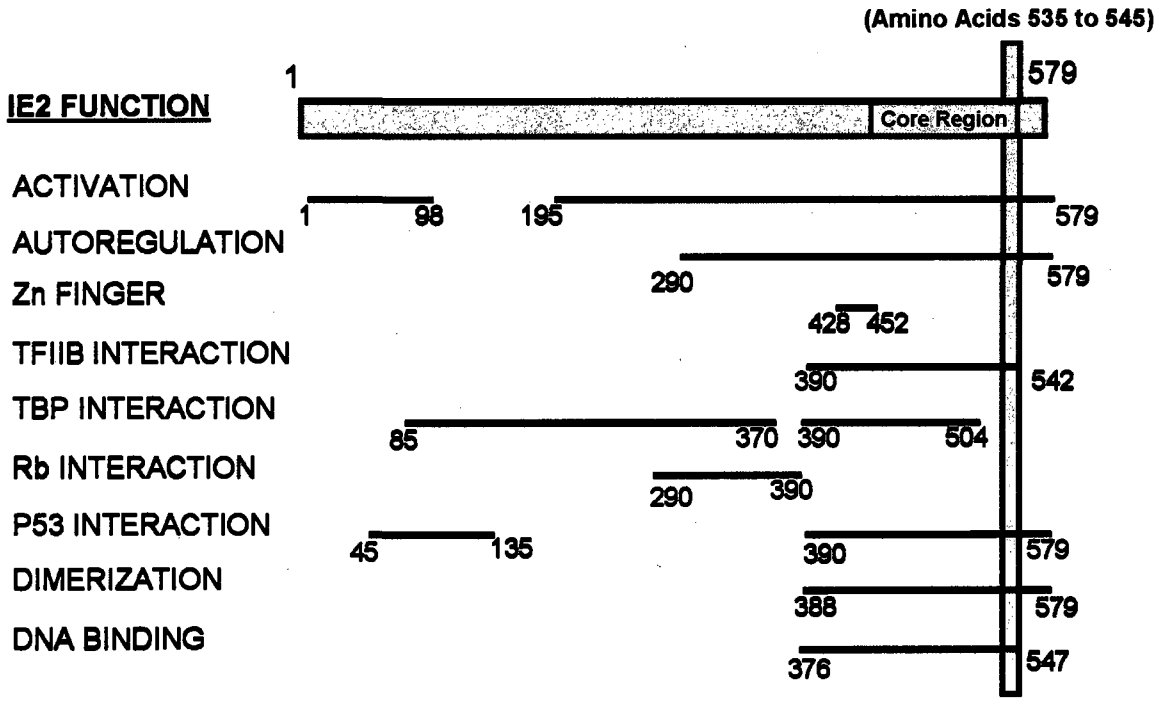


FIG. 6. Structure-Function Analysis of the IE2 Protein. Mutational analyses of the IE2 genes in transient transfection assays have identified a number of functional domains in IE2 gene region. This dissertation focuses on the region between amino acids 535 and 545 of IE2. This region overlaps several functional domains of IE2 including sequences involved in transactivation (46, 84, 108, 122, 127, 134, 154), interactions with cellular transcription factors (17, 22, 58, 127), dimerization (82, 83) and DNA binding (20, 48, 69, 74, 83, 107). The IE2 gene contains the following structural domains: a nuclear localization sequence, a leucine rich region, and a putative zinc finger domain (35, 84, 108, 134).

the importance of the "core" region in IE2 function in transient transfections and during virus infection. Petrik *et al.* demonstrated that a double mutation in IE2 at amino acid 535 and 537 prevented early gene activation of UL44, UL54 and IRL7 promoters in the context of the viral genome (105). IE2 protein mutated at amino acid 535/537 was still able to bind and autoregulate the MIE promoter, suggesting the domains involved in IE autorepression and early gene activation are distinct (105). IE2 protein mutated at amino acid 535/537 was also unable to bind to the UL4 and UL112 promoters. In 2004, White *et al.* generated internal amino acid mutations or deletions in IE2 between amino acids 356 to 359, 427 to 435, and 505 to 511 within HCMV bacterial artificial chromosomes (BAC) (149). These mutations inhibited expression of the UL44 and UL112-113 early genes during infection (149). In addition, these mutations did not result in viable virus (149). However, White *et al.* did not address functional properties of IE2 which may have been impaired with mutations in IE2 (105). In addition, in both of the aforementioned studies the authors did not look directly at the effects of single amino acid mutations on transcriptional regulation. Thus, it is possible that more than one functional property of IE2 was disrupted. The experiments presented in the dissertation will examine transcriptional regulation of IE and early viral promoters by IE2 protein mutated in single amino acids and identify IE2 functional properties disrupted by these mutations.

## Regulation of the MIE Promoter

The major IE promoter (MIEP) is regulated by a complex interplay between viral and cellular proteins. Prior to synthesis of viral gene products, the MIEP is primarily

regulated by cellular transcription factors, such as NF-κB, CREB, SP1, ERF, and YY1. The IE1 and pp71 viral proteins increase activation of the MIE promoter leading to increased MIE gene products (4, 12, 21, 75, 84, 134, 137).

In contrast, IE2 negatively regulates expression of the MIE gene products (20, 48, 69, 74, 83, 107). Negative regulation is typically required to prevent overexpression of viral genes and subsequent inhibition of virus replication. Since IE2 regulates a variety of cellular and viral promoters, including genes involved in cell cycle regulation, uncontrolled expression of IE2 could be problematic to virus replication. IE2 is able to repress its own expression through direct binding to the *cis* repression sequence (CRS), located between the TATA box and the transcription start signal of the MIEP (31, 64, 70, 109, 144). This interaction has been confirmed by Xu, *et al.* using ChIP assays in HCMV infected cells (153). It is thought that IE2 inhibits transcription from the MIEP by blocking RNA polymerase recruitment and preventing assembly of the transcription initiation complex (72). Additional studies have demonstrated that transcription of the MIE gene products are also controlled by a number of cellular transcription factors (37, 48, 76, 90, 99, 124, 151), The presence of proteins such as CREB/ATF, SP1, AP1, and NF-κB at the promoter function to enhance transcription of the MIE gene products (35, 116, 142). A number of cellular factors also repress transcription of MIE gene products, such as MBF2, YY1, MRF, and ERF (37, 48, 76, 90, 99, 124, 151).

## Regulation of Early Viral Promoters

The UL54 early gene promoter encodes the viral DNA polymerase and is required for virus replication (102). This promoter is a well-characterized, prototypical early

promoter which is regulated by actions of both viral and cellular transcription factors (59, 61, 62, 65, 81). Recent studies suggest, but have not directly shown, that IE2 functions in cooperation with cellular factors, TBP, ATF-1 and SP1, and viral factors, such as the UL112-113 gene products, to facilitate transactivation of UL54 promoter (17, 43, 57, 59, 61, 62, 80).

During HCMV infection, SP1 activity is significantly upregulated within the cell (80, 158). The SP1 protein recognizes specific GC-rich elements in viral promoters to allow direct DNA binding and to assist in transcriptional activation (29, 30). A SP1 protein binding site on UL54 was mapped to an inverted repeat domain within the promoter using transient assays (59, 62). Generation of recombinant viruses containing mutations in the putative SP1 binding site of UL54 located upstream of a CAT gene demonstrated a delay in UL54 activation at early times in infection, suggesting a role of SP1 in UL54 expression (59, 62).

Studies have revealed that the C-terminus of Sp1 is able to interact directly with the IE2 protein at viral gene promoters in transient assays (80, 158). In addition, several reports demonstrate that IE2 can interact with SP1 and increase transactivation of viral promoters using *in vitro* binding assays and reporter gene assays (59, 62, 81). IE2 has also been shown to interact with TBP (43, 152) and plays an important role in stabilizing the binding of TBP to the TATA box (57, 58). These studies suggest that IE2 may function as a scaffolding protein at the UL54 promoter in a complex with TBP and SP1.

Viral transcription factors have also been implicated in the regulation of the UL54 promoter. Expression of the IE1 gene product enhances UL54 activation in the presence of IE2 during transient assays (59). In addition to the MIE gene products, the UL112-113

locus encodes four phosphoproteins early in infection which serve of transactivators of the UL54 promoter (53). Studies show that the UL112-113 promoter is controlled by an essential ATF/CREB and a nonessential IE2 binding site (113).

## Summary

Previous studies in our laboratory identified a region in the C-terminus of IE2 important in transcriptional regulation of HCMV promoters (134). In addition, other laboratories have investigated regions within IE2 in order to identify regions of the protein essential for virus replication and transcriptional regulation (3, 105, 149). However, these studies did not assess functional defects of IE2 transcriptional regulatory properties and/or utilized IE2 protein containing multiple mutations. The work presented in this study uses both transient transfections and assessment in the context of the viral genome in order to more precisely define regions and functional properties of IE2 required for transcriptional regulation of viral early promoters and replication.

# CHAPTER II

## OBJECTIVES OF THIS WORK

The objective of this study is to investigate the role of IE2 on transcriptional regulation of HCMV early promoters. Expression of the MIE, UL54, and UL112-113 genes are required for successful virus infection. Previous research on transactivation of the early promoters has primarily been performed in transient assays. This project will assess viral gene regulation in both transient assays and in the context of the HCMV genome in order to further characterize the function of viral and cellular factors in early gene transactivation. These results will be significant in understanding the regulation of early viral genes essential for replication and successful virus infection. The specific aims of this study were to:

### Specific Aims

1. **Identify the regions of IE2 required for transcriptional regulation of viral promoters in transient assays.**

   Transcriptional activation of the early viral genes, such as UL54 and UL112-113, is essential for HCMV replication in the cell. IE2 is a key transcriptional regulator of the early viral genes during virus infection. IE2 is able to autoregulate its own expression by repression of the major IE (MIE) promoter. Previous studies have also identified regions of IE2 important in transcriptional regulation at the C-terminus of IE2. Site-directed mutagenesis was used to generate single amino acid mutations within this region of the IE2 protein at amino acids 535 to 545. Luciferase reporter

assays were performed to assess the ability of IE2 to activate early gene promoters and repress the MIE promoter. These assays were performed in the presence or absence of other MIE gene products to assess IE2-specific properties. Real time PCR and western blot analysis were used to assess differences in IE2 RNA and protein expression levels, respectively.

2. **Characterize the effects of mutations in IE2 on replication and transcription regulation of viral promoters during HCMV infection.**

Due to the essential nature of the IE2 protein for virus replication, it is difficult to generate recombinant virus through traditional methods. To assess the effects of mutations in the IE2 C-terminus on virus replication, we generated HCMV recombinant bacterial artificial chromosomes (BACs) containing mutations in the IE2 protein using homologous recombination. Recombinant HCMV BACs were transfected into human fibroblasts and monitored for cytotoxity and plaque formation to assess the ability to form infectious virus. Virus replication and DNA replication assays were performed to ensure manipulation of the original BAC construct did not influence virus replication using parental and wildtype revertant virus. To determine the effects of mutations in IE2 on transcriptional regulation of viral promoters, RNA was harvested from BAC-transfected fibroblasts at various times post transfection and assessed for viral gene expression by real time RT-PCR analysis.

3. **Characterize the effects of mutation in IE2 on functional properties of IE2 important in UL54 activation.**

IE2 regulates transcription of viral genes through several potential mechanisms. In order to characterize functional properties of IE2 required for UL54 activation, we used *in vitro* GST pulldown assays to assess the ability of mutated IE2 protein to interact with TBP, a member of the basal transcription machinery. Chromatin immunoprecipitations (ChIP) were performed to investigate recruitment of the IE2 protein and RNA polymerase II to the UL54 promoter during transient transfections.

# CHAPTER III

## MUTATIONS IN IE2 DISRUPT TRANSCRIPTIONAL REGULATION OF VIRAL PROMOTERS IN TRANSIENT TRANSFECTIONS

### Introduction

IE2 is a potent transcriptional regulator of viral and cellular genes and is essential for viral replication (45, 74, 86, 107, 109, 134). This protein regulates a number of HCMV promoters encoding proteins required for virus replication, including the MIE, UL112/113 and UL54 promoters. Regulation of viral and cellular genes is thought to be mediated by direct binding of IE2 to DNA and/or protein interactions of IE2 with cellular factors, such as members of the basal transcription machinery, TFIIB and TBP (43, 44, 57, 58). IE2 is also able to interact with cellular transcription factors to regulate viral gene transactivation, including SP1, CREB, AP1 (68, 119, 120, 146). In addition, IE2 has been found to interact with histone modifiers such as P/CAF and HDAC3, suggesting IE2 is involved in chromatin modification to facilitate transcriptional activation (14, 98, 159).

IE2 can also regulate early gene transcription by direct binding to DNA (2, 18, 48, 121, 122). IE2 is able to repress its own expression through direct binding to the CRS, located between the TATA box and the transcription start signal of the MIEP (31, 64, 70, 109, 144). It is thought that IE2 inhibits transcription from the MIEP by blocking RNA polymerase II recruitment and preventing assembly of the transcription initiation complex (72).

The purpose of the experiments outlined in this chapter was to investigate regions

of the IE2 protein essential for transcriptional regulation of viral promoters. The C-terminus of IE2 has been previously investigated by mutational analysis; however, few studies have used single amino acid mutagenesis to begin narrowing down specific functions involved in the regulation of individual promoters (3, 5, 105, 149). Therefore, we sought to investigate the effects of single amino acid mutations in IE2 on transcriptional regulation of the viral gene promoters by the following two approaches. First, to determine the effects of mutations in IE2 on transcriptional regulation of essential genes, we generated single amino acid mutations in IE2 at a region surrounding amino acid 540 and evaluated the effects of the mutated constructs on the MIE, UL54 and UL112-113 early gene regulation. This region was chosen due to it conserved sequence homology with CMV from other species (Fig. 7) and previous studies in our laboratory demonstrating that insertion of a four amino acid linker sequence at amino acid 540 resulted in decreased UL54 activation in transient transfections (105, 134). This approach allowed us to identify specific amino acid mutations which disrupted transcriptional regulation of essential early genes. Second, we investigated the effects of specific amino acid mutations on IE2 RNA and protein expression. Since the MIE promoter is auto-regulated by IE2 it is possible that mutations which inhibit transactivation of early genes may also interfere with IE2-mediated repression of the MIE promoter. This approach allowed us to determine if mutations in IE2 affect expression of the MIE gene products during transient transfections. These experiments will be significant in the identification of regions within IE2 important for transcriptional regulation of early and/or IE genes.

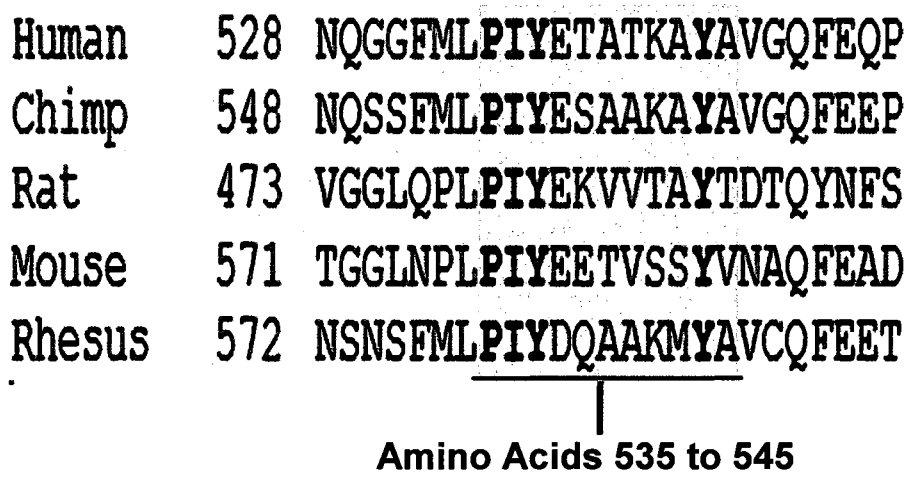


FIG. 7. Sequence similarity between CMV species. The HCMV IE2 protein shares sequence similarity at amino acids 535 to 545 in the C-terminus (shaded area) with a number of other CMV species suggesting this region may be important in virus infections. This figure was adapted from (105).

## Materials and Methods

### Mammalian Cells

Primary human foreskin fibroblasts (HFF) were obtained from Clonetics Corporation and grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) with 10% Newborn Calf Serum (Invitrogen), 2 mM L-glutamine, 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma). Cells were split at a ratio of 1:2 once a week.

### Plasmids Used for Evaluation of Transcriptional Regulation

*IE2 Plasmids*

For transient expression of IE2 and other MIE gene products in culture, the pSVHk plasmid was generated by deletion of a KpnI restriction enzyme site in the pSVH plasmid (27). This plasmid contains the entire UL122-123 coding sequence under the control of the native MIE promoter. pMCRS86 was received as a gift from Jay Nelson (Oregon Health and Science University). pMCRS86 contains the IE2 cDNA sequence under the control of a mutated MIE promoter, allowing for transient expression of the IE2 protein alone. The promoter contains a mutation within the *cis* repression sequence (CRS) mutated between nucleotide -2 and -12 to prevent auto-regulation by IE2. For correction of aberrant mutations in IE2 within the original pMCRS86 construct, the SmaI-Bsu36I fragment from pSVHk was extracted and transferred into pMCRS86 (as described in (5)).

Mutagenesis of IE2 in the pSVHk and pMCRS86 constructs was achieved using Stratagene's Site-directed PCR mutagenesis kit XL according to the manufacturer's

instructions and using a PCR extension time of 7 minutes. Primer sequences for individual mutations are shown in TABLE 1.

The pSVOd plasmid was used as an empty control vector and has been previously described (91).

*Luciferase Reporter Plasmids*

For luciferase reporter assays, the promoter regions of the MIE, UL54 and UL112-113 promoter were cloned upstream of a luciferase reporter gene to generate pMIEP-Luc, pPol-Luc and pUL112-113-Luc, respectively. pMIEP-Luc and pUL54-Luc were generated by cloning of a HindIII fragment from pCAT760, kindly provided by Mark Stinski (137), and pPolCAT (134) into pGL3-Basic (Promega), respectively. pUL112-113-Luc was generated by PCR amplication of p729CAT, kindly provided by Deborah Spector (University of California, San Diego, CA), and TA cloning into pCR4-TOPO (Invitrogen) according to the manufacturer's instructions. The UL112-113 promoter was cloned into pGL3-Basic (Promega) following HindIII and BglII digestion (131).

*Real Time PCR Plasmids*

IE2 and glyceraldehyde 6-phosphate dehydrogenase (G6PD) plasmids were generated by PCR amplification of cDNA from HCMV-infected transfected primary fibroblasts using IE2 and G6PD primers described in White *et al.* to generate IE2-TOPO and G6PD-TOPO (149). PCR fragments were TA cloned into pCR4-TOPO according to the manufacturer's instructions.

TABLE 1. Primers used for site-directed PCR mutagenesis.

| Primer | Primer Sequence [a,b] | Mutation |
|---|---|---|
| P535A-2 | **5′** GTGGGTTCATGCTGGCTATCTACGAGACGGC **3′** | Pro 535→Ala |
| I536A | **5′** GGTTCATGCTGCCTGCCTACGAGACGGCCA **3′** | Ile 536→Ala |
| Y537A | **5′** CATGCTGCCTATCGCCGAGACGGCCACG **3′** | Try 537→Ala |
| E538A | **5′** GTTCATGCTGCCTATCTACGCGACGGCCACGAAGG **3′** | Asn 538→Ala |
| T539A | **5′** CCTATCTACGAGGCGGCCACGAAGGC **3′** | Try 539→Ala |
| A540V | **5′** TATCTACGAGACGGTCACGAAGGCCTACGC **3′** | Ala 540→Val |
| T541A | **5′** CTATCTACGAGACGGCCGCGAAGGCCTA **3′** | Try 541→Ala |
| K542A | **5′** TATCTACGAGACGGCCACGGCGGCCTACGC **3′** | Asn 542→Ala |
| A543V | **5′** GGCCACGAAGGTCTACGCCGTGGG **3′** | Ala 543→Val |
| Y544A | **5′** CCACGAAGGCCGCCGCCGTGGGGC **3′** | Try 544→Ala |
| A545V | **5′** CGAAGGCCTACGTCGTGGGGCAGTTTGA **3′** | Ala 545→Ala |

[a] All primers were phosphorylated on the 5' end.
[b] Only forward primer sequence shown for each primer set and mutations in IE2 are underlined.

## Molecular Cloning and Generation of Plasmid DNA

*Restriction Enzyme Digestion of Plasmid DNA.*

For cloning of plasmid DNA, restriction enzyme digestions were performed according to the manufacturers' recommendation (New England Biolabs). For confirmation of restriction enzyme digestion patterns, approximately 500 ng of DNA was digested for 2-3 hours with 1.5-3 units of enzyme in a total reaction volume of 10-15 µl. Following confirmation of digestion patterns on a 1.2% agarose gel, approximately 10 µg of plasmid DNA was digested with 30-50 units of enzyme for 4-5 hours. Following electrophoresis, agarose gels were stained in 10 mg/ml of ethidium bromide and imaged using Kodak Gel Logic 200 Imaging System.

*Isolation of DNA fragments for cloning*

For cloning of plasmid DNA, the restriction enzyme reactions described above were separated by gel electrophoresis on a 0.8% low melt agarose gel. Following electrophoresis, agarose gels were stained in 10 mg/ml of ethidium bromide. The fragment of interest was excised from the gel and processed with a QIAquik DNA extraction kit (Qiagen) according to the manufacturer's instructions.

*Ligation of DNA fragments for Cloning*

For ligation of DNA fragments to generate new plasmids, ligation reactions were performed at molar ratio of 1:3 between insert and vector fragments according to the manufacturer's instructions of the T4 DNA ligase (Invitrogen). Briefly, approximately 100 ng of vector DNA was combined with 3-fold molar amount of insert DNA, 1 unit of

T4 DNA ligase, and 1X T4 DNA ligation buffer. The reaction was incubated overnight at 4°C. Following incubation, 1-5 µl of the reaction mix was used for transformation into chemi-competent XL1 blue cells.

*Generation of Chemi-competent XL1 Blue cells for Transformation and Propagation of Plasmid DNA*

For preparation of chemically competent XL1-Blue cells for transformation of plasmid DNA, an overnight culture of XL-1 cells was inoculated from a single colony into 5 mls of Luria broth (LB) media and grown overnight at 37°C. Two hundred and fifty milliliters of LB was then inoculated with 3 mls of the overnight culture and grown at 37°C with shaking until $OD_{550}$ of 0.4-0.5. Cells were chilled by swirling in an ice-water bath 1-2 minutes then centrifuged at 5000 X *g* for 10 minutes at 4°C. The supernatant was then removed and the pellet was gently re-suspended in 100 ml cold 100 mM $CaCl_2$. Cells were then chilled on ice for 30 minutes, with swirling every 5 to 10 minutes. Following centrifugation at 5000 X *g* for 10 minutes at $4^0$C, the cells were then re-suspended in 8 ml Calcium/Glycerol. Cells were separated into 250 µl aliquots, frozen in a dry-ice ethanol bath, and stored at -80°C.

*Transformation of Plasmid DNA*

For transformation and amplification of plasmid DNA, chemi-competent XL1 blue cells were thawed on ice for 10-20 minutes. After cells had thawed, 100 µl of competent cells to was added to 10-100 ng of DNA to be transformed in a pre-chilled eppendorf tube. Cells plus DNA were incubated on ice for 30 minutes followed by a heat

shock at 42°C for 2.5 minutes. To allow cells to recover after the heat shock, 1 ml of LB of was added to the cells plus DNA and incubated at 37°C for 60-90 minutes with shaking. Following recovery at 37°C, 50-200 µl of cells were plated onto LB-agar containing appropriate antibiotic and incubated at 37°C overnight.

*DNA Isolation and Purification from Bacterial Cells*

For amplification of plasmid DNA, a single colony was used to inoculate a 5 ml culture of LB with the appropriate antibiotic. The following day, 4 mls of the culture was centrifuged at 5,000 X *g* for 5 minutes and the supernatant was removed. Small scale DNA purifications were performed using Qiagen's MiniPrep kit following the manufacturer's instructions. Following confirmation of small scale cultures by PCR analysis and restriction enzyme analysis as previously described, 0.5 ml of the residual 5 ml culture was diluted 1:500 and grown overnight in 250 mls of LB with the appropriate antibiotic. The 250 ml culture was centrifuged at 6,000 X *g* for 15 minutes and the supernatant was removed. Large scale DNA purifications were performed using Qiagen's MaxiPrep kit according to the manufacturer's instructions.

*Sequencing of Plasmid DNA*

To confirm that manipulations of plasmid DNA did not alter the nucleotide sequence and/or generated the designed mutations, sequencing reactions were performed using BigDye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's instructions with the following exception: the BigDye Ready Reaction mix was diluted 1:8 for each reaction. Sequencing was performed by an ABI Primer 3130 Genetic

Analyzer. Sequencing of the IE2 gene was confirmed with the following primers: SeqPrimer1 (5′ AAT TGC ACA CCC ACC AAT TA 3′), SeqPrimer 2 (5′ AAG AAG AAG AGC AAA CGC ATC 3′), SeqPrimer 3 (5′ GAT TAT TAA ACC GCC CGT G 3′), SeqPrimer4 (5′ CGA CGT TCC TGC AGA CTA TG 3′), SeqPrimer5R (5′ CCT CGG TCA CTC GTT CAA AAG 3′). These primers bind at codons corresponding to amino acids 501, 335, 194, 60, and 72, respectively.

**Transfection of Human Foreskin Fibroblasts with Plasmid DNA**

Primary fibroblast cells were transfected with plasmids at 80% confluency by the DEAE-dextran technique (103). Twenty-four hours prior to transfection, cells were seeded at 0.25 million cells/well or 1 million cells/plate for a 6-well plate or 100 mm plate, respectively. Briefly, 2.5 or 10 μg of total DNA was used for each well in a 6-well plate or 100 mm plate, respectively. An equal amount of pEGFP-C3 (BD Biosciences Clontech) which results in expression of a green fluorescent protein (GFP) was transfected into a separate well of fibroblasts as a control for transfection efficiency. Cells were incubated in 1ml of 1X TBS (30 mM Tris, pH 7.5, 150 mM NaCl) and 0.5 mg DEAE-dextran (diluted in 50 μl of 1X TBS) for 30 minutes. Following incubation at 37°C, 10 mM Chloroquine was added to cells for 2.5 hours. Cells were washed in 1X TBS and new media was added. The cells were harvested 48 hours after the media was changed.

**Analysis of Early Gene Activation using Luciferase Assays**

Luciferase reporters and expression plasmids were transfected into human

fibroblasts using DEAE dextran transfection methods as previously described. Cell extracts were harvested using Cell Culture Lysis Buffer (CCLR) following manufacturer's instructions (Promega). Luciferase assays were performed according to the manufacturer's instructions (Promega) using 10 µl of protein extract in 50 µl of luciferase assay buffer. A Turner Designs 20/20 luminometer was used under the following conditions: 2 seconds of delay time, 10 seconds of integrate time, and the mode was set to Standard. Two to three independent experiments were performed in duplicate and expressed as mean + standard deviation. Significance was determined by a P-value $< 0.05$ using a two-tailed Student's T-test.

**Analysis of Protein Expression Levels**

*Bradford Assays*

Protein concentrations were determined using Biorad's Bradford reagent assay according to the manufacturer's instructions. Experiments were performed in duplicate and averaged for final concentration.

*Western Blot Analysis*

For western blot analysis, cells were harvest in 600 µl of Tris/SDS Buffer (30 mM Tris, 1% SDS). Protein concentration was determined by Bradford assays as described above, equal concentrations of protein were resuspended in 5X SDS-PAGE loading dye (25% glycerol, 5% SDS, 125 mM Tris, pH 6.8, 0.004% Bromophenol Blue), and boiled for 5 minutes. Proteins were separated according to their molecular weight through a 12.5% SDS-PAGE gel at 10 mAmps overnight. To assess molecular weight, 10 µl of

rainbow high molecular weight marker was added for each gel (Amersham). Separated proteins were transferred to nitrocellulose membranes through a semi-dry transfer cassette at 134 mAmp for 1 hr and 30 minutes at room temperature. The following electroblotting buffers were used: Anode Buffer #1 (0.3 M Tris, pH 10.4, 20% Methanol), Anode Buffer #2 (25 mM Tris pH 10.4, 20% Methanol) and Cathode Buffer (25 mM Tris pH 9.4, 40 mM 6-Aminohexanoic Acid, 20% Methanol). The membrane was washed in 1X PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM $KH_2PO_4$, 7.81 mM $NaHPO_4$) for 5 minutes after the transfer. The membrane was blocked overnight in 5% nonfat milk (diluted in PBS) to prevent nonspecific binding to the membrane. The membrane was then washed four times in 1X PBS plus 0.1% Tween (PBST, 5 minutes/wash) and the primary antibody was added at the specified dilutions and incubated for 1 hour at room temperature. For detection of the MIE proteins, we used a 1:2000 dilution of the MAB810 monoclonal antibody (Millipore, 1 mg/ml). For detection of actin as control for cell number, we used a 1:1000 dilution of actin antibody A1978 (Sigma). The membrane was washed four times for 5 minutes in PBST. The secondary antibody was diluted 1:15000 in 5% milk and incubated 1 hour at room temperature. The following secondary antibodies were used: IRDye 800CW goat, anti-mouse IgG and IRDye 680 goat, anti-Rabbit IgG (Licor). The membrane was washed four times for 5 minutes in PBST. Proteins were detected using the Odyssey Infrared Imaging (Licor) according to the manufacturer's instructions.

**Analysis of RNA expression in Transient Transfected Cells**

*RNA Isolation*

To determine RNA expression levels, primary fibroblasts cells were transfected with 10 μg of total DNA by the DEAE-dextran transfection as described above. Samples were harvested 48 hours post-transfection using RNeasy kit (Qiagen) according to the manufacturer's instructions. During purification, RNA was treated with DNAse I using Qiagen's DNAse I Kit according to manufacturer's instructions for on-column digestion and resuspended in RNase-free water. RNA concentration and quality were determined by 260 and 280 nm absorbance readings.

*First Strand cDNA Synthesis*

Superscript III first strand synthesis supermix kit (Invitrogen) was used to generate cDNA for real time PCR using 1-5 μg of total RNA primed with 250 ng of random hexamers according to the manufacturer's instructions.

*Real-Time PCR*

cDNA and DNA levels were assayed by Real time PCR using iQ SYBR green supermix (Invitrogen) in a total of 25 μl, containing 12.5 μl of mastermix, 300 nM of specific primers, and 1-5 μl of cDNA. Primers used to amplify the IE2 and GAPDH genes have been previously described (149). Real time PCR reactions were performed with a Biorad iCycler system using the standard curve method of quantification. Each PCR was performed as follows: initial uracil DNA glycosylase decontamination at 50°C for 2 min, a 2 min hot-start denaturation at 95°C, and 39 amplification cycles (15 s at

95°C, 30 s at 60°C). Melting Curve analysis was performed to ensure the specificity of PCR product and lack of primer-dimer artifacts. The melting temperature of the final double-stranded DNA products was determined by gradual heating from 55°C to 95°C for 80 cycles with a 0.5°C increase after each cycle.

*Quantification of DNA and RNA by Real Time PCR*

The PFAFFL mathematical model was used for quantification of real time PCR reactions (106). Briefly, G6PD-TOPO and IE2-TOPO plasmids were used to generate standards for calculation of PCR efficiencies for each primer set. Serial dilutions of the plasmids from $10^9$ to $10^3$ copies were used for determination of the PCR efficiency for each primer set. Each reaction was performed in duplicate and error bars reflect standard deviations within each experiment. Significance was determined by a P-value $< 0.05$ using a two-tailed Student's T-test. The ratio of target gene mRNA expression relative to the internal control was calculated as described in (106) using the following equation (where E is the efficiency of real time PCR amplification for the specified gene):

$$\text{Relative Expression} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (wildtype} - \text{mutant)}}}{(E_{\text{reference}})^{\Delta Ct \text{ reference (wildtype} - \text{mutant)}}}$$

In this equation, target represents the gene of interest (IE2) and reference represents the internal reference gene or calibrator (G6PD).

## Results

### Mutations in IE2 Inhibit Early Gene Activation

Previous studies in our laboratory identified a region of IE2 at amino acid 540 that when mutated by insertion of a linker coding for 4 amino acids resulted in both loss of MIE promoter repression and UL54 activation (134). However, a study in 2007 demonstrated that a double mutation at P535A/Y537A in IE2 inhibited gene activation of viral early promoters, but not repression of the MIE promoter, suggesting separation of the IE2 transactivation and autoregulation functions (105). To more closely address the discrepancies between these two studies and uncover regions of IE2 important in transcriptional regulation, we generated single amino acid mutations in IE2 between amino acid 535 and 545 using site directed mutagenesis as described in Materials and Methods. Amino acids were mutated to either an Alanine, or Valine, because these residues are structurally neutral, thereby reducing steric or electrostatic effects. We chose to analyze UL54 and UL112-113 transcriptional activation due to the fact that these promoters are required for HCMV infection and are well-characterized in the literature. Primary fibroblasts were co-transfected with a luciferase reporter plasmid containing the luciferase gene upstream of the UL54 or UL112-113 early promoters and an IE2 expression construct in a pSVHk vector by DEAE-dextran transfection (Fig. 8 and 9). The pSVHk vector contains genomic DNA from the entire UL122-123 region (under the control of the MIE promoter) with single amino acid mutations in the C-terminus of IE2. As a control, luciferase constructs were co-transfected with an empty vector, indicative of basal levels of promoter activation. We found that mutations at

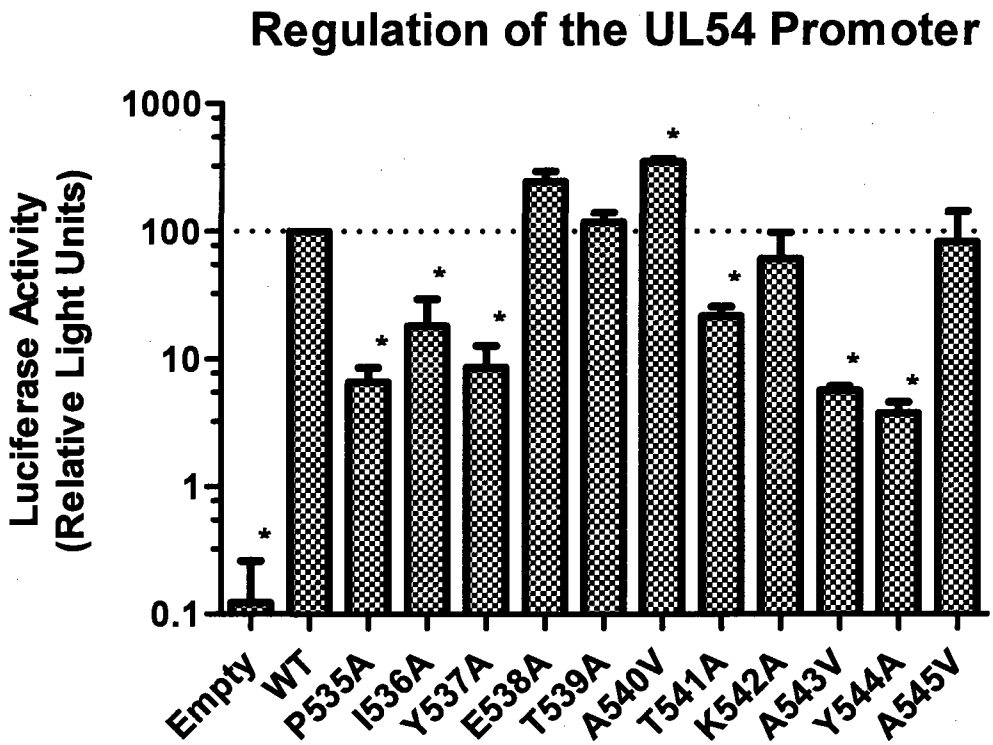


FIG. 8. Mutations in IE2 inhibit UL54 promoter activity. The pPol-Luc reporter construct was co-transfected into primary fibroblasts with IE2 wildtype or mutant expression plasmids in pSVHk vector. The pSVHk plasmid contains the UL122-123 genomic region, expressing the MIE proteins under the control of the native MIE promoter. Cells were harvested for luciferase activity 48 hours after transfection. Luciferase assays were performed in duplicate with 2-3 independent experiments and luciferase activity was expressed as mean ± SD. An asterisk (*) indicates statistically significant difference ($P<0.05$), wildtype versus mutant IE2.

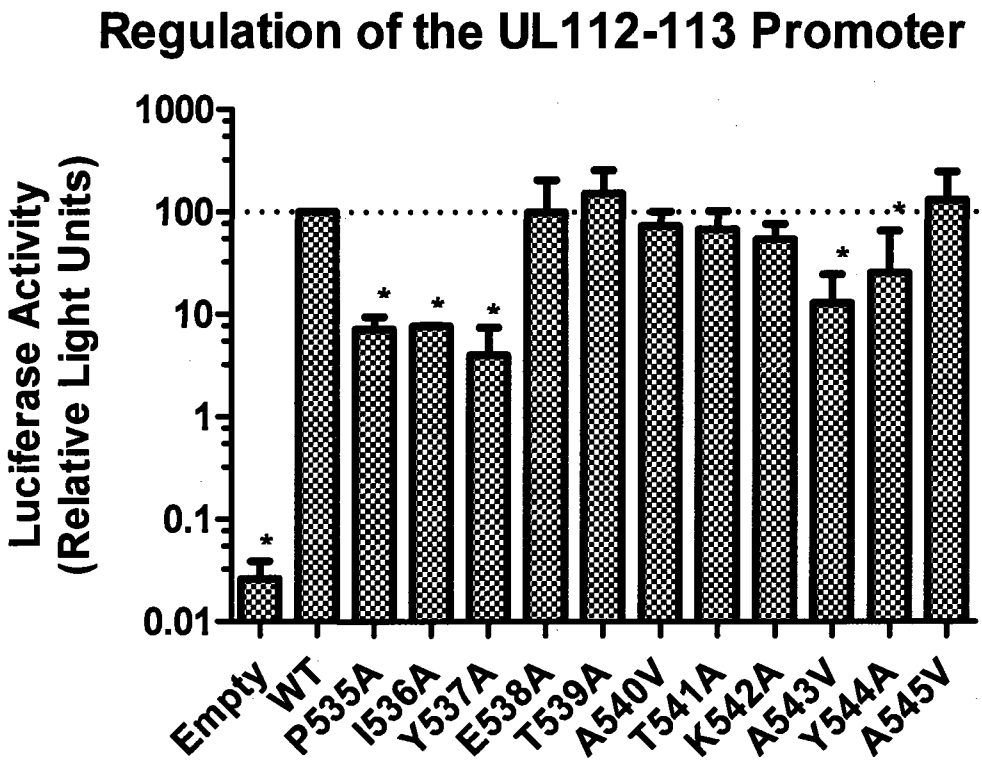


FIG. 9. Mutations in IE2 inhibit UL112-113 promoter activity. The pUL112-113-Luc reporter construct was co-transfected into primary fibroblasts with IE2 wildtype or mutant expression plasmids in pSVHk vector. The pSVHk plasmid contains the UL122-123 genomic region, expressing the MIE proteins under the control of the native MIE promoter. Cells were harvested for luciferase activity 48 hours after transfection. Luciferase assays were performed in duplicate with 2-3 independent experiments and luciferase activity was expressed as mean ± SD. An asterisk (*) indicates statistically significant difference ($P<0.05$), wildtype versus mutant IE2.

amino acids 535, 536, 537, 543 and 544 significantly downregulated luciferase expression from the UL112-113 and UL54 promoters when compared to wildtype IE2. Interestingly, a mutation at amino acid 541 demonstrated a significant decrease in UL54 activation but not UL112-113 activation. It is unlikely that the results we observe in transcriptional regulation are due to structural instability since other mutations in IE2 between amino acids 538 to 542 had minimal effects on early gene regulation. These results are consistent with previous reports suggesting that amino acids in this region of IE2 are important for transcriptional regulation of early genes. However, the results of the analysis of the mutation at Threonine 541 demonstrate that a single amino acid mutation may have distinct effects on early gene activation at different promoters.

**Mutations in IE2 Alleviate IE2-Mediated Autorepression**

IE2 is able to autoregulate its own expression by binding to the CRS of the MIE promoter. To determine whether IE2 mutant proteins unable to regulate early gene transcription also lose IE2 autoregulatory functions, we investigated the effects of mutations in IE2 on MIEP autorepression by luciferase assays as described above. We do not see repression of the MIE promoter with the wildtype (WT) IE2 since other MIE proteins are present to balance MIE promoter regulation. Mutations at 535, 536, 537, 543 and 544 resulted in significantly higher levels of MIE promoter activation when compared to wildtype IE2, suggesting a loss in IE2 auto-repressive properties (Fig. 10). The mutation at amino acid T541A resulted in a slight, but not significant, increase in MIEP activity. These results suggest that mutations which disrupt the transactivation of the UL112-113 and UL54 promoters are likely to disrupt the ability of IE2 to

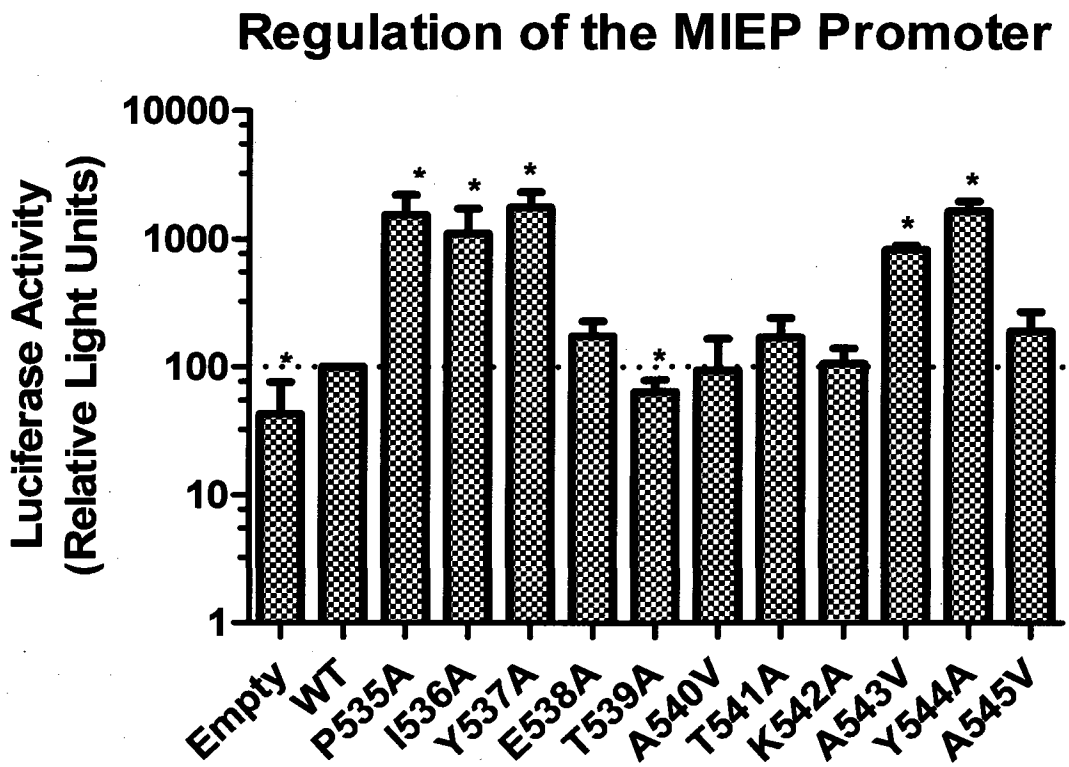


FIG. 10. Mutations in IE2 relieve alleviate MIE promoter repression. The pMIEP-Luc reporter construct was co-transfected into primary fibroblasts with IE2 wildtype or mutant expression plasmids in pSVHk vector. The pSVHk plasmid contains the UL122-123 genomic region, expressing the MIE proteins under the control of the native MIE promoter. Cells were harvested for luciferase activity 48 hours after transfection. Luciferase assays were performed in duplicate with 2-3 independent experiments and luciferase activity was expressed as mean ± SD. An asterisk (*) indicates statistically significant difference ($P<0.05$), wildtype versus mutant IE2.

autoregulate the MIEP. However, the IE2 mutation at T541A emphasizes the fact that individual promoters may be regulated through different mechanisms by IE2.

**Inhibition of Viral Gene Regulation is Specific for the IE2 Gene Product**

The IE2 gene product is expressed as a splice variant from the UL122-123 gene region of HCMV. The MIE gene region encodes a number of additional splice variants, including the IE1 protein (132, 136). To determine effects specific for the IE2 protein, luciferase assays were performed by co-transfection of IE2 wildtype and mutant expression plasmid in a pMCRS86 vector with luciferase reporter constructs under the control of the UL112-113 or UL54 promoters. The pMCRS86 vector expresses the IE2 cDNA under the control of a mutated MIE promoter designed to prevent autoregulation of the transfected plasmid. We chose to continue analysis with only the P535A and Y544A mutants since these mutants displayed similar transcriptional regulatory patterns although the physical mutations are distinct from one another. Consistent with studies in the context of the entire genomic region, mutations in IE2 at amino acids 535 and 544 impaired transcriptional regulation of the MIE, UL54 and UL112-113 promoter between 9- and 62-fold in the absence of other viral gene products (Fig. 11). These results confirm that the effects observed in transcriptional regulation of the MIE and early promoters with these two mutations are specific for the IE2 protein.

**Mutations in IE2 Lead to increased IE2 RNA and Protein Expression**

Differences observed in promoter regulation by the IE2 mutant protein may be

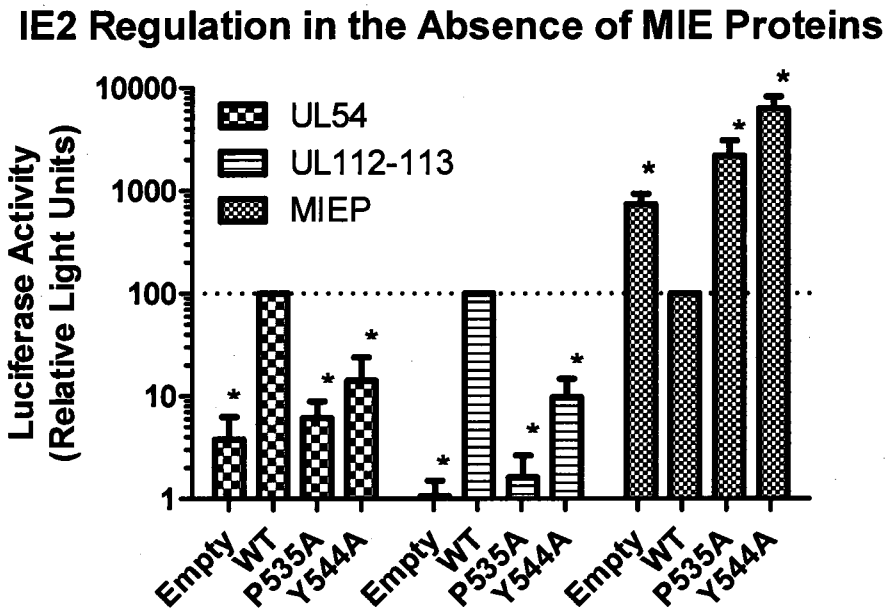


FIG. 11. Regulation of HCMV promoters by IE2 mutants in absence of other MIE proteins. The pMIEP-Luc, pPol-Luc or UL112-112 reporter constructs were co-transfected into primary fibroblasts with IE2 wildtype or mutant expression plasmids in the pMCRS86 vector. The pMCRS86 plasmid expresses IE2 cDNA under the control of a MIE promoter with mutations in the CRS to prevent autoregulation to IE2. Cells were harvested for luciferase activity 48 hours after transfection. Luciferase assays were performed in duplicate with 2-3 independent experiments and luciferase activity was expressed as mean ± SD. An asterisk (*) indicates statistically significant difference ($P<0.05$), wildtype versus mutant IE2.

due to variations in the amount of IE2 RNA and protein present in the cell. In addition, loss of activation or autorepression may be due to decreased levels of the mutant IE2 protein. To exclude these possibilities, accumulation of IE2 RNA and protein levels in transfected cells was determined by real time (RT)-PCR and western blot analysis, respectively. Our results demonstrate that mutations in IE2 lead to increased levels in IE1 and IE2 mRNA levels compared to wildtype, consistent with loss of autoregulation, as shown in Fig. 12. Western blot analysis coincided with mRNA expression showing increased protein accumulation of the IE1 and IE2 gene products in primary fibroblast (Fig. 13). These observations confirm our previous results indicating that IE2 mutations at amino acids 535 and 544 disrupt autoregulation of the MIEP and, subsequently, result in increase levels of IE2 (and IE1) RNA and protein levels.

## Discussion

The goal of specific aim 1 in this dissertation was to identify regions of IE2 important in transcriptional regulation of viral gene promoters. Previous studies identified a region in IE2 at amino acid 540 important in both repression of the MIEP and activation of the UL54 promoter mutated by linker insertion (134). More recently, Petrik, *et al.* determined that a double mutation at amino acid 535 and 537 resulted in changes to IE2- mediated activation of UL4 and UL112-113 early gene promoters (105). However, in this study, the authors were unable to uncover any changes to regulation of the MIE promoter, concluding that the domains involved in autoregulation and transactivation of the IE2 do not overlap (105). Although these results are contradictory in respect to functional domains in the IE2 C-terminus, they emphasize the importance of this region

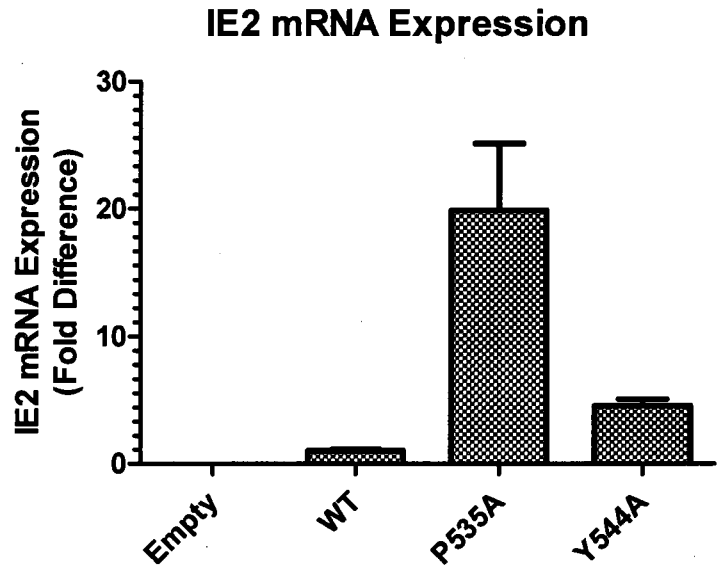


FIG. 12. Mutations in IE2 increase IE2 mRNA expression in transient assays. Primary fibroblasts were transfected with 10 μg of wildtype or mutant IE2 expression plasmids in the pSVHk vector. The pSVHk plasmid contains the UL122-123 genomic region, expressing the MIE proteins under the control of the native MIE promoter. Total RNA was harvested at 48 hours post transfection, reverse transcribed to cDNA and expression levels were determined by real time PCR. Real time PCR analyses were performed in duplicate and relative expression was expressed as mean ± SD.

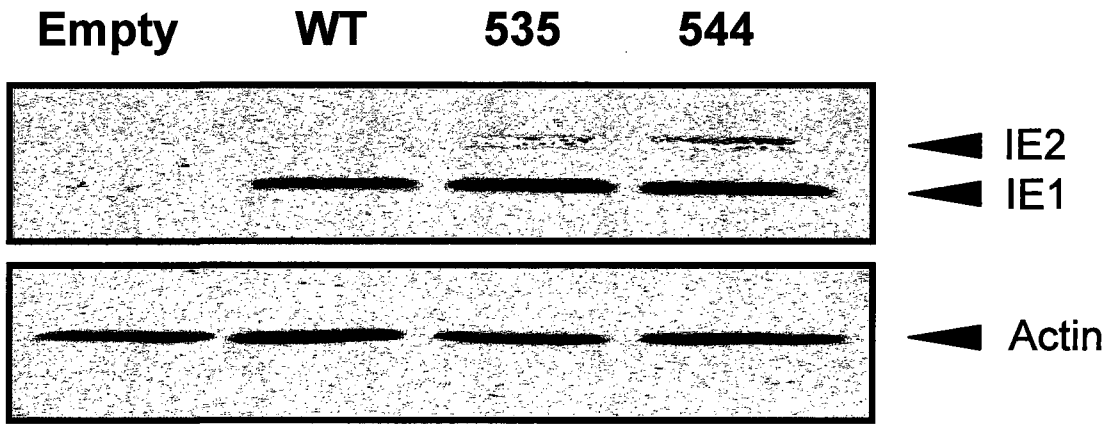


FIG. 13. Mutations in IE2 increase protein expression in transient assays. Primary fibroblasts were transfected with 10 μg of wildtype or mutant IE2 expression plasmids in the pSVHk vector. The pSVHk plasmid contains the UL122-123 genomic region, expressing the MIE proteins under the control of the native MIE promoter. Total protein was harvested at 48 hours post transfection and separated on a 12.5% SDS-PAGE. Western blot analysis was performed using antibodies to the MIE proteins and actin.

in transcriptional regulation of HCMV promoters.

**Potential Effects of Single Amino Acid Mutations**

Several studies have sought to investigate functional domains of IE2 important in transcriptional regulation of early viral genes. Many of these studies have approached this problem using IE2 mutant protein contain more than one amino acid mutation or large deletions. In this study, we have used single amino acid mutations to determine the effects of mutations in the C-terminus of IE2 on transcriptional regulation. It is not unexpected for single amino acid mutations in the C-terminus of IE2 to have the ability to significantly alter protein function. Three-dimensional modeling of the IE2 C-terminus suggests that this region is dynamic in structure and may be altered by subtle changes in the protein sequence (5).

The region between amino acids 535 and 545 of HCMV is highly conserved among different species of CMV, especially at amino acids 535, 536, 537 and 544 (Fig. 7). Mutations at these highly conserved amino acids disrupted transactivation of viral early promoters and autoregulation of the MIE promoter. In addition, the mutations at amino acids 535 and 544 led to increased mRNA and protein levels of mutant HCMV proteins. These results suggest that these residues are critical for IE2 function. It is important to note that increases that mRNA level increases are much higher than the increase in IE2 protein levels with the mutation at amino acid 535. This result suggests that IE2 protein may be degraded with mutations in IE2 and will be evaluated in future studies using assays such as pulse-chase experiments.

**IE2 Transactivation and Autoregulatory Domains Overlap**

In order to address the discrepancy in the location of IE2 functions domains involved in transcriptional regulation, IE2 recombinant proteins were generated containing single amino acid mutations at amino acids 535 to 545 to either an Alanine or Valine residue. Luciferase assays were used to determine the ability of mutated IE2 to activate early viral gene promoters, UL54 and UL112-113, or repress the MIE promoter in transient assays. The IE2 protein was expressed either in the context of the UL122-123 gene loci where other MIE gene products were all co-expressed or IE2 cDNA was expressed under the control of a mutated MIE promoter. The results presented in Fig. 12 demonstrate that a number of the mutations generated between amino acids 535 and 545 resulted in both a loss of IE2 autoregulatory and transactivation properties in primary fibroblasts when functioning in the presence of other MIE proteins.

In this dissertation, we have focused on the IE2 mutations at amino acid 535 and 544. The P535A and Y544A mutations lead to a significant decrease in activation of both the UL54 and UL112-113 promoters. This result was consistent in both the presence and absence of other MIE proteins. In addition, both mutations also resulted in loss of repression of the MIE promoter in the presence and absence of other MIE proteins. These results support Stenberg, *et al.* suggesting the IE2 domains involved in transcriptional activation and repression overlap each other (134).

The MIE gene region encodes for a number of splice variants. In order to determine if mutations in the IE2 gene affect viral gene regulation, cells were cotransfected with an IE2 plasmid expressing IE2 cDNA and luciferase reporter construct. Our results confirm that the effects observed in transcriptional regulation of

the MIE and early promoters with these two mutations are specific for the IE2 protein and result in similar phenotypes of transcriptional regulation (Fig. 11).

**The T541A Mutant Differentially Regulates Early Gene Promoters**

In our studies, we observed that a single amino acid mutation at T541A resulted in distinct effects on early gene activation at different promoters. Specifically, while the mutation at amino acid 541 has no effect on UL112-113 activation or MIE promoter repression, this mutation leads to a significant decrease in UL54 activation *in vitro*. In the AD169 laboratory strain of HCMV, the amino acid 541 is originally an Alanine residue (5). In addition, in other CMV species this amino acid is typically an Alanine or Valine residue (Fig. 7). This suggests that reversion of this amino acid from a Threonine to an Alanine restores this residue to a more conserved amino acid residue. The two early promoters studied in this dissertation are regulated by two different mechanisms. The UL54 promoter is primarily regulated by cellular and viral transcription factors (59, 61, 62, 81). The UL112-113 promoter is regulated by nonessential DNA binding of IE2 in addition to transcription factors. Unfortunately, the exact mechanism(s) of IE2 transcriptional regulation remains unclear. In addition, due to the numerous functional properties available to IE2 for transcriptional regulation, it is likely that individual promoters require separate and overlapping functions for efficient activation. To obtain a more comprehensive understanding of how individual promoters are regulated, exploration of this mutation in IE2 will be the focus of future studies.

**Summary**

Our results suggest that mutations which disrupt the transactivation of the UL112-113 and UL54 promoters are likely to disrupt the ability of IE2 to autoregulate the MIEP. These results are consistent with previous studies in our laboratory which suggest that the domain involved in IE2 activation of the UL54 promoter overlaps the domain required for repression of the MIE promoter. In addition, mutations in IE2 which prevented regulation of the MIE promoter, also led to increased levels of RNA and protein expression. Overall, these results are consistent with our hypothesis that single amino acid mutations within IE2 are able to alter transcriptional regulation of HCMV immediate early and early gene promoters.

# CHAPTER IV

## MUTATIONS IN IE2 INHIBIT REPLICATION AND TRANSCRIPTIONAL REGULATION OF VIRAL PROMOTERS DURING HCMV INFECTION

### Introduction

Expression of the HCMV IE2 protein is required for virus replication and activation of essential viral genes (45, 74, 86, 107, 109, 134). Our data in transient assays supports a model where mutations in IE2 at amino acid 535 and 544 result in loss of both IE2 transactivation and autoregulatory functions. Since the IE2 protein with mutations at these amino acids is no longer able to activate promoters essential for virus DNA replication in transient assays, we hypothesize that these mutations will disrupt the ability of IE2 to activate early promoters during infection and prevent HCMV replication.

Due to the difficulty in generating recombinant virus mutated in an essential gene most of the structure-function studies of IE2 have been performed in transient transfections (3, 14, 57, 62). In order to circumvent the difficulties in mutational analysis of essential HCMV genes, more recent studies have employed the use of HCMV bacterial artificial chromosomes (BACs) to evaluate mutations in the context of the genome (1, 9, 92, 143). Although laboratories have been able to successfully generate recombinant viruses from HCMV BACs mutated in IE2, lack of complementary characterization of the mutant IE2 protein *in vitro* have made these analyses difficult to interpret (45, 117, 149).

BACs are able to stably maintain up to 300 kilobasepairs of DNA, allowing genetic cloning of the large HCMV genome. The HCMV BAC contains the HCMV viral

genome, a bacterial selectable marker and an origin of replication (*ori*) to allow propagation of the DNA in bacteria. Mutagenesis of these BAC constructs require several considerations, including awareness of the BAC vector sequence, source of the mutated DNA sequence, and the method of recombination (reviewed in (1, 143)).

The strategy used in this dissertation is based on the Red system of recombination mediated by the cI-recombination proteins, *exo*, *bet* and *gam* (155). DY380 *E. coli* cells contain a defective cI-prophage carrying the cI-recombination genes under the control of the temperature-sensitive I-cI857 repressor (71). Cells are incubated at 42°C allowing inactivation of the cI repressor and production of the cI-recombination proteins to produce recombination-competent cells. The HCMV BAC construct utilized and modified in this dissertation uses Cre-mediated loxP recombination to remove the backbone of the BAC DNA and remove the antibiotic gene inserted for screening the modified BAC construct.

Characterization of mutations in IE2 within the context of the viral genome is important in order to verify the results seen in transient transfections. Several studies have shown that results demonstrated by transient transfections may not necessarily reflect processes that occur during virus infection. For example, the modulator region of IE2 is located upstream of the CRS and contains binding sites to YY1 (48, 78, 97, 124). Transient studies show that this region is important in autoregulation of the MIEP (48, 78, 97, 124). However, deletion of the modulator region in recombinant viruses does not alter transcription of the MIE gene products (89). Another example is found in the functional analysis of the ICP0, an HSV-1 IE protein. Transient assays demonstrate that ICP0 alone is able to induce ICP8 mRNA (112). However, when assessed in the viral

genome, ICP0 appears to have little effect on ICP8 mRNA expression (26). These studies emphasize differences between transient assays and characterization of promoter regulation in the context of a viral infection

In order to investigate transcriptional regulation of viral promoters in the context of a viral infection, we took the following three approaches. First, recombinant HCMV BACs containing mutations in IE2 were generated using homologous recombination and confirmed by several approaches as described in Materials and Methods. Second, recombinant BAC DNA was transfected into primary fibroblasts and evaluated for the ability to produce infectious virus. Third, recombinant BAC DNA was transfected into primary fibroblasts and assessed for IE2-mediated activation of IE and early genes. These experiments provide a more biologically relevant understanding of the role of IE2 in transcriptional regulation of viral genes and replication within HCMV infected cells.

## Materials and Methods

### Plasmids used for generation and analysis of HCMV BACs

*IE2 Plasmids*

Mutagenesis of IE2 in the pSVHk and pMCRS86 constructs was achieved using Stratagene's Site-directed PCR mutagenesis kit XL according to the manufacturer's instructions and using a PCR extension time of 7 minutes. The following primer sequences were used (mutations in IE2 are underlined, only forward primer is shown): P535Af-3, GTG GGT TCA TGC TGG CGA TCT ACG AGA CGG, and Y544A-2, CCA CGA AGG CCG CGG CCG TGG GGC.

*Generation of HCMV BACs*

To generate pIE2-Zeo-TOPO for homologous recombination with the HCMV BAC, the Zeocin gene was first amplified from pCMV/Zeo (Invitrogen) using BamHI-LoxP-Zeo-LoxP-BglII forward and reverse primers: 5′-AGA TCT ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TGG AAC GGA CCG TGT TGA C-3′ and 5′-GGA TCC ATA ACT TCG TAT AGC ATA CAT TAT ACG AAG TTA TCA AGT TTC GAG GTC GAG GTG-3′, respectively (BamHI and BglII recognition sites are underlined). These primers add a BamHI and LoxP site to the 5′ end and a BglII and LoxP site to the 3′ end. This fragment was then cloned into pCR4-TOPO according to the manufacturer's instructions (Invitrogen) to generate pZeo-TOPO. Subsequently, the IE2 fragment was PCR amplified from pMCRS86 using PstI-435-IE2-579-BamHI primers which amplifies the IE2 region between amino acid 435 and 579 adding a PstI site next to aa435 and a BamHI site after 579. The forward and reverse PstI-435-IE2-579-BamHI primers are 5′-CTG CAG AAC CTG GCC CTC TCC ACT C-3′ and 5′-GGA TCC ACT TAC TGA GAC TTG TTC CTC AGG TCC-3′, respectively (PstI and BamHI recognition sites are underlined). This PCR fragment was then subcloned into pCR4-TOPO to generate pIE2-TOPO. pIE2-Zeo-TOPO was generated by cloning the IE2 fragment of pIE2-Zeo into the pZeo-TOPO construct using PstI and BamHI restriction enzyme digestion.

*Real Time PCR*

IE2-TOPO and G6PD-TOPO plasmids were generated by PCR amplification and TOPO cloning as previously described.

**Mammalian Cells**

Primary human foreskin fibroblasts (HFF) were grown and maintained as previously described in Chapter 3.

**Molecular Cloning and Generation of Plasmid DNA**

Molecular cloning techniques and generation of plasmid DNA were performed as previously described in Chapter 3.

**Generation of HCMV BACs**

*Generation of the Exon5 Deletion BAC*

The Exon5 Deletion BAC was generated by PCR amplification of the kanamycin resistance marker from pGEM-oriV/kan-1 (kindly provided by Hua Zhu, University of Medicine and Dentistry of New Jersey, Newark, NJ) using the following Exon5_449-Kan-580 forward and reverse primers containing sequence homology to the kanamycin gel and a 20 bp overhang with homology to the C-terminus of IE2 between amino acids 449 and 579: 5′ CCA TGG CCC TCT CCA CTC CCT TCC TCA TGG AGC ACA CCA TGG CTC TTG TTG GCT AGT GCG TA 3′ and 5′ CAC TAT GTA CAA GAG TCC ATG TCT CTC TTT CCA GTT TTT CTC TGC CAG TGT TAC AAC CAA 3′. This PCR fragment was gel-purified and transformed into electro- and recombination-competent DY380 cells containing the HCMV Towne-BAC to generate the HCMV ΔExon5 BAC. Positive clones containing successful deletion of the amino acids 449 to 579 and replacement of this region with the kanamycin resistance marker were confirmed by growth on LB agar plates containing kanamycin and chloramphenicol, PCR analysis

and restriction enzyme digestion.

*Generation of the HCMV wildtype-reverent and mutant BACs*

The IE2 Wildtype-Revertant and mutant BACs were generated by PCR amplification of wildtype or mutant IE2-Zeo-TOPO (described previously) using the following Exon5 449-Kan-580 Repl forward and reverse primers containing IE2 overhangs with sequence homology to the HCMV IE2 region flanking amino acid 544 to 579: 5′ CAA CCT GGC CCT CTC ACT CCC TTC CTC ATG GAG CAC ACC ATG CCC GTG ACA CAT 3′ and 5′ CGG GGA ATC ACT ATG TAC AAG AGT CCA TGT CTC TCT TTC CAG TTT TTC AGA TCT ATA ACT TCG TAT AAT G 3′. This PCR fragment was gel-purified and transformed into electro- and recombination-competent DY380 cells containing the HCMV ΔExon5 BAC. Positive clones were confirmed by growth on LB agar plates containing zeocin and chloramphenicol, PCR analysis, southern blot analysis and restriction enzyme digestion.

**BAC Cloning and Manipulation**

*Generation of Electro-competent and Recombination-competent DY380 cells for Transformation and Propagation of BAC DNA*

For preparation of electro- and recombination-competent DY380 cells for BAC transformations, an overnight culture of DY380 cells (with or without BAC construct) was inoculated from a single colony into 5 mls of LB media with appropriate antibiotic. The following day, 100 mls of LB was inoculated with 5 mls of the overnight culture (1:20) and grown at 32°C to an $OD_{600}$ of 3.5-4.0. In order to prepare recombinant-

competent cells, bacteria were incubated at 42°C in a water bath with shaking for 10 minutes. Cells were then chilled on ice for 30 minutes. The cells were centrifuged at 4,000 X *g* for 15 minutes at 0°C. The supernatant was then removed and the cells were re-suspended in 100 mls of ice cold sterile water. This wash was repeated two more times. Cells were then re-suspended in 10 mls of 10% glycerol and spun at 5,000 X *g* for 15 minutes at 0°C. Last, cells were re-suspended in 300 µl of 10% glycerol, separated into 40 µl aliquots, and stored in -80°C (or used immediately for electroporation).

*Transformation of BAC DNA*

For transformation of BAC DNA into electro-competent DY380 cells, cells were first thawed on ice. Approximately 200 ng of DNA was incubated with thawed cells for and mixed gently with a pipette tip. Cells plus DNA were transferred to a 0.1 cm electroporation cuvette and pulsed at 1.6 kV, 25 µF with pulse controller of 200 Ω using a Gene Pulser XCell Electroporation System (Biorad). Following electroporation, 1 ml of LB media was added to each cuvette and transferred to a round bottom polypropylene tube. Cells were incubate at 32°C for 1 hour without shaking, then transferred to a 1.5 ml eppendorf tube and centrifuged at maximum speed for 1 minute. Last, cells were resuspended into 0.1 ml of LB media and plated on LB agar containing the appropriate antibiotic.

*DNA Amplification and Purification from Bacterial Cells*

For amplification of BAC DNA, a 5 ml overnight culture was grown in LB with the appropriate antibiotic. The following day, the overnight culture was centrifuged for 5

min at 5000 X *g*. DNA was isolated as described in Copeland, *et al.* with slight modifications (147). Briefly, the supernatant was removed and the pellet was resuspended in 250 µl of Buffer P1 with RNase (Nucleobond). The cells were incubated for 5 minutes at RT. After the 5 minute lysis, 250 µl of Buffer P2 was then added for 5 minutes at RT, followed by 250 µl of Buffer N3 for 5 min on ice. Cells were centrifuge at 6,000 X *g* for 5 min at RT. The supernatant was transferred to a new tube and the centrifugation was repeated at 6,000 X *g* for 5 min at RT. The supernatant was transferred to a new tube and 750 µl of isopropanol was added to the supernatant. The solution was mixed by inversion and incubated on ice for 10 min. Cells were centrifuged at 6,000 X *g* for 10 min at 4°C. The supernatant was decanted and the pellet was washed in 500 ul of 70% ethanol. The sample was centrifuge at 14,000 X *g* for 5 min at 4°C. The supernatant was decanted and pellet was allowed to air dry for at least 10 min. The pellet was then resuspended in 50 µl of TE.

Following confirmation of small scale cultures in BAC DNA, the 5 ml overnight culture was diluted 1:1000 and grown overnight in 250 mls of LB with the appropriate antibiotic. Two 250 ml cultures were combined and centrifuged at 6,000 X *g* for 15 minutes and the supernatant was removed. Large scale DNA purifications were performed using Clontech's Nucleobond BAC 100 kit according to the manufacturer's instructions.

**Confirmation of HCMV BAC DNA**

*Southern Blot Analysis of HCMV BACs*

To confirm that generation of HCMV BAC DNA did not result in any large scale

rearrangements of the genome, DNA was assessed by southern blot analysis. First, 5 μg of BAC DNA was digested with SmaI restriction enzyme overnight and separated on a 1.0% agarose gel. DNA fragments were visualized by ethidium bromide staining and transferred onto a Hybond nylon membrane (Amersham) overnight by capillary methods. The membrane was then baked for two hours at 80°C in a vacuum oven. The following primers were used to amplify IE2 from the pSVHk vector: 86P449_FSouthern, CCC GTG ACA CAT CCA CCC AAA GTG, and 86E579_RSouthern, CTG AGA CTT GTT CCT CAG GTC CTG G. To generate a probe for IE2, the PCR product was DIG-labeled using the DIG High Prime Starter Kit according to the manufacturer's instructions (Roche) and quantitated by dot blot analysis. The DNA membrane was hybridized overnight with labeled probe, washed to remove non-specifically bound probe, and detected by anti-DIG antibody followed by CPSD staining according to the manufacturer's instructions. Membrane was exposed to X-ray film for 15-25 minutes at room temperature.

*Confirmation of BAC DNA by restriction enzyme digestion*

In order to confirm that no large scale deletions or mutations had occurred to the BAC DNA during mutagenesis, BAC DNA was digested with appropriate restriction enzymes. Digestion was performed as previously described for plasmid DNA in Chapter 3 with the following exception: approximately 1 μg of BAC DNA was digested with 10-20 units of enzyme for 16-18 hours.

*Confirmation of BAC DNA by PCR analysis*

The following primers were used for confirmation of HCMV BAC constructs within the IE2 gene, Downstream IE2 Primer Forward, 5′ GAT GTC TCG CAG GGT GGG TAG ATG 3′, Upstream IE2 Primer Reverse, 5′ GCA TGT TCC GCA ACA CCA ATC G 3′, Internal IE2 Primer Forward, 5′ AAG ACC TGG ACA CCC TGA GCC TG 3′, and Internal IE2 Primer Reverse, 5′ CAG GCT CAG GGT GTC CAG GTC TTC 3′.

*Sequencing of BAC DNA*

To confirm that manipulations of BAC DNA did not alter the nucleotide sequence and/or generated the designed mutations, sequencing of the mutated region was performed using BigDye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's instructions for analysis of BAC DNA. Sequencing was performed by an ABI Primer 3130 Genetic Analyzer. Sequencing of the IE2 gene was confirmed using the sequencing primers described previous for sequencing of plasmid DNA.

**Transfections of BAC DNA**

For BAC transfections in primary fibroblasts, 10 μg of BAC DNA and 1 μg of pp71 DNA was transfected into HFF by the calcium phosphate method. Cre Recombinase (1 μg) was transfected with BACs in DNA and virus replication assays (86). Media was changed every 4-5 days until plaque outgrowth occurred. Cells were monitored for 3 or more weeks for plaque formation.

**Growth of Recombinant Viruses**

*Harvesting Reconstituted Virus*

Following BAC transfections into primary fibroblasts, reconstituted virus was harvested from the supernatant of BAC transfected cells when 90-100% of cells displayed cytopathic effects (CPE) and stored at -80°C.

*Generation of Virus Stocks*

For generation of virus stocks, supernatant from BAC transfected cells were thawed at room temperature, diluted in DMEM media at 1:1000, and used to inoculate a T150 flask of primary fibroblasts at 100% confluency. Cells were incubated at 37°C for two hours. After two hours, inoculum was removed and new media was added. Cells were monitored daily for CPE and supernatants were harvested at 100% CPE. Media was changed every 5 days.

*Titering of Virus Stocks*

To determine the concentration of virus stocks, serial dilutions were generated and used to inoculate individual wells within 6-well plates containing primary fibroblasts at 100% confluency. Cells were incubated at 37°C for two hours, followed by an overlay with 1% agarose, 2X MEM, and 1% penicillin-streptomycin. Cells were monitored daily for plaque formation and a new overlay was added every 5 days. The following equation was used to determine virus stock concentration in plaque forming units (pfu)/ml of supernatant:

$$\text{pfu/ml} = \frac{\text{Number of plaques}}{D * V}$$

In this equation, D is the dilution factor and V is the volume of diluted virus added to each well.

**Analysis of Nucleic Acid Expression**

*TriZol extractions of RNA, DNA and protein*

Primary fibroblasts were transfected with approximately 10 μg of HCMV BAC DNA and 1 μg of HA-pp71 by calcium phosphate transfection as described above. Samples were harvested at various times postinfection using Trizol reagent (Invitrogen) according to the manufacturer's instructions. During purification of RNA, isolated RNA was treated with DNAse I using Qiagen's DNAse I Kit according to manufacturer's instructions. DNA and RNA concentration and quality were determined by 260 and 280 nm absorbance readings.

*First Strand cDNA Synthesis*

Superscript III first strand synthesis supermix kit (Invitrogen) was used to generate cDNA for real time PCR using 1-5 μg of total RNA primed with 250 ng of random hexamers according to the manufacturer's instructions.

*Real-Time PCR*

cDNA and DNA levels were assayed by Real time PCR using a SYBR green Real time PCR kit (Applied Biosystems) as previously described.

*Quantification of DNA and RNA by Real Time PCR*

Plasmids containing the UL54-CAT promoter, GAPDH promoter, IE2 gene, and G6PD gene were used to generate standard curves to quantitate relative amounts of cDNA in each sample. Serial dilutions of the plasmids from $10^9$ to $10^3$ copies were used for determination of the PCR efficiency for each primer set. Each reaction was performed in duplicate and error bars reflect standard deviations. Significance was determined by a P-value $< 0.05$ using a two-tailed Student's T-test. The ratio of target gene mRNA expression relative to the internal control was calculated as described in (106) using the following equation (where E is the efficiency of real time PCR amplification for the specified gene):

$$\text{Relative Expression} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (wildtype – mutant)}}}{(E_{\text{reference}})^{\Delta Ct \text{ reference (wildtype – mutant)}}}$$

In this equation, target represents the gene of interest (IE2) and reference represents the internal reference gene or calibrator (G6PD).

*Viral DNA replication assays*

To determine the effects of mutation in IE2 on viral DNA replication, BAC-transfected cells were harvested at various time points using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Real time PCR analysis was performed as described above using equal amounts of total DNA (100 ng). The primer sequence used for amplification of gB has been previously described (105).

*Virus replication assays*

To determine the effects of mutations in IE2 on HCMV replication, supernatant from BAC-transfected cells was harvested at various time points and stored in -80°C. Virus replication assays were performed as described in Lorz, *et al* (77). Briefly, 20 μl of viral supernatant was incubated with 160 μl of 10 mg/ml proteinase K for 1 hour at 56°C. DNA was denatured by incubation at 95°C for 5 minutes. Viral DNA levels in the supernatant was assessed by real time PCR analysis as described previously using 10 μl of the extract as a DNA template and primers for gB DNA as described in Petrik, *et al.* (105).

## Results

### Generation of HCMV Towne BACs mutated in the IE2 gene

Based on the results of our mutational analyses of IE2 in transient transfections, we speculated that mutations at amino acid 535 and 544 would demonstrate impaired transcriptional regulation in the context of the viral genome. We chose to continue evaluation of these mutants to test our hypothesis that they would be lethal to virus replication and to more closely investigate the transcriptional regulatory properties of IE2 that are essential for activation of early genes. It is important to note that although IE2 mutagenesis of the BAC construct resulted in an Alanine residue, consistent with *in vitro* studies, additional basepairs were mutated at amino acid 535 and 544 to ensure mutated IE2 did not revert back to wildtype during infection (see Materials and Methods) (149).

Mutagenesis of the HCMV Towne BAC DNA was used to evaluate the effects of mutations in IE2 on replication and transcriptional regulation in the context of the viral

genome. The HCMV Towne BAC clone was kindly provided by Dr. Hua Zhu (University of Medicine and Dentistry of New Jersey, Newark, NJ) (86). This BAC construct replaces the US1 to US12 region of HCMV, which is dispensible for infection, with a F-plasmid sequence (86). The F-plasmid sequence contains a GFP expression cassette, a chloramphenicol resistance marker, and an origin of replication for amplification in DY380 *E. coli* (86). DY380 cells were generated by modification of DH10B to express phage derived recombination genes under the control of a temperature-sensitive repressor, cI, and were kindly provided by Dr. Hua Zhu (71).

Alterations in the endogenous IE2 gene of HCMV BACs was accomplished using a two step PCR approach (Fig. 14). In the first PCR reaction, the kanamycin resistance marker was PCR amplified from pGEM-oriV/kan-1 (kindly provided by Hua Zhu) using primers containing sequence homology to the kanamycin gene and a 20 bp overhang with homology to the C-terminus of IE2 between amino acids 449 and 579 (Fig. 14). This PCR fragment was gel-purified and transformed into electro- and recombination-competent DY380 cells containing the wildtype HCMV Towne-BAC. Recombination of IE2 overhangs with the IE2 region resulted in deletion of amino acids 449 to 579 of IE2 to generate the HCMV ΔExon5 BAC (Fig. 14). Positive clones containing successful deletion of the amino acids 449 to 579 and replacement of this region with the kanamycin resistance marker were confirmed by growth on LB agar plates containing kanamycin and chloramphenicol, PCR analysis and restriction enzyme digestion.

Following generation and confirmation of the HCMV ΔExon5 BAC, the plasmid pIE2-Zeo-TOPO (containing Exon 5 of IE2 located upstream of Zeocin) was mutated by site-directed PCR at amino acid P535A or Y544A and used to generate HCMV mutant

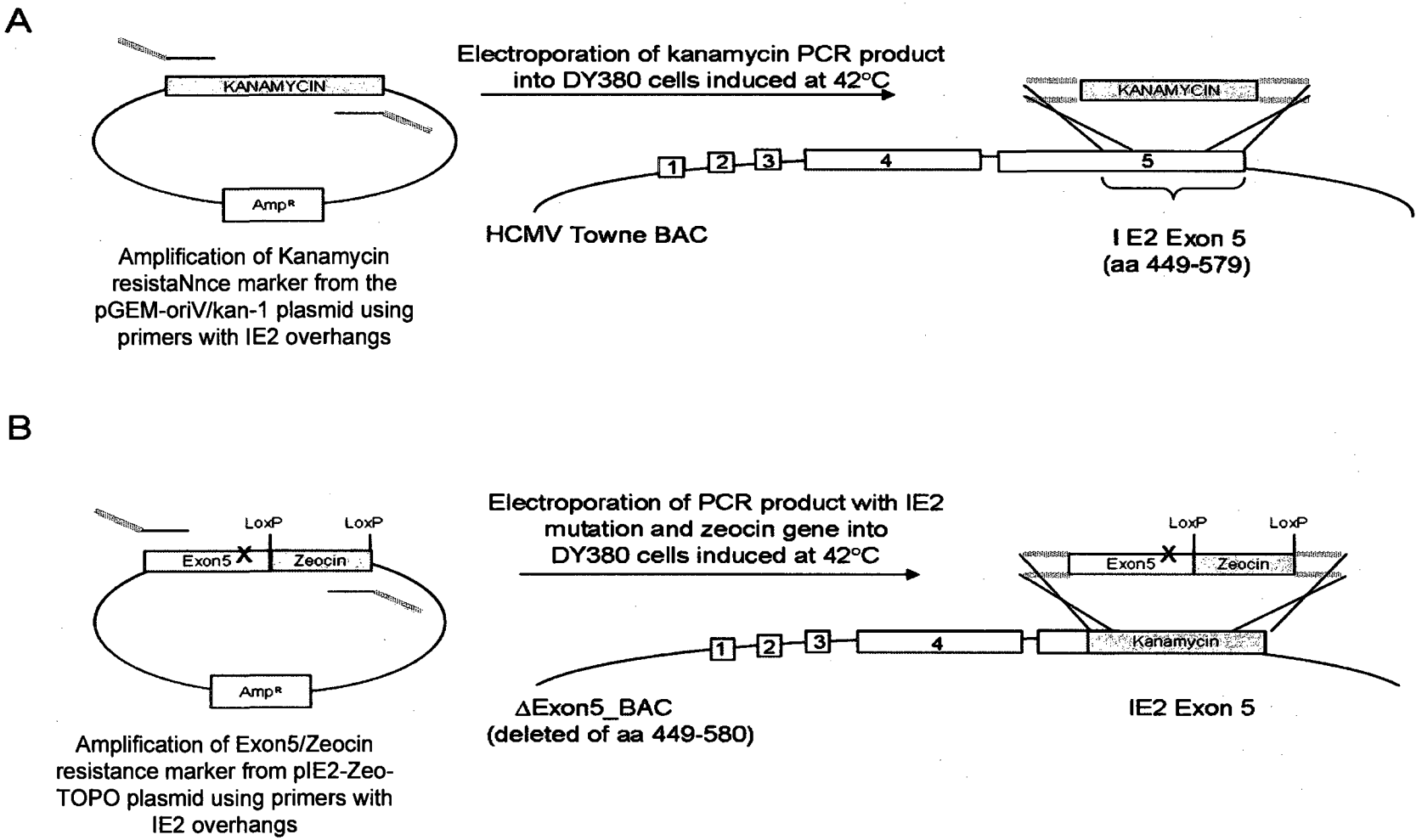


FIG. 14. Generation of HCMV Towne BACs which express the IE2 protein mutated at amino acid 535 or 544.

FIG. 14, Continued. Mutations in IE2 within HCMV BACs were generated by two steps. (A) First, amino acids 449 to 579 of IE2 was deleted from HCMV BACs by homologous recombination with a PCR product containing the kanamycin gene flanked by regions homologous to IE2 sequence outside of amino acids 449 and 579. (B) The HCMV BAC mutated in the IE2 focal region was generated by homologous recombination of the HCMV deletion BAC with a mutated PCR product containing the IE2 region between amino acids 449 and 579 upstream of a zeocin gene. Please refer to Materials and Methods for additional details. The symbol X represents mutations in IE2.

BACs (Fig. 14A). In the second PCR reaction, the IE2 region between amino acid 449 and 579 and the zeocin resistance marker flanked by loxP sites was amplified from wildtype or mutated pIE2-Zeo-TOPO (Fig. 14B). This was achieved using primers recognizing the pIE2-Zeo-TOPO fragment and containing IE2 overhangs with sequence homology to the HCMV IE2 region flanking amino acid 544 to 579. Amplification of wildtype pIE2-Zeo-TOPO plasmid containing the endogenous IE2 sequence was used to generate a wildtype- revertent (WT-Rev) BAC. Generation of a WT-Rev BAC is important to make sure that any additional DNA introduced in the generation of the HCMV BAC DNA during mutagenesis does not interfere with the IE2 phenotype. The PCR fragment was gel purified and transformed into DY380 cells containing the HCMV ΔExon5 BAC to generate recombinant BAC DNA. Positive clones were confirmed by growth on LB agar plates containing zeocin and chloramphenicol. In addition, BAC DNA was further of confirmed by PCR analysis, southern blot analysis and restriction enzyme digestion (Fig. 15 and 16). Comparison of the EcoRI/SalII restriction digestion pattern demonstrated introduction of the zeocin gene and confirms large scale rearrangements did not occur during the recombination procedure (Fig. 15 and 16).

**Wildtype (WT) and WT-Rev virus display similar growth and viral DNA replication kinetics**

During the generation of HCMV recombinant BAC DNA, we introduce DNA sequences not present into the original construct. Specifically, a BamHI restriction enzyme site and single loxP remain in the DNA following the IE2 stop codon when transfected in the presence of Cre Recombinase. To ensure that these sites do not

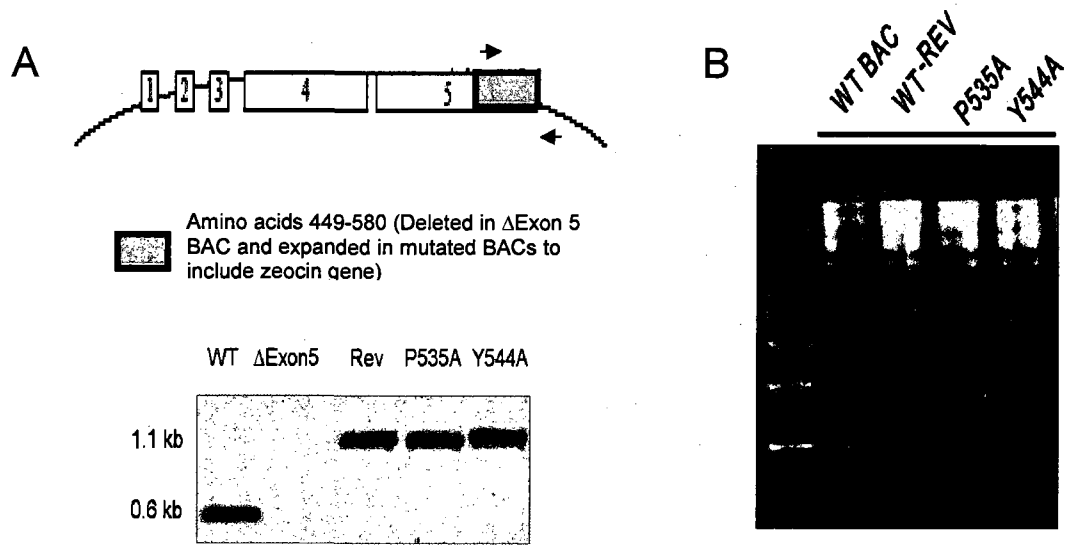


FIG. 15. Confirmation of Mutant HCMV BACs by PCR and restriction enzyme analysis. To confirm generation of mutant HCMV BACs the HCMV BAC screening primers (A) were generated to flank amino acid 449 to 579 of IE2 and used to PCR amplify the IE2 gene region. The gel fragments were separated on a 1.2% agarose gel. HCMV WT BAC results in a approximately 0.6 kb fragment using the indicated primers (arrows). With the introduction of the Zeocin, we see an increase in the size of this fragment to 1.1 kb in the WT-Rev, P535A and Y544A BAC constructs. With deletion of the IE2 C-terminus, we see no PCR product. (B) For restriction enzyme analysis, HCMV BACs were digested with SalI and HindIII to confirm large scale rearrangements did not occur with mutations in HCMV BACs. Following digestion, fragmented DNA was run on a 0.8% agarose gel.

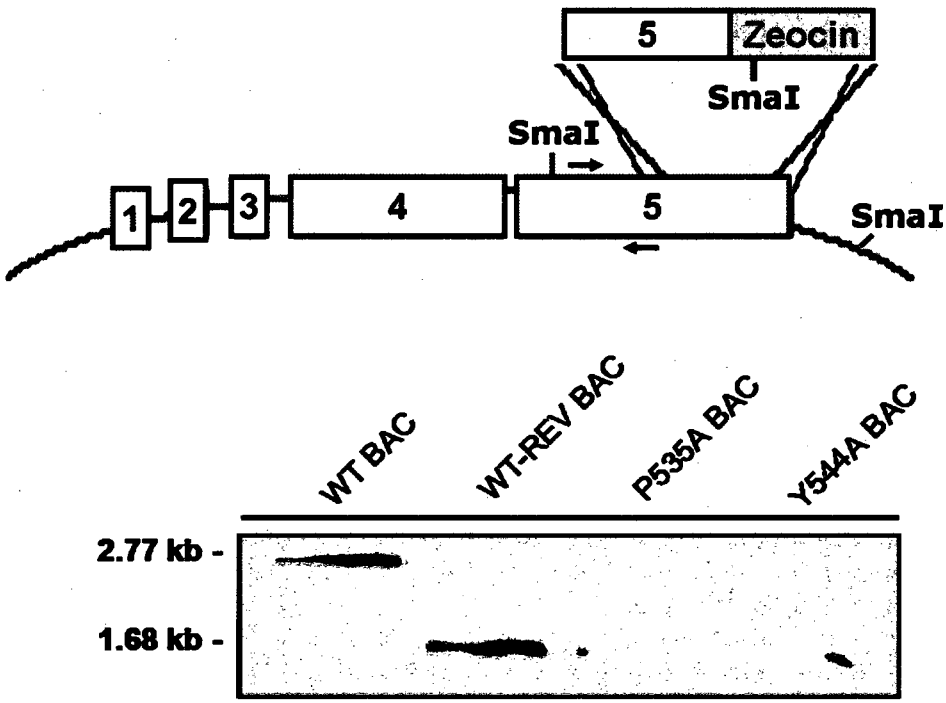


FIG. 16. Confirmation of Mutant HCMV BACs by Southern Blot Analysis. To confirm generation of mutant HCMV BACs, HCMV BACs were digested with SmaI restriction enzyme and separated by electrophoresis on a 1% agarose gel. DNA was transferred to a nylon membrane and probed by southern blot analysis as described in the Materials and Methods. As shown in the diagram above, genomic IE2 contains a two SmaI recognition sites resulting in a 2.77 kb fragment (WT BAC). The cloning of the zeocin gene at the C-terminus of IE2 introduces an additional SmaI recognition site, resulting in a 1.68 kb fragment as seen with WT-Rev, P535A and Y544A BACs. The arrows shown this diagram, indicate the primers used for southern probe generation which bind at amino acid 449 and 579 of IE2.

interfere with viral growth, we compared virus replication and viral DNA synthesis of the WT and WT-Rev BACs. WT and WT-Rev BAC DNA was transfected into primary fibroblasts and virus titers were determined for reconstituted virus. Primary fibroblasts were infected with 0.5 pfu/cell of virus and harvested daily for DNA. Virus supernatant was harvested and digested with proteinase K to determine levels of infectious virus in the supernatant. As shown in Fig. 17, wildtype and revertant viruses had similar levels of gB DNA in the supernatant confirming there was no difference in virus replication between WT and WT-Rev virus. To assess differences in viral DNA replication, genomic DNA was harvested from cells and assessed for gB DNA levels by real time PCR. Similar levels of IE2 gB DNA were found in the cells of WT and WT-Rev virus (Fig. 18). These results demonstrate that the additional sequences found in the WT-Rev virus, as well as the recombinant viruses with mutations in the IE2 gene, do not affect replication of HCMV.

**WT-Rev and mutant BAC DNA have similar transfection efficiencies**

We have demonstrated in the previous section that the WT and WT-Rev virus have similar replicative capacities during infection. Thus, further studies compared mutant phenotypes to that of the WT-Rev BAC. To ensure that there were no differences in transfection efficiency between WT-Rev and mutant BACs, BAC DNA was transfected into primary fibroblasts with Cre Recombinase and harvested at 1 day after transfection for genomic DNA. DNA levels were assessed by real time PCR as previously described. As shown in Fig. 19, we saw no noticeable differences in DNA levels at 1 day after transfection indicating the BAC transfection efficiency between WT-

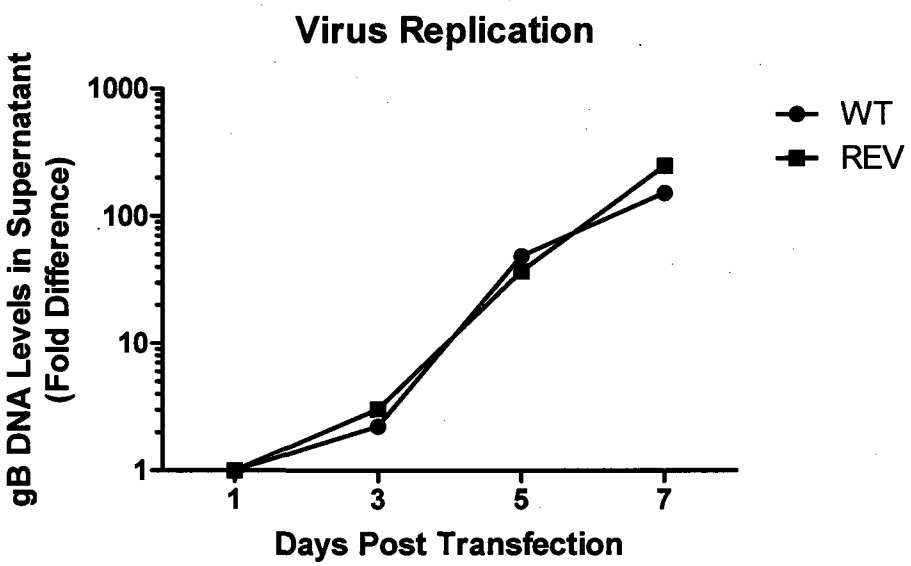


FIG. 17. Growth Curve Kinetics of HCMV WT and WT-Rev virus. WT-Rev and mutant HCMV BACs were transfected into primary fibroblasts by calcium phosphate transfections. Virus supernatants were harvested for genomic DNA at indicates times. following transfection and assessed for gB DNA levels by real time PCR.

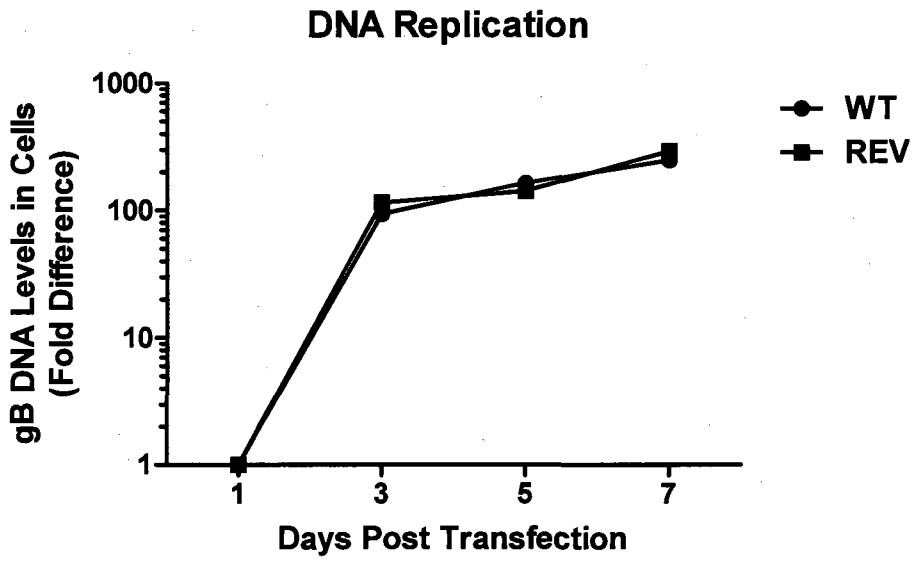


FIG. 18. Replication of HCMV WT and WT-Rev viral DNA in primary fibroblasts. WT-Rev and mutant HCMV BACs were transfected into primary fibroblasts by calcium phosphate transfections. Genomic DNA was harvested from primary fibroblasts at the indicated times following transfection and assessed for gB DNA levels by real time PCR.

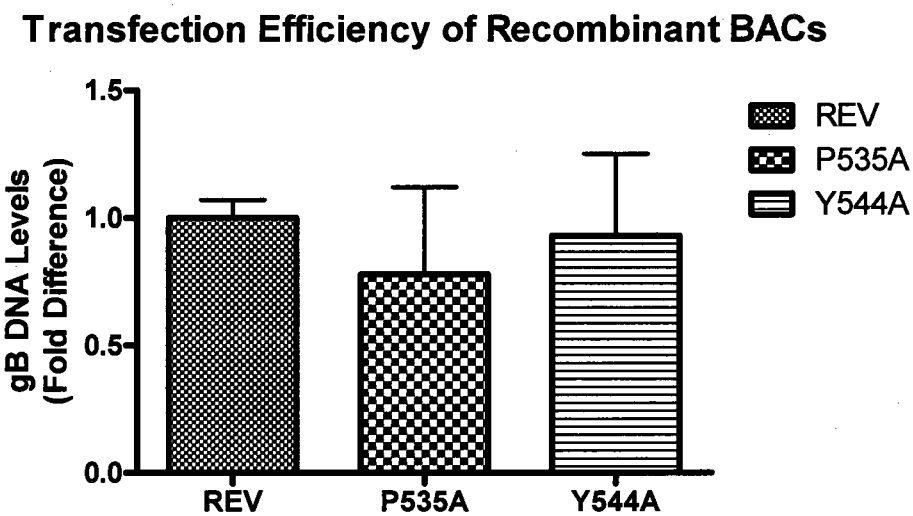


FIG. 19. gB DNA levels at 1 day post transfection as an indicator of transfection efficiency. WT-Rev and mutant HCMV BACs were transfected into primary fibroblasts by calcium phosphate transfections. Cells were harvested for genomic DNA at one day following infection and assessed for gB DNA levels by real time PCR. DNA levels are expressed as mean ± SD within a representative experiment.

Rev and mutant BACs.

**Mutations in amino acid 535 and 544 inhibit virus replication**

The experiments performed in transient transfections suggest that mutations at 535 and 544 inhibit activation of the UL54 and UL112-113 promoters. Since both of these proteins are essential for virus replication, we predicted that generation of these mutations in the HCMV BAC would not yield viable virus. Wildtype, WT-Rev, and mutant BACs were transfected into primary fibroblasts along with pp71 and Cre recombinase expression plasmids to investigate the effects of these mutations on virus replication. Recombinant BACs mutated at amino acid 535 and 544 were unable to generate viable virus when compared to WT or WT-Rev virus (data not shown). Plaques appeared after approximately 6 days in cells transfected with WT and WT-Rev BACs. Cells transfected with the IE2 mutated BACs were monitored and maintained for up to 6 weeks following several repetitions with no indication of virus replication. These results confirm that mutations in IE2 prevent the generations of viable virus when compared to WT or WT-Rev virus.

**Mutations in IE2 reduce activation of early gene promoters**

One possible reason for the lack of viable HCMV infectious virus production is an inability to efficiently replicate viral DNA. To determine the stage in which virus replication may be blocked, viral RNA of BAC transfected cells were assessed by real time PCR. Total RNA was isolated from cells harvested at various days post transfection and reverse transcribed into cDNA. UL54 and UL112-113 mRNA transcript levels were

then measured by real time PCR. Unlike our results *in vitro*, we observed differences in regulation of UL54 and UL112-113 promoters between recombinant viruses containing mutations in IE2 at amino acid 535 and 544 (Fig. 20). It is important to note that since virus replication began at 6-7 days post transfection, that promoter regulation at 1 and 4 days post transfection represent transcriptional activation or repression. RNA levels at 7 and 10 days post transfection most likely represent differences in virus replication.

In fibroblasts transfected with the WT-Rev HCMV BAC, we observed a 6-fold increase in UL54 transcript levels 4 days following transfection (Fig. 20). Since we do not begin to see high levels of viral particles in the cell until 4-5 days after transfection, this most likely represents an increase in UL54 activation. We continue to see a rise in UL54 transcript levels at 7 days post transfection, indicative of virus replication and spread to adjacent cells (Fig. 20).

Interestingly, the mutation in IE2 at amino acid 535 displays wildtype-Rev levels of UL54 activation at 1 and 4 days following transfection suggesting an ability to activate the UL54 promoter (Fig. 20). However, at 7 days post transfection the UL54 activation levels reduced significantly (Fig. 20). In contrast, cells transfected with recombinant BAC DNA mutated at amino acid 544 display significantly lower levels of UL54 transcripts at all days post transfection suggesting an inefficiency to activate the UL54 promoter at all time-points tested (Fig. 20). The ability of the IE2 protein mutated at amino acid 535 to efficiently regulate UL54 activation, suggests that the mutation at 544 may disrupt a property of IE2 required for UL54 activation which is maintained with a mutation at 535. Similar to transient transfections, IE2 protein with mutations in amino acids 535 and 544 were unable to activate the UL112-113 promoter

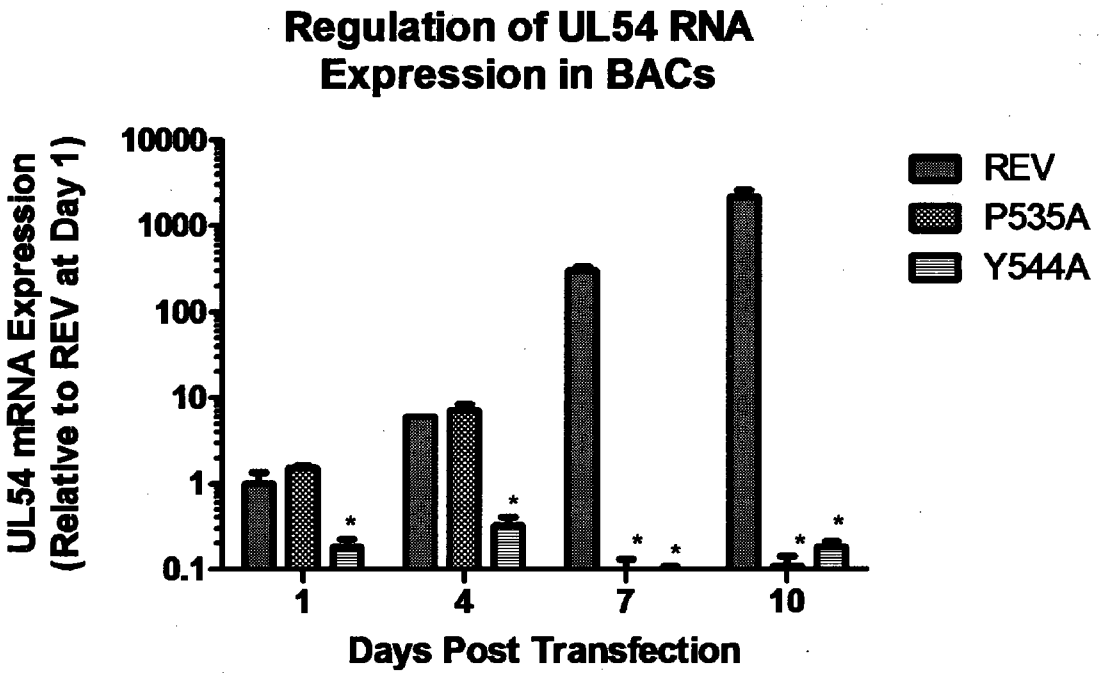


FIG. 20. Regulation of the UL54 promoter in BAC transfected cells. Total RNAs were isolated from BAC transfected cells as described in Materials and Methods and analyzed by quantitative real-time RT-PCR with using primers specific for UL54 cDNA. Experiments were performed in duplicate with 2 independent experiments and mRNA expression levels were expressed as mean ± SD. An asterisk (*) indicates statistically significant difference ($P<0.05$), wildtype-Rev versus mutant IE2.

after day 1 as shown in Fig. 21. The failure of both mutant IE2 proteins to activate the UL112-113 promoter suggests both mutations disrupt a functional property of IE2 required for UL112-113 activation.

**Mutations in IE2 prevent repression of MIE promoter early in infection.**

The IE2 protein has been shown to autorepress the MIE promoter (20, 48, 69, 74, 83, 107). To determine the effects of mutations in IE2 at amino acid 535 and 544 on MIE autorepression in BAC transfected cell, we evaluated MIE mRNA expression at various times after transfection, as previously described. Following transfection of wildtype reverent BAC, mRNA expression levels of IE2 remain stable at 1 and 4 days post transfection, then begin to increase at 7 and 10 days post infection due to virus spread (Fig. 22). Similar to results observed in transient transfections (Fig. 10), 1 day following transfections of BAC DNA, mutations in IE2 at amino acid 535 and 544 were unable to repress the MIE promoter resulting in a 65- and 4-fold increase in IE2 mRNA levels, respectively (Fig. 22). These results confirm our transient assays demonstrating that mutations in IE2 result in loss of MIE autorepression and UL54 activation.

## Discussion

Herpesviruses are significant pathogens for animal and humans, emphasizing the need for characterization of viral genes that regulate replication. Analysis of functional properties both *in vitro* and *in vivo* is essential for understanding the distinct function of individual gene products. The most common strategy used to evaluate the function of a

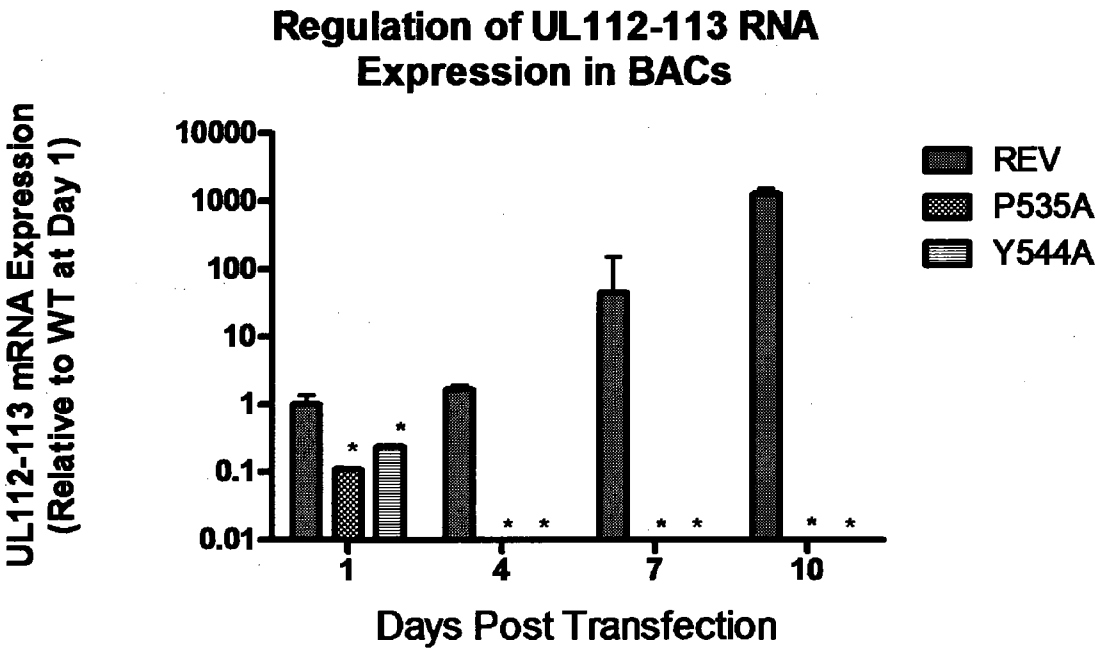


FIG. 21. Regulation of the UL112-113 promoter in BAC transfected cells. Total RNAs were isolated from BAC transfected cells as described in Materials and Methods and analyzed by quantitative real-time RT-PCR with using primers specific for UL112-113 cDNA. Experiments were performed in duplicate with 2 independent experiments and mRNA expression levels were expressed as mean ± SD. An asterisk (*) indicates statistically significant difference ($P<0.05$), wildtype-Rev versus mutant IE2.

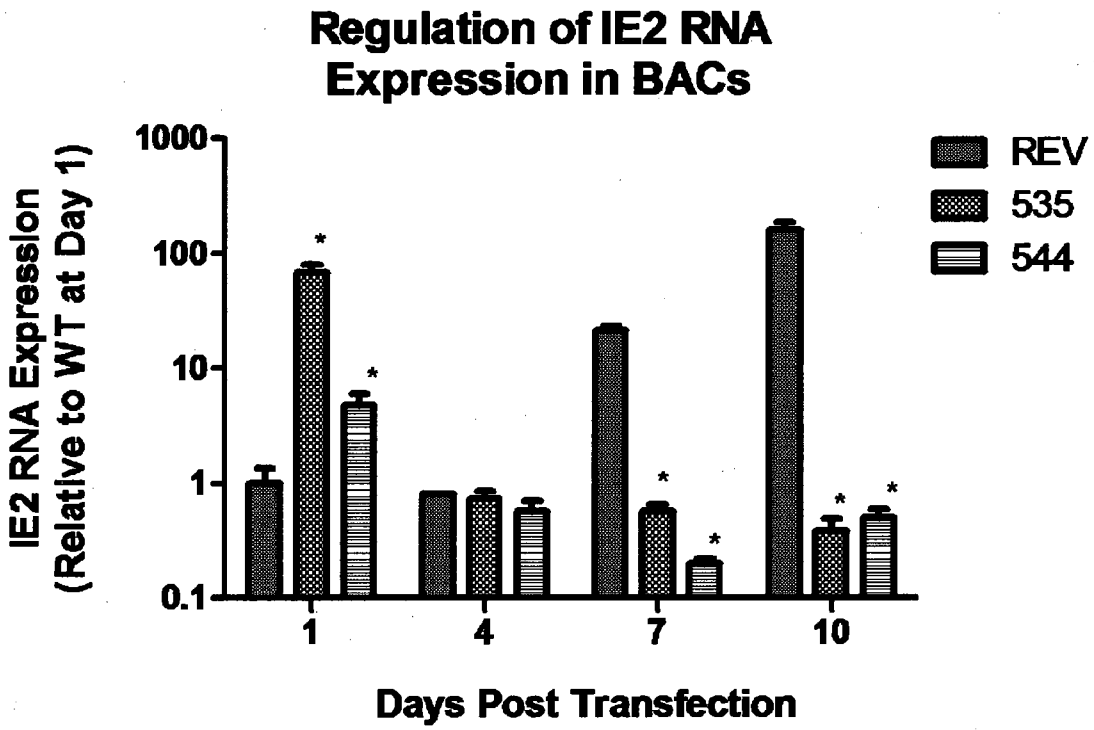


FIG. 22. Regulation of the MIE promoter in BAC transfected cells. Total RNAs were isolated from BAC transfected cells as described in Materials and Methods and analyzed by quantitative real-time RT-PCR with using primers specific for UL54 cDNA. Experiments were performed in duplicate with 2 independent experiments and mRNA expression levels were expressed as mean ± SD. An asterisk (*) indicates statistically significant difference ($P<0.05$), wildtype-Rev versus mutant IE2.

viral protein in the context of a viral infection is to generate mutations within the gene and determine the effects of those mutations on the phenotype of the mutated virus. Due to the critical role of IE2 in HCMV infection, it is difficult to generate recombinant viruses containing mutations in IE2. To circumvent this problem, we utilized HCMV Towne BAC constructs. These constructs allow mutagenesis of the viral genome and expression in human fibroblasts.

Our transient data suggests that mutations in the C-terminus of IE2 result in loss of IE2 transcriptional regulation of immediate early and early viral promoters. However, excluding the MIE gene products, these experiments are performed in the absence of other HCMV gene products which may influence promoter regulation in the presence of mutant or wildtype IE2 protein. The use of HCMV BACs will allow us to assess viral gene regulation in the context of the HCMV genome.

**Generation of HCMV BACs mutated in the IE2 gene**

To determine the effects of mutations in IE2 on transcriptional regulation and virus infection, we mutated HCMV recombinant BAC DNA. The generation of single amino acid substitutions in IE2 required a complex process of BAC mutagenesis by homologous recombination and confirmation of BAC DNA fidelity by PCR amplification, southern blot analysis and restriction enzyme digestion. These methods of confirmation were important to ensure that mutagenesis of the IE2 protein did not result in aberrant mutations within the HCMV genome. Based on these methods, we were able to successfully confirm the generation of mutations in IE2 at amino acids P535A and Y544A.

**HCMV Wildtype and WT-Rev BACs have a Similar Phenotype**

To ensure any sequence DNA introduced into the HCMV BAC DNA during BAC generation did not influence functions and/or properties of IE2, a wildtype-revertent BAC (WT-Rev) was generated, as previously described. Similar to WT BAC DNA, the revertant was able to successfully replicate in fibroblasts and produce infectious particles. To ensure there were no significant differences in viral DNA replication, we isolated DNA from WT or WT-Rev infected cells and analyzed for gB levels by real time PCR. A similar methodology was used to evaluate virus replication by analysis of gB levels in the supernatant at indicated times post infection. As shown in Fig. 17 and 18, we observed no noticeable differences in virus replication or DNA replication between WT and WT-Rev virus. These results confirm that the WT-Rev BAC behaves similar to WT and allowed us to continue experiments using WT-Rev BAC as a control.

**Mutations in IE2 Inhibit Virus Replication**

HCMV recombinant viruses were transfected into primary fibroblasts and monitored for plaque formation as an indicator of virus replication. BAC constructs containing mutations in IE2 were unable to form plaques even after 6 weeks compared to WT or WT-Rev virus where plaques became visible after approximately 6 days (data not shown). Levels of viral DNA were assessed at 1 day after transfection to ensure all recombinant BACs had similar transfection efficiency (Fig. 19). Real time PCR analysis demonstrated that there were no significant differences in transfection efficiency between wildtype revertant and mutant BACs. Thus, we can conclude that mutations in IE2 at amino acid 535 and 544 prevent efficient HCMV replication.

**UL112-113 Early Gene Activation is Impaired with mutations in IE2 within HCMV BACs**

With the absence of infectious viral particles from recombinant viruses with mutation in IE2, we next sought to determine the effects of these mutations on transcriptional regulation of early promoters. Our studies in transient assays suggested that the mutations generated in IE2 would prevent IE2-mediated transactivation of early gene promoters. To evaluate promoter activation, we isolated RNA at various times after BAC transfection, synthesized cDNA using random hexamers, and performed real time PCR analysis. When compared to wildtype revertant at all time points, minimal to no UL112-113 mRNA was detected with mutations in IE2 at both amino acid 535 and 544.

The UL112-113 locus encodes four phosphoproteins early and late in infection which are required for virus replication and serve as transactivators of the UL54 promoter (53). Studies show that the UL112-113 promoter is controlled by an essential ATF/CREB and a nonessential IE2 binding site (113). The ATF/CREB binding is required for activation early in infection (8 to 24 hours), whereas IE2 binding has only a moderate transactivation function (113). Late in infection (72 hours), ATF/CREB binding becomes less important and the mechanism of transcriptional regulation is unclear (113). Previous studies demonstrate that IE2 can interact with ATF/CREB family members (80). Since IE2 is unable to activate UL112-113 early in infection, it is possible that IE2 is required for recruitment of ATF/CREB to the UL112-113 promoter and that our mutations in IE2 disrupt this interaction. Future studies will assess mutated IE2 for its ability to bind ATF/CREB family members. Since UL112-113 is essential for replication (102), it is likely that loss of UL112-113 activation is a significant reason for

loss of virus replication for recombinant HCMV BACs.

**Regulation of UL54 Early Gene Activation by mutated in IE2 within HCMV BACs**

The UL54 promoter is regulated by a number of viral and cellular transcription factors (59, 61, 62, 81). Transient transfections demonstrate that mutations in IE2 at both amino acid 535 and 544 prevent UL54 activation (Fig. 8). Consistent with our *in vitro* analysis, mutagenesis of IE2 at amino acid 544 results in a significant reduction in UL54 promoter activation. However, in the context of the viral genome it appears that the mutation in IE2 at 535 does not impair UL54 activation. This discrepancy may be due to the presence of additional HCMV viral gene products which may be able to compensate for a functional defect in IE2 generated by a mutation at amino acid 535. In addition, it is possible that the mutation at amino acid 535 may disrupt a property of IE2 not essential for activation of UL54 during virus infection.

Previous studies suggest the UL54 promoter is primarily regulated by a SP1 binding site at early times in infection and an ATF-1 binding late in infection (59, 61, 62, 81). The IE2 protein is hypothesized to form a bridge between TBP and SP1 to stabilize the complex at the UL54 promoter. IE2 is also thought to interact with ATF-1 at the promoter to enhance transcription (61). In addition, IE2 interacts with HDAC2, a cellular repressor of the UL54 promoter, during virus infection to prevent UL54 repression (14). Due to the numerous roles IE2 may play in regulating the UL54 promoter, it is unclear which property may be leading to the phenotype demonstrated by the mutation at amino acid 544. Chapter 5 will begin addressing this question using transient assays.

In addition to direct effects of IE2 on UL54 activation, it is also important to note

that the UL112-113 gene products function to enhance UL54 activation in the presence of MIE gene products in transient assays. Thus, a decrease in UL54 activation may be partially due to loss of UL112-113 promoter activation, described in the previous section. However, activation of UL54 in the presence of a mutation in IE2 at amino acid 535, still occurs in the absence of UL112-113 activation. This suggests that expression of the UL112-113 gene products may not be required for activation of the UL54 promoter. In future studies, it will be important to investigate expression of other viral proteins, such as the IRS1/TRS1 gene products.

**Mutations is IE2 Disrupt Autorepression of the MIE promoter at Early Times in Infection**

IE2 is able to autorepress it own promoter, the MIE promoter. Consistent with transient studies, we observe differences in MIE promoter regulation with mutations in IE2 at 535 and 544. At 1 day following BAC transfections, the level of IE2 RNA present in the cell is 50 and 60-fold higher than wildtype for P535A and Y544A, respectively. However, these levels decrease to wildtype levels by 4 days and remain low at 7 and 10 days after transfection indicative of loss of virus replication. These results indicate that mutations in this region of IE2 alleviate repression of the MIE promoter early in infection. Since the MIE promoter is primarily down-regulated by binding of IE2 to the promoter, it will be interesting to see if IE2 binding is disrupted by mutations in IE2.

Since IE2 activates a number of viral and cellular promoters, it is not surprising that increased expression of the MIE gene products would lead to impaired transcriptional regulation and replication. Although the mechanism of impairment is

unclear, increased expression may lead to a dominant negative phenotype or sequester other factors important in transcriptional regulation. It is also possible that although levels of IE2 mRNA increase with mutations in IE2, protein expression levels may not. Evaluation of IE2 protein expression will be addressed in future studies. Lastly, even in the situation of increased IE2 protein levels, it is likely that mutated IE2 is nonfunctional and unable to activate essential genes, as demonstrated in UL54 and/or UL112-113 regulation.

A recent study evaluated the effects of a double mutation in the HCMV BAC at amino acid 535 and 537 (105). Although the study described in this dissertation utilized HCMV BACs containing single amino acids, it is possible that the mutation at P535A would display a phenotype similar to the A535/537 mutation. Consistent with our findings for the mutation at amino acid 544, the study evaluating A535/Y537 demonstrated that this mutated BAC was unable to activate transcriptional regulation of the UL112-113 and UL4 early genes. However, our mutation at 535 retained UL54 activation early in infection (Fig. 20). Since the double mutation at 535/537 and our mutation at amino acid 544 both disrupt Tyrosine residues, it may be possible that these amino acids have a functional role of IE2-mediated transcriptional regulation facilitated by biochemical properties or posttranslational modification of Tyrosine residues.

Contrary to our findings, previous reports indicate that mutations at the P535/Y537A residues in HCMV BACs autorepress the MIE promoter similar to wildtype. In our study, both the mutations at amino acid 535 and 544 resulted in a significant increase of IE2 mRNA one day after transfection, suggesting a loss of autorepression. One possible explanation for this discrepancy is a difference in the time

at which RNA expression was assessed between the two studies. Isolation of P535/537A RNA occurred at 48 hour following transfection and mutants in this study were isolated at 1 day post infection. However, due to the significant level of IE2 RNA present at 1 day following transfection (68.5-fold increase), it is unlikely that levels could be reduced to this extent after only one day. A more likely explanation for this difference is the type of mutations made in the recombinant BAC. For example, the Tyrosine 537 mutation may partially compensate for the Proline 535 mutation with respect to autorepression.

**Summary**

The use of recombinant HCMV BACs have provided a more biologically relevant analysis of functional properties of IE2 that may be important for transcriptional regulation. These studies support the hypothesis that IE2 regulates different early viral promoters by both independent and overlapping mechanisms. For example, a mutation in IE2 amino 544 can disrupt autoregulatory, and UL54 and UL112-113 transactivation functions of IE2, but a mutations at 535 disrupts only IE2 autoregulation and UL112-113 transactivation. To elucidate functional properties of IE2 required for transcriptional regulation of individual promoters, comprehensive functional assays, evaluating IE2 interactions with cellular and viral proteins, and interactions with DNA will be required both *in vitro* and in the context of a virus infection.

# CHAPTER V

## IE2 RECRUITMENT TO THE UL54 PROMOTER AND INTERACTIONS WITH TBP ARE IMPORTANT FOR UL54 ACTIVATION

### Introduction

The UL54 promoter is thought to be regulated by both viral and cellular transcription factors (59, 61, 62, 81). The IE2 protein regulates transcription through the following potential mechanism(s): binding directly to the promoter region, interactions with cellular factors at the promoter, or working in cooperation with other viral promoters such as IE1. At the UL54 promoter, studies have shown that IE2 is able to sequester cellular repressors, such as HDAC2 to prevent UL54 repression (98). *In vitro* studies also suggest that IE2 interacts with the both the SP1 and TBP cellular factors to enhance UL54 activation in transient assays (59, 62). Last, studies suggest IE2 may bridge SP1 and TBP at the UL54 promoter, stabilizing the complex and thereby enhancing transcription (59, 62). Many studies using mutagenesis to map functional domains of IE2 have neglected to investigate the functional properties of IE2 disrupted by mutagenesis in IE2.

In this dissertation, we have investigated the effects of mutations in IE2 on transcriptional regulation of viral genes in transient assays and in the context of the viral genome. Mutations in IE2 at amino acid 535 and 544 prevented activation of the UL54 and UL112-113 promoters in transient assays. In the context of the viral genome, the mutation at amino acid 544 inhibited activation of the UL54 and UL112-113 promoters, consistent with *in vitro* studies. In contrast, cells transfected with BACs containing a mutation at 535 did not activate UL112-113, but expressed similar levels of UL54

mRNA. In addition, both mutations resulted in a loss of autorepression of the MIE promoter in transient assays and in the context of a virus infection.

There are several potential methods used to assess functional properties of IE2 that may be disrupted by mutations. IE2 is able to regulate UL54 activation by interactions with cellular proteins and recruitment to the viral promoter. Co-immunoprecipitations (ChIP) assays and GST pulldowns are commonly used to assess protein-protein interactions. Gel shift assays are commonly used to evaluate the ability of proteins to bind DNA. However, gel shifts are only able to detect direct DNA binding. Chromatin immunoprecipitations can be used to evaluate recruitment of proteins to a promoter of interest and thereby assess direct and indirect protein-DNA interaction.

To investigate properties of IE2 important in transcriptional regulation the UL54 promoter, we investigated IE2 functional properties of the mutated proteins using the following two approaches. First, GST pulldown assays were used to evaluate IE2-protein interactions in the presence of IE2 mutations. Second, ChIP assays were performed to determine whether the mutated IE2 protein is able to be recruited to the UL54 promoter. These experiments are significant in defining functional properties of IE2 that may be required for transcriptional regulation.

## Materials and Methods

### Mammalian Cells

HeLa cells were obtained from ATCC and grown in Iscove's Medium with 10% fetal bovine serum (FBS, Invitrogen), 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma). HeLa cells were split at a ratio of 1:5 at 90% confluency.

**Plasmids Used for Evaluation of IE2 Functional Properties**

*GST Expression Plasmids*

To assess IE2 protein interaction with cellular transcription factors *in vitro*, GST fusion proteins were generated. For generation of a plasmid expressing TBP protein fused to a GST tag, the TBP sequence was PCR-amplified from primary human fibroblast cDNA using primers as described in Inada, *et al* (50). The sequence was TA subcloned into the pSC-A vector according to manufacturer's directions (Stratagene), and then cloned into pGEX-6P-1 using EcoRI digestion to generate pGEX-TBP. Plasmids were confirmed by sequencing using 3′ pGEX and 5′ pGEX sequencing primers (Amersham).

*In Vitro Transcription/Translation Plasmids*

The plasmid pBS-IE2 was received as a gift from Jay Nelson and used to express IE2 for *in vitro* transcription/translation assays. For correction of aberrant mutations in IE2, the SmaI-Bsu36I fragment from pSVHk was extracted and transferred into pBS-IE2 (as described in (5)).

*Real Time PCR Plasmids*

G6PD-TOPO plasmids were generated by PCR amplification and TOPO cloning as previously described. Similarly, pUL54-CAT-TOPO plasmids were generated as previously by PCR amplification of pUL54-CAT using the following forward and reverse primers amplifying the promoter region of pUL54-CAT: 5′-GCC GCT GCA GAA CCT CTT TC -3′ and 5′-CCT TAG CTC CTG AAA ATC TCC C-3′, respectively.

**Molecular Cloning and Generation of Plasmid DNA**

Molecular cloning techniques and generation of plasmid DNA were performed as previously described in Chapter 3.

**GST Pulldown Assays**

*In vitro transcription/translations (IVTT)*

Generation of recombinant IE2 and mutant IE2 proteins *in vitro* were performed using the TnT Coupled Reticulocyte Lysate System (Promega) with 30 μCi (Specific Activity >1000Ci/mM) of $^{35}$S-methionine (Amersham) according to manufacturer's directions using T7 RNA polymerase. Expression of the translated proteins and input quantification was tested using 5% of total reaction and was resolved on a 10-20% Gradient Tris-HCL Ready Gel (Bio-Rad) after boiling proteins for 5 minutes in the presence of 1X SDS-PAGE Loading Dye (5% glycerol, 1% SDS, 80 mM Tris-HCl, pH 6.8, Bromophenol blue) and 2 μl of ß-mercaptoethanol . After electrophoresis the gel was dried at 85°C for 1 hour and exposed to phosphor screen. The image was visualized using a Typhoon 9410 Variable Mode Imager. Protein quantification was performed using ImageQuant TL Software. The use of radioactive materials in this dissertation was approved by the Eastern Virginia Medical School Radiation Safety Committee.

*Propagation and Purification of GST and GST Fusion Proteins.*

Overnight cultures of *E. coli* BL21 containing one of the GST fusion constructs were diluted 1:20 in LB with ampicillin and grown to an $OD_{600}$=0.6. IPTG was added to a final concentration of 0.1 mM then incubated at 30°C for 3 hours with shaking.

Bacteria were pelleted and resuspended in 25 mls of Buffer A (20 mM HEPES, pH 7.2, 100 mM KCl, 0.2 mM EDTA, 20% Glycerol, 1% Triton X-100). Bacteria were sonicated four times for 30 seconds on ice at 10 Watts using a Fisher Scientific Sonic Dismembrator Model 100 (1/8 inch tip), then centrifuged at 19,800 X *g* for 10 minutes.

GST fusion proteins were precipitated using Glutathione sepharose (GE Healthcare) according to manufacturer's instructions. Briefly, 400 µl of Glutathione Sepharose was incubated with purified GST protein for 2 hours at 4°C (with rotation). The GST protein complexed to glutathione sepharose was washed five times in NETN Buffer (0.5% NP-40, 20 mM Tris, pH 8.0, 100 mM sodium chloride, 1 mM EDTA) following centrifugation for 5 minutes at 500 X *g*. The pellet was resuspended in 150 µl of NETN and an aliquot was resolved on a 10-20% Gradient Tris-HCL Ready Gel (Bio-Rad) by brief centrifugation, following by boiling for 5 minutes in the presence of SDS-PAGE Loading Dye and 2 µl of ß-mercaptoethanol. The gel was stained overnight in SYPRO Ruby Protein Gel Stain (Invitrogen) diluted 1:1 in water. The following day, the gel was visualized on the Kodak Gel Logic 200 Imaging System. Protein quantification was performed using ImageQuant TL Software.

*GST Pulldown Assays.*

GST protein was first equilibrated with 500 µl Binding Buffer (25 mM HEPES, pH 7.5, 12.5 mM magnesium chloride, 20% glycerol, 0.1% NP-40, 150 mM potassium chloride, 0.15mg/ml bovine serum albumin, 1 mM DTT) and spun at 500 X *g* for 5 minutes at 4°C. The GST pellet was then resuspended in 200 µl fresh binding buffer. GST pulldowns were performed by incubation of the purified GST fusion

protein/Glutathione Sepharose complexes with 20 µl of radiolabeled protein for 2 hours at 4°C (with rotation). Following incubations, complexes were purified by centrifugation at 500 X *g* at 4°C for 5 minutes. Complexes were washed 5 additional times in 1 ml of NETN and spun at 500 X *g* at 4°C for 5 minutes. Protein complexes were denatured by boiling and resolved by SDS-PAGE on 12.5% acrylamide gels. After electrophoresis the gel was dried at 85°C for 1 hour and exposed to a phosphor screen. Image was visualized using a Typhoon 9410 Variable Mode Imager. Protein quantification was performed using ImageQuant TL Software.

**Transfection of HeLa Cells for Chromatin Immunoprecipitations**

HeLa cells (1.5 X $10^6$ cells per 150 mm dish) were transfected by the calcium phosphate technique (41). Briefly, each plate was transfected with 80 µg of total DNA. For each sample, 50 µl of 2.5M Calcium Chloride ($CaCl_2$) was added to a 6 ml tube, followed by 1500 µl of 2X HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4$, 12 mM dextrose, 50 mM HEPES, pH 7.5). This reaction was incubated 30 minutes at room temperature then added to cells dropwise. Media was changed 20 hour after transfection and cells were harvested for chromatin immunoprecipitations (ChIP) assays 24 hours after the media was changed.

**Chromatin Immunoprecipitations (ChIP)**

*Harvesting cells and Crosslinking for ChIPs*

In order to assess the effects of mutations in IE2 on recruitment of IE2 or RNA polymerse II to the UL54 promoter, HeLa cells were transfected with IE2 expression

constructs in the pMCRS86 vector and pUL54-CAT by calcium phosphate transfections. Transfected cells were crosslinked with 1% formaldehyde for 10 min at 37°C. Media was removed and cells were washed twice in cold 1X PBS containing Complete EDTA-free protease inhibitors (Roche). Cells were scraped into a 2.0 ml eppendorf tube and centrifuged at 500 X *g* for 4 minutes. The cell pellet was resuspended in 800 µl of SDS Lysis Buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1) containing protease inhibitors and incubated on ice for 10 minutes. Cells were either sonicated immediately or stored at -80°C.

*Fragmentation of total DNA by sonication of HeLa Cells*

To assess protein recruitment at individual promoters, DNA was fragmented by sonication to a size of 500-1000 basepairs. Using a Fisher Scientific Sonic Dismembrator Model 100 (1/8 inch tip), cells were sonicated on ice for 12 pulses at 30 seconds/pulse and 10 Watts. Cell lysates were centrifuge at a maximum speed for 10 minutes. The supernatant was diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl) containing protease inhibitors and used immediately or stored at -80°C.

To confirm DNA fragment sizes following sonication, a 400 µl aliquot was incubated with 20 µl of 5M NaCl at 65°C overnight. The next day, DNA was purified using Qiagen's PCR purification kit according to the manufacturer's instructions and electrophoresed on a 1.2% agarose stained in ethidium bromide as previously described.

*Immunoprecipitation of Protein-DNA Complexes*

Monoclonal antibodies against IE2 (MAB810) and RNA polymerase II (05-623, Millipore) were used to immunoprecipitate chromatin. To subtract background levels, mouse IgG2a and mouse IgG (Sigma) isotype matched controls, respectively, were included with each experiment. ChIP assays were performed by incubating a 400 µl aliquot of sonicated DNA in ChIP dilution buffer with 5 µg of the specified antibody or isotype matched control antibody overnight at 4°C (with rotation). An additional 400 µl of the sonicated DNA diluted in ChIP dilution buffer was stored at -80°C for quantification of the amount of input DNA present in each experiment. Following the overnight incubation, immunoprecipitation reactions were incubated with 60 µl of Salmon Sperm DNA/Protein A Agarose Slurry for one hour at 4°C with rotation. Agarose was pelleted by centrifugation at 500 X *g* at 4°C for 1 minute. The supernatant was removed and the agarose was washed by rotation for 5 minutes at 4°C with 1 ml of each of the following buffers in the order listed:

A) Low Salt Buffer (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), one wash

B) High Salt Buffer (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), one wash

C) LiCl Buffer (0.25M LiCl,1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1), one wash

D) 1X TE (10mM Tris-HCl, 1mM EDTA,pH 8.0), two washes

Protein-DNA complexes were then eluted from agarose beads by the addition of 250 µl

of elution buffer (1%SDS, 0.1M $NaHCO_3$), vortexed for 15 seconds then rotated for 15 minutes at room temperature. The reaction was centrifuged for 1 minute at 500 X *g* and the eluate transferred to a new tube. The remaining agarose beads were washed once more in 250 µl of elution buffer and eluates were combined. To reverse crosslinks, protein-DNA complexes were incubated with 25 µl of 5M NaCl overnight at 65C. DNA was isolated as previously described.

*Real-Time PCR*

DNA levels were assayed by Real time PCR using iQ SYBR green supermix (Invitrogen) in a total of 25 µl, containing 12.5 µl of mastermix, 300 nM of specific primers, and 1-5 µl of cDNA. PCR Primers, pUL54-CAT forward and reverse (described in *Real Time PCR Plasmids*), were designed to detect the promoter region fragment in the ppol-CAT plasmid as described above. Real time PCR reactions were performed with a Biorad iCycler system using the standard curve method of quantification. Each PCR was performed as follows: initial uracil DNA glycosylase decontamination at 50°C for 2 min, a 2 min hot-start denaturation at 95°C, and 39 amplification cycles (15 s at 95°C, 30 s at 58.7°C). Melting Curve analysis was performed to ensure the specificity of PCR product and lack of primer-dimer artifacts. The melting temperature of the final double-stranded DNA products was determined by gradual heating from 55°C to 95°C for 80 cycles with a 0.5°C increase after each cycle.

*Quantification of DNA and RNA by Real Time PCR*

Serial dilutions of the plasmid, pUL54-CAT-TOPO, from $10^9$ to $10^3$ copies were

used for determination of the PCR efficiency for each primer set. Each reaction was performed in duplicate and error bars reflect standard deviations within each experiment. Significance was determined by a P-value < 0.05 using a two-tailed Student's T-test. The ratio of target gene mRNA expression relative to the internal control was calculated as described in (106) using the following equation (where E is the efficiency of real time PCR amplification for the specified gene):

$$\text{Relative Expression} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (wildtype – mutant)}}}{(E_{\text{reference}})^{\Delta Ct \text{ reference (wildtype – mutant)}}}$$

In this equation, target represents the promoter region of interest (UL54-CAT) and reference represents the internal reference gene or calibrator (Input).

## Results

### Mutations in IE2 inhibit in IE2 association with TBP *in vitro*

IE2 is able to regulate viral gene expression by direct or indirect binding to the viral promoters or interactions with cellular and viral factors at the promoter (115). Previous studies suggest that IE2 may be recruited to the UL54 promoter due to interactions with cellular factors, such as TBP (59, 62). To determine if our mutations in the C-terminus of IE2 disrupt the interaction of IE2 with TBP, we performed GST pulldown assays. In order to synthesize radiolabeled wildtype and mutated IE2 protein we used the TnT Rabbit Reticulocyte Protein Labeling Kit (Promega) to perform *in vitro* transcription and translation. The IE2 wildtype and mutated plasmids containing the IE2 cDNA under the control of the T7 bacteriophage promoter were used as DNA templates

for coupled *in vitro* transcription and translation in the presence of $^{35}$S-labeled methionine. Successful labeling and expression of IE2 wildtype or mutant protein was confirmed prior to GST pulldown assays (Fig. 23A). As a positive control, a luciferase plamid was *in vitro* transcribed and translated to generate a monomeric protein of 61 kDa. A negative, mock control was also included in which no DNA template was added to the reaction. GST and GST-TBP proteins were generated by expression in BL21 bacterial cells and purified using glutathione sepharose beads. GST proteins were separated on a 10-12.5% Gradient SDS-PAGE gel to confirm protein expression. As shown in Fig. 23B, we were able to successful generate GST and GST-labeled TBP proteins. Lower molecular weight proteins were expressed which are most likely GST-TBP breakdown products.

GST pulldown assays were performed by incubating the IE2 protein with equal concentrations of GST or GST-TBP protein and purification of protein complexes by glutathione sepharose beads (Fig. 24). Consistent with previous studies (17), our experiments show that wildtype IE2 protein is able to interact with TBP (Fig. 24). The IE2 mutated protein, Proline 535, also associates with TBP levels similar to wildtype (Fig. 24). In contrast, the mutation at Tyrosine 544 showed reduced binding with TBP, demonstrating a potential loss in IE2 interaction with TBP (Fig. 24). BACs containing IE2 with a mutation at amino acid 544 were unable to activate the UL54 promoter in BAC transfected cells, when compared to WT-Rev and recombinant BAC containing mutations amino 535, suggesting that interactions with TBP may facilitate this difference in regulation (Fig. 20).

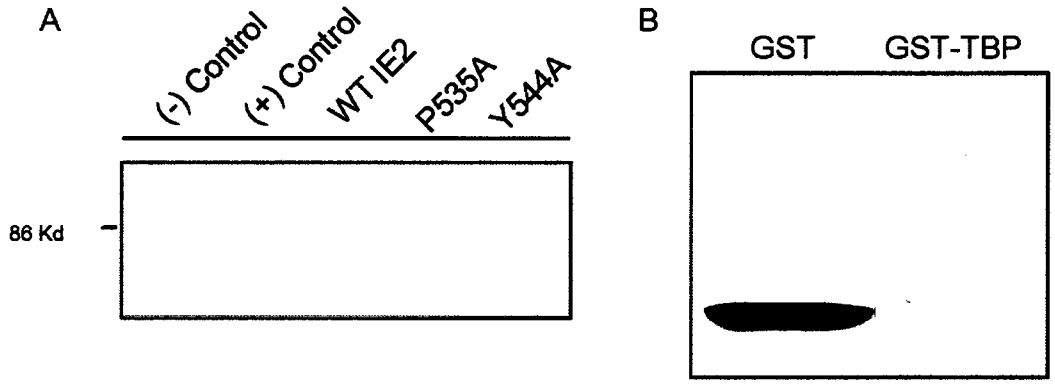


FIG. 23. Generation of Protein for GST Pulldown Assays. (A) Wildtype and mutated $^{35}S$ labeled IE2 protein was generated by *in vitro* transcription/translation reactions. Protein expression of translated protein and input quantification was tested using 5% of the total reaction and was resolved on a 10-20% Gradient Tris-HCL Ready Gel. A positive and negative control were included. (B) GST fusion proteins were precipitated using Glutathione Sepharose (GE Healthcare) according to manufacturer's instructions. A 10% aliquot was resolved on a 10-20% Gradient Tris-HCL Ready Gel and stained overnight in SYPRO Ruby Protein Gel Stain before visualization on the Kodak Gel Logic 200 Imaging System.

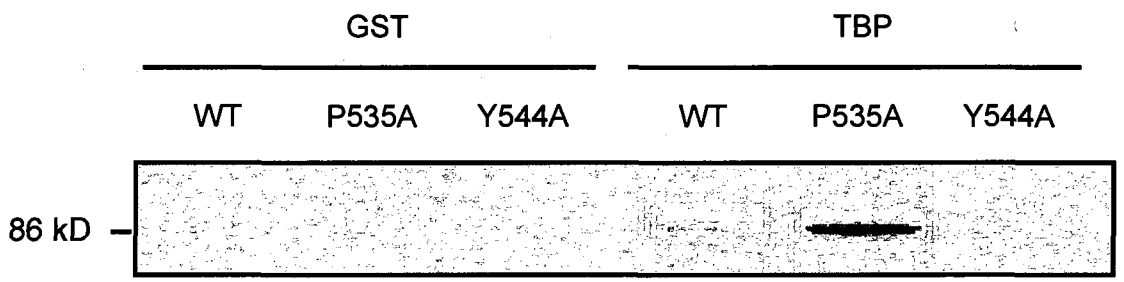


FIG. 24. Mutation in IE2 Inhibit Interactions with TBP. Wildtype and mutated $^{35}S$ labeled IE2 protein was incubated with bacterially expressed GST or GST-TBP. GST complexes were purified by glutathione sepharose beads. Input amounts are shown on Fig. 23. Following extensive washings, immunoprecipitated proteins were eluted from beads, separated on a 12.5% SDS-PAGE and visualized on a Typhoon imager.

**Mutations in IE2 prevent IE2 recruitment to the UL54 Promoter**

Studies have shown that IE2 expression is important for efficient activation of the UL54 promoter (59). IE2 has also been shown to interact with several transcription factors and members of the basal transcription machinery (43, 44, 57, 58). To date, studies have not looked at recruitment of IE2 to the UL54 promoter in transiently transfected cells. To determine if IE2 is present at the UL54 promoter and if mutations in IE2 alter recruitment to the promoter in transiently transfected cells, HeLa cells were transfected with the UL54-CAT plasmid and mutant IE2 cDNA expression vectors. HeLa cells were used for transfections instead of primary fibroblasts because they have an increased transfection efficiency than primary fibroblasts. Cells were cross-linked and harvested for ChIP assays. Following immunoprecipitations with the appropriate antibodies, cells were washed intensively, cross-links were reversed, and isolated DNA was assessed by real time PCR analysis. An isotype matched control antibody was included with each experiment. For each antibody tested, background levels of isotype matched control antibodies were below the level of detection by real time PCR (data not shown).

For these experiments, we continued to assess the mutations at amino acids 535 and 544 to determine if mutations in this region affect the transcriptional regulatory properties of IE2. ChIP assays with the IE2 antibody demonstrate that the mutation at amino 535 and 544 resulted in a significant loss of IE2 protein recruitment to the UL54 promoter compared to the wildtype protein (Fig. 25).

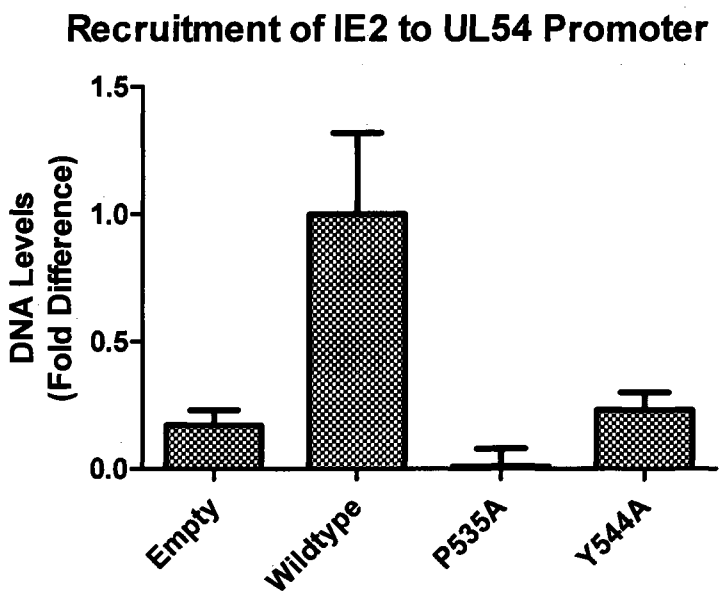


FIG. 25. Recruitment of Mutant IE2 Protein to the UL54 promoter. HeLa cells were transfected with 40 μg UL54-CAT plasmid and 40 μg of parental or mutant IE2 plasmid. After 48 hours, cells were cross-linked and chromatin immunoprecipitations were performed using the indicated antibody. Primers to UL54-CAT were used in Real Time PCR analysis to detect the UL54 promoter as described in Materials and Methods. Background levels of an isotype match control were below the level of detection. Real time analysis was performed in duplicate and DNA levels were expressed as mean ± SD.

**Recruitment of Basal Transcription Machinery to the UL54 Promoter**

Since our previous studies have shown that mutated IE2 is unable to activate the UL54 promoter, we sought to determine whether the basal transcription machinery was recruited to the UL54 promoter in the presence of mutated IE2 using ChIP assays. As shown in Fig. 26, neither mutation in IE2 had an effect on RNA polymerase II recruitment. These results suggest that IE2 recruitment to the UL54 may not be important for recruitment of the RNA polymerase II and IE2 may therefore function downstream of RNA polymerase II recruitment.

## Discussion

IE2 possesses several functional properties that facilitate transcriptional regulation of viral promoters during infection (3). First, IE2 has the potential to bind directly to DNA through specific sites on promoter regions, such as the *cis* repression sequence (2, 18, 48, 121, 122). Second, IE2 is able to interact with members of the basal transcription machinery such as TBP or RNA polymerase II (37, 48, 76, 90, 99, 124, 151). Third, IE2 is able to interact with cellular transcription factors at the promoter, such as SP1 or ATF (17, 22, 58, 127). Last, IE2 is able to work in cooperation with other viral proteins such as IE1 or the pp71 tegument protein (4, 12, 21, 75, 84, 134, 137). Research is still unclear regarding which of the aforementioned properties are required for regulation of individual promoters.

The UL54 promoter encodes the viral DNA polymerase and is essential for virus replication (102). However, our understanding of regulation of this promoter in the context of a virus infection is limited. Transient assays suggest the IR1 element is

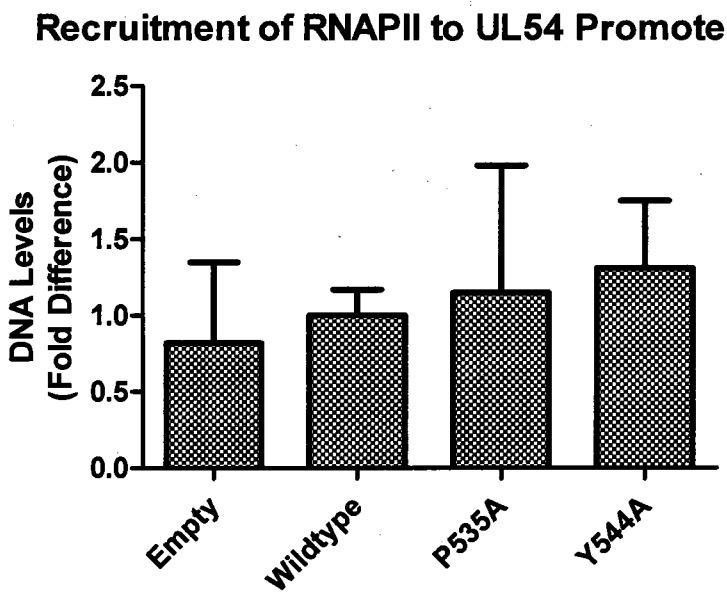


FIG. 26. Recruitment of RNA Polymerase II to the UL54 promoter *in vitro*. HeLa cells were transfected with 80 μg of IE2 or mutant IE2 plasmid. After 48 hours, cells were cross-linked and chromatin immunoprecipitations were performed using the indicated antibody. Primers to UL54-CAT were used in Real Time PCR analysis to detect the UL54 promoter as described in Materials and Methods. Background levels of an isotype match control were below the level of detection. Real time analysis was performed in duplicate and DNA levels were expressed as mean ± SD.

required for UL54 activation by the MIE and UL112-113 gene products (59). This suggests that these viral proteins may interact with or form a complex with the SP1 cellular transcription factor found to bind to the IR1 binding site. Mutagenesis of this region in HCMV recombinant virus indicates that this region of UL54 is critical at early (between 15-24 hours post infection), but not late times (72-96 hours post infection), in infection (59). Studies using a recombinant virus mutated in the putative ATF-1 binding site reveals a role for ATF in UL54 promoter activation at both early late times in infection (61). Mutagenesis is this region reduced UL54 activation significantly at both early and late times in infection.

IE2 interactions with cellular transcription factors may also facilitate transactivation of the UL54 promoter. IE2 is able to bind the TBP and RNA polymerase II (43, 44, 57, 58). IE2 is also able to interact with the SP1 and ATF-1 general transcription factors. Last, IE2 binds histone modifiers such as HDAC2 to regulate transcription (98) . However, these interactions have not been demonstrated at the UL54 promoter either during transient transfections or viral infections.

**IE2-TBP Protein Interaction Inhibited by Mutation at Tyrosine 544 in IE2**

One mechanism IE2 utilizes to regulate early gene promoter is through interactions with members of the basal transcription machinery. At the UL54 promoter, transient transfection studies suggest IE2 acts as a bridge between the SP1 transcription factor and TBP. To determine if mutations in IE2 disrupt interactions with TBP, GST-pulldowns were performed using $^{35}$S-labeled IE2 protein. As shown in Fig. 24, mutations in IE2 at amino acid 544 results in a loss of this interaction at the UL54 promoter. The

mutation at amino acid 535 did not disrupt this interaction with TBP *in vitro*.

Although the TBP interaction domain of IE2, does not map to this amino acid 544 of IE2, studies suggest that dimerization of IE2 may be important for IE2-TBP interactions (127). Since amino acid 544 does overlap the dimerization domain of IE2, it is possible that this mutation may inhibit IE2 dimerization. Future studies will assess the ability of mutated IE2 to dimerize.

It is interesting that although the mutations in IE2 at 535 and 544 have similar phenotypes of transcriptional regulation, the functional properties of transcriptional regulation differ. These results confirm the hypothesis that the IE2 protein may have more than one property required for transcriptional regulation of the UL54 promoter. In addition to regulating UL54 expression through a direct interaction with TBP, studies reveal that IE2 may be able to bind cellular transcription factors, such as SP1 or ATF, interact with viral proteins, and/or sequester repressors of the UL54 promoter, such as HDAC2 (98). It is possible that the mutations at 535 and 544 may disrupt one or more of these mechanisms. Future studies will assess additional IE2-protein interaction which may be disrupted by these mutations. In addition, the functional IE2 properties have been addressed *in vitro* within this study. Therefore, it is possible that other viral proteins present during HCMV infection may influence the ability of IE2 to interact with proteins at the promoter. To more closely uncover functional properties required for regulation during infection, future studies should be performed using HCMV BACs.

**Mutations in IE2 Disrupt IE2 recruitment to the UL54 Promoter**

Previous studies indicate that the MIE proteins enhance transcription of the UL54

promoter during transient transfections (134). However, it is unclear whether this regulation is mediated by IE2 recruitment to the UL54 promoter or through IE2-mediated regulation of cellular factors. ChIP assays demonstrate that wildtype IE2 is recruited to the UL54 promoter through either direct or indirect interactions at the promoter. However, mutagenesis of IE2 at amino acids Pro 535 and Tyr 544 prevent recruitment of IE2 to the UL54 promoter. These results are consistent with data demonstrating that a double mutation at Pro 535 and Tyr 537 prevents recruitment of IE2 to the UL4 early promoter. It is possible that these mutations disrupt the ability of IE2 to interact with another viral or cellular factor required for promoter regulation.

It is difficult to draw a definitive conclusion regarding the mutation at Proline 535 due to the differences in transcriptional regulation in transient transfections and in the context of the genome. While the loss of IE2 recruitment is consistent with inhibition of UL54 activation *in vitro*, this observation is not seen in the context of the viral infection. One possible explanation is that other viral proteins may be able to compensate for an inability of IE2 to bind the UL54 promoter in the context of a viral infection. In contrast, the mutation at 544 may have a more lethal phenotype which cannot be compensated for. Future studies of IE2 recruitment in the context of a viral infection will help confirm the functional properties of IE2 in transcriptional regulation during virus infection

**RNA polymerase II recruitment to the UL54 is Independent on IE2 Recruitment**

We have shown in Chapter 3 and 4 that mutations in IE2 disrupt transcriptional activation of the UL54 promoter in transient assays and/or in the context of the viral genome. Due to this loss of UL54 transactivation, we hypothesized that RNA

polymerase II would not be recruited to the UL54 promoter in the presence of mutated IE2. In addition, previous studies suggest that the IE2 protein may stabilize and/or recruitment TBP and RNA polymerase II to the UL54 promoter (59, 62). Interestingly, mutations in the IE2 protein did not alter recruitment of the RNA polymerase II to the promoter region. In addition, RNA polymerase II is recruited even in the absence of IE2 expression. Although these results are paradoxical, it is not surprising since the double mutation described previously did not alter recruitment of TBP or RNA polymerase II to the early gene promoters tested (105). Similarly, our data suggests that IE2 recruitment to the UL54 promoter is not required for RNA polymerase II recruitment. However, since our data demonstrates that these mutations do alter transcriptional activation of IE2, it is likely that mutations at 535 and 544 disrupt other potential mechanism(s) of IE2-mediated transactivation, such as sequestering repressors of UL54 or altering transcriptional regulation following the formation of the pre-initiation complex.

**Summary**

Our analysis of mutations in IE2 at amino acid 535 and 544 has begun narrowing down functional properties of IE2 required for transcriptional regulation of the UL54 promoter. For example, the mutation at amino acid 544, but not 535, impairs IE2-TBP interactions *in vitro*. Similarly, HCMV BACs with mutations in IE2 at amino acid 544, but not 535, impair UL54 activation. These studies suggest that IE2-TBP interaction may be important in UL54 transcriptional regulation in the context of HCMV infection. In addition, mutations at both 535 and 544 disrupt IE2 recruitment to the UL54 promoter, suggesting IE2 recruitment is not important for UL54 promoter activation. This

hypothesis is further supported by the fact that RNA polymerase II is recruited to the UL54 promoter regardless of whether IE2 is recruited. However, additional studies will be required to pinpoint the exact functional mechanism(s) disrupted by mutations in IE2 to begin identifying functional properties required for promoter regulation. More specifically, future studies should assess the ability of mutated IE2 protein to interact with viral and cellular factors, as well as DNA, *in vitro* and in the context of a viral infection.

# CHAPTER VI

## CONCLUSIONS

The IE2 protein plays a key role in transcriptional regulation of early gene promoters by functioning to recruit cellular and viral transcription factors as well as members of the basal transcription machinery. More importantly, current research suggests that individual promoters are regulated by distinct transcriptional regulatory properties. The studies described in this dissertation have three primary aims (1) characterization of IE2 functional domains involved in transcriptional regulation during transient transfections (2) characterization of IE2 functional domains important in viral replication and transcriptional regulation during infection, and (3) identification of IE2 transcriptional regulatory properties which may be inhibited in IE2 mutations resulting in impaired virus replication and transcriptional regulation.

In this study, we have shown that mutations in the C-terminus of IE2, between amino acid 535 and 545, prevent transcriptional regulation of essential viral promoters (UL54, UL122-123, and UL112-113) in transient transfections. In addition, these mutations alter IE2 mRNA or protein expression. Previous studies have shown that biological processes characterized *in vitro* may not be reproducible *in vivo*. In addition, it is difficult to generate viable virus with a mutation in an essential gene. To overcome this obstacle and assess the effects of the mutations in IE2 on virus replication, recombinant viruses were generated using bacterial artificial chromosomes (BAC). Mutations in amino acids 535 and 544 resulted in nonviable recombinant virus when compared to wildtype. This is most likely due to an inability to differentially activate

viral promoters essential for virus replication, including UL112-113 and/or UL54, as demonstrated using recombinant HCMV BACs. Mutations at Proline 535 or Tyrosine 544 impaired UL112-113 and/or UL54 early gene activation in the context of the viral genome. Thus, we are able to conclude that mutations in the C-terminus of IE2 result in loss of early gene activation of essential genes and prevent virus replication.

Both of our mutations in IE2 of HCMV BACs resulted in a loss of MIE repression. However, since we saw differential regulation of early gene promoters with different mutations in IE2, our results support the hypothesis that both independent and overlapping IE2 functional domains of IE2 are required for regulation of individual viral promoter. For example, the autoregulatory domain of IE2 may overlap a functional domain of IE2 important for activation of the UL112-113 promoter, but not the UL54 promoter.

Potential mechanism(s) by which IE2 is able to regulate viral promoters include (1) interactions with transcription factors, (2) interactions with members of the basal transcription machinery, (3) binding directly to DNA, (4) functioning in cooperation with viral proteins, and/or (5) sequestering of cellular repressors. In this study, we begin narrowing down functional properties of IE2 important in UL54 activation in transient assays. We demonstrate that IE2 is recruited to the UL54 promoter during transient transfection and suggest IE2 recruitment may not be required for RNA polymerase II recruitment. We also show TBP-IE2 interactions are disrupted by mutations in IE2 suggesting another mechanism of transcriptional regulation that may be required for UL54 promoter activation.

Future studies will expand of analysis of IE2 functional properties and identify

properties of IE2 impaired by IE2 mutagenesis both *in vitro* and during virus infection. Specifically, future studies will begin to assess additional functional properties of IE2, such as interactions with histone modifying enzymes and evaluate these properties in the context of the virus in order to gain a more biologically relevant assessment. Furthermore, to gain a better understanding of which functional properties of IE2 are required for transcriptional regulation of early gene promoters a more global assess of gene regulation would be used. Microarray analysis of HCMV gene expression would allow us to assess which functional properties of IE2 are required for transcriptional regulation of individual promoters. Last, further analysis of other HCMV mutated BACs, such as a Threonine 541 mutation in IE2, will further strengthen the hypothesis that IE2 differentially regulates individual promoters based on independent functional properties.

Overall, these result concluded in the dissertation will be significant in gaining a better understanding of HCMV replication and regulation of viral genes essential for virus replication. This will be important in the development of novel therapies for CMV treatment and prevention.

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