UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Functional Roles of the Human Cytomegalovirus IE2 86 kDa Protein in HCMV-Infected Cells

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

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2007

The dissertation of Elizabeth A. White is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2007

## DEDICATION

For the people who have mattered the most: Mom, Dad, Catherine, and Tony.
You have all my love and gratitude.

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## FIELD OF STUDY

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**ABSTRACT OF THE DISSERTATION**

Functional Roles of the Human Cytomegalovirus IE2 86 kDa Protein

in HCMV-Infected Cells

by

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Doctor of Philosophy in Biology

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Professor Deborah H. Spector, Chair

The human cytomegalovirus (HCMV) IE2 86 kDa protein is an essential viral regulatory factor that has been shown in transient transfection and in vitro assays to transactivate early viral promoters and to interact with many viral and cellular proteins. To understand the functions provided by this protein in the HCMV-infected cell, we have constructed and characterized a family of recombinant viruses

containing changes to the IE2 gene and other parts of the HCMV major immediate early (IE) region. The study of these HCMV mutants has allowed us to confirm that several of the predicted functions of IE2 86 are relevant in the virus-infected cell and has identified new functions for the protein.

Introducing small deletions into the C-terminus of IE2 86 resulted in viruses that do not support early gene expression, replicate, or repress the major IE promoter. Surprisingly, these constructs also mediated up-regulation of several delayed early and late viral genes, suggesting that IE2 functions are required for the proper regulation of late viral gene expression. By constructing several viruses that do not express the IE2 40 and IE2 60 kDa proteins, which are present in infected cells at late times post infection, we continued to investigate the regulation of late gene expression by IE2. Again, we found that the IE2 40 and IE2 60 proteins are required for proper late gene expression and for repression of the major IE promoter.

Interactions between the virus and the host cell are also crucial for proper HCMV replication, and a recombinant virus with a deletion in exon 3 of the major IE region demonstrated that IE2 86 is important not only for transactivation of viral early promoters, but also for dysregulation of the host cell cycle. Cells infected with this recombinant virus fail to exhibit diffuse PML staining at early times post infection and express less cyclin E protein and produce less infectious virus than do wild-type virus-infected controls. The experiments described in this dissertation demonstrate that IE2 86 is a multifunctional protein that contributes many functions, several of which were not previously identified, to HCMV replication in infected cells.

# CHAPTER 1

# INTRODUCTION

## HUMAN CYTOMEGALOVIRUS

Human cytomegalovirus (HCMV), a betaherpesvirus, is a prevalent human pathogen. Betaherpesviruses comprise a subset of the herpesvirus family and share the large, double stranded DNA genome and enveloped particle common to the herpesviruses, but are distinguished by slow replicative cycles and a restricted host range. In contrast, alphaherpesviruses, including Herpes simplex virus (HSV) and Varicella-zoster virus (VZV), have a shorter life cycle and a less restricted host range. The third group, gammaherpesviruses, includes Epstein-Barr virus, and members of this subfamily replicate slowly and tend to lytically or latently infect lymphoid cells (180). A series of observations in the first half of the 20$^{th}$ century led to the realization that the appearance of cytomegalic cells, those with characteristic inclusions isolated from one of several tissues, resulted from a viral infection (161). The causative viral agent, human cytomegalovirus, was independently isolated by three groups (64, 183, 199), reviewed in (229) in the mid-1950's.

In the human host, HCMV is able to infect a wide range of tissues in organs including brain, liver, lung, salivary gland, and many more. HCMV infection is medically relevant primarily in two populations: congenitally infected infants and those with compromised immune systems, and infection with the virus can have

severe consequences for members of both populations. HCMV is the most prevalent viral cause of birth defects, and symptoms of congenital infection can include hearing loss, mental retardation, and even death of the fetus. HCMV infection of the developing fetus is most likely to occur if the mother experiences primary infection during pregnancy, and in such cases the rate of transmission to the fetus is between 20 to 40%. Between 0.5 to 2.4% of infants are congenitally infected with HCMV, and 10 to 15% of these display symptoms of infection (25, 161).

The structure of HCMV particles is at least partly understood. The HCMV DNA genome is contained in a protein capsid, and the capsid is in turn surrounded by a more nebulous collection of proteins referred to as the tegument or matrix. Five proteins make up HCMV capsids, and these are the major capsid protein (MCP, UL86), minor capsid protein (mCP, UL85), minor capsid binding protein (mCBP, UL46), smallest capsid protein (SCP, UL48-49), and fragments of the assembly protein (UL80) (32, 81). The structure of the capsid, both alone and in combination with tegument proteins, has been determined by electron cryomicroscopy (40, 52). In a proteomics study, purified virus particles were subjected to tandem mass spectrometry analysis to identify all proteins associated with the virus particle. This work identified 14 proteins as components of the tegument, although several uncharacterized proteins present in virions could also be part of the tegument (224). The phosphoprotein product of the UL83 gene, pp65, is the most abundant component of the tegument, and other tegument proteins relevant to the dissertation are pp28 (encoded by UL99) and pp71 (encoded by UL82). The virus particle is surrounded by a host cell-derived lipid envelope, and several virus-encoded glycoproteins are

embedded in the envelope. Some of these are required for virus growth in cultured cells, and the most abundant glycoprotein in the virion is gM (32, 224). The viral genome contained in this particle is a linear, double-stranded DNA molecule of approximately 235 kbp (147). There are two main segments of the viral genome, the unique long (UL) and unique short (US) regions, and each of these is flanked by shorter DNA segments that are inverted repeating sequences. These are denoted the internal or terminal regions flanking the long (IRL/TRL) or short (IRS/TRS) genome segments. Viral genes are named according to their location in the genome.

HCMV replication begins with the entry of the virus into the host cell, uncoating of the virus particle, and translocation of the viral genome to the nucleus (Figure 1.1). Then, the ordered and tightly controlled process of viral gene expression can begin (reviewed in (73). As for other herpesviruses, HCMV gene expression follows a temporally defined set of steps leading to viral DNA replication and to the assembly and release of infectious virus. The first event in this cascade is the expression of the viral immediate-early (IE) genes, which by definition does not require any de novo host or viral protein synthesis. IE gene expression can first be detected as early as several hours post infection (p.i.), and the products of these genes have several functions that will be discussed further below. One key role for the IE products is to transactivate the promoters of the viral early genes. The levels of some early transcripts, such as the HCMV 2.2 kb family of transcripts (UL112-113), peak by about 8 h p.i. Other early genes, including the HCMV 2.7 kb major early transcript (TRL4), are expressed to lower levels at this time and increase in abundance

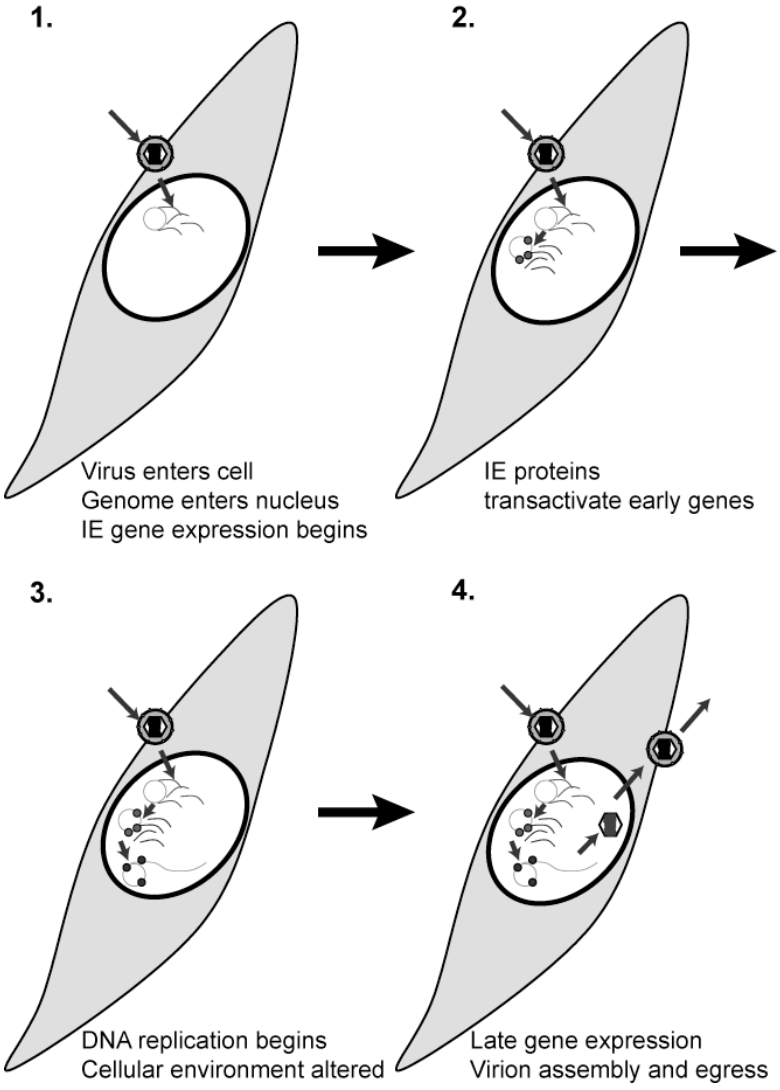



**Figure 1.1 Major events in HCMV replication.** The lytic life cycle of human cytomegalovirus begins with entry of virus particles into a permissive cell. Following uncoating of the particle, the viral genome is translocated to the nucleus. There, immediate-early (IE) genes begin to be transcribed, and this step does not require new host or viral protein synthesis. Several IE proteins, once synthesized, return to the nucleus to assist in the transactivation of viral early promoters. Early gene products mediate the replication of the viral DNA and contribute to the alteration of several processes in the infected host cell. Following DNA replication, late viral genes are expressed, viral capsids are assembled, DNA is packaged into capsids, and virions mature and exit the host cell. In a permissive cell, the life cycle takes between 72 to 96 hours from the time of infection to the release of virus.

as the infection progresses. A third class of viral early genes is not expressed until just before viral DNA replication begins, and these are often referred to as delayed early genes. One such delayed early transcript is the abundant HCMV 1.2 kb RNA (TRL7). HCMV DNA replication begins approximately 48 to 72 h p.i. and is mediated by the products of several early genes. Finally, late viral genes are expressed after DNA replication has begun. These genes encode many of the proteins that form the structural elements of the virus particle.

The approximately 150 predicted HCMV ORFs have been classified as IE, early, or late (48). In general, IE transcripts are those that are present 13 h p.i. in cells that were infected in the presence of cycloheximide to inhibit host and viral protein synthesis. Early viral RNAs are isolated from cells treated with ganciclovir for 72 h, so that IE RNAs and proteins can be made and early genes can be expressed, but viral DNA replication cannot occur. Transcripts that are expressed with late kinetics are isolated from untreated cells at 72 h p.i., and these can be further classified as delayed early or late based on their differential sensitivity to ganciclovir (48). Genes of a given kinetic class are not clustered by location or polarity of the ORF in the genome, but the majority of the US region ORFs are expressed with early kinetics. Of the approximately 150 HCMV ORFs, 63% are expressed with early or early-late kinetics, 32% are expressed with late kinetics, and only about 5% of ORFs are transcribed at IE times (48).

Each HCMV ORF has been characterized as essential or dispensable for virus growth in tissue culture cells (70, 249). Two studies used large-scale mutagenesis of the HCMV genome to examine 162 ORFs in one case and 156 in the other, and

reached similar conclusions. Less than 30% of the ORFs examined are required for growth in tissue culture, and about half of the genes are dispensable. The remaining approximately 20% of loci contribute to virus replication but are not strictly required. When one of these open reading frames is disrupted, the resulting viruses replicate slowly or to lower titers compared to wild-type virus. When any one of four open reading frames (UL9, UL20a, UL23, and US30) is disrupted, the recombinant viruses grow better than wild-type virus in tissue culture (70). The majority of the essential ORFs encode core proteins common to all herpesviruses, and most of the dispensable genes are unique to cytomegaloviruses. The fact that these unique genes have been preserved and continue to be present in the genomes of clinical isolates of virus points to their importance *in vivo* and indicates that they have functions not easily elucidated in tissue culture.

The two most commonly studied laboratory-adapted strains of HCMV are AD169 and Towne. These are a strain derived from the adenoids of an infected child and a candidate vaccine strain isolated from the urine of an infected infant, respectively (171, 183). Both have been passaged repeatedly and are thought to have lost sequences, compared to clinical isolates, because of this serial passage. A clinical strain of HCMV, Toledo, contains a large segment of DNA that encodes 19 potential ORFs (UL133-UL151) not present in the AD169 genome. Towne encodes one ORF homologous to UL147 in Toledo as well as three additional, unique ORFs, UL152-UL154, but lacks most of the additional genes encoded by Toledo (47). It is clear, then, that the very useful strains studied in the laboratory setting are different than virus newly isolated from infected patients. HCMV replication and propagation in

tissue culture is best achieved in primary human fibroblasts derived from skin or lung, and the majority of the experiments described in this dissertation were conducted using primary human foreskin fibroblasts (HFF). With regard to the differences between clinical and laboratory strains mentioned above, it should be noted that newly isolated virus seems to replicate better in endothelial cells than the laboratory strains do (reviewed in (147).

Concurrent with this complex pattern of viral gene expression, conditions in the infected host cell are significantly altered as the infection proceeds. These changes result in the inhibition of apoptosis, disruption of ND10 sites, a block in host cell DNA synthesis, and an overall arrest in the host cell cycle. Several of these effects and the HCMV proteins that are know to be involved in promoting each are discussed further below.

**HCMV Regulatory Factors**

The viral IE gene products are the primary regulators of HCMV early gene expression, and they must act to begin the cascade of viral gene expression described above. Since they are such crucial regulatory factors, the IE proteins have been studied extensively. The main sites of IE transcription are the UL122-123 (major immediate early, MIE), UL36-38, US3, and IRS1/TRS1 open reading frames (Figure 1.2). The predominant and best-characterized members of this group are the products of the major IE region: the IE1 72 and IE2 86 kDa proteins and related products. These major IE proteins and their functions in the HCMV-infected cell are the focus of this work.

**Structure and function of the IE1 72 and IE2 86 kDa proteins.** A single, five-exon transcript from the major IE region (UL122-123) is differentially spliced and translated to give two predominant products: the IE1 72 kDa protein (exons 1-4, UL123) and IE2 86 kDa protein (exons 1-3 and 5, UL122) (Figure 1.2). Translation of each mRNA initiates in exon 2, and the two proteins share 85 amino acids (aa) at their amino termini (211-213). At the beginning of the infection, IE1 72 is the more abundant product at both mRNA and protein levels. IE1 72 has modest transactivating effects, including the ability to transactivate the major immediate early promoter (MIEP). IE2 86 is a stronger transactivator, both of the MIEP and of heterologous promoters, and evidence demonstrating these activities is discussed below. Both the regions unique to IE1 and to IE2 encode additional, minor transcripts, some of which are cell-type specific (109, 122, 174, 196, 209). Two of these transcripts are indicated (Figure 1.2). The start site of the 1.5 kb transcript has been mapped (209) and the start site of the 1.75 kb transcript(s) is predicted based on local sequence features.

Since the expression of the IE1 and IE2 proteins is tightly regulated and since these factors are strong transactivators, many studies have aimed to show how they control the progression of the infection. These studies have been conducted largely in vitro and in transient assays, and much of this work has provided the foundation for this dissertation. IE2 86 has no homology to other known viral or cellular proteins, so efforts to understand its functions have not been able to make many comparisons to studies on other regulatory factors.

First, a large body of work examined the ability of IE2 86 to transactivate and repress gene expression via protein-protein and protein-DNA interactions. IE2 86

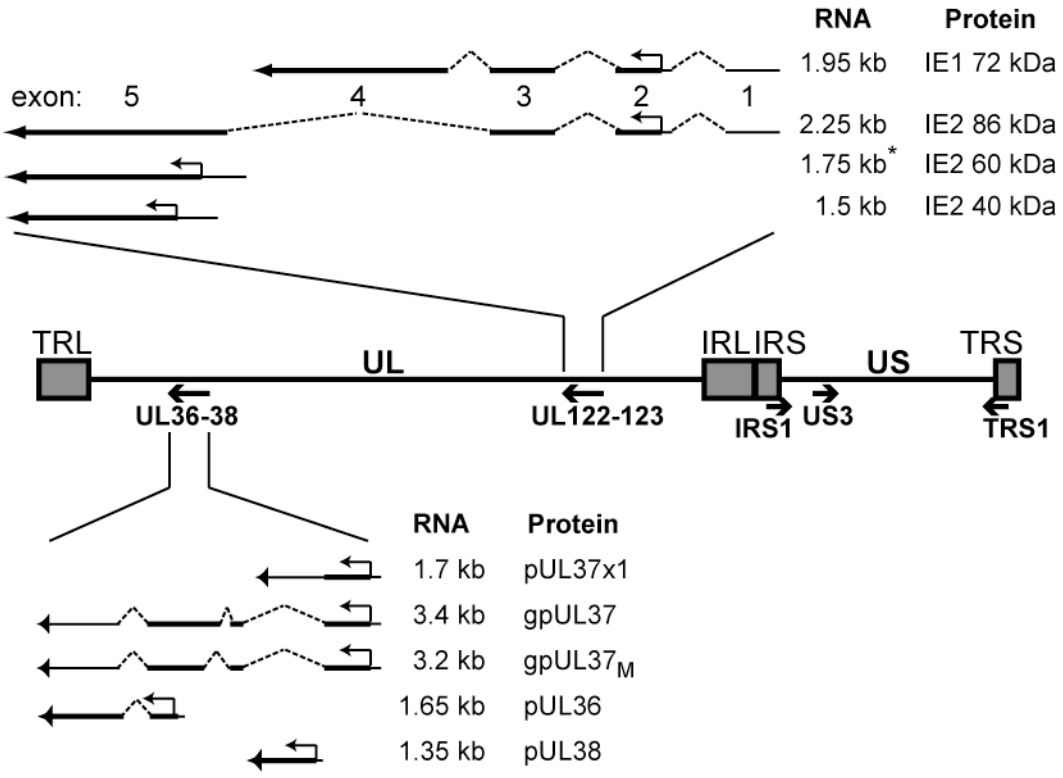



**Figure 1.2 Predominant immediate-early loci in the HCMV genome.** The sites of IE transcription discussed in the text and their relative locations in the HCMV genome are indicated. The UL122-123 genes produce two major transcripts with IE kinetics, and these encode the IE1 72 and IE2 86 kDa proteins. Two additional transcripts from UL122 are expressed at late times and encode IE2 40 and IE2 60 kDa proteins. Four IE transcripts and one early transcript are expressed from the UL36-38 region, and the alternative splicing patterns used to generate these RNAs and the proteins they encode are indicated. Asterisk (*), approximate predicted size of the IE2 60 transcript.

binds to itself, to the product of the viral UL84 gene, and to multiple cellular proteins. These host factors include components of the basal transcription complex TBP, TFIIB, and multiple TBP-associated factors (TAFs), Rb, p53, and transcription factors including Sp1, Tef-1, c-Jun, JunB, ATF-2, NF-κB, protein kinase A-phosphorylated delta CREB, p300, CBP, P/CAF, Nil-2A, CHD-1, Egr-1, and UBF (24, 36, 46, 56, 57, 74, 77, 80, 88, 89, 116, 127, 139, 140, 193-195, 200, 204, 205, 227, 242, 248) (unpublished results of F. Ruchti and D.H. Spector). These studies have been conducted in transfected cells or in in vitro binding assays, and very few results to date suggest which of these protein-protein interactions are also relevant in the virus-infected cell. In HCMV-infected cells, IE2 86 interacts with the viral protein UL84 and has also been reported to interact with the cellular proteins HDAC3 and mdm2 (157, 251) (unpublished results of E.A. White, R.L. Sanders, and D.H. Spector).

IE2 86 is thought to bind to DNA through interactions with the minor groove (129, 226), a notable example being its binding to the 14 bp *cis*-repression signal (CRS) between the TATAA box and transcription start site in the major immediate early promoter (MIEP). It has been shown that this interaction with DNA is the mechanism by which IE2 86 negatively regulates its own transcription (54, 102, 129, 135, 141, 167). In addition, IE2 86 binds to similar 14 bp sites upstream of the TATAA box in early promoters including the UL112-113 (2.2 kb RNA), TRL7 (1.2 kb RNA), and UL4 promoters (11, 50, 102, 194, 195).

Many studies have defined the motifs and domains in IE2 86 that allow these protein-protein and protein-DNA interactions and have located the residues of IE2 86 that are likely to be posttranslationally modified. The ability of IE2 86 to interact with

other proteins maps broadly to the majority of the region not shared with IE1 72, amino acids 86-542 (56, 200). A subset of this region, aa 388-542, is required for IE2 86 to dimerize (6, 56, 77). The DNA binding capability of IE2, which allows regulation of early promoters as well as autoregulation, is also the result of sequences present in the C-terminal half of the protein between residues 290-579 (56, 128, 194). Regions spanning the length of the protein appear to be important to the ability of IE2 86 to transactivate heterologous promoters and HCMV early promoters, with the regions between aa 1-98 and 170-579 required for activation (97, 142, 168, 195, 200, 210, 247). In particular, activation of the UL112-113 promoter requires sequences from aa 26-85 and aa 290-579 of IE2 86, while activation of the 1.2 kb RNA promoter requires these and sequences from aa 86-135 (195, 200). While interactions between IE1 72 and cellular proteins including p107 (111) have also been demonstrated, the IE1 products have not been shown to bind to DNA.

Both IE1 72 and IE2 86 proteins carry extensive posttranslational modifications, but there is little evidence to suggest how these modifications contribute to the proteins' functions. Both proteins are phosphorylated and modified by ubiquitin-like proteins: IE1 72 by SUMO-1 and IE2 86 by SUMO-1 and Ubc9 (9, 95, 96, 100, 206, 243). Both in vitro and in vivo, IE2 86 is phosphorylated on multiple residues (91). Site-directed mutagenesis of consensus MAP kinase motifs at amino acids 27, 144, 233-234, and 555 of IE2 86 to alanine resulted in some cases in a protein with a stronger capacity to transactivate than the wild-type in transient assays. In the context of the viral genome, these mutations have no effect on virus replication in fibroblasts (96).

There are slight sequence variations in the UL122 ORF in the Towne and AD169 strains of HCMV (16). The two resulting forms of the protein have three single-site amino acid differences and also differ in length by one residue due to a run of serines beginning at amino acid 258 that is seven residues long in Towne and eight residues long in AD169. One study examined differences in IE2 86 protein phosphorylation resulting from these changes. In particular, changing the threonine present at residue 541 in Towne IE2 86 to the alanine found in AD169 protein at the same position appears to have little effect on the phosphorylation pattern. The Towne and AD169 clones were also compared to an IE2 86 cDNA clone (210) that has been used extensively and contains some of the features of each strain. In transient assays, the ability of the IE2 86 variants to transactivate the promoters tested differed by up to two-fold, with AD169 IE2 86 typically the strongest transactivator and the cDNA clone the weakest. The recombinant viruses constructed for study in this dissertation were generated using an AD169-based construct.

Numerous studies have shown that the major IE proteins function together and separately to activate their own promoter and heterologous promoters. These have been conducted primarily using transient transfection of effector plasmids expressing IE1 72 and IE2 86 proteins and target plasmids expressing reporters driven by a range of viral promoters. In particular, these include the 1.2 and 2.7 kb RNA and UL112-113 (2.2 kb RNA) early promoters and sequences driving expression of genes involved in viral DNA replication (60, 124, 194, 195). The results of these studies indicate that IE2 86 contributes the most to the activation observed and in some cases, increases the level of reporter expression 40- to 80-fold over expression in the absence

of IE1 72 or IE2 86. IE1 72 alone is a much weaker activator, but can function with IE2 86 to provide a modest increase in reporter gene expression relative to that seen with IE2 86 alone.

**Additional Immediate Early Proteins Have Regulatory Roles.** In addition to UL122-123, the UL36-38 and IRS1/TRS1 families and the US3 locus encode IE proteins with regulatory functions (Figure 1.2). Knockout mutants constructed to date suggest that with the possible exception of UL37 exon 1, these IE loci are not necessary for the expression of early genes and the progression of the infection (23, 26, 70, 162, 249). The IRS1 and TRS1 ORFs encode three proteins: two expressed from a promoter located in the repeated region flanking the US segment of the genome with an ORF continuing into the unique region, and a third, smaller, protein designated pIRS1$^{263}$ that is expressed from an internal promoter in the unique region of the IRS1 gene (181). In transient expression assays, either the IRS1 or TRS1 protein is required for complementation of HCMV origin-dependent DNA replication. In further transient studies Romanowski and colleagues demonstrated that either pIRS1 or pTRS1 has the ability to up-regulate transcription from the MIEP slightly and that this is increased with the addition of the UL69 protein (160, 181). None of these three products appears to be essential for viral replication in tissue culture, since recombinant viruses lacking the unique regions of the IRS or TRS1 open reading frames are viable (23, 112). The mutant lacking the IRS1 products grows normally while the TRS1 deletion mutant exhibits a multiplicity-dependent phenotype. The amount of virus released from cells infected with the TRS1 deletion mutant at a low multiplicity of infection (MOI) is reduced drastically compared to that released from

wild-type infected cells, but cells infected at a high MOI produce only slightly less virus than the wild-type. TRS1 mutant virus replication proceeds normally through DNA replication, but pp65 is mis-localized in mutant-infected cells.

The UL36-38 gene products were identified in a study that identified eleven loci required for complementation of DNA replication (160). Individually, these genes are dispensable for growth in culture with the exception of the UL37 exon 1 protein, which is required only in the absence of UL36 (70, 249). Four of the five transcripts from this region are expressed with IE kinetics, three from the UL37 promoter and one coding for the UL36 product (51, 82, 126) (Figure 1.2). The 3.4 kb spliced transcript from the UL37 promoter is present only at IE times and encodes an integral membrane glycoprotein, gpUL37 (126, 219, 220). An alternative splice of this transcript generates the 3.2 kb mRNA coding for gpUL37$_M$ (59), and these proteins traffic through the endoplasmic reticulum (ER) to mitochondria. gpUL37 is able to transactivate the hsp70 promoter in transient assays, but the deletion of UL37 exon 2 or exon 3 does not affect the ability of the virus to replicate in culture (26, 58, 82, 221, 250). In contrast, the high degree of conservation of the UL37 exon 1 sequence in clinical isolates and high-passage laboratory strains has been viewed as evidence that this region is necessary for replication *in vivo* and in cultured cells, and this prediction is supported by the impaired replication of UL37 exon 1 mutants (70, 82, 92, 249). The UL37 exon 1 sequence is also conserved among primate cytomegaloviruses (144). As noted above, the protein product of UL37 exon 1, vMIA, has anti-apoptotic functions. In transient transfection assays, a construct expressing UL37 exon 1 can activate the UL54, UL44, and other early viral promoters. Although

this activation is observed when constructs expressing IE1 72 and IE2 86 are used in this assay (58), adding UL37 exon 1 and IE1/IE2 expression vectors together results in a synergistic activation effect. The final IE transcript from the UL36-38 region is the UL36 RNA, which encodes a protein (vICA) that is also involved in preventing apoptosis in infected cells (198). The fifth transcript from this region is the product of the UL38 gene and is expressed with early kinetics.

A fourth site transcribed with IE kinetics is the US3 gene, which specifies at least three alternatively spliced RNAs coding for related proteins. It is the target of complex positive and negative regulatory control (18-21, 49, 222) and like much of UL36-38, it is dispensable for growth in tissue culture (112). Proteins generated from this region help the virus to evade the host immune response by functioning to keep MHC class I molecules retained in the ER and unable to traffic to the plasma membrane (10, 113). US3 transcripts are abundant during the first three hours of infection and decrease by five hours p.i. Transcription is regulated by a combination of elements located near the promoter including a silencer, enhancer, and transcriptional repressive element (tre) that has a sequence similar to that of the CRS element involved in control of major IE region expression (18, 37, 38, 49, 131, 222). The protein product of the UL34 gene binds the tre element and represses the transcription of US3 (130). The US3 proteins seem to possess limited intrinsic transactivating capability, as they have only been shown to induce the cellular hsp70 promoter in transient assays (60, 221, 250).

**Mutational analysis of the major IE products in the viral genome.** The studies described above laid the groundwork for elucidating the critical domains and

functions of the IE proteins, in particular the products of the major IE genes. More recent studies have used recombinant viruses with mutations in the UL122-123 ORFs to understand the functions provided by IE1 72 and IE2 86 not only in transient assays, but in the virus-infected cell where all of the relevant factors are present.

One of the recombinant viruses that has proved very important to understanding the functions of the IE proteins in the infected cell is cr208, a mutant virus that lacks UL123 exon 4 and is therefore unable to express full-length IE1 72 (79, 84, 148). cr208 is viable, exhibiting minimal growth defects in cells infected at a high-multiplicity and striking deficiencies in cells infected at low MOI. This result indicates that under the proper conditions, IE1 72 is not required for HCMV replication. Fibroblasts infected with 0.4 pfu/cell of cr208 express IE2 86 about as frequently and to similar levels as wild-type virus-infected cells, but are deficient in the expression of delayed early proteins and RNAs. Apparently, while high levels of IE2 86 or another factor are able to compensate for the loss of IE1 72 during a high-multiplicity infection, the efficient activation of early genes in cells infected at low-multiplicity requires the full-length IE1 72 protein. The IE1 deletion mutant virus can be complemented in *trans* (179). Adding wild-type or mutant forms of IE1 72 to cr208 virus-infected cells shows that aa 476-491 tether IE1 72 to chromatin but are not required for complementation, while aa 421-475 comprise an acidic domain necessary for complementation and restoration of wild-type titers during low-multiplicity growth. A human fibroblast cell line expressing IE1 72 allows the propagation of cr208 and related viruses (148), but to date no analogous complementing cell line that expresses IE2 86 has been isolated. The ways in which IE2 86 alters the host cell

cycle are introduced below, and these provide some explanation for the difficulty of generating such a cell line.

In the absence of a complementing cell line, the construction and propagation of recombinant viruses with mutations in essential genes like IE2 86 lagged behind the IE1 72 studies described above. The advent of bacterial artificial chromosomes (BACs) as vectors for the cloning of herpesvirus genomes has largely allowed this problem to be circumvented (4). The advantages of BAC-mediated mutagenesis and some of the techniques used to manipulate the HCMV BAC are introduced below.

Several groups have used BAC cloning to construct HCMV IE2 86 mutants. A recombinant virus with most of the unique region of the IE2 gene (ORF UL122) deleted is defective in early gene expression and does not produce infectious progeny, demonstrating that IE2 86 is required for the activation of early genes and for viral replication (143). A viable mutant with a deletion spanning residues 136-290 of IE2 86 expresses IE and early genes and replicates its DNA comparably to the wild-type virus, but is delayed in the expression of a subset of late genes (188). Heider and colleagues used BAC cloning to create a temperature-sensitive IE2 86 mutant virus that contains the point mutation C510G and is able to transactivate the UL112-113 promoter at 32.5°C, but not at 39.5°C (95). Additionally, it exhibits increased transcription from immediate early loci. This dissertation describes a family of recombinant viruses derived from BACs, uses them to delineate the functions of IE2 86 that are provided by specific regions of the protein, and begins to show how these functions alter the progression of the infection and conditions in the virus-infected cell.

**HCMV Encoded MicroRNAs.** MicroRNAs (miRNAs) have been recently understood to be important regulators of cellular gene expression and appear to contribute to the regulation of viral genes as well (17). miRNAs are a subset of the small single stranded RNAs that are expressed in numerous species. They are 20 to 24 bases long, and each is initially expressed in the nucleus as part of a longer primary transcript (pri-miRNA). A hairpin loop sequence containing the miRNA is recognized and cleaved by the RNase III Drosha complex, then transported to the cytoplasm where the resulting pre-miRNA is further processed by a second endonuclease, Dicer, to produce a double stranded RNA (dsRNA). At least one strand of the dsRNA is loaded into the RNA induced silencing complex (RISC). Transcripts are targeted by RISC depending on the complementarity between the miRNA and the target transcript. In cases of complete homology, the target transcript is cleaved, while incomplete homology can lead to either the degradation of the transcript or the inhibition of its translation (13, 246). In either case, the interaction between the miRNA and its target has been thought to lead exclusively to decreased expression of the target gene(s). For the vast majority of identified miRNAs, validated target transcripts and regulatory functions are unknown (110).

Virus-encoded miRNAs have been recently identified as additional elements that could regulate the expression of both viral and cellular genes in virus-infected cells (156, 166, 191, 214). The genomes of several DNA viruses, including SV40, KSHV, MHV68, HSV-1, and EBV encode miRNAs (41-43, 65, 86, 87, 165, 215). It has been predicted that HCMV encodes at least eleven miRNAs, and this estimate could be higher depending on the algorithm used to identify the miRNAs. The

expression of at least five of these miRNAs has been verified by Northern blotting RNA isolated from HCMV-infected cells (71, 85, 165). Most of the HCMV-encoded miRNAs are expressed with early kinetics during the lytic infection and could therefore be involved in many of the virus-induced processes that occur during and following early times p.i.

## INTERACTIONS BETWEEN HCMV AND THE HOST CELL

Viral gene products from every kinetic class contribute to the HCMV-mediated disruption of host cellular processes. Some of these, including pp71 (ppUL82) and UL69 proteins, are delivered with the virus particle – although these genes are expressed later in the viral life cycle, their protein products begin to exert their effects as soon as the particle enters the host cell. The protein encoded by UL69 functions as a transactivator and contributes to the arrest of HCMV-infected cells in a G1-like phase of the cell cycle (45, 93, 138, 236, 237). The ppUL69 protein delivered with the particle appears to be sufficient to mediate this effect: if the viral genome does not contain the UL69 gene but the protein is present in the tegument, the effects on the host cell still occur. One function of the pp71 protein that is particularly relevant in the context of this dissertation is its ability to stimulate IE gene expression and to enhance the infectivity of HCMV DNA (14, 31, 136). The protein also contributes to changes in the host cellular environment and has the ability to mediate proteasomal degradation of Rb and related proteins (117-119).

IE proteins mediate several important changes to the host cell including disruption of ND10 sites, inhibition of apoptosis, and down-regulation of the host interferon response. In uninfected cells, a number of proteins including the growth suppressors promyelocytic leukemia protein (PML), Sp100, HP1, and Daxx are localized to ND10 domains (also referred to as PODs [PML oncogenic domains] or PML bodies). Upon infection with HCMV, a subset of viral genomes are deposited at ND10 sites immediately following infection and the ND10 sites are rapidly dispersed (8, 107, 120, 125). It is these genomes that provide the template for the initial viral transcription events (106, 107). The transcripts produced at these sites are consequently in close proximity to spliceosome assembly factor SC35 domains, which may further aid in the rapid expression of IE genes following infection. One IE protein, IE1 72, is required for disruption of the ND10 sites, but ND10 disruption is not required for the infection to progress (5, 8, 107). pp71 is required for the proteasome-mediated degradation of Daxx that begins 2 h p.i. in HCMV-infected cells. This degradation is required for efficient IE gene expression and is thought to increase gene activity by eliminating Daxx-mediated histone deacetylase recruitment to promoters (185, 239).

Two viral IE proteins from the UL36-38 region of the genome block apoptosis in the infected cell. One (vMIA) is the protein product of UL37 exon 1, and the second (vICA) is encoded by UL36. vMIA blocks apoptosis by acting at a stage between the activation of caspase 8 and the release of cytochrome c into the cytoplasm (82, 144). vICA is dispensable for virus growth in culture and acts to block the cleavage and subsequent activation of pro-caspase 8 (162, 198).

When virus particles contact the host cell, RNAs corresponding to some of the host-cell encoded alpha- or beta-interferon (IFN-a/β)- inducible genes begin to be expressed (27, 35, 155, 253, 254). The viral tegument protein pp65 helps to dampen this effect (1, 34, 35), as do IE1 72 and IE2 86 proteins. IE2 86 blocks the production of IFN-β and several chemokines (217, 218), while IE1 72 acts further downstream to block type I interferon-mediated signaling and the induction of multiple interferon-responsive genes (163).

Several studies have examined other contributions of IE2 86 to changes in the host cellular environment, although few of these have been conducted in the context of the virus-infected cell. The expression of IE2 in uninfected cells leads to up-regulation of cyclin E expression and modulates the expression of multiple other E2F-responsive genes (201, 231, 233), but a study conducted to address the same question in HCMV-infected cells suggests that during the infection, early gene expression is required for this effect to occur (145). Multiple publications have reported that when it is expressed in uninfected cells, IE2 86 can contribute to cell cycle arrest (24, 152, 158, 197, 202, 232), although these vary in their description of the state in which cells arrest. Perhaps the most relevant reports are those that characterize cell cycle arrest in HCMV-infected cells (98, 108, 186). Together, these studies suggest that infected cells are arrested in a state that has some similarities to a normal G1 phase and some similarities to a normal S phase, but is not identical to either one.

Early HCMV proteins provide a large and diverse set of functions to alter conditions in the infected host cell, ensuring that virus replication is favored over that of the host. HCMV infection results in the stimulation of many genes encoding

proteins that are required for host cell DNA synthesis and cell cycle control (35). Cells infected with HCMV are not able to replicate their own DNA, and DNA replication appears to be blocked at several steps. First, the assembly of pre-replication complexes at the replication origins is inhibited, both through delayed expression of the Mcm proteins and defective loading of the Mcm proteins onto the cellular DNA (22, 234). Next, the expression of cyclin A and its associated kinase activity is inhibited (108), and this also likely inhibits the replication of host cellular DNA.

Other host cellular protein levels increase at early times p.i. HCMV induces elevated levels of cyclin E and cyclin B and their associated kinase activities (30, 108, 145, 186, 189). Cyclin E is induced at the RNA level, and this up-regulation requires the expression of viral early genes (145, 186). In contrast, multiple posttranscriptional pathways are used in the activation of Cdk1/cyclin B1 complexes. These include the accumulation of cyclin B1 because of increased synthesis and reduced degradation of the protein and the accumulation of cdk1 in its active form in virus-infected cells, and these pathways appear to require at least some early viral gene expression (189).

The expression and activity of p53 is also altered by HCMV infection, and p53 protein levels are elevated in HCMV-infected cells relative to uninfected controls (108, 150). Levels of the cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$ ($p21^{cip1}$), which are regulated by p53 as well as by numerous other pathways, however, are lower in HCMV-infected cells than in uninfected cells (53, 78). This suggests that the p53 present in infected cells has been at least partially inactivated. p53 interacts with IE2 86, and this interaction has been implicated in the increased levels of p53 in infected

cells (24, 101, 149, 202, 223, 251). p53 is sequestered into replication centers in infected cells, (75, 182), raising the question of whether its presence is advantageous for the virus or not, but it has recently been shown that HCMV replication is impaired in p53-deficient human fibroblasts. This suggests that the presence of wild-type p53 contributes to HCMV replication (44).

These events lead the cell to a fully activated and permissive state, but it is clear that the virus has primed the cell for its own replication at the host's expense. It has sufficiently dysregulated the cell cycle and signaling pathways to ensure that the cell can no longer divide, and host cellular resources are therefore diverted to promote viral DNA replication and virus production.

## BACTERIAL ARTIFICIAL CHROMOSOME-MEDIATED MUTAGENESIS OF THE HCMV GENOME

Most of the studies described above that allowed the characterization of the IE2 86 protein were conducted in transient transfection experiments and by in vitro synthesis and analysis of mutant proteins. This approach is limited by the inability to study the effects of these mutations in the context of the virus-infected cell, where all of the relevant viral and cellular factors are present. One very useful way to understand the function of a viral protein in the context of the infected cell is to generate a recombinant virus that does not express the protein of interest or expresses a mutated version of it, but forward genetic studies like these require methods for easy manipulation of the viral genome. Since the mutation rate for herpesviruses during

normal replication is low, the isolation of fortuitously occurring mutations in relevant genes is not sufficient.

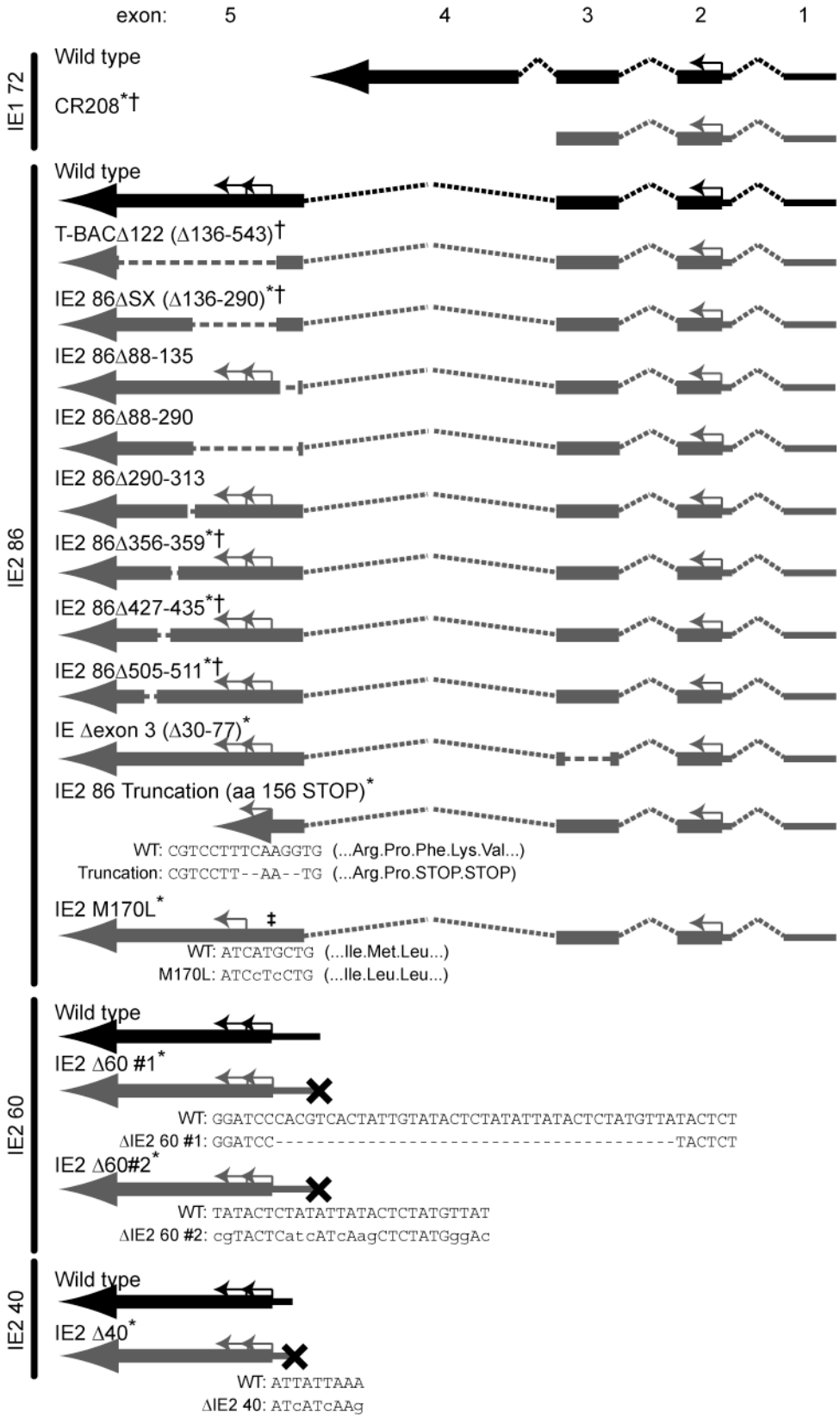
Early efforts to construct HCMV recombinants used viral DNA cotransfected into permissive cells along with a plasmid containing a marker cassette. The marker encodes a gene like β-galactosidase flanked with HCMV sequences homologous to the intended insertion point, so that recombinant viruses can be selected against the background of wild-type virus (83, 146, 203, 238). Disadvantages to this method include potential difficulties in generating recombinants and in separating them from wild-type virus, and it is not possible to use this method to introduce a mutation that results in a non-viable virus. A next-generation approach to the construction of HCMV recombinants used cosmid cloning, involving cotransfection of a set of overlapping cosmid clones containing the entire viral genome into permissive cells (121). One cosmid would contain the mutated sequences to be incorporated into the virus in place of wild-type sequences. This method has the advantage that it does not generate a mixture of wild-type and recombinant viruses, but usually about eight cosmids are cotransfected together and therefore the frequency of the correct recombination events occurring simultaneously tends to be low. Also, only viable recombinant viruses can be generated by this method.

The restrictions inherent in these methods were significantly eased by the cloning of herpesviral genomes (including the HCMV genome) as bacterial artificial chromosomes (BACs) (4, 26, 146, 225). The HCMV BAC is a circularized version of the viral genome that also carries bacterial selectable marker and origin of replication sequences necessary and sufficient for the propagation of the DNA in *E. coli*. The

bacterial sequences can either take the place of a segment of the viral genome or be carried in addition to the complete viral genome. In either case, since the essential parts of the viral genome are present in a single BAC molecule, mutations can be made and characterized entirely in bacteria regardless of the viability of the resulting virus. The mutated genome is transfected into cells permissive for the HCMV infection and studied in tissue culture. Reconstitution of virus from the clone is achieved by transfecting the altered genome and a construct expressing pp71 into cells permissive for HCMV infection (14). In addition to the ability to perform highly specific site-directed mutagenesis of a viral gene of interest, this approach can also be used to insert other cellular genes under the control of heterologous promoters into regions of the HCMV genome that are tolerant of additional sequences.

Several techniques are useful for manipulating the HCMV BAC in *E. coli*. An early approach required transformation of a shuttle vector containing the desired mutation into bacteria that already harbored the HCMV BAC. Via a series of selections, bacteria containing a cointegrate BAC that contained both wild-type and mutant sequences could be isolated. The cointegrate could then be 'resolved' so that wild-type sequences were removed and the desired mutant sequences remained (146). Next-generation approaches use either recombination mediated by phage recombinases or site-directed transposition to more easily manipulate the HCMV genome in bacteria (4, 33, 90, 225). HCMV genomes derived from a BAC can also be isolated from infected eukaryotic cells and reintroduced into *E. coli* for further manipulation, if necessary. The various methods available for BAC manipulation and the relative speed with which recombinant DNA can be generated using BAC cloning

**Figure 1.3 Major IE region mutants relevant to the dissertation.** Deletions and alterations contained in viruses discussed in the text are indicated. Transcripts that are primarily affected by the mutation are indicated at left. Thin horizontal lines indicate noncoding exon sequences and thick horizontal lines indicate coding exon sequences. Angled dashed lines indicate splicing events. Small arrows indicate the locations of initiating methionines for proteins encoded by the major IE region. Horizontal dashed lines indicate regions deleted in the genome of a recombinant virus. The double dagger (‡) marks the location of the M170L point mutation. An asterisk (*) indicates that a rescued version of this virus has been generated. A dagger (†) indicates that this virus was not cloned by the dissertation author. T-BACΔ122 has been described by Marchini et al. (143). cr208 was initially generated by Greaves and Mocarski (84). Characterization of IE2 86ΔSX was first reported by Sanchez and colleagues (188).


exon: 5 4 3 2 1
IE1 72
Wild type
CR208*†
IE2 86
Wild type
T-BACΔ122 (Δ136-543)†
IE2 86ΔSX (Δ136-290)*†
IE2 86Δ88-135
IE2 86Δ88-290
IE2 86Δ290-313
IE2 86Δ356-359*†
IE2 86Δ427-435*†
IE2 86Δ505-511*†
IE Δexon 3 (Δ30-77)*
IE2 86 Truncation (aa 156 STOP)*
WT: CGTCCTTTCAAGGTG (...Arg.Pro.Phe.Lys.Val...)
Truncation: CGTCCTT--AA--TG (...Arg.Pro.STOP.STOP)
IE2 M170L*
‡
WT: ATCATGCTG (...Ile.Met.Leu...)
M170L: ATCcTcCTG (...Ile.Leu.Leu...)
IE2 60
Wild type
IE2 Δ60 #1*
WT: GGATCCCACGTCACTATTGTATACTCTATATTATACTCTATGTTATACTCT
ΔIE2 60 #1: GGATCC----------TACTCT
IE2 Δ60#2*
WT: TATACTCTATATTATACTCTATGTTAT
ΔIE2 60 #2: cgTACTCatcATcAagCTCTATGggAc
IE2 40
Wild type
IE2 Δ40*
WT: ATTATTAAA
ΔIE2 40: ATcATcAAg

have made it the method of choice for constructing mutant herpesviruses. All of the HCMV recombinants generated by the dissertation author and discussed in this work were cloned as BACs.

**GOAL OF THE WORK**

The studies introduced above demonstrated several important points. First, HCMV gene expression is tightly controlled through the combined actions of numerous virus- and cell-encoded factors. One viral protein, IE2 86, contributes many of these regulatory functions and is essential for HCMV replication. Years of previous work defined the functions of IE2 86 in vitro and in transient assays, but not in the context of the virus-infected cell where each relevant viral and cellular factor is present. With this in mind, the goal of the studies presented in this dissertation is to delineate the functions provided by IE2 86 in fully permissive cells infected with HCMV. The approach has been to generate recombinant viruses with mutations in IE2 86 and to characterize these viruses in cultured cells. By constructing a library of IE2 86 mutant viruses and defining their phenotypes, it has been possible both to verify several previously suggested functions of IE2 86 in the infected cell and to discover new functions. It has also been possible to map these functions to specific regions of the protein.

Since IE2 is essential to the progression of the infection and since efforts to construct an IE2 86 expressing cell line capable of complementing *ie2* mutants have so far been unsuccessful, the ability to construct and propagate viruses with mutations in

IE2 86 was at first limited. The ability to clone HCMV recombinants as BACs was a tremendously helpful development in this regard, since this technology can be used to generate and characterize both viable and non-viable viruses resulting from changes in the IE2 86 gene.

This dissertation describes the construction and characterization of a family of HCMV recombinants with mutations in the major IE region, particularly in the IE2 86 gene. Members of this family and other relevant viruses with changes in the same region are shown (Figure 1.3). By characterizing non-viable viruses, we have taken advantage of several of the features of the BAC cloning system to show that several small regions of IE2 86 are essential for proper function of the protein and for HCMV replication (Chapter 2). Unexpectedly, mutation of these small regions implicated IE2 86 in the control of late viral promoters. Continuing on this theme of the functions of IE2 late in the infection, a family of recombinant viruses that do not express smaller forms of IE2 helped to identify the functions of these proteins at late times (Chapter 3). Aspects of this research also examined the contributions of IE1 72 and IE2 86 to the control of host cellular processes in the virus-infected cell. A recombinant virus with a deletion in exon 3 of the major IE region is viable, but expresses altered forms of both IE1 72 and IE2 86 proteins (Chapter 4). Characterization of this mutant showed that the major IE proteins contribute not only to the regulation of viral gene expression, but also to the dysregulation of several host cellular processes observed in HCMV-infected cells. These studies, their key conclusions, and some related observations, are summarized in the conclusion of the dissertation (Chapter 5). Finally, experiments suggesting that an HCMV-encoded microRNA could contribute

to the regulation of the major IE region are described in the appendix. This work has answered some questions regarding the functions of IE2 86 in the HCMV-infected cell, but by uncovering new functions for the protein, has raised many more.

# CHAPTER 2

# SMALL INTERNAL DELETIONS IN THE HUMAN CYTOMEGALOVIRUS IE2 GENE RESULT IN NON-VIABLE RECOMBINANT VIRUSES WITH DIFFERENTIAL DEFECTS IN VIRAL GENE EXPRESSION


## ABSTRACT

The human cytomegalovirus IE2 86-kDa protein is a key viral transactivator and an important regulator of HCMV infections. We used the HCMV genome cloned as a bacterial artificial chromosome (BAC) to construct four HCMV mutants with disruptions in regions of IE2 86 that are predicted to be important for its transactivation and autoregulatory functions. Three of these mutants have mutations that remove amino acids 356 to 359, 427 to 435, and 505 to 511, which disrupt a region of IE2 86 implicated in the activation of HCMV early promoters, a predicted zinc finger domain, and a putative helix-loop-helix motif, respectively, while the fourth carries three arginine to alanine substitution mutations in the region of amino acids 356 to 359. The resulting recombinant viruses are not viable, and using quantitative real-time reverse transcription-PCR and immunofluorescence we have determined the location of the block in their replicative cycles. The IE2 86Δ356-359 mutant is able to support early gene expression, as indicated by the presence of UL112-113 transcripts and UL112-113 and UL44 proteins in cells transfected with the mutant BAC. This mutant does not express late genes and behaves nearly

indistinguishably from the IE2 86R356/7/9A substitution mutant. Both exhibit detectable up-regulation of major immediate early transcripts at early times. The IE2 86Δ427-435 and IE2 86Δ505-511 recombinant viruses do not activate the early genes examined and are defective in repression of the major immediate-early promoter. These two mutants also induce expression of selected delayed early (UL89) and late genes at early times in the infection. We conclude that these three regions of IE2 86 are necessary for productive infections and for differential control of downstream viral gene expression.

## INTRODUCTION

Human cytomegalovirus (HCMV), a betaherpesvirus, has a double-stranded DNA genome of approximately 230 kbp that encodes at least 150 open reading frames (67). Infection with HCMV has serious consequences for immunocompromised patients and is the leading viral cause of birth defects (for a review, see reference (161). HCMV gene expression is separated into three temporal categories (for a review, see reference (147). Immediate-early (IE) genes are the first to be activated and do not require de novo host or viral protein synthesis for their expression. Early genes represent a broadly defined class whose transcription is typically regulated by the interaction of IE gene products with cellular factors. Viral DNA replication follows early gene expression. Finally, late viral genes, many of which encode structural proteins, are expressed.

Since IE gene expression begins this cascade of events, the regulation and products of IE genes have been studied extensively. The major immediate-early (MIE) gene, made up of open reading frames UL122 and UL123, is a region of particular interest. It consists of five exons that are transcribed, differentially spliced, and translated to give two predominant products: the IE1 72 kDa protein (exons 1 to 4) and IE2 86 kDa protein (exons 1 to 3 and 5). Translation of each transcript initiates in exon 2, and the two proteins share 85 amino acids (aa) at their amino termini (211-213), reviewed in reference (76). The IE2 region also encodes an additional product expressed later in the infection and a splice variant present in infected human monocyte-derived macrophages (109, 122, 174, 209). IE1 72 is the more abundant

product at both mRNA and protein levels and has modest transactivating effects, including the ability to transactivate the major immediate early promoter (MIEP) (for reviews, see references (76, 147). Numerous in vitro and in vivo studies have shown that IE2 86 is a strong transactivator and also represses its own promoter (54, 55, 97, 135, 167, 169, 210). Other immediate early genes include IRS1, TRS1, the UL36 to -38 genes, and US3. Many of these, including US3, UL36, and IRS1, are not required for HCMV replication in cultured cells (23, 26, 112, 162). A virus lacking TRS1 exhibits normal gene expression during IE and early times post infection but is defective in late stages of replication (23). An IE1 mutant virus is viable but shows growth defects during a low-multiplicity infection (79, 84, 148). In contrast, the failure of a virus lacking most of the IE2 gene to support early gene expression and replicate indicates that IE2 86 is essential for productive infection (143).

Significant efforts have been directed towards defining the elements that provide IE2 86 with its strong regulatory capabilities, and IE2 86 is thought to transactivate and repress via protein-protein and protein-DNA interactions. IE2 86 binds to the product of the viral UL84 gene and to multiple cellular proteins. These host factors include components of the basal transcription complex TBP and TFIIB, numerous cellular transcription factors, Rb, p53, and others (24, 46, 56, 57, 74, 77, 80, 88, 89, 115, 127, 140, 193-195, 200, 204, 227). In addition, IE2 86 is modified by multiple ubiquitin-like proteins (9, 100). IE2 86 is thought to bind DNA through interactions with the minor groove, a notable example being its binding to the 14 bp *cis*-repression signal (CRS) between the TATAA box and transcription start site in the major immediate early promoter (MIEP). It has been shown that this binding is the

source of IE2 86's negative regulatory effect on its own transcription (54, 102, 129, 135, 141, 167). In addition, IE2 86 binds to similar 14-bp sites upstream of the TATAA box in early promoters including the UL112-113 (2.2-kb RNA) and 1.2-kb RNA promoters (11, 50, 194, 195).

Multiple studies have aimed to define motifs and domains in IE2 86 that allow these protein-protein and protein-DNA interactions and the location of residues likely to be posttranslationally modified. The ability of IE2 86 to interact with other proteins maps broadly to the majority of the region not shared with IE1 72 (aa 86 to 542) (56). It should be noted to avoid confusion that the amino acid numbers in the text correspond to IE2 86 in the Towne strain. AD169 has an additional serine after amino acid 264. A subset of this region, aa 388 to 542, is required for IE2 86 to dimerize (6, 56, 77). The DNA binding capability of IE2, which allows regulation of early promoters as well as autoregulation, is also the result of sequences present in the C-terminal half of the protein, between residues 290 and 579 (56, 128, 194). Various structures and functional regions have been more finely mapped. There are three potential sites for phosphorylation by casein kinase II located between amino acids 203 and 277 (168). Harel and Alwine (91) demonstrated that both in vitro and in vivo, IE2 86 is phosphorylated on multiple residues. They performed site-directed mutagenesis of consensus mitogen-activated protein (MAP) kinase motifs at aa 27, 144, 233 to 234, and 555 to alanine and found that in transient expression assays, some of these changes resulted in a protein with a stronger capacity to transactivate than the wild type. IE2 86 has at least two nuclear localization signals, at aa 145 to 151 and 321 to 328, a leucine-rich region predicted to form a helix-loop-helix between

aa 463 and 573, and a putative zinc finger domain at aa 428 to 452 (77, 142, 168, 210). Although IE2 mutants with changes in the zinc finger domain fail to bind to DNA or autorepress their own transcription, in transient expression assays they are able to drive transcription from the major IE promoter (241). The insertion of four amino acids at residue 356 or 540 reduces at least 6-fold the capacity of the protein to stimulate the viral DNA polymerase (UL54) promoter; however, these mutations have the opposite effect on the MIEP and result in a 10-fold increase in its activation (210).

Until recently, these mapping studies were conducted primarily with expression vectors in transient expression assays or with bacterially expressed, purified IE2 86 mutant proteins in in vitro assays due to the difficulty of examining the mutations in the context of the viral genome. While our group and others have attempted to construct an IE2 86 expressing cell line capable of complementing *ie2* mutants, to date none has been isolated. In the absence of such a tool, it is difficult to propagate recombinant viruses with mutations in essential genes like IE2 86; however, the advent of bacterial artificial chromosomes (BACs) as vectors for the cloning of herpesvirus genomes has largely allowed this problem to be circumvented. Since the majority of the viral genome is present in the BAC, mutations can be made and characterized entirely in bacteria, regardless of the viability of the resulting virus. The altered genome is then transfected into cells permissive for HCMV infection along with a construct expressing pp71 (ppUL82), allowing reconstitution of virus from the clone. pp71 expression has been shown to increase the infectivity of transfected HCMV DNA (14). Murine cytomegalovirus was the first herpesvirus to be cloned as a BAC, and the technique has been successfully extended to include HCMV, herpes

simplex virus type 1, Epstein-Barr Virus, and others (26, 68, 146, 184), reviewed in references (4, 225). Several groups have since used this approach to construct HCMV IE2 86 mutants. Marchini et. al showed that a recombinant virus with most of the IE2 gene (open reading frame UL122) deleted is defective in early gene expression and does not produce infectious progeny (143). Members of our group generated a viable mutant with a deletion of IE2 86 residues 136 to 290 and showed that this virus expresses IE and early genes and replicates its DNA comparably to the wild type but has delayed expression of selected late genes (188). Heider and colleagues used BAC cloning to create a temperature-sensitive IE2 86 mutant virus containing the point mutation C509G (C510G in AD169) and showed that it is able to transactivate the UL112-113 promoter at 32.5°C, but not at 39.5°C. Additionally, it exhibits increased transcription from immediate early loci (95).

For this work, we have constructed and characterized four HCMV recombinant viruses with mutations in the IE2 86 gene: internal deletions of amino acids 356 to 359, 427 to 435, or 505 to 511, or substitutions of alanine for arginine at positions 356, 357, and 359. These mutations were selected on the basis of the IE2 86 domain mapping and functional studies discussed above and are all located in the C-terminal region important for protein-protein interactions and DNA binding. The IE2 86Δ356-359 and IE2 86R356/7/9A mutations remove or disrupt amino acids implicated in the activation of the UL112-113 and UL54 promoters, while the zinc finger and helix-loop-helix motifs are disrupted by the IE2 86Δ427-435 and IE2 86Δ505-511 mutations, respectively. Although none of the mutants are viable, they display differential phenotypes. We find that these mutants have varying degrees of increased

IE1 72 and IE2 86 expression compared to the wild type virus at early times post infection. The IE2 86Δ356-359 mutant shows wild type levels of IE1 72, IE2 86, and early gene products, but it is defective in the expression of late genes. IE2 86Δ427-435 and IE2 86Δ505-511 express increased levels of selected late transcripts at early times but do not express early gene products.

## MATERIALS AND METHODS

**Cells.** Human foreskin fibroblasts (HFFs) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 200 U penicillin, 200 µg streptomycin, 1.5 µg Amphotericin B, and 50 µg gentamicin per ml and were grown as described previously (216).

**Molecular cloning.** Construction of the shuttle plasmids for use in the BAC mutagenesis procedure began with the plasmid pHCMV *Eco*RI J (216), which contains the HCMV strain AD169 MIE region. The 10-kbp J fragment was liberated from this plasmid by *Eco*RI digestion, gel purified, and blunt ended with Klenow enzyme. *Kpn*I linkers were ligated onto both ends of the fragment, and it was subcloned into the unique *Kpn*I site present in pST76K_SR (a gift of Martin Messerle, Ludwig-Maximilians-Universität München), resulting in the shuttle plasmid wtJ-pST76K_SR. The pST76K_SR parental plasmid contains a kanamycin resistance marker, the *SacB* gene under the control of the lac promoter, and a gene encoding a temperature sensitive RecA mutant that provides recombinase activity at 30°C but not above 37°C. This plasmid also contains an origin of replication and a *rep ts* gene, which permit replication at 30°C but not above 37°C.

For construction of a template for site-directed mutagenesis, an 875 bp *Fse*I-*Stu*I fragment isolated from pHCMV *Eco*RI J was cloned into pFastBacI (Invitrogen, Carlsbad, CA). Mutagenic oligonucleotide primers were used in conjunction with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to create four plasmids, each containing one of the following deletions or a 3 aa-substitution in the

UL122 ORF: Δ356-359, Δ427-235, Δ505-511, or R356/357/359A. The numbering used here indicates the amino acids removed from the IE2 86 protein, and the plasmids are pFB-86Δ356-359, pFB-86Δ427-435, pFB-86Δ505-511, and pFB-86R356/7/9A. These constructs were cycle sequenced using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (United States Biochemical, Cleveland, OH) to verify the presence of the correct mutations. The sequences of the mutagenic primers (Integrated DNA Technologies, Coralville, IA) are as follows: for pFB-86Δ356-359, sense: 5' GCACACCCAACGTGCAGACTGTCAAGATTGACGAGGTGAG 3', antisense: 5' CTCACCTCGTCAATCTTGACAGTCTGCACGTTGGGTGTGC 3'. For pFB-86Δ427-435, sense: 5' GTGAAGAGTGAGGTGGATGCGGTGCTGGCCCTCTCCACTCCCTTCCTC 3', antisense: 5' GAGGAAGGGAGTGGAGAGGGCCAGCACCGCATCCACCTCACTCTTCAC 3'. For pFB-86Δ505-511, sense: 5' GCCACCCCCGTGGACCTGTTGGGCCTGATGCAAAAGTTTCCCAAACAG 3', antisense: 5' CTGTTTGGGAAACTTTTGCATCAGGCCCAACAGGTCCACGGGGGTGGC 3'. For pFB-86R356/7/9A, sense: 5' CCCAACGTGCAGACTGCGGCGGGTGCCGTCAAGATTGACGAG 3', antisense: 5' CTCGTCAATCTTGACGGCACCCGCCGCAGTCTGCACGTTGGG 3'.

pFB-86Δ356-359, pFB-86Δ427-435, pFB-86Δ505-511, and pFB-86R356/7/9A were digested with *Fse*I and *Stu*I and the resulting 863-, 848-, 854-, and 875-bp fragments were gel purified. The fragments were then ligated with the 15.5-kbp

fragment resulting from an *Fse*I and *Stu*I digest of wtJ-pST76K_SR to produce the four shuttle plasmids containing the 12-, 27-, and 21-bp deletions or the three substitutions in the IE2 86 coding region. These shuttle plasmids were sequenced as described above to confirm that the mutations were present and that the reading frame had been preserved.

**BAC mutagenesis.** Mutagenesis of the wild-type HCMV strain AD169 BAC pHB5, obtained from M. Messerle (26), was performed by using the shuttle plasmids described above. Briefly, *E. coli* strain DH10B containing pHB5 was transformed by electroporation with one of the shuttle plasmids. Transformants were selected on Luria-Bertani (LB)-chloramphenicol (30 μg/ml) or -kanamycin (30 μg/ml) plates at 30°C, then replated onto LB-chloramphenicol and -kanamycin plates and grown at 43°C to select for bacteria in which a BAC-shuttle plasmid cointegrate had formed. The resulting clones were transferred to LB-chloramphenicol plates and grown at 30°C to allow recombination and resolution of the cointegrates. Two rounds of sucrose selection and the confirmation of kanamycin sensitivity were used to isolate mutants, and the resulting BACs (p86Δ356-359, p86Δ427-435, p86Δ505-511, and p86R356/7/9A) were sequenced as above to confirm that each was a pure mutant containing the correct sequences.

Rescued BACs were generated from the deletion mutants by the same method. *E. coli* strain DH10B was transformed with one of the three deletion mutant BACs (p86Δ356-359, p86Δ427-435, or p86Δ505-511) and was made electrocompetent. The resulting bacteria harboring a mutant HCMV BAC were each transformed with wtJ-pST76K_SR. Clones containing a rescued BAC were isolated by the selection

procedure described above, and the presence of wild type IE2 86 sequences in the resulting BACs, p86Δ356-359rescue, p86Δ427-435rescue, and p86Δ505-511rescue, was verified by sequencing. Wild-type, mutant, and rescue BAC DNAs were amplified and purified as described previously (188). Each BAC was subjected to digestion with *Eco*RI and gel electrophoresis to ascertain that no major alterations to the DNA were sustained during the cloning procedure.

**Electroporation time course with BAC viruses.** HFFs ($3.2 \times 10^6$) were transfected by electroporation with 2 μg BAC DNA and 1 μg pcDNApp71tag (a gift of Bodo Plachter, University of Mainz) for RNA isolation or with 6.25 μg BAC DNA and 3.75 μg pcDNApp71tag for immunofluorescence and genome quantification in a BTX ECM-600 electroporator (Genetronics, Inc.). Briefly, confluence synchronized HFFs (186) were trypsinized, pelleted, washed in MEM plus 100 mM HEPES with no antibiotics or serum, and resuspended in the same medium at a concentration of $4 \times 10^6$ cells/ml. Eight-hundred-microliter samples of resuspended cells were added to 4-mm-gap cuvettes containing BAC DNA and pcDNApp71tag and were mixed. Cells and DNA were electroporated at 300 V, 2500 μF, and 72 W, and the resulting pulse lengths were typically between 26 and 28 ms. Immediately after electroporation, cells were recovered in MEM supplemented as described above plus 100 mM HEPES and were seeded into 75-$cm^2$ flasks or onto sterile coverslips in 12-well dishes for immunofluorescence assays (IFAs). Mock-treated cells received the pp71 expression vector alone. One, 3, 6, and 9 days postelectroporation, cells were harvested from flasks for RNA analysis or fixed on coverslips for IFAs.

**Quantitative real-time RT-PCR.** Total RNA was isolated from cell pellets by using a NucleoSpin RNA II kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The concentration of each sample was determined by UV spectrophotometry. Quantitative real-time reverse transcription (RT)-PCR was performed in an Applied Biosystems ABI Prism 7700 Sequence Detection System, using the TaqMan One-Step RT-PCR master mix reagents kit (Applied Biosystems) and oligonucleotide primers and TaqMan dual-labeled (5'-FAM and 3'-black hole quencher) probes (Integrated DNA Technologies, Coralville, IA) designed with Primer Express software (Applied Biosystems) (Table 1). Each probe spanned a splice junction in the transcript of interest. RT-PCR reactions contained 50 ng of total RNA each and were performed in duplicate. Additionally, the RNA isolated one day post electroporation from cells that received pHB5 BAC DNA was used to generate a standard curve for each gene examined. The standard curve was used to calculate the relative amount of specific RNA present in a sample, from which the fold induction of transcription of the gene was calculated by comparison to wild-type values at one day postelectroporation. As an additional control for the inclusion of equal amounts of RNA in each reaction, samples were analyzed with primers and TaqMan probe specific to the cellular housekeeping gene glucose-6-phosphate dehydrogenase (G6PD).

**Normalization of transfected viral genomes.** At 24 h postelectroporation, cells seeded on coverslips were washed in phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde in PBS, and then stained for MIE protein expression as described below. Cells in flasks were harvested and nuclear DNA was isolated as

**Table 2.1** Quantitative real-time RT-PCR primer and TaqMan probe sequences.

| Transcript | Primer/probe sequences | | |
|---|---|---|---|
| | Forward primer | Reverse primer | TaqMan probe |
| IE1 | CAAGTGACCGAGGATTGCAA | CACCATGTCCACTCGAACCTT | TCCTGGCAGAACTCGTCAAACAGA |
| IE2 | TGACCGAGGATTGCAACGA | CGGCATGATTGACAGCCTG | TGGCAGAACTCGGTGACATCCTCGCC |
| UL112-113 | TGACGGACGTGGCCG | CAATCATTGAGCATTTTGGTCAA | CCGACGGAATCCGCGGCGCCTCAG |
| UL89 | GGCGCTTTTTGCCAGTTG | ACCAGCAGCAAGTGGAAGTTTT | TACAACACCAACAGCATCCGAGGAC |
| R160461 | CAGTCCGCCGCCCAA | AGATTTCTGTTCGTAAACTTATCCGTT | TGCGCGTCCCAGGTACCACCCGTCGA |
| UL77 | CGTTGCCCGGGAACG | GGTGTGAAAGCGGATAAAGGG | ACCTAGCTACTTTGGAATCACGCAGAACGA |
| G6PD | TCTACCGCATCGACCACTACC | GCGATGTTGTCCCGGTTC | ATGGTGCTGAGATTTGCCAACAGGA |

previously described (207). Fifty nanograms of nuclear DNA was analyzed by quantitative real-time PCR with primers and TaqMan probe specific for the unspliced UL77 gene. The ratio of viral DNA recovered to the number of IE-expressing cells by immunofluorescence varied by less than twofold among the transfected BACs tested.

**Immunofluorescence.** At 24 h or 9 days postelectroporation, cells seeded on coverslips were washed in PBS and fixed in 2% paraformaldehyde in PBS. Cells were permeabilized in 0.2% Triton-X 100 and stained as previously described (188). After 30 min of blocking in 10% normal goat serum in PBS, cells were incubated with a primary antibody in 5% normal goat serum at the following dilutions: CH16.0 monoclonal antibody (MAb) specific to the region shared by IE1 72 and IE2 86, 1:1000; IE1 72-specific MAb p63-27, 1:500; IE2 86-specific MAb IE 2.9.5, no dilution; IE2 86 rabbit polyclonal antibody 1218, 1:250; UL112-113 MAb m23, 1:5; UL44 MAb, 1:1000; and pp28 MAb 41-18, no dilution. Monoclonal antibodies p63-27, 2.9.5, and 41-18 were gifts from William Britt (University of Alabama, Birmingham), m23 was a gift from Masaki Shirakata (Tokyo Medical and Dental University), and the polyclonal antibody directed against IE2 86 was a gift from Jay Nelson (Oregon Health Sciences University). CH16.0 and anti-UL44 were purchased from the Goodwin Institute (Plantation, Fla.). After three washes in PBS, coverslips were incubated with Hoechst and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) (Southern Biotech, Birmingham, AL) (for CH16.0, UL112-113, UL44, and pp28 stains); FITC-conjugated goat anti-mouse IgG2a and tetramethyl rhodamine isocyanate (TRITC)-conjugated goat anti-mouse IgG3 (for IE1 and IE2 stains of IE2 86Δ356-359 and IE2 86Δ505-511 BAC-

electroporated cells); or TRITC-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (for IE1 and IE2 stains of IE2 86Δ427-435 BAC-electroporated cells), diluted 1:75 to 1:200 (Jackson ImmunoResearch Laboratories, West Grove, Pa). Coverslips were washed and mounted onto slides with SlowFade antiphotobleaching reagent (Molecular Probes, Eugene, Oreg.). Images were captured using a Nikon Eclipse E800 microscope and Photometrics CoolSnap fx CCD camera (for IE2 86Δ427-435 electroporations) or a Leica DMRB microscope and Hammamatsu C5810 Color 3CCD Camera (for IE2 86Δ356-359 and IE2 86Δ505-511 electroporations) by use of Metamorph software (Universal Imaging Corp., Downington, PA) and were processed with Adobe Photoshop.

## RESULTS

**Recombinant HCMV mutant and rescue BAC production.** To construct the four mutant BACs, we used site-directed mutagenesis to make in-frame deletions of aa 356 to 359, 427 to 435, or 505 to 511 from the coding region of IE2 86, or mutation of the arginines at positions 356, 357, and 359 to alanines (Figure 2.1A). We chose to focus on deletions rather than amino acid substitutions to reduce the possibility of mutants reverting to the wild-type sequence, as we find that in our hands, substitution mutants with changes in the IE2 86 sequence rapidly undergo reversion at one or more of the altered sites. While the parent BAC used in this study was derived from HCMV strain AD169, the numbering we used reflects the more commonly used Towne amino acid sequence, which differs from AD169 primarily by the deletion of one residue in the set of serines at aa 258 to 264 (16). The intermediate constructs were sequenced to confirm the presence of the correct deletion and preservation of the reading frame, and a portion of the IE2 gene containing the deletion was then cloned into the shuttle vector wtJ-pST76K_SR in place of the corresponding wild type sequence. *E. coli* strain DH10B containing the wt HCMV BAC pHB5 was transformed with a shuttle vector, and mutant BACs were isolated as described in Materials and Methods. The BACs were isolated and checked for the presence of the correct sequences by DNA sequencing. A comparison of the *Eco*RI restriction digest patterns of the newly constructed mutants to that of pHB5 was also used to confirm that no large-scale rearrangements of the BAC DNA had occurred during the cloning procedure (Figure 2.1B). To confirm that the phenotypes observed

in this study were not due to mutations that had occurred elsewhere in the HCMV genome, we rescued the deletion mutant BACs, returning each to the wt as described in Materials and Methods.

Each of these deletions removes or disrupts a region predicted to be needed by IE2 86 to transactivate downstream promoters. The aa 356 to 359 deletion removes a region that has been shown to be important for the activation of the viral early promoter for the UL54 gene (210), and the arginine to alanine substitution mutant similarly disrupts this region without introducing a deletion that might result in a large-scale alteration of the IE2 86 structure. Removal of aa 427 to 435 disrupts a predicted zinc finger, which when altered in other studies resulted in a protein that can activate transcription of the MIE region but cannot bind DNA or autorepress transcription (115, 141). Finally, the aa 505 to 511 deletion mutant is missing part of a leucine-rich region that is predicted to form a helix-loop-helix structure (77).

To determine the effects of these deletions on the viability of the virus, we transfected the wild-type BAC, mutant BACs, and BACs generated by rescue of the deletion mutants into HFFs along with a pp71 expression construct and observed the cultures for development of a cytopathic effect. Beginning at 6 days postelectroporation, plaques were visible in cultures that received the wild type or each of the rescued BACs. In contrast, plaques were not observed in cultures transfected with any of the mutants for several repetitions of the experiment. Since this result indicates that the residues that were deleted from IE2 86 are required for a productive infection, we proceeded to determine at what stage during the infection the life cycle of each of these mutants is blocked.

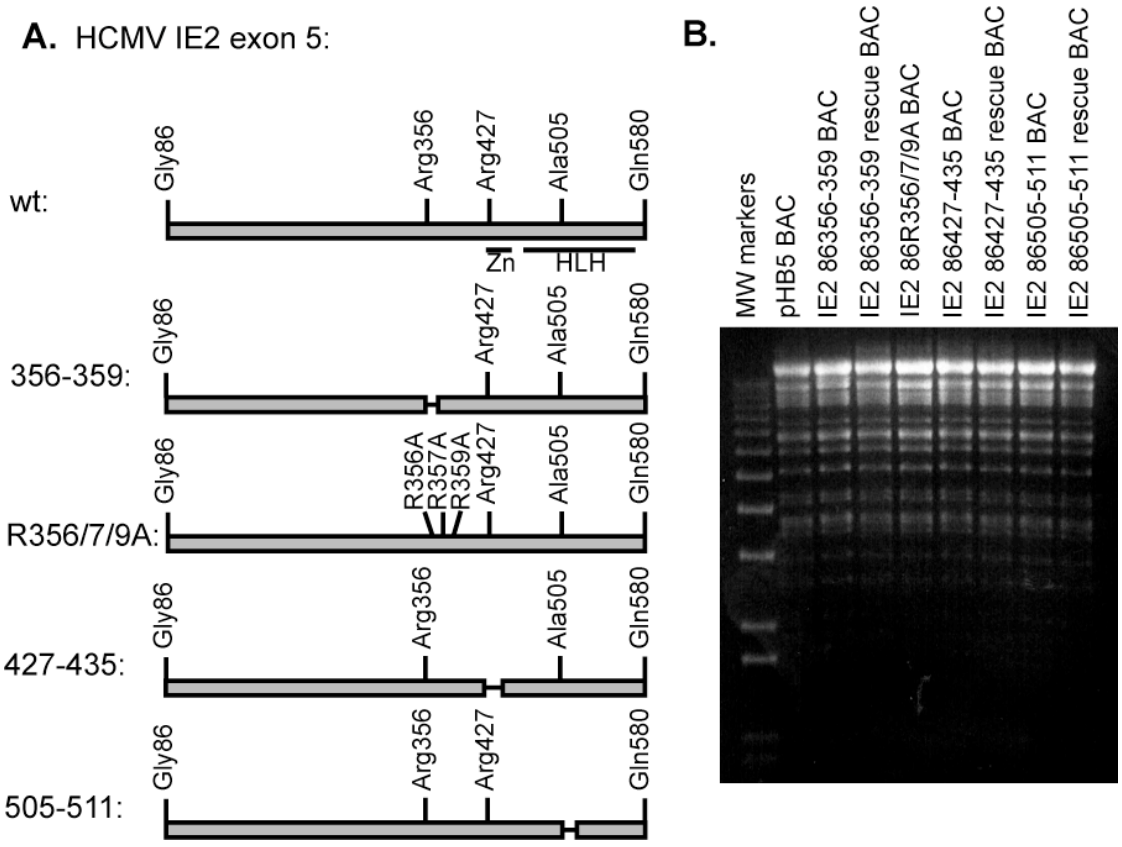
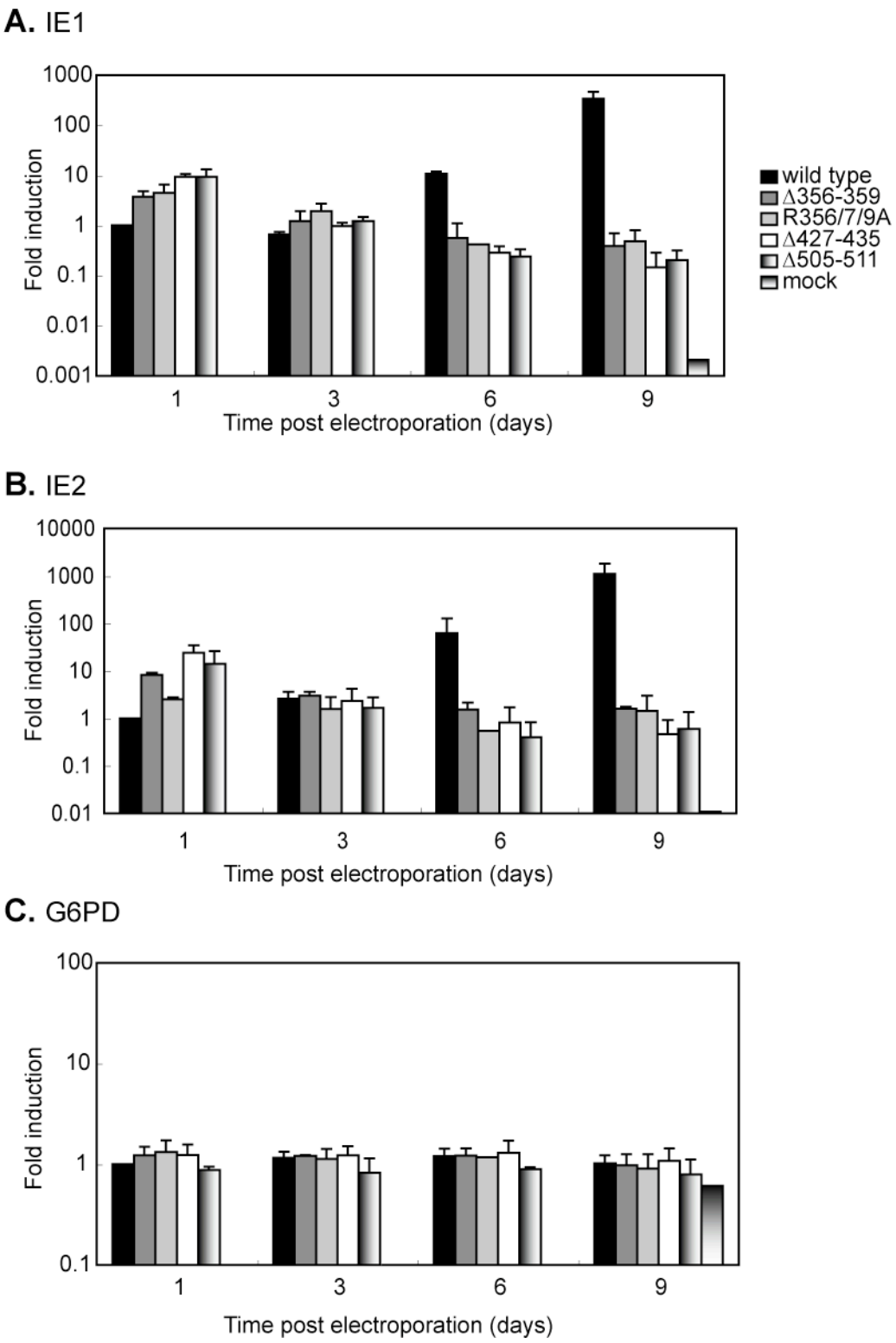



**Figure 2.1 Construction of the HCMV IE2 86 deletion mutant BACs.** (A) Schematic of exon 5 of the HCMV MIE region. For construction of the four mutants, IE2 86Δ356-359, IE2 86R356/7/9A, IE2 86Δ427-435, and IE2 86Δ505-511, regions of the exon were deleted or amino acids were substituted as shown. Zn, zinc finger domain; HLH, helix-loop-helix motif. (B) *Eco*RI digests of the HCMV BAC clones. 3 micrograms of wild-type (pHB5), mutant, or rescued mutant BAC DNA was cut with *Eco*RI, and the resulting digests were separated on a 0.6% agarose gel in 1× Tris-borate-EDTA. MW, molecular weight markers.

**IE gene expression is increased in IE2 86 mutant-electroporated cells.** To investigate the kinetics of IE gene expression from the IE2 86 deletion mutants, we electroporated the wild type, mutant, and rescued BACs into $G_0$ confluence-synchronized primary human fibroblasts along with a pp71 expression vector to promote viral replication. A mock sample received the pp71 construct alone. Total RNA was isolated from cells harvested 1, 3, 6, and 9 days post electroporation, and IE1 and IE2 transcript levels were measured by quantitative real-time RT-PCR. Each RT-PCR reaction was performed in duplicate and standardized to threshold cycle values obtained for reactions containing a known amount of RNA. In addition, RNA samples were analyzed by real-time RT-PCR with G6PD-specific primers and probe to ensure that RNA concentrations in all samples were approximately equal (Figure 2.2C). Rescued viruses behaved essentially like wild type for each target analyzed by RT-PCR or stained by immunofluorescence.

All three mutants displayed, to various degrees, up-regulation of IE1 72 and IE2 86 transcripts at early times postelectroporation. The IE2 86Δ356-359 and IE2 86R356/7/9A mutants show a pattern of IE1 72 gene expression that was most similar to that of the wild type at early times postelectroporation (Figure 2.2A). Both mutants expressed three to four times more IE1 72 1 day after electroporation than the wild type and IE2 86Δ356-359 rescue viruses did, and the two mutants continued to express slightly more IE1 72 transcript than wild type 3 days after electroporation. After six days, visible plaques indicated that the wild type and IE2 86Δ356-359 rescue viruses had spread to infect neighboring cells in the second round of infection. IE1 72 transcript levels increased to approximately 12 times their day one values as a result of

**Figure 2.2 Increased IE1 and IE2 transcription at IE times in cells electroporated with the IE2 86 mutant BACs.** $G_0$-synchronized HFFs were electroporated with pHB5, IE2 86Δ356-359, IE2 86R356/7/9A IE2 86Δ427-435, or IE2 86Δ505-511 BAC and pcDNApp71tag and were harvested at the indicated time points post electroporation. Total cellular RNA was analyzed by quantitative real-time RT-PCR as described in Materials and Methods to measure the presence of IE1 72 (A) or IE2 86 (B) transcripts. Values displayed in the graphs are the averages of two to four independent experiments, with each conducted using duplicate reactions. Error bars indicate one standard deviation from the means of the combined experiments. To ensure that an equal amount of RNA was included in each reaction, we analyzed samples with G6PD-specific primers and probe (C).


A. IE1
1000
100
10
1
0.1
0.01
0.001
Fold induction
1
3
6
9
Time post electroporation (days)
wild type
Δ356-359
R356/7/9A
Δ427-435
Δ505-511
mock
B. IE2
10000
1000
100
10
1
0.1
0.01
Fold induction
1
3
6
9
Time post electroporation (days)
C. G6PD
100
10
1
0.1
Fold induction
1
3
6
9
Time post electroporation (days)

this spread. In contrast, IE1 72 expression from the IE2 86Δ356-359 and IE2 86R356/7/9A mutants decreased after day 3 postelectroporation, consistent with the lack of spread of these two recombinants.

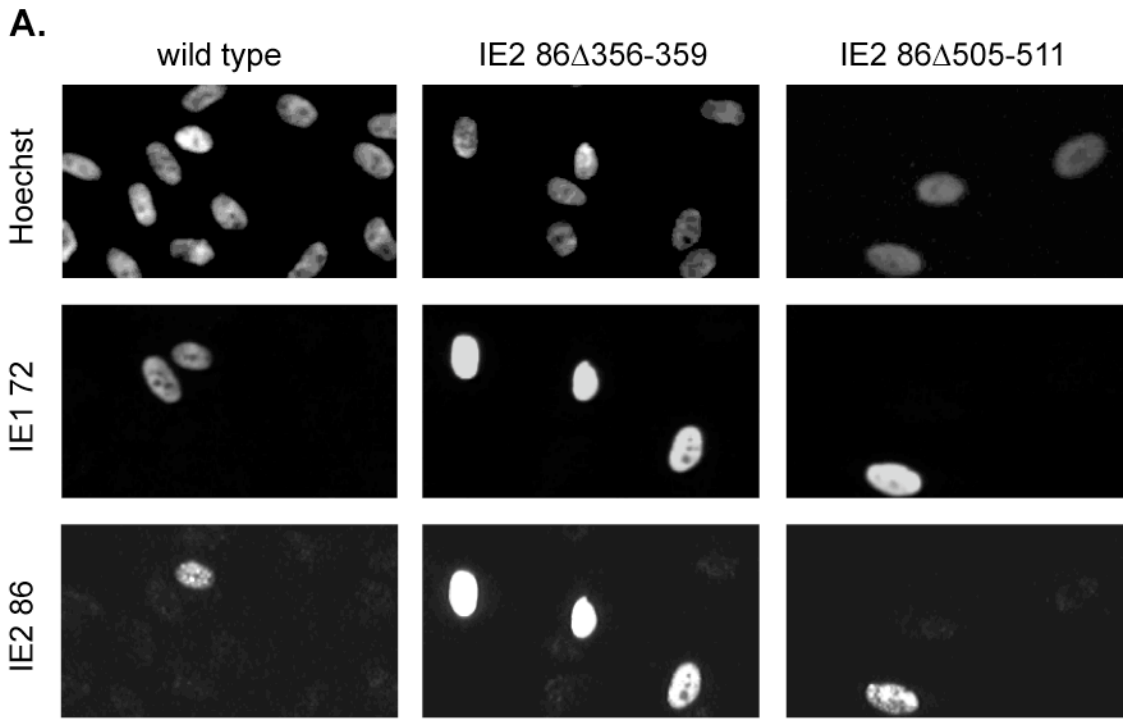
The pattern of IE1 72 gene expression by the IE2 86Δ427-435 and IE2 86Δ505-511 mutants was similar, but was up-regulated more compared to the wild type than was that of the aa 356 to 359 region mutants 1 day after electroporation of the cells. Specifically, the mutants expressed approximately 10-fold more IE1 72 at this time (Figure 2.2A). IE1 72 expression in the cells that received these mutant BACs then fell to levels approximating those in cells with the wild-type and rescue viruses by day 3 and continued to decrease for the duration of the time course. In contrast, IE1 72 transcript levels in the cells electroporated with wild-type and rescue viruses were induced 75- to 300-fold over their day 1 levels by the end of the time course.

IE2 expression by the four mutants and corresponding rescue viruses was characterized similarly after electroporation into HFFs (Figure 2.2B and data not shown). Like IE1 72, IE2 86 expression 1 day after electroporation was slightly elevated in the cells that received IE2 86Δ356-359 (8-fold up-regulation) or IE2 86R356/7/9A (2.5-fold up-regulation) relative to those that received the wild type and rescue viruses. IE2 86Δ427-435 and IE2 86Δ505-511 mutants similarly produced 14 to 24 times more IE2 RNA at this time than did the wild-type virus. IE2 expression fell to wild-type levels by day 3 postelectroporation for all four viruses and then decreased or remains level throughout the time course.

To determine the transfection efficiencies and relative expression levels of IE1 and IE2 in individual cells, we further examined the cultures by IFAs with antibodies specific for IE1 and IE2. At 1 day postelectroporation, single IE1- and IE2-positive cells were present in cultures electroporated with each of the IE2 deletion mutants (Figure 2.3 and data not shown). The number of positive cells was approximately equal to the number of IE1- and IE2-positive cells electroporated with the wild-type BAC. By the end of the time course, IE1- and IE2-positive plaques were evident in wild-type transfected cultures. In cells that were electroporated with the IE2 mutants, only single IE1 and IE2 positive cells were observed, indicating that no spread of the virus from the first transfected cell to neighboring cells had occurred (Figure 2.4).

As an additional control to ensure that changes in gene expression observed in mutant BAC-electroporated cells were not due to variations in transfection efficiency, we performed test transfections of each of the wild-type or deletion mutant BACs and harvested cells for isolation of nuclear DNA and for IFA 24 h postelectroporation, before replication of the viral genome had begun. Equal amounts of DNA isolated from the nuclei of wild-type or deletion mutant-electroporated cells were used as templates in quantitative real-time PCRs (data not shown) with primers and probes specific for the portion of the HCMV genome encoding the unspliced UL77 gene. Cells fixed for immunofluorescence were stained with an antibody detecting the region common to IE1 72 and IE2 86 proteins, and the number of cells expressing IE proteins by IFA was compared to the relative amount of transfected viral genome determined by quantitative PCR. The ratio of DNA transfected to IE positive cells

**Figure 2.3 IE1 72 and IE2 86 expression in wild-type- and IE2 86 mutant BAC-electroporated cells at 1 day postelectroporation.** Cells were seeded onto sterile coverslips in 12-well dishes after electroporation with pcDNApp71tag and pHB5, IE2 86Δ356-359, IE2 86Δ505-511 (A), or IE2 86Δ427-435 (B), and 24 h later they were fixed with 2% paraformaldehyde in PBS. Cells were stained with Hoechst dye to visualize nuclei, anti-IE1 72 monoclonal antibody p63-27, and either anti-IE2 86 monoclonal antibody IE 2.9.5 (A) or anti-IE2 86 rabbit polyclonal antibody (B), followed by appropriate FITC- or TRITC-conjugated secondary antibodies (see Materials and Methods). Magnification, ×400.


A.
wild type
IE2 86Δ356-359
IE2 86Δ505-511
Hoechst
IE1 72
IE2 86

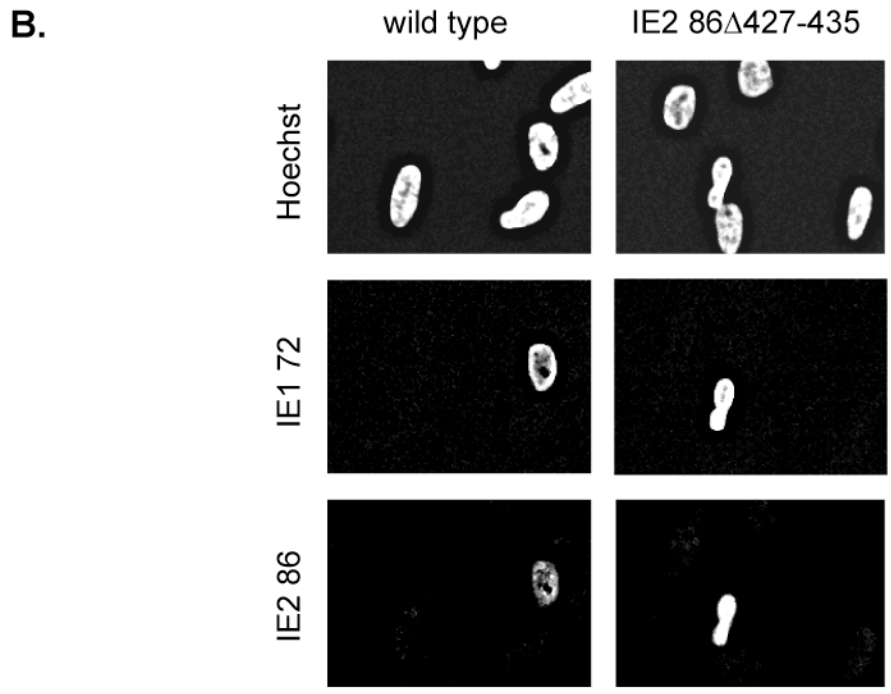
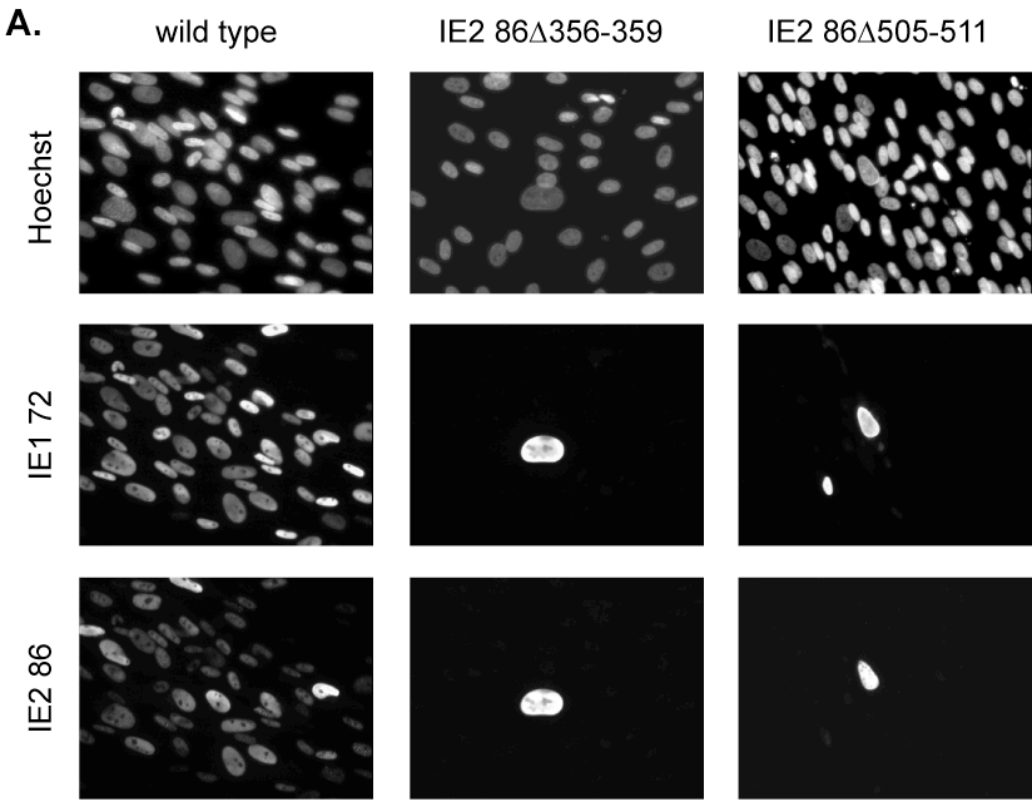
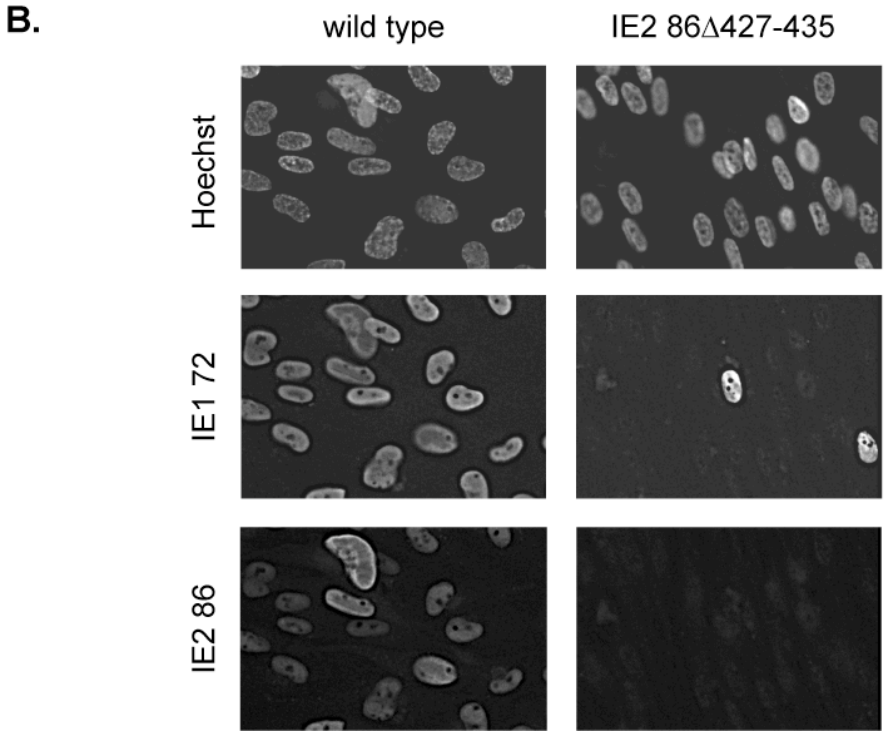

B.
wild type
IE2 86Δ427-435
Hoechst
IE1 72
IE2 86

**Figure 2.4 IE1 72 and IE2 86 expression in wild-type- and IE2 86 mutant BAC-electroporated cells at 9 days postelectroporation.** Cells were seeded onto sterile coverslips in 12-well dishes after electroporation with pcDNApp71tag and pHB5, IE2 86Δ356-359, IE2 86Δ505-511 (A), or IE2 86Δ427-435 (B), and 9 days later they were fixed with 2% paraformaldehyde in PBS. Coverslips were processed as described (in the legend for Fig. 3 and Materials and Methods). Magnification, ×400.


A.
wild type
IE2 86Δ356-359
IE2 86Δ505-511
Hoechst
IE1 72
IE2 86


B.
wild type
IE2 86Δ427-435
Hoechst
IE1 72
IE2 86

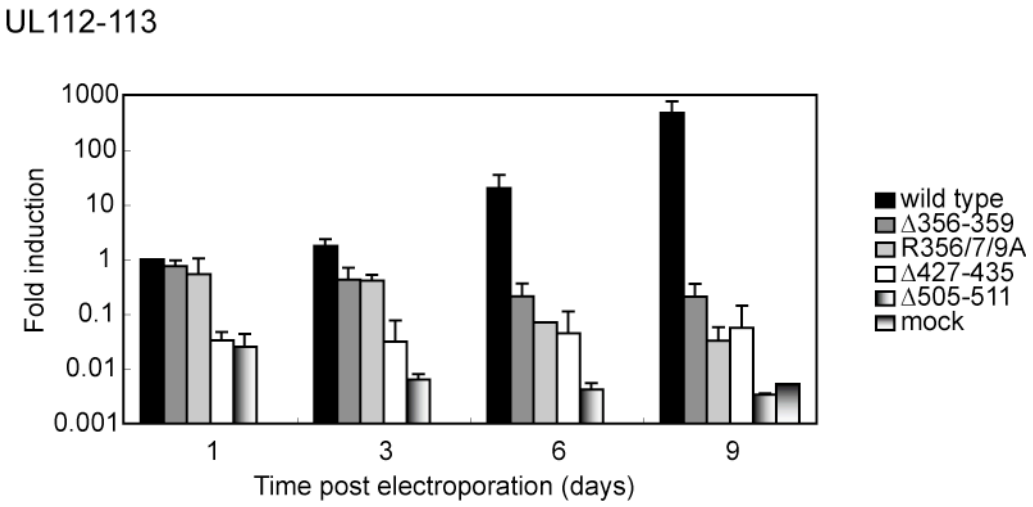



**Figure 2.5 Reduced UL112-113 transcription in IE2 86 mutant BAC-electroporated cells.** Total cellular RNAs were isolated from electroporated cells as described in Materials and Methods and the legend for Fig. 2 and were analyzed by quantitative real-time RT-PCR with UL112-113 specific primers and a TaqMan dual-labeled probe. Values displayed in the graphs are the averages of two to four independent experiments, with each conducted using duplicate reactions. Error bars indicate one standard deviation from the means of the combined experiments.

varied less than twofold among the four viruses tested, indicating that transfection efficiency does not significantly impact the observed changes in viral gene expression.

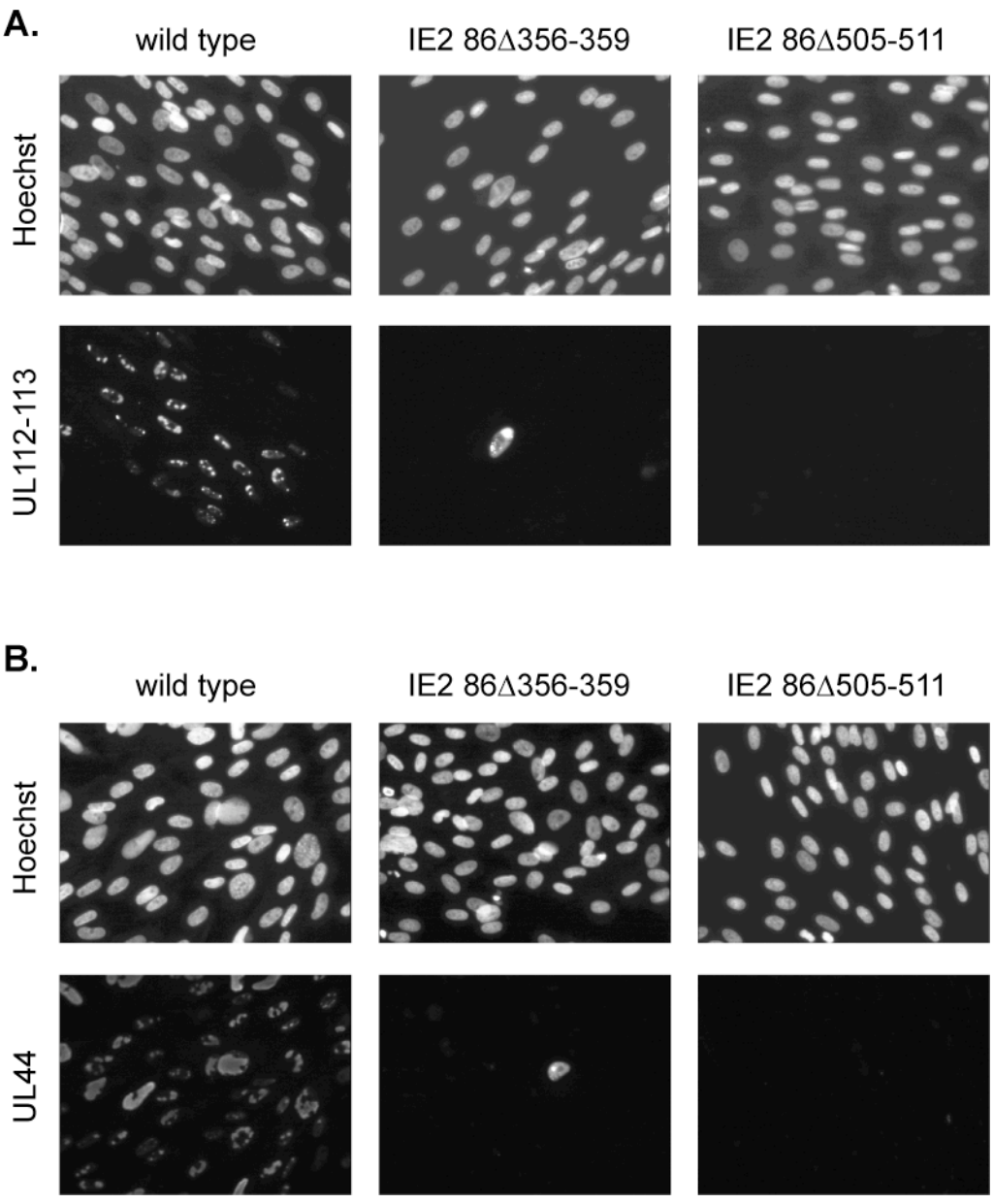
**Early gene expression levels are low in IE2 86Δ356-359- and IE2 86R356/7/9A-transfected cells and are severely reduced in IE2 86Δ427-435- and IE2 86Δ505-511-transfected cells.** Transcription of the UL112-113 region, which is expressed with early kinetics, was also measured by using real-time RT-PCR (Figure 2.5). The IE2 86Δ356-359 and IE2 86R356/7/9A mutants expressed near-wild-type levels of UL112-113 transcripts 1 day after electroporation. After day 3, wild type and rescue virus transcript levels increased dramatically, while in cells electroporated with either mutant, the amount of UL112-113 transcript decreased after day 1 and remained low throughout the time course. In contrast, in cells electroporated with IE2 86Δ427-435 and IE2 86Δ505-511, the amount of UL112-113 RNA present after 1 day was >10-fold less than that in cells electroporated with the wild-type or rescued BAC DNA. By day 3 or 6, UL112-113 induction remained approximately constant or had decreased further in IE2 86Δ427-435 and IE2 86Δ505-511-electroporated cells. In contrast, the wild-type and rescue viruses had spread rapidly and progressed through a second round of replication as demonstrated by the 2- to 3-log induction of UL112-113 expression in each of these cultures.

To examine early gene expression in the individual cells following electroporation with the mutant BACs, we used immunostaining directed against the UL112-113 and UL44 proteins. Consistent with the UL112-113 RT-PCR data, we observed UL112-113 expression by IFA in a few IE2 86Δ356-359 transfected cells 9 days after electroporation, but not in cells that received IE2 86Δ427-435 or IE2

86Δ505-511 BAC DNA (Figure 2.6A and data not shown). The pattern of expression of UL112-113 in cells that were electroporated with the IE2 86Δ356-359 mutant was comparable to that in cells that were electroporated with the wild-type BAC, but remained restricted to single isolated cells due to the inability of the mutant virus to spread to neighboring cells. Similarly, UL44-positive plaques were evident in cells 9 days after electroporation with wild-type BAC, but UL44 expression was restricted to single isolated cells following electroporation with IE2 86Δ356-359 and was not observed after electroporation with the other deletion mutants (Figure 2.6B and data not shown). IE2 86Δ356-359 DNA replication in BAC-transfected cells, however, was not detectable by slot blot analysis (data not shown).

**Delayed early and late gene expression is increased at early times and decreased at late times in IE2 86Δ427-435 and IE2 86Δ505-511 electroporated cells.** The expression of UL89, the DNA terminase that is involved in viral genome maturation, begins at early times and increases through late times postinfection. The R160461 gene is expressed exclusively with late kinetics, and both the UL89 and R160461 transcripts are spliced (175). We measured the induction of UL89 and R160461 transcripts in cells that were electroporated with each of the four IE2 mutant BACs at various times postelectroporation. As seen previously for the characterization of IE and early gene expression, the IE2 86Δ356-359 and IE2 86R356/7/9A mutants were most similar to the wild type, with slightly elevated levels of expression of both genes 1 day after electroporation (figure 2.7). These mutants showed variable increases in the amount of both UL89 and R160461 RNAs between

**Figure 2.6 UL112-113 and UL44 expression in IE2 86Δ356-356 BAC-electroporated cells.** Cells were seeded onto sterile coverslips in 12-well dishes after electroporation with pcDNApp71tag and pHB5, IE2 86Δ356-359, or IE2 86Δ505-511, and 9 days later they were fixed with 2% paraformaldehyde in PBS. Coverslips were stained with Hoechst dye to visualize nuclei and with anti-UL112-113 monoclonal antibody m23 (A) or anti-UL44 monoclonal antibody (B) followed by FITC-conjugated anti-mouse secondary antibody. The blank fields for UL112-113 and UL44 staining of IE2 86Δ505-511 electroporated cells are included for comparison and are representative of the lack of UL112-113- and UL44-positive cells that were electroporated with either the IE2 86Δ427-435 or IE2 86Δ505-511 BACs. Magnification, ×400.


A.
wild type
IE2 86Δ356-359
IE2 86Δ505-511
Hoechst
UL112-113
B.
wild type
IE2 86Δ356-359
IE2 86Δ505-511
Hoechst
UL44

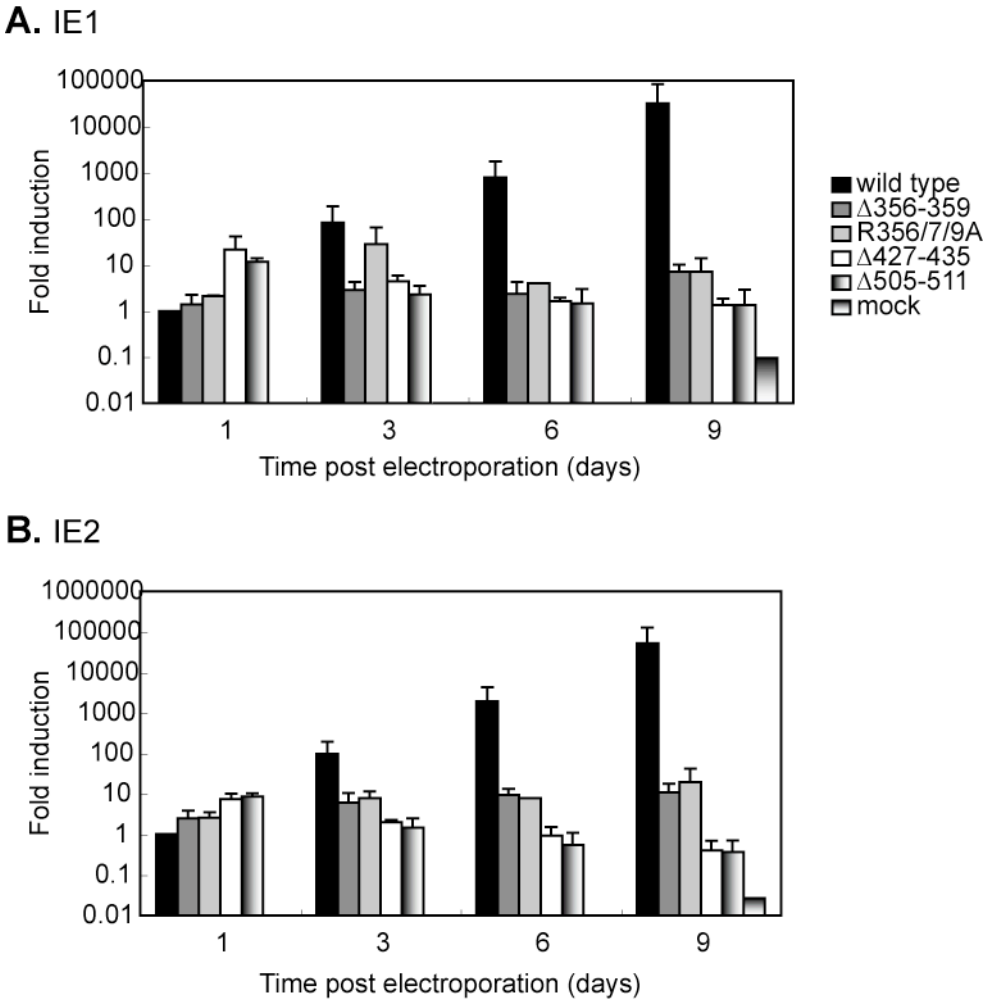



**Figure 2.7 UL89 and R160461 transcription is increased at IE times in mutant BAC-electroporated cells.** Total cellular RNA was isolated from electroporated cells as described in Materials and Methods and the legend for Fig. 2 and were analyzed by quantitative real-time RT-PCR with UL89 (A)- or R160461 (B)-specific primers and TaqMan dual-labeled probes. Values displayed in the graphs are the averages of two to four independent experiments, with each conducted using duplicate reactions. Error bars indicate one standard deviation from the means of the combined experiments.

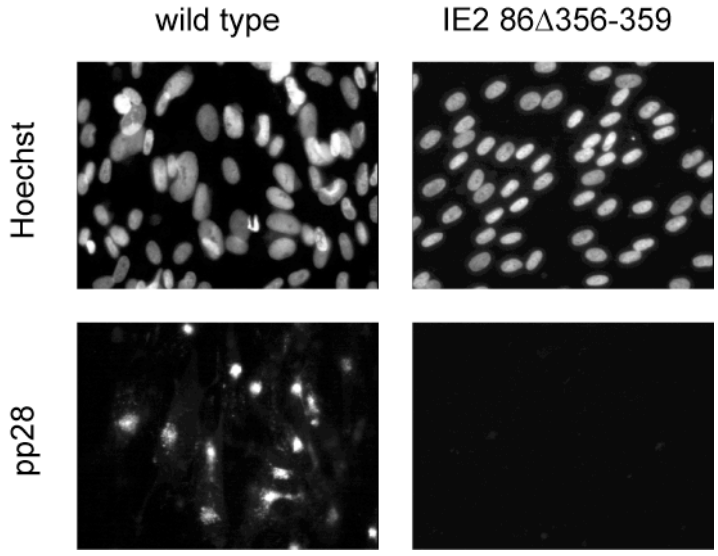



**Figure 2.8 pp28 expression in mutant BAC-electroporated cells.** Cells were seeded onto sterile coverslips in 12-well dishes after electroporation with pcDNApp71tag and pHB5 or IE2 86Δ356-359, and 9 days later, they were fixed with 2% paraformaldehyde in PBS. Coverslips were stained with Hoechst dye to visualize nuclei and with anti-pp28 monoclonal antibody 41-18 followed by FITC-conjugated anti-mouse secondary antibody. The blank field indicating the lack of pp28 staining in the IE2 86Δ356-359-electroporated cells is included for comparison and is representative of pp28 staining in cells electroporated with the IE2 86Δ427-435 or IE2 86Δ505-511 BAC. Magnification, ×400.

days 1 and 3 and then maintained this level throughout the time course. In contrast, by the end of the time course in cells electroporated with the wild-type BAC, the levels of UL89 and R160461 transcripts were nearly 1,000- or 10,000-fold higher than at day 1. This strong induction results from normal late gene expression in addition to spread of the wild-type and rescue viruses through the cultures.

Surprisingly, at 1 day postelectroporation of the IE2 86Δ427-435 or IE2 86Δ505-511 mutants, UL89 and R160461 expression levels were 11- to 21-fold higher than that observed in cells electroporated with the wild-type and rescued viruses. This suggests that these mutants either activate or fail to repress expression of these genes at very early times in the viral life cycle (Figure 2.7). The presence of a consensus CRS element in the UL89 promoter suggests that the defect may be lack of repression, and this will be discussed below. In contrast to the case of the aa 356 to 359 region recombinant viruses, as the time course progressed, these deletion mutants did not maintain elevated levels of UL89 and R160461 transcripts. Rather, the concentrations of the UL89 and R160461 RNAs decreased gradually through the experiment, in each case approaching the wild-type day 1 levels of expression by the last time point examined. In the case of all four mutant viruses, both transcripts were still clearly present above the limit of detection when compared to the uninfected control at 9 days post electroporation.

We also assessed the expression of pp28, which is encoded by UL99 and expressed with late gene kinetics. Because there are multiple colinear transcripts from this region, we used immunofluorescence to examine its expression (2). While pp28 is strongly expressed in wild-type electroporated cells 9 days postelectroporation, none

of the mutant viruses can support pp28 expression, even in single cells (Figure 2.8 and data not shown). This is consistent with the finding that gene expression by all of these mutants is blocked before the replication of the viral DNA.

## DISCUSSION

The HCMV IE2 86 protein has been extensively characterized in efforts to better understand its role as an essential regulatory factor in the viral life cycle. Much of this work has been performed in vitro, using mutational analysis to define regions of the protein that are required for the transcription of other viral genes. These studies showed that both protein-protein and protein-DNA interactions are involved in IE2 86's transactivation capabilities, but the assays were performed either with either bacterially expressed, purified IE2 86 protein or IE2 86 expression vectors in transient transfection experiments. In either case, the mutant protein was not examined in the context of the infected cell. We have taken advantage of the cloning of the HCMV genome as a BAC to construct recombinant viruses with mutations in the IE2 86 gene residing in the viral genome. The mutations remove aa 356 to 359, 427 to 435, and 505 to 511 or mutate arginines at residues 356, 357, and 359 to alanine and were chosen based on previous studies from our laboratory and others suggesting that these regions are particularly important to the ability of IE2 86 to transactivate other viral promoters. The region near aa 356 to 359 has been shown to affect both the transactivation and autoregulatory functions of IE2 86. In transient transfection assays, the insertion of four residues between amino acids 359 and 360 both reduces the ability of IE2 86 to transactivate the UL54 (DNA polymerase) promoter and limits its capacity to repress its own promoter (210). Similarly, an IE2 86 mutant in which the three residues from aa 358 to 360 are changed to an alanine, serine, and alanine has been examined by use of transient assays, in vitro work, and yeast one- and two-

hybrid systems. This mutant appears able to dimerize, but has little to no DNA binding capacity (as assayed by an electrophoretic mobility shift assay and yeast one-hybrid experiments, respectively) and does not repress transcription from the MIEP in transient transfection assays (6, 226).

The deletion of aa 427 to 435 removes residues involved in the formation of a predicted zinc finger, while the removal of aa 505 to 511 disrupts a putative helix-loop-helix. The zinc finger has been altered by the mutation of histidines 446 and 452 to leucine, resulting in a protein which can neither repress transcription of the MIEP in vitro nor bind DNA in an electrophoretic mobility shift assay (141). The helix-loop-helix region was recently analyzed by mutation of cysteine 509 to glycine (C510G in AD169) in an HCMV BAC, resulting in a temperature-sensitive virus (95). At the nonpermissive temperature, cells infected with this virus contain elevated levels of IE transcripts but no detectable early (UL44 and UL54) RNAs. Correspondingly, the C509G mutant does not replicate its DNA. In all three cases, it is important to note that three of the mutants we characterize here contain internal deletions in the IE2 gene rather than the insertions or point mutations described in other reports. We chose to introduce deletions based on the observation that in our system, single and multiple point mutations in the IE2 coding region undergo spontaneous reversion to the wild type in the infected cell, but we have also included the construction and analysis of a substitution mutant, IE2 86R356/7/9A, and shown that it behaves essentially the same as the corresponding deletion mutant. In this case, it appears that the phenotype observed is not due to a simple change in protein structure generated by deletion of these amino acids.

In the present report, we show that the three regions of IE2 86 described above are crucial to its function as a key regulator of HCMV infection. Mutation of any of these regions results in a recombinant virus that is not viable and is blocked in its replication early in the viral life cycle. To determine the location of these blocks, we used quantitative real-time RT-PCR and IFA to examine the expression of selected IE, early, and late genes at the RNA and protein levels. To ensure that only viral RNA and not input or replicated viral DNA was detected during RT-PCR analysis, we examined the transcription of selected spliced viral genes only and used real-time PCR probes spanning splice junctions in each target.

All four mutants up-regulated IE1 72 and IE2 86 expression compared to the wild type at early times. During the first round of infection, IE2 86Δ356-359 and IE2 86R356/7/9A expressed transcripts from the IE1 and IE2 regions at slightly elevated levels relative to those observed for the wild type (Figure 2.2). Immunostaining for IE1 72 and IE2 86 also indicated that protein levels in the mutants and wild type were comparable (Figure 2.3 and data not shown). In contrast, RT-PCR analysis of UL112-113 transcripts indicated near-wild-type transcription of this locus in IE2 86Δ356-359- or IE2 86R356/7/9A-electroporated cells (Figure 2.5). By IFA, both UL112-113 and UL44 were detectable in single cells that received the deletion mutant (Figure 2.6). We could not, however, detect DNA replication by slot blotting (data not shown) with cells transfected with the IE2 86Δ356-359 BAC. The transcription of delayed early (UL89) and late (R160461) loci by the IE2 86Δ356-359 or IE2 86R356/7/9A mutants was induced only slightly and remained low throughout the time course, and a representative late protein (pp28) was not detectable by immunostaining in IE2

86Δ356-359 electroporated cells (Figures 2.7 and 2.8). Taken together, these data suggest that the IE2 86Δ356-359 mutant can support IE and some early gene expression, but is blocked before DNA replication begins. The IE2 86R356/7/9A mutant behaved very similarly, indicating that the changes in gene expression induced by the deletion of these crucial amino acids are not simply the result of a structural alteration resulting from the deletion. These data are consistent with previous work in which this region of IE2 86, when disrupted and tested in transient assays, resulted in the decreased activation of early promoters and the 10-fold up-regulation of MIE transcription (210).

IE gene expression in the IE2 86Δ427-435 and IE2 86Δ505-511 electroporated cells was also different from that observed in cells containing the wild-type virus. At the earliest time examined postelectroporation, IE1 and IE2 transcripts were >10-fold higher in cells that received the mutant BACs than in those transfected with the wild type or rescued construct (Figure 2.2). While this induction was not observed for UL112-113 transcription, a similar pattern was seen when UL89 and R160461 transcripts were examined by RT-PCR. Early in the time course, UL89 and R160461 transcription was significantly increased compared to that of the wild type. This effect was most apparent at the transcriptional level at 1 day post electroporation, less apparent after 3 days, and not detectable later in the time course as a result of spread of the wild-type and rescue viruses through the culture. While this phenotype was also detectable in cells transfected with the IE2 86Δ356-359 and IE2 86R356/7/9A BACs, these more N-terminal disruptions resulted in an intermediate phenotype and a less striking up-regulation of selected delayed early and late genes.

Since the IE2 86Δ427-435 and IE2 86Δ505-511 mutations disrupt the zinc finger and helix-loop-helix motifs in the DNA binding domain of IE2 86, we speculate that the deleted regions, and possibly the amino acids near residue 356, are required for IE2 86 to bind the CRS. The deletion of these amino acids results in the loss of the capacity of IE2 86 to repress its own promoter and other CRS-containing promoters. This hypothesis is supported by the presence of a consensus CRS element directly upstream of the UL89 transcription start site. Many CRS elements are also located near the proposed site of initiation of R160461 transcription (175). These sequences are both upstream and downstream of the proposed TATAA box, although they do not appear to be in a location that would be expected to affect transcription. However, since the start site of the transcript is based on the sequence of a cDNA clone generated from infected cell RNA, it is possible that R160461 transcription initiates at a nearby, but different, site and may therefore be regulated by the same mechanism. Analysis of UL83 transcription at 1 day postelectroporation provided further support for this idea. UL83 encodes the matrix phosphoprotein pp65 and is expressed with delayed early kinetics, but it lacks a CRS element in its promoter region. We observed that at early times, UL83 transcripts were present at approximately equal levels in cells electroporated with the wild type, any of the three IE2 deletion mutants, or the rescued BACs (data not shown). Although we have not examined all possible late gene targets of regulation by IE2 86, these data suggest that the presence of a CRS upstream of the promoter of a delayed early or late gene may determine whether this locus will be overexpressed at early times in the mutant-electroporated cell. There are

other possibilities, including alternative regulation of spliced late genes by the mutant virus, and experiments to resolve this question are in progress.

In contrast, UL112-113 transcription is severely reduced in cells electroporated with the IE2 86Δ427-435 and IE2 86Δ505-511 mutants. The cells electroporated with these mutants contain <10% the amount of UL112-113 RNA in the wild-type-electroporated cells 1 day after transfection, and the expression of this transcript dropped to the limit of detection by days 3 and 6 for the IE2 86Δ427-435 and IE2 86Δ505-511 mutants, respectively (Figure 2.5). This result is also reflected at the protein level, since neither one of the early genes examined (UL112-113 and UL44) was detectable by immunostaining (Figure 2.6 and data not shown). Therefore, introducing either of these mutations results in a recombinant virus blocked even earlier in its replication than the IE2 86Δ356-359 virus.

These results indicate that the three regions of IE2 86 examined are essential for viral replication and the progression of a productive infection. Results to date suggest that disruption of some or all of these regions can decrease the ability of IE2 86 to bind to DNA and result in a protein that is able to activate early promoters minimally or not at all. The IE2 86Δ356-359 mutation has a less severe phenotype than the other deletion mutants examined, as the recombinant virus containing this deletion drives some early gene transcription and supports the expression of both UL112-113 and UL44 proteins.

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The text of this chapter, in full, is a reprint of the material as it appears in *Journal of Virology*, 78:1817-1830, 2004. White E.A., C.L. Clark, V. Sanchez, and D.H. Spector. Small internal deletions in the human cytomegalovirus IE2 gene result in nonviable recombinant viruses with differential defects in viral gene expression. The dissertation author was the primary investigator and author of this paper.

# CHAPTER 3

# THE IE2 60 KDA AND 40 KDA PROTEINS ARE DISPENSABLE FOR HUMAN CYTOMEGALOVIRUS REPLICATION, BUT ARE REQUIRED FOR EFFICIENT DELAYED EARLY AND LATE GENE EXPRESSION AND PRODUCTION OF INFECTIOUS VIRUS


## ABSTRACT

The human cytomegalovirus (HCMV) IE2 86 kDa protein is an essential transactivator of viral and cellular gene expression. Additional proteins of 60 and 40 kDa are expressed from the IE2 gene at late times p.i. and are identical to the C-terminus of IE2 86. We have constructed HCMV recombinants that express wild-type full-length IE2 86 but do not express the IE2 40 and IE2 60 kDa proteins. Each of these recombinants is viable, indicating that neither the 60 kDa nor the 40 kDa protein is required for virus replication, either alone or in combination. Cells infected with the IE2 60 and IE2 40 deletion mutants, however, exhibit decreased expression of selected viral genes at late times. In particular, expression of the viral DNA replication factor UL84 is affected by the deletion of IE2 40 and expression of the tegument protein pp65 (ppUL83) is affected by the deletion of both IE2 40 and IE2 60. IE2 60 and IE2 40 are also required for the production of normal levels of infectious virus. Finally, IE2 40 appears to function as a repressor of major immediate-early transcription in the infected cell. These results begin to define functions for the IE2 60- and IE2 40-kDa

proteins and indicate that these products contribute both to the expression of selected viral genes and to the overall progression of the infection.

## INTRODUCTION

Human cytomegalovirus gene expression, like that of all herpesviruses, occurs as a tightly controlled series of events beginning with expression of the immediate-early (IE) genes. These go on to activate expression of early viral genes, allowing replication of the viral genome and subsequent transcription of late, primarily structural, genes. Two important IE genes, UL122 and UL123, comprise the major IE region. This segment of the HCMV genome encodes two predominant products, the IE1 72 kDa (ppUL123) and IE2 86 kDa (ppUL122) proteins. These are expressed from alternatively spliced forms of a five-exon transcript; the IE1 72 mRNA consists of exons 1 to 4, and the IE2 86 mRNA contains exons 1 to 3 and 5. Translation of both proteins begins in exon 2, so that IE1 72 and IE2 86 have an identical 85 amino acid (aa) N-terminus and unique C-terminal domains. Both proteins have been characterized extensively, most recently in studies that have used recombinant viruses containing deletions in the major IE region to elucidate the functions of IE1 72 and IE2 86 in the HCMV-infected cell. These studies indicate that the IE1 72-kDa protein contributes to virus replication during low, but not high, multiplicity infections and is therefore non-essential (79, 84, 148). In contrast, IE2 86 is strictly required for HCMV replication, and even small deletions or changes to the sequence of the protein can result in a virus that does not replicate (143, 230).

Both IE1 72 and IE2 86 are transcriptional activators, and their ability to promote viral gene expression has been studied in a number of transient transfection assays. IE2 86-mediated transactivation of the 1.2- and 2.7-kb RNA and UL112-113

(2.2-kb RNA) early promoters and of promoters driving genes involved in viral DNA replication has been particularly well-characterized (60, 124, 194, 195). IE2 86 also functions as a repressor of transcription: it binds to DNA through interactions with the minor groove (129, 226) and down-regulates its own expression via site-specific binding to the 14 bp *cis*-repression signal (CRS) between the TATAA box and transcription start site in the major immediate-early promoter (MIEP) (54, 102, 129, 135, 141, 167). This DNA binding capability allows regulation of early promoters as well as autoregulation, and it is encoded by aa 290 to 579 of IE2 86 (56, 128, 194). Regions spanning the length of the protein appear to be important for IE2 86 to transactivate heterologous promoters and HCMV early promoters, with the regions between aa 1 to 98 and 170 to 579 required for activation (142, 168, 195, 200, 210, 247). Both proteins have been shown to interact in vitro with multiple viral and cellular factors, although fewer of these interactions have been confirmed in the HCMV-infected cell (for a review, see reference (76)). The only viral protein identified to date that interacts with IE2 86 is UL84. UL84 protein is present in replication centers in the nuclei of infected cells and can promote *ori*Lyt-dependent DNA replication in the presence of core replication proteins from HCMV and Epstein-Barr virus (133, 134, 190, 204, 245). UL84 is required for viral DNA replication and for the production of infectious virus and appears to have UTPase activity (61, 62, 244).

Additional spliced RNAs are expressed from both the IE1 and IE2 genes in infected fibroblasts. Spliced RNAs predicted to encode 19- and 17-kDa forms of IE1 are detectable in infected fibroblasts (12, 196). These contain exons 1, 2, 3, and

portions of exon 4 resulting from different splice patterns than those that generate the IE1 72 RNA. A spliced RNA predicted to encode a 9-kDa form of IE1 has also been detected in infected fibroblasts and contains exons 1, 2, a 5' segment of exon 3, and an alternatively spliced portion of exon 4 (12). Reports of protein expression from these transcripts vary. The IE1 17 protein has been detected only following transfection of CV-1 cells, but not in infected fibroblasts. While Shirakata et al. used an antibody directed against the region shared by IE1 and IE2 to detect the IE1 19 protein in infected fibroblasts, studies by Awasthi and colleagues suggest that this protein is in fact a breakdown product of full-length IE1 72 (12, 196). They detect IE1 19 protein only in transfected, not infected, cells, and were not able to detect IE1 9 protein in either assay.

Other splicing events in exon 5 generate RNAs from the full-length transcript that could encode 55- and 18-kDa proteins. The IE2 18 RNA is expressed in HCMV-infected monocyte-derived macrophages, but not in infected fibroblasts except in the presence of cycloheximide (122). An IE2 55 RNA is expressed only at IE times in HCMV-infected fibroblasts, and the corresponding IE2 55-kDa protein is detected in infected cells only after release from a cycloheximide block (209).

In contrast, other forms of IE2 are translated from unspliced RNAs that are expressed with late kinetics from different, downstream, promoters (174). These encode proteins of 60 and 40 kDa that are detectable at late times in infected fibroblasts. The 40-kDa protein has been more extensively characterized than the 60 kDa form. The IE2 40-kDa protein is colinear with the C-terminus of IE2 86 and is predicted to be expressed from a 1.5-kb RNA (170, 174, 209). Other, slightly larger,

forms of this RNA are present in the cytoplasm of HCMV-infected cells as well (209). The 60-kDa protein can be detected with an antibody that recognizes both IE2 86 and IE2 40, suggesting that IE2 60 is also identical to a C-terminal portion of IE2 86 (170). Translation of IE2 60 is predicted to begin at methionine 170 of IE2 86, and IE2 40 translation initiates at methionine 242 of IE2 86. The numbering used here reflects amino acid numbers in the Towne strain of HCMV. At late times p.i., both IE2 60 and IE2 40 are expressed to higher levels than IE2 86. In transient assays, IE2 40 represses the major IE promoter in much the same way as IE2 86, and it activates a version of this promoter lacking the crs when expressed in combination with IE1 72 (109). These smaller forms of IE2 have not yet been characterized in the HCMV-infected cell.

Work from our laboratory previously showed that the IE2 86ΔSX virus, which contains an internal deletion in the IE2 gene spanning aa 136 to 290, is viable. IE2 86ΔSX replicates slowly and to lower titers than wild-type virus, but supports early gene expression to approximately wild-type levels. Notable molecular defects in IE2 86ΔSX virus-infected cells include decreased expression of UL83 (pp65) RNA and protein and slightly decreased expression of UL99 (pp28) protein, but not RNA (188). IE2 86 is expressed to lower levels in IE2 86ΔSX virus-infected cells. Also, as a result of the deletion of aa 136 to 290 from IE2 86, the 60-kDa and 40-kDa forms of the protein are not expressed, demonstrating that these forms are not required for the progression of a productive infection. In the present study, we constructed recombinant viruses that do not express the IE2 40 and IE2 60 proteins and made rescued versions of each mutant virus. Using this family of HCMV recombinants, we

show that neither the IE2 60 nor the IE2 40 protein is essential for virus replication, but that deletion of both forms leads to a ten-fold drop in the production of infectious virus. As predicted by the transient assays, IE2 60 and IE2 40 contribute to regulation of the major IE promoter. Other functions of these proteins were not predicted by transient assays. These newly uncovered functions include regulation of pp65 expression via a decrease in transcript levels and control of UL84 protein expression through what appears to be a posttranscriptional mechanism.

## MATERIALS AND METHODS

**Cells.** Human foreskin fibroblasts (HFF) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 200 U penicillin, 200 µg streptomycin, 1.5 µg Amphotericin B, and 50 µg gentamicin per milliliter, and grown as described (216).

**BAC mutagenesis.** Construction of the p40, p60, and p40+60 deletion mutant viruses began with the plasmid pSP-J(BglII-StuI), which contains the approximately 2000 base pair (bp) fragment generated by digesting the HCMV AD169 genomic *EcoR*I J region (216) with *Bgl*II and *Stu*I. Mutagenic oligonucleotide primers were used in conjunction with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to create the plasmids pSP-J(BglII-StuIΔ40), pSP-J(BglII-StuIΔ60), or pSP-J(BglII-StuIΔ40+60). These are identical to pSP-J(BglII-StuI) but contain changes to the predicted TATAA box for the 1.5-kb RNA (Δ40), an upstream AT-rich region described in the text (Δ60), or both sites (Δ40+60). These constructs were sequenced to verify the presence of the correct mutations. DNA sequencing was performed by Eton Bioscience, San Diego, CA. Sequences of mutagenic primers (Integrated DNA Technologies, Coralville, IA) for the IE2 40 TATAA mutation are sense, 5' CCTTTCAAGGTGATCATCAAGCCGCCCGTGCCTCCC 3'; antisense, 5' GGGAGGCACGGGCGGCTTGATGATCACCTTGAAAGG 3'; and for the upstream AT-rich site mutation are sense, 5' GGATCCCACGTCACTATTGCGTACTCATCATCAAGCTCTATGGGACACTCT GTAATCC 3'; antisense, 5'

GGATTACAGAGTGTCCCATAGAGCTTGATGATGAGTACGCAATAGTGACG TGGGATCC 3'.

One or both of the mutations was introduced into the UL122-123 coding region using the Counter Selection BAC Modification kit (Gene Bridges, Dresden, Germany). Briefly, oligonucleotide primers were used to amplify a marker cassette containing the neomycin resistance and RpsL genes and to simultaneously introduce 50 nt of homology to the UL122-123 region onto either end of the cassette. Sequences of primers (Integrated DNA Technologies, Coralville, IA) are sense, 5' GATAGAGGAAGTTGCCCCAGAGGAAGAGGAGGATGGTGCTGAGGAACCC AGGCCTGGTGATGATGGCGGGATCG 3'; antisense, 5' CCTTCTCGTTGTCCAACTCGGAGATGCGTTTGCTCTTCTTCTTGCGGGGTTC AGAAGAACTCGTCAAGAAGGCG 3'. The linear product was recombined into the UL122-123 region contained in the wt HCMV strain AD169 BAC pHB5 (gift of M. Messerle) (26) and the resulting intermediate construct pHB5(IEexon5-RpsLneo) was selected on the basis of resistance to kanamycin. Next, pSP-J(BglII-StuIΔ40), pSP-J(BglII-StuIΔ60), and pSP-J(BglII-StuIΔ40+60) were used as a template in conjunction with the following primers (Integrated DNA Technologies, Coralville, IA): sense, 5' CAGGAAGAAAGTGAGCAGAGTGATG 3'; antisense, 5' AGCGATTGGTGTTGCGGAAC 3' to amplify a linear fragment containing the mutated UL122-123 region. This fragment was recombined into pHB5(IEexon5-RpsLneo), replacing the RpsLneo cassette, and the resulting IE2 Δ40, IE2 Δ60, and IE2 Δ40+60 BACs were selected on the basis of increased streptomycin resistance.

The altered region from each was amplified and sequenced to confirm that the intended deletion had been introduced into the BAC.

A rescued BAC was generated from the each of the mutant BACs by the reverse procedure. The RpsL-neomycin marker cassette was introduced into each of the three mutant BACs using the procedure and primers listed above. pSP-J(BglII-StuI) was then used as a template in conjunction with the primers described above to produce a linear fragment containing the wild-type HCMV sequence. This fragment was inserted into each of the RpsL-neomycin containing intermediate BACs by homologous recombination. These rescued BACs were designated IE2 40 rescue, IE2 60 rescue, and IE2 40+60 rescue.

Wild-type, mutant, and rescued BAC DNAs were amplified and purified as described (188). Each BAC was digested with *Hind*III and separated by field inversion gel electrophoresis to ensure that no major alterations to the DNA were sustained during the cloning procedure.

**Reconstitution of virus, determination of virus titers, and growth curves.** Wild-type, IE2 Δ40, IE2 Δ60, IE2 Δ40+60, IE2 40 rescue, IE2 60 rescue, and IE2 40+60 rescue BACs were transfected into HFF by electroporation as previously described (230) and monitored for plaque development. When all cells in a culture exhibited cytopathic effect, supernatants were harvested and used to infect fresh cells. Stocks of wild-type, mutant, and rescued mutant viruses were harvested and titered by plaque assay. To analyze the kinetics of virus replication, confluence-synchronized HFF were infected at an MOI of 5 pfu/cell. Supernatant from infected cells was harvested daily, replaced with fresh media, and titered by plaque assay.

**Time course of virus infection.** HFF were grown to and maintained at confluence for three days prior to infection to allow synchronization in a $G_0$ state. At the time of infection, cells were released from $G_0$ by trypsinization, infected at an MOI of 5 pfu/cell, and re-plated at a lower density. Cells were re-fed daily, then harvested by trypsinization and frozen at the indicated times p.i.

**Quantitative real-time RT-PCR analysis.** Real-time reverse transcription PCR (RT-PCR) and data analysis were performed essentially as previously described using primers and probes directed against the HCMV genes IE1 72, IE2 86, UL83, UL84, and the cellular housekeeping gene glucose-6-phosphate dehydrogenase (G6PD) (230). RNA was isolated with a NucleoSpin II kit (Clontech, Mountain View, CA) and subsequently treated with DNase using a Turbo DNA-free kit (Ambion, Austin, TX) to remove any residual DNA contamination and to allow the analysis of unspliced viral transcripts. Sequences of the IE1 72, IE2 86, and G6PD primers and TaqMan probes have been previously described (230). The sequence of the primers and probe for the UL83 assay are: sense, 5' TCTTCCTGGAGGTACAAGCCA 3'; antisense, 5' CAGCCACGGGATCGTACTG 3'; probe, 5' [6-FAM]-ACGCGAGACCGTGGAACTGCG-[black hole quencher-1]-3'. The sequence of the primers and probe for the UL84 assay are: sense, 5' AGACATTGGGACCCTCCGTC 3'; antisense, 5' GCGGTGATTCGTTCGGG 3'; probe, 5' [6-FAM]-TGGACGATTGGAGCTAG-[black hole quencher-1]-3'.

**Western blotting.** Cells were lysed in reducing sample buffer (50 mM Tris [pH 6.8], 0.2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM ß-glycerophosphate, 1 mM

phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 100 μM pepstatin A) and protein content was determined by a Bradford assay. Equal amounts of protein were separated on SDS-PAGE gels and transferred to nitrocellulose. Membranes were stained with amido black to ensure equal protein loading. After blocking in 5% nonfat dried milk in TBS-T (Tris buffered saline [pH 7.4] with 0.05% Tween-20), blots were incubated with primary antibodies in 5% nonfat dried milk in TBS-T, diluted as follows: CH16.0 MAb, 1:10,000; IE2 MAb 8140, 1:1,000; UL44 MAb, 1:5,000; UL57 MAb, 1:10,000; pp65 MAb, 1:10,000; UL84 MAb, 1:2,000 to 1:10,000; pp28 MAb, 1:10,000; β-actin MAb AC-15, 1:10,000. CH16.0, anti-UL44, anti-UL57, anti-pp65, and anti-pp28 were purchased from the Goodwin Institute (Plantation, FL) and from Virusys (Sykesville, MD). Anti IE2 was purchased from Clontech (Mountain View, CA). Anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO). Anti-UL84 was the gift of Dr. Greg Pari (University of Nevada, Reno). Membranes were washed in TBS-T and incubated in horseradish peroxidase (HRP)-coupled anti-mouse antibody (Calbiochem, San Diego, CA), diluted 1:2000. After washing in TBS-T, proteins were detected using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

## RESULTS

**Construction of IE2 60, IE2 40, and IE2 60+40 deletion mutant viruses.** In addition to full-length IE2 86, the UL122 gene encodes additional, smaller proteins that are identical to the C-terminus of IE2 86. Two of these proteins have molecular masses of 40 kDa and 60 kDa and are expressed at late times p.i. from one or more transcript(s) distinct from the full-length IE2 86 mRNA (170, 174, 209). The 1.5-kb transcript expressed from this region is the predominant IE2 RNA present in the cytoplasm of infected cells at late times p.i. (209). The experiment that mapped the 1.5-kb species identified several other IE2 transcripts also present in the cytoplasm at late times, and these were approximately 130 to 280 bases longer than the 1.5-kb RNA. Translation of the 60-kDa protein initiates at methionine 170 of IE2 86, while translation of the 40-kDa protein begins at methionine 242, and both of these residues are present in the 1.5-kb RNA as well as in the longer species. The sequence of the promoter region and the sites that were changed to eliminate the expression of these proteins are shown in Figure 1.

Previous work from our laboratory described the construction and characterization of IE2 86ΔSX-EGFP, a viable HCMV recombinant with a deletion spanning aa 136 to 290 of IE2 86 (188). The initiating methionines for both the IE2 40- and IE2 60-kDa proteins are not encoded by this virus, and the mutant virus replicates slowly, grows to lower titers than wild-type virus, and exhibits

**A.**

GAGGCAAGAG CACCCACCCT ATGGTGACTA GAAGCAAGGC TGACCAGtat atatatatac
agttactgtt atggatccca cgtcactatt gtatactcta tattatactc tatgttatac
tctgtaatcc tactcaataa acgtgtcacg cctgtgaaac cgtactaagt ctcccgtgtc
ttcttatcac catcagGTGA CATCCTCGCC CAGGCTGTCA ATCATGCCGG TATCGATTCC
AGTAGCACCG GCCCCACGCT GACAACCCAC TCTTGCAGCG TTAGCAGCGC CCCTCTTAAC
AAGCCGACCC CCACCAGCGT CGCGGTTACT AACACTCCTC TCCCCGGGGC ATCCGCTACT
CCCGAGCTCA GCCCGCGTAA GAAACCGCGC AAAACCACGC GTCCTTTCAA GGTGATTATT
AAACCGCCCG TGCCTCCCGC GCCTATCATG CTGCCCCTCA TCAAACAGGA AGACATCAAG
CCCGAGCCCG ACTTTACCAT CCAGTACCGC AACAAGATTA TCGATACCGC CGGCTGTATC
GTGATCTCTG ATAGCGAGGA AGAACAGGGT GAAGAAGTCG AAACCCGCGG TGCTACCGCG
TCTTCCCCTT CCACCGGCAG CGGCACGCCG CGAGTGACCT CTCCCACGCA CCCGCTCTCC
CAGATGAACC ACCCTCCTCT

**B.**

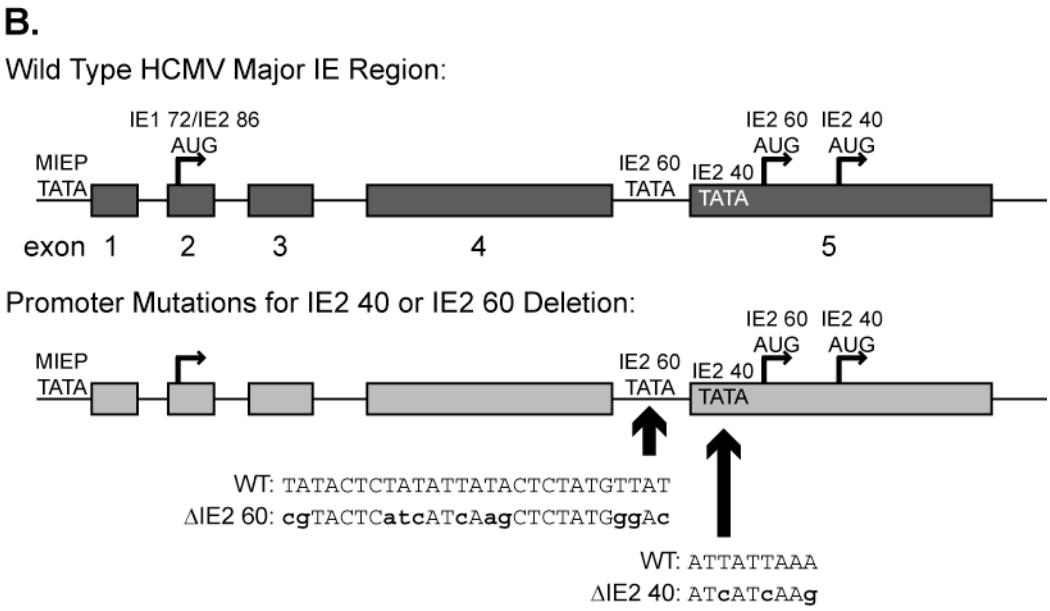



**Figure 3.1 Construction of the IE2 40 and IE2 60 deletion mutant viruses.** (A) Partial sequence of the wild-type HCMV major IE region from the 3' end of exon 4 through the 5' portion of exon 5. Exon sequences are capitalized and intron sequences are in lower case. The regions that were changed to eliminate expression of the IE2 40 and IE2 60 proteins are underlined. The codons for the initiating methionines of each protein are boxed. (B) Schematic of the major IE region. The relative locations of the exons and the TATAA boxes and initiating methionines for IE1 72, IE2 86, IE2 60, and IE2 40 are indicated. The sequences that were altered to remove the predicted TATAA boxes for the late RNAs are indicated. For the mutations, nucleotides that are identical to the wild-type sequence are capitalized and altered nucleotides are in lower case. Diagram is not to scale.

distinct defects in viral gene expression. At the outset, the present study had two goals. First, we aimed to delineate the functions of the smaller, C-terminal 40-kDa and 60-kDa forms of IE2 and their roles in HCMV-infected cells at late times p.i.. Second, we wanted to determine which of the effects observed in the IE2 86ΔSX virus-infected cell were due to the deletion of aa 136 to 290 in full-length IE2 86 versus the elimination of IE2 40 and IE2 60 expression.

To answer these questions, we constructed recombinant HCMV bacterial artificial chromosomes (BACs) encoding viruses that do not express one or both of the IE2 40 and IE2 60 proteins. These BACs were generated as previously described (Materials and Methods), using the AD169 BAC pHB5 (gift of Martin Messerle) and homologous recombination in *E. coli*. Initially, we constructed a recombinant BAC, designated IE2 Δ40, in which the TATAA box driving expression of the predominant 1.5-kb RNA is rendered nonfunctional using silent mutations at the wobble bases. The amino acid sequence of the full-length IE2 86 protein expressed by this recombinant is identical to wild-type. We noted that although this mutation eliminates all IE2 40 protein expression in the IE2 Δ40 virus-infected cell, some residual IE2 60 protein is still expressed (Figure 2, top panel). In order to characterize HCMV replication in the absence of all IE2 40 and IE2 60 protein, we therefore introduced a second mutation into the BAC. This change is in an AT-rich region that could serve as a TATAA box to drive the expression of the slightly longer transcripts that were also detected in the study that mapped the start site of the 1.5-kb RNA (Figure 1A) (209). We introduced this change into the HCMV BAC either alone (IE2 Δ60) or in combination with the initial mutation (IE2 Δ40+60). The altered sequence is in the intron between exons 4

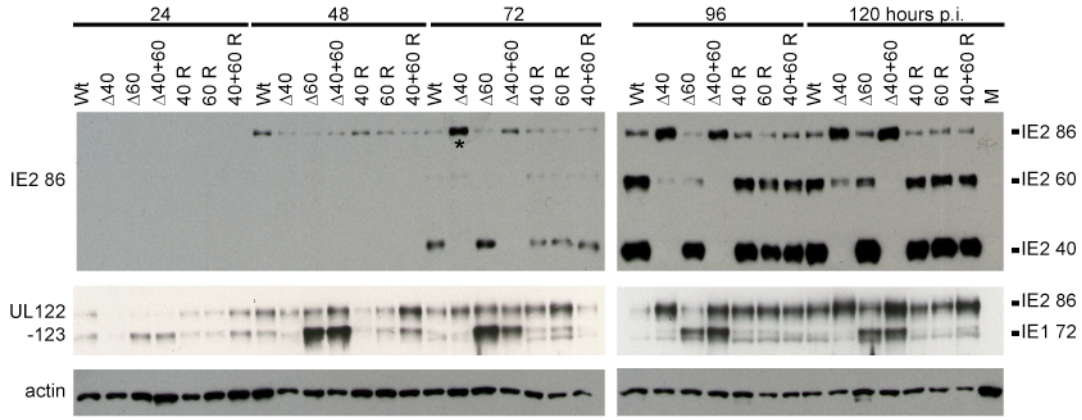



**Figure 3.2 Major immediate-early protein expression is altered following infection with IE2 Δ40 and IE2 Δ60 viruses.** $G_0$-synchronized HFF cells were infected with 5 pfu/cell of wild-type (Wt), mutant (Δ40, Δ60, or Δ40+60), and rescued mutant (40 R, 60 R, or 40+60 R) virus or mock-infected (M) and harvested at the indicated times p.i. Equal amounts of cell lysates, in micrograms, were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. IE1 72, IE2 86, IE2 60, and IE2 40 protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading. The asterisk (*) indicates that the Δ40 72h lane is slightly overloaded in the IE2 Western blot (top panel) relative to the IE2 86 and IE1 72 Western blot (middle panel).

and 5 and while several residues have been altered, neither the size of the intron nor the coding sequence of any form of IE1 or IE2 protein has been changed. Introducing both TATAA mutations into the HCMV BAC (IE2 Δ40+60) eliminated all detectable expression of IE2 40 and IE2 60 proteins (Figure 2, top panel). When only the change to the 5' TATAA site was included (IE2 Δ60), we still detected some expression of IE2 60 protein. This is consistent with the behavior of the IE2 Δ40 recombinant, which suggested that some IE2 60 protein was translated from the predominant 1.5-kb RNA.

To further ensure the integrity of the three recombinant BACs, we constructed a rescued version of each BAC in which the altered sequences were returned to wild-type. The resulting six recombinant BACs and the wild-type pHB5 parent were digested with HindIII and fragments were separated by field inversion gel electrophoresis to confirm that no large-scale deletions or rearrangements had occurred during the cloning procedure. The restriction digest pattern of each of the recombinant BACs was the same as the restriction digest pattern of the wild-type BAC (data not shown). The wild-type, mutant, and rescued mutant BACs were transfected into human foreskin fibroblasts (HFF) and virus stocks were reconstituted as previously described (Materials and Methods).

**IE2 60- and 40-kDa proteins contribute to virus replication.** The ability to reconstitute virus from each of these mutant BACs indicated that neither the 60- nor 40-kDa forms of IE2 86 were strictly required for HCMV replication in cultured cells. To understand how these proteins contribute to virus replication, we first constructed single-step growth curves following high-multiplicity infection of HFFs. Following

high-multiplicity infection (MOI 5) of HFFs, the amount of virus produced reached its peak 5 to 6 days p.i. for wild-type and for each of the three deletion mutant viruses (Figure 3). The peak titers for the mutant viruses were lower than for the wild-type and rescued viruses. This indicated that during high MOI growth, the 60- and-40 kDa proteins did not contribute to the timing of virus production, but were required for the virus to replicate to maximal titers. In particular, cells infected with IE2 Δ40+60 virus produced an average of 12 times less virus than wild-type at 5 days p.i. and up to 40 times less virus at 6 days p.i.

**IE1 72 and IE2 86 protein levels are altered by deletion of IE2 60 and IE2 40 kDa proteins.** Since the replication of the IE2 60 and IE2 40 deletion mutant viruses is impaired at high MOI as well as at low MOI, we began by looking for defects in viral gene expression that might contribute to reduced production of infectious virus when IE2 60- or 40-kDa proteins are not expressed. We infected HFF cells at a multiplicity of 5 pfu/cell with wild-type, mutant viruses, and rescued mutant viruses, and harvested infected cells at the indicated times p.i. Cells were processed for Western blots and blots were probed with antibodies that recognize the HCMV major IE proteins. Having already characterized the expression of IE2 40 and IE2 60 proteins in the recombinant virus-infected cells (Figure 2, top panel), we next assayed the expression of both IE1 72 and IE2 86 proteins in the mutant virus-infected cells using an antibody directed against the shared exon 2 region of these proteins (Figure 2, middle panel). We detected expression of both IE1 72 and IE2 86 in cells infected with wild-type virus, the three deletion mutant viruses, and the three rescued mutant viruses. Using either antibody, we detected increased expression of full-length IE2 86

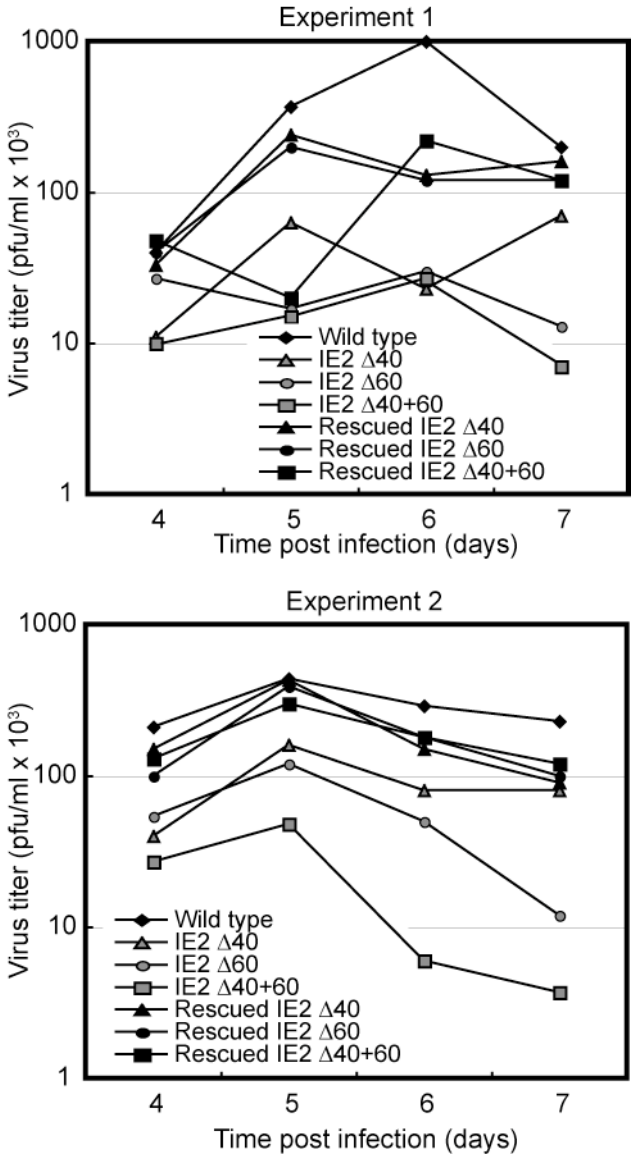



**Figure 3.3 Deletion of IE2 40 and IE2 60 reduces virus production following high-multiplicity infection.** HFFs were infected with 5 pfu/cell of wild-type, mutant, or rescued mutant virus. Infected cell supernatants were collected at the indicated times, and titers were determined as described in Materials and Methods. The two graphs indicate the titers determined in two independent experiments. All plaque assays were performed in duplicate.

relative to wild-type when IE2 40 was deleted, either alone or in combination with IE2 60. Furthermore, the expression of IE1 72 increased relative to wild-type when IE2 60 was deleted, either alone or in combination with IE2 40.

**UL122 and UL123 RNAs increase upon deletion of IE2 40.** To understand which of these changes in protein expression resulted from differences at the RNA level, we next examined the levels of UL123 (IE1 72) and UL122 (IE2 86) transcripts following a high multiplicity (MOI = 5) infection. Since the differences in the amount of protein were most apparent at late times p.i., we examined RNA levels at 72, 96, and 120 h p.i. Total RNA was isolated from cells infected with wild-type, mutant, and rescued mutant viruses and assayed by real time RT-PCR using primers and TaqMan probes that detect IE1 72 and IE2 86 transcripts (230). The TaqMan probe used in the IE1 assay was complementary to the splice junction between exons 3 and 4 and the TaqMan probe used in the IE2 assay was complementary to the splice junction between exons 3 and 5. Neither probe, therefore, detected the unspliced late transcripts.

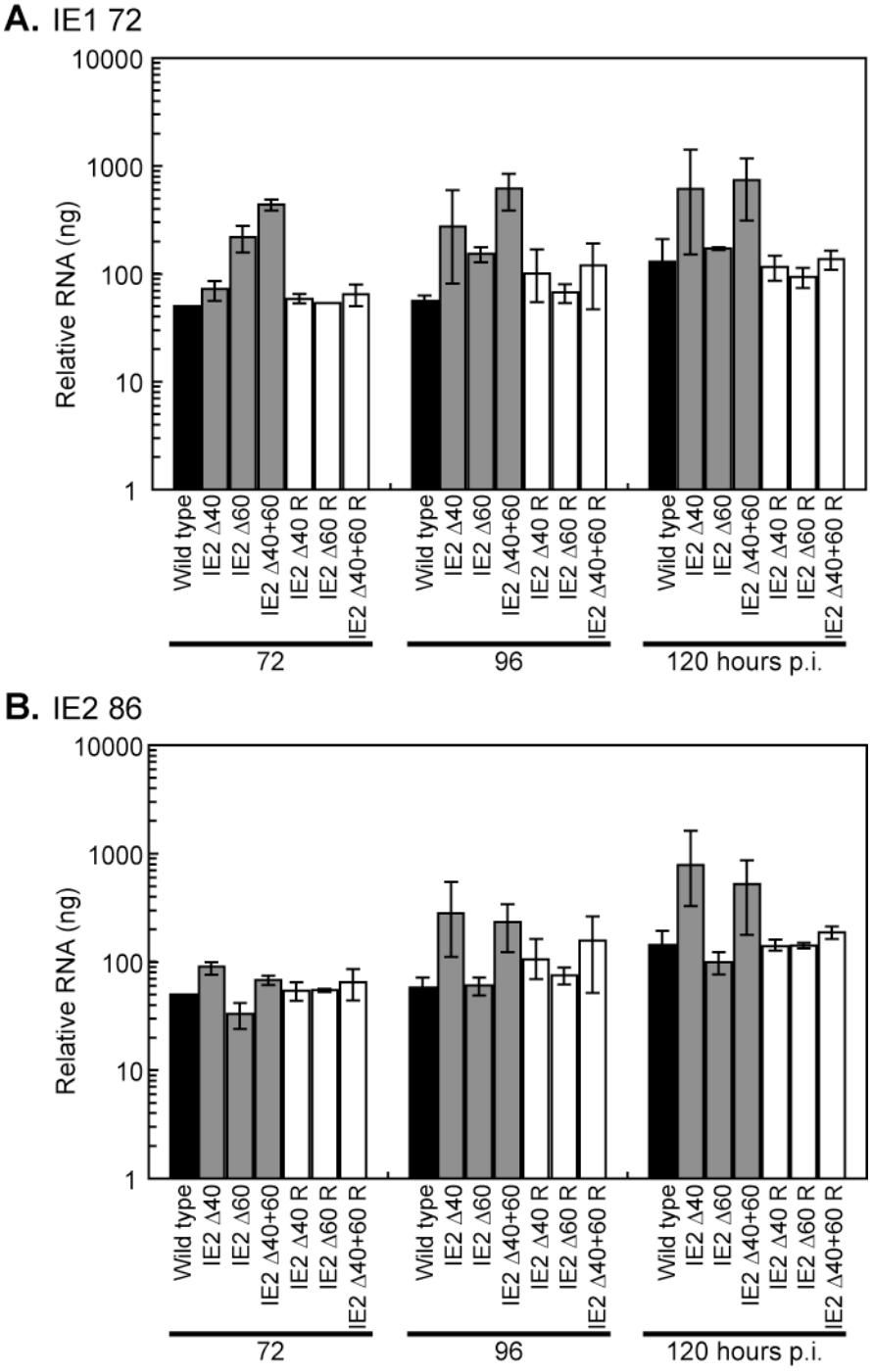
The differences in IE1 72 and IE2 86 RNA levels in the wild-type and deletion mutant virus-infected cells were consistent with the hypothesis that IE2 40 can function to repress transcription from the major IE promoter. Although there was variability between experiments, at 96 and 120 h p.i., cells infected with IE2 Δ40 and IE2 Δ40+60 mutant viruses expressed more IE1 72 RNA and IE2 86 RNA than did wild type virus-infected cells (Figure 4). There was up to 11 times more IE1 72 transcript and 4 times more IE2 86 transcript present in IE2 Δ40+60 virus-infected cells than in wild-type virus-infected cells. We believe that in infected cells, as in

transient assays, the IE2 40 protein functions at late times to down-regulate expression of IE1 72 and IE2 86 transcripts. When IE2 40 is not expressed, we therefore observe an increase in the levels of these major IE transcripts.

These results are consistent with the increase in IE2 86 protein observed when IE2 40 is not expressed, but differ from the observation that cells infected with IE2 Δ60 (and IE2 Δ40+60) mutant virus over-express IE1 72 protein compared to wild-type. Cells that are infected with the IE2 Δ60 virus express slightly more IE1 72 RNA than do wild-type virus-infected cells, particularly at 72 h p.i., but the change in IE1 72 transcript levels does not appear sufficient to explain the difference in protein expression. Possible reasons for this discrepancy are discussed below.

**Early viral protein expression is not altered by the deletion of IE2 60 or IE2 40 proteins.** IE2 40 and IE2 60 proteins are expressed with late kinetics, and the low titers observed for the deletion mutants are therefore unlikely to be due to defects in early gene expression. To confirm that defective early gene expression was not the source of the lower titers produced by the mutant viruses, we examined the levels of two early proteins by Western blotting (Figure 5A). The expression of UL44 and UL57 proteins did not appear to be altered significantly by deletion of IE2 40 or IE2 60, particularly at the later time points examined. UL44 encodes the HCMV polymerase processivity factor and is expressed with early kinetics (72, 104, 228). UL57 encodes a single stranded DNA binding protein and is also expressed with early kinetics (123). This result suggests that even late in the infection, when the effects of the lack of IE2 40 and IE2 60 might be most apparent, the expression of viral early proteins proceeds normally.

**Figure 3.4 IE1 72 and IE2 86 RNA levels are altered in deletion mutant virus-infected cells.** $G_0$-synchronized HFF cells were infected with 5 pfu/cell of wild-type virus, mutant viruses (IE2 Δ40, IE2 Δ60, or IE2 Δ40+60), and rescued mutant viruses (IE2 Δ40 R, IE2 Δ60 R, or IE2 Δ40+60 R) or mock-infected and harvested at the indicated times p.i. Total RNA was analyzed by quantitative real-time RT-PCR as described in Materials and Methods to measure the relative levels of (A) IE1 72 or (B) IE2 86 transcripts. The values plotted on the graphs are the average of two or three independent experiments, and range bars indicating the highest and lowest values obtained in the independent experiments are shown. To ensure that an equal amount of RNA was included in each reaction, samples were analyzed with G6PD-specific primers and probe. Values shown in the graphs have been standardized to G6PD levels. When mock-infected cell RNA was analyzed with either one of the TaqMan probes, amplification was near or below the limit of detection. In addition, there was no amplification when the samples were treated with RNase prior to PCR analysis.


A. IE1 72
Relative RNA (ng)
10000
1000
100
10
1
Wild type
IE2 Δ40
IE2 Δ60
IE2 Δ40+60
IE2 Δ40 R
IE2 Δ60 R
IE2 Δ40+60 R
72
96
120 hours p.i.
B. IE2 86
Relative RNA (ng)
10000
1000
100
10
1
Wild type
IE2 Δ40
IE2 Δ60
IE2 Δ40+60
IE2 Δ40 R
IE2 Δ60 R
IE2 Δ40+60 R
72
96
120 hours p.i.

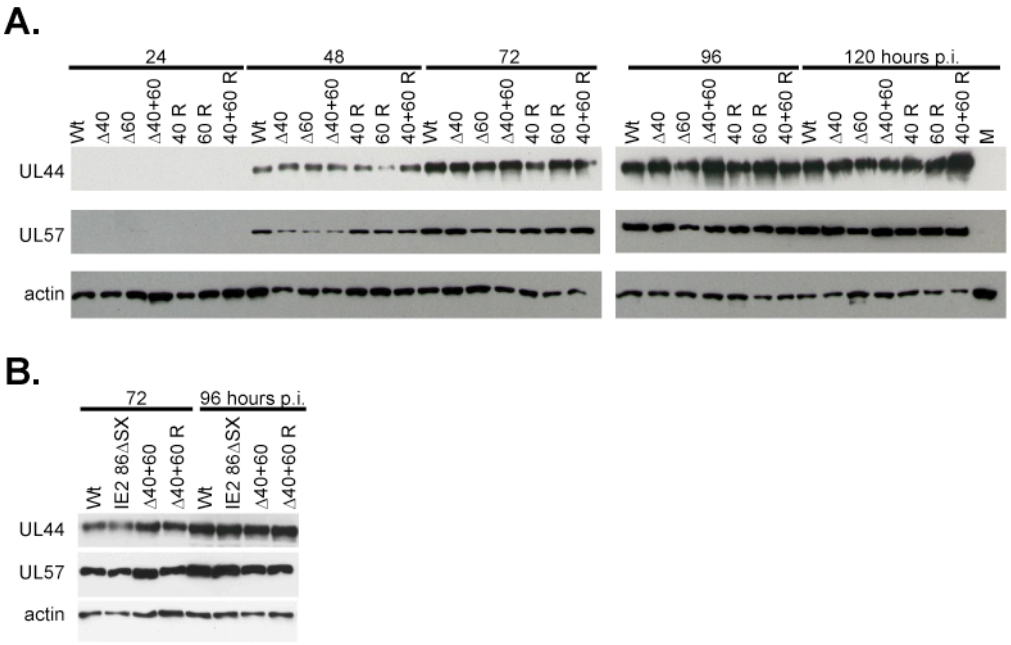



**Figure 3.5 Early viral protein expression is not altered following infection with IE2 Δ40 and IE2 Δ60 viruses.** (A) $G_0$-synchronized HFF cells were infected with 5 pfu/cell of wild-type (Wt), mutant (Δ40, Δ60, or Δ40+60), and rescued mutant (40 R, 60 R, or 40+60 R) virus or mock-infected (M) and harvested at the indicated times p.i. Equal amounts of cell lysates, in micrograms, were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. UL44 and UL57 protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading. (B) $G_0$-synchronized HFF cells were infected with 5 pfu/cell of wild-type (Wt), IE2 86ΔSX-EGFP, IE2 Δ40+60 (Δ40+60), or rescued IE2 Δ40+60 (40+60 R) virus and harvested at the indicated times p.i. Equal amounts of cell lysates, in micrograms, were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. UL44 and UL57 protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading.

The IE2 86ΔSX-EGFP recombinant virus does not express IE2 40 or IE2 60 and also lacks the segment of the open reading frame that codes for aa 136 to 290 of IE2 86. IE2 86ΔSX-EGFP also expresses enhanced green fluorescent protein (EGFP) fused to the C-terminus of the mutated IE2 86 protein. IE2 86ΔSX-EGFP as well as its wild-type parent virus, WT IE2 86-EGFP, have been previously characterized (188). IE2 86ΔSX-EGFP grows indistinguishably from IE2 86ΔSX, a recombinant virus in which the mutated IE2 86 protein has not been fused to EGFP, and WT IE2 86-EGFP grows indistinguishably from the wild-type pHB5-BAC derived virus used in this study (E.A. White and D.H. Spector, unpublished results). We wanted to distinguish between the effects observed in IE2 86ΔSX-EGFP-infected cells that are due to the lack of aa 136 to 290 versus those that result from the absence of IE2 60 and IE2 40. In a separate experiment, we compared early gene expression in cells infected with IE2 86ΔSX-EGFP, IE2 Δ40+60 virus, and the rescued IE2 Δ40+60 virus. As previously reported, the deletion of aa 136 to 290 in the IE2 86ΔSX-EGFP virus did not affect early gene expression. At the 72 h and 96 h p.i. time points, where the effect of deleting the late forms of IE2 might be most apparent, UL44 protein levels were comparable in IE2 Δ40+60 virus-infected cells and IE2 86ΔSX-EGFP infected cells. This suggested that neither IE2 40 and IE2 60 nor aa 136 to 290 of IE2 86 contribute directly to UL44 expression (Figure 5B).

To further verify that viral early gene expression proceeds normally in IE2 40 and IE2 60 deletion mutant virus-infected cells, we examined the expression of representative early proteins in cells infected with wild-type, IE2 Δ40, IE2 Δ60, and IE2 Δ40+60 viruses by immunofluorescence analysis. Gene expression in cells

infected with any one of the four viruses appeared comparable; in each case, all the cells that expressed IE1 72 protein also expressed UL44 protein by 48 h p.i. (data not shown). Consistent with the Western blot results, this observation suggested that early gene expression, as judged by UL44 positivity in individual infected cells, was not affected by the deletion of IE2 40 or IE2 60.

**IE2 60 and 40 kDa proteins contribute to the expression of some late viral genes.** Since IE2 40 and IE2 60 proteins are expressed with late kinetics, we predicted that the deletion mutants would be most likely to show defects in the expression of late viral genes. We therefore examined the expression of several viral genes that are expressed with delayed early or late viral kinetics. First, we measured the levels of UL84 and pp65 proteins by Western blotting following a time course of infection with wild-type virus, deletion mutant viruses, and rescued mutant viruses, in HFF (Figure 6A). UL84 protein binds to IE2 86 and participates in replication of the viral DNA (61, 160, 204). Although UL84 is initially expressed at early times in the virus-infected cell, the early rise in UL84 RNA is not sustained and UL84 transcript levels are low between 6 to 24 h p.i. Maximum UL84 transcription is observed after 72 h p.i., and the study that initially characterized this expression suggested that the peak production of UL84 might depend on DNA replication (94). UL83 encodes pp65 protein, which is a component of the HCMV tegument (147).

Of the three mutant viruses, the IE2 Δ40+60 mutant virus was the most impaired in the expression of UL84 and pp65. Cells infected with the IE2 Δ40+60 mutant virus began to exhibit decreased expression of UL84 and pp65 proteins as early as 72 h p.i. compared to wild-type virus infected cells. This lower level of

expression was also apparent at 96 and 120 h p.i. In the experiment shown in figure 6A, there is approximately 2-fold more pp65 in wild-type virus-infected cells than in IE2 Δ40+60 mutant virus-infected cells at 72 h and 96 h p.i. and approximately 4 times more at 120 h p.i. The deletion of IE2 40 alone also resulted in a small decrease in pp65 protein, but this effect was more modest.

The deletion of IE2 40 also affected the expression of UL84. Quantification of the UL84 Western blots shown indicates that in the absence of IE2 40 or both IE2 40 and IE2 60, there is a 2- to 4-fold drop in UL84 protein expression at 96 to 120 h p.i. These results indicated that the absence of IE2 40 protein was sufficient to reduce the expression of UL84 protein compared to wild-type. In contrast, decreased levels of pp65 were observed primarily when both IE2 60 and IE2 40 were not expressed. It is possible, then, that pp65 expression does not depend on IE2 40 and that the small amount of IE2 60 protein expressed in the IE2 Δ40 virus-infected cell is sufficient to mediate wild-type levels of pp65 expression.

Decreased expression of pp65 protein had been previously identified as a hallmark of the IE2 86ΔSX infection, but in the previous study the expression of UL84 had not been examined (188). We therefore wanted to compare the expression of these delayed early and late factors in cells infected with the IE2 86ΔSX-EGFP and IE2 Δ40+60 viruses. Again, we compared these two mutants at the 72 h and 96 h p.i. time points that had shown some of the most significant changes in the experiment described above. We observed that UL84 and pp65 expression was equally affected by the IE2 86ΔSX mutation and the IE2 Δ40+60 deletions (Figure 6B). This result suggested that the defect in late protein expression observed for the IE2 86ΔSX-EGFP

virus was due to the lack of expression of IE2 40 and IE2 60 proteins, not due to the additional deletion of aa 136 to 290 of IE2 86. IE2 86ΔSX was previously shown to express slightly lower than wild-type levels of pp28 protein in a low-multiplicity infection (188). To see whether pp28 protein levels differed in IE2 86ΔSX versus IE2 Δ40+60 virus-infected cells, we examined the expression of pp28 protein in this high-multiplicity experiment. At the higher multiplicity, we did not detect significant differences in pp28 protein expression in wild-type, IE2 86ΔSX, IE2 Δ40+60, or rescued IE2 Δ40+60 virus-infected cells.

**UL83 expression, but not UL84 expression, is affected at the transcriptional level.** Again, to determine which of the observed changes in protein expression at late times p.i. were due to changes in RNA levels**,** we measured the levels of UL83 (pp65) and UL84 transcripts by real-time RT-PCR following high multiplicity infection (Figure 7A). As a control for RNA contamination, samples were also treated with RNase prior to analysis by real-time RT-PCR. No amplification was observed in the RNase-treated samples. It had previously been shown that following low multiplicity infection, decreased pp65 protein levels in IE2 86ΔSX-EGFP virus-infected cells corresponded to a decrease in the UL83 transcript, which encodes pp65 (188). Here, UL83 levels following a high multiplicity infection behaved in the same way and were between 7- to 12-fold lower in IE2 86ΔSX-EGFP virus-infected cells than in wild-type virus-infected cells. Using the IE2 Δ40+60 mutant virus, we saw that this effect could be entirely due to the lack of IE2 40 and IE2 60 expression in IE2 86ΔSX-EGFP infected cells. IE2 Δ40+60 virus-infected cells expressed up to 12-fold

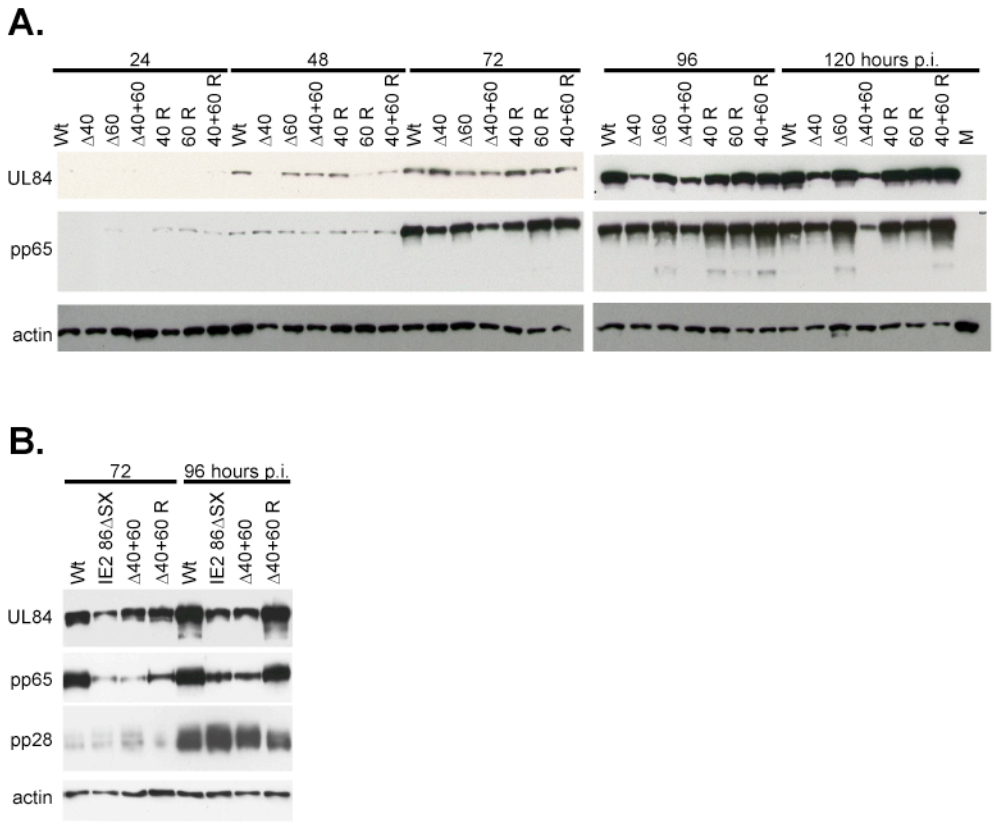
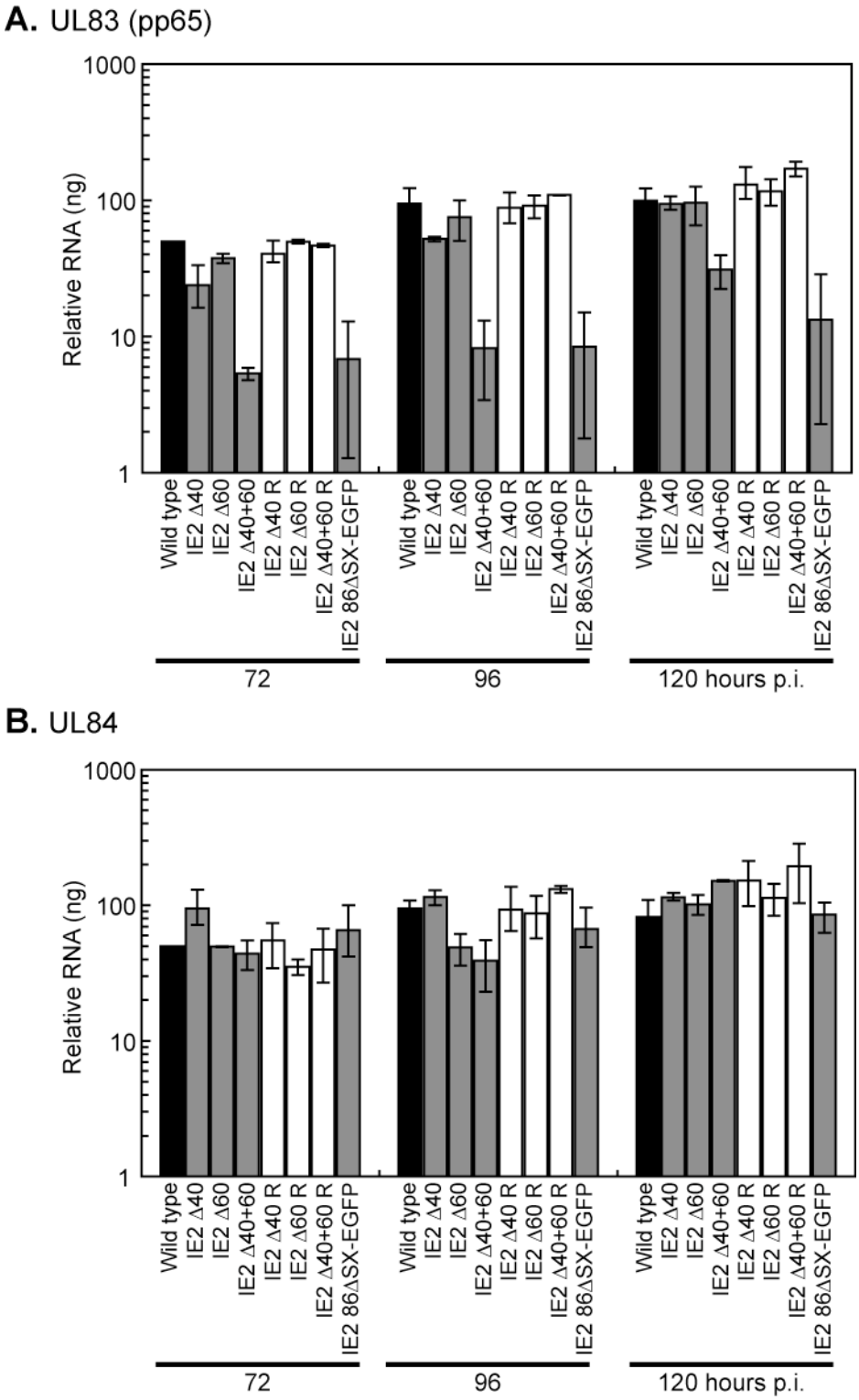



**Figure 3.6 UL84 and pp65 protein expression is reduced following infection with IE2 Δ40 and IE2 Δ60 viruses.** (A) $G_0$-synchronized HFF cells were infected with 5 pfu/cell of wild-type (Wt), mutant (Δ40, Δ60, or Δ40+60), and rescued mutant (40 R, 60 R, or 40+60 R) virus or mock-infected (M) and harvested at the indicated times p.i. Equal amounts of cell lysates, in micrograms, were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. UL84 and pp65 protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading. (B) $G_0$-synchronized HFF cells were infected with 5 pfu/cell of wild-type (Wt), IE2 86ΔSX-EGFP, IE2 Δ40+60 (Δ40+60), or rescued IE2 Δ40+60 (40+60 R) virus and harvested at the indicated times p.i. Equal amounts of cell lysates, in micrograms, were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. UL84, pp65, and pp28 protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading.

**Figure 3.7 UL83, but not UL84, RNA levels are altered in deletion mutant virus-infected cells.** $G_0$-synchronized HFF cells were infected with 5 pfu/cell of wild-type virus, IE2 86ΔSX-EGFP virus, mutant viruses (IE2 Δ40, IE2 Δ60, or IE2 Δ40+60), and rescued mutant viruses (IE2 Δ40 R, IE2 Δ60 R, or IE2 Δ40+60 R) or mock-infected and harvested at the indicated times p.i. Total RNA was analyzed by quantitative real-time RT-PCR as described in Materials and Methods to measure the relative levels of (A) UL83 or (B) UL84 transcripts. The values plotted on the graphs are the average of two or three independent experiments, and range bars indicating the highest and lowest values obtained in the independent experiments are shown. To ensure that an equal amount of RNA was included in each reaction, samples were analyzed with G6PD-specific primers and probe. Values shown in the graphs have been standardized to G6PD levels. When mock-infected cell RNA was analyzed with either one of the TaqMan probes, amplification was near or below the limit of detection. In addition, there was no amplification when the samples were treated with RNase prior to PCR analysis.


A. UL83 (pp65)
Relative RNA (ng)
1000
100
10
1
Wild type
IE2 Δ40
IE2 Δ60
IE2 Δ40+60
IE2 Δ40 R
IE2 Δ60 R
IE2 Δ40+60 R
IE2 86ΔSX-EGFP
72
96
120 hours p.i.
B. UL84
Relative RNA (ng)
1000
100
10
1
Wild type
IE2 Δ40
IE2 Δ60
IE2 Δ40+60
IE2 Δ40 R
IE2 Δ60 R
IE2 Δ40+60 R
IE2 86ΔSX-EGFP
72
96
120 hours p.i.

less UL83 RNA than did wild-type virus-infected cells, and this result is consistent with our observation that pp65 protein levels were lower in IE2 Δ40+60 virus-infected cells than in wild-type virus-infected cells.

Finally, we wanted to determine whether changes in UL84 transcript levels might similarly cause the differences we observed in UL84 protein abundance. In contrast to the UL83 result, UL84 RNA levels were not significantly affected by infection with any of the deletion mutant viruses or with IE2 86ΔSX-EGFP (Figure 7B). In the experiment shown, there were small fluctuations in transcript levels, but the mutant virus-infected cells expressed an amount of RNA that was at most 2.4-fold different than the level in the wild-type virus-infected cell, and this difference was not observed at every time point. This result suggested that the lower levels of UL84 protein observed in IE2 Δ40 and IE2 Δ40+60 virus-infected cells did not result from decreased expression of the UL84 transcript.

## DISCUSSION

The IE2 60- and IE2 40-kDa proteins are abundantly expressed in the HCMV-infected cell and are colinear with the C-terminus of the essential viral regulatory protein IE2 86, but their contributions to the HCMV infection have not been well understood. An earlier study showed that the IE2 40 protein could function both as an activator and a repressor of the major IE promoter in a transient transfection assay (109). Due to its methodology, this work could not identify functions of IE2 40 that are separate from the functions provided by full-length IE2 86 or confirm the role of IE2 40 in the HCMV-infected cell, but it did raise interesting questions about how IE2 40 and IE2 60 might contribute to virus replication. A study from our laboratory suggested that IE2 40 and IE2 60 were not essential for HCMV growth in cultured cells, but the recombinant virus used in this study carried an additional deletion of aa 136 to 290 of IE2 86 that complicated attempts to assign functions to the smaller proteins (188). The slow growth of this recombinant also suggested that IE2 40 and IE2 60 might, individually or in combination, contribute to the virus' ability to replicate to wild-type levels and with wild-type kinetics. Finally, the IE2 40 and IE2 60 proteins are unique among the factors expressed from the major IE region. Unlike the proteins that arise from splice variant transcripts under unique conditions (such as release from a cycloheximide block) and are expressed in cell types other than fibroblasts, the expression of IE2 40 and IE2 60 is robust, easily detectable in infected fibroblasts, and occurs with late kinetics. These observations suggested to us that these proteins might provide functions at late times during the HCMV infection that

had not been previously identified. This idea was supported by our previous observation that mutations in IE2 86 led to altered regulation of late viral genes (230), suggesting that IE2 86 or related proteins could directly regulate late viral gene expression in HCMV-infected cells.

To further investigate these questions, we constructed and characterized recombinant viruses that lack the ability to express one or both of the IE2 40 and IE2 60 proteins. Neither small form of IE2 is required for HCMV replication to proceed, but both contribute to efficient virus growth. We began by measuring replication of the mutant viruses following high-multiplicity infections and demonstrated that their growth is impaired. The IE2 Δ40+60 mutant virus, which does not express either small protein, shows the largest defect in replication. IE2 Δ40+60 replicates to titers that are about 10-fold lower than the titers of wild-type or rescued mutant virus in a matched infection. However, the kinetics of virus replication are the same for the mutant and wild-type viruses under these conditions.

Further analysis of the events in the viral life cycle that might be impaired and therefore contribute to this decrease in replication revealed two main effects: altered expression of full-length IE2 86 and IE1 72 proteins in deletion mutant virus-infected cells and reduced levels of two viral proteins that are produced late in the infection. These two effects and their proposed causes are discussed more fully below.

First, we observed that in mutant virus-infected cells, IE1 72 and IE2 86 expression was altered at both the RNA and protein levels. The most striking difference is that when changes were made to the 1.5 kb RNA promoter and IE2 40 protein is not expressed, we observed an increase in IE2 86 and IE1 72 RNA levels.

This behavior is consistent with the prediction that IE2 40 functions as a repressor of the major IE promoter (109). When IE2 40 is not expressed, we expect that overall levels of the major IE transcript will increase and that there will be a corresponding increase in IE1 72 and IE2 86 RNAs. Indeed, we observed these predicted changes in RNA levels.

Unexpectedly, the increase in the major IE RNA is reflected at the protein level for IE2 86, but not for IE1 72. IE1 72 protein levels are more affected by changes to the upstream AT-rich site, either when the change is part of the IE2 Δ60 virus or the IE2 Δ40+60 virus. Although it is not yet clear why the change to the upstream AT-rich site more directly impacts IE1 72, we note that the mutation that has altered that site has not only changed a possible promoter for the ~1.7 kb late IE2 RNAs, but has also changed the sequence of the 3' untranslated region for the IE1 72 message. It is possible that introducing this change into the IE1 72 RNA has increased its translation relative to wild-type, but that its transcription has not been significantly altered. Experiments to address these questions are in progress.

Two viral genes that are expressed with delayed early kinetics behaved differently in cells infected with the mutant viruses. These effects were most apparent in cells infected with the IE2 Δ40+60 mutant virus, which does not express any detectable IE2 40 or IE2 60 protein. The expression of the UL83 gene, which encodes pp65, was decreased in IE2 Δ40+60 virus-infected cells at the level of both RNA and protein synthesis. This is the same result that had previously been reported for the IE2 86ΔSX-EGFP virus and therefore demonstrates that full expression of pp65 depends on the IE2 40 and IE2 60 proteins and not on aa 136 to 290 of IE2 86. There are two

ways in which these proteins could be responsible for pp65 expression. It is possible that some expression of either IE2 40 or IE2 60 protein is required for full expression of pp65, and so we detect the drop in pp65 RNA and protein levels only when neither late form of IE2 is expressed. However, we cannot rule out the alternative possibility that a small amount of IE2 60 protein alone is sufficient to support the full expression of pp65, and that we therefore detect wild-type levels of pp65 protein and RNA in both IE2 Δ40 and IE2 Δ60 virus-infected cells.

In contrast, IE2 40 and IE2 60 must contribute differently to the regulation of UL84 expression. While UL84 protein is less abundant in cells infected with IE2 Δ40+60 mutant virus or with IE2 86ΔSX-EGFP virus relative to wild-type virus-infected cells, we do not see a significant drop in UL84 RNA in mutant virus-infected cells. A similar drop in UL84 protein, but not RNA, is also observed for the IE2 Δ40 virus, indicating that deletion of the 40-kDa protein is sufficient to mediate this effect. Reducing levels of the IE2 60 protein alone does not alter UL84 expression, although whether this is due to the higher abundance of IE2 40 than IE2 60 protein, or due to the difference in their sequences, is not clear. It appears that while UL83 expression is regulated by IE2 40 or IE2 60 at the level of transcription (or RNA stability), UL84 is regulated by IE2 40 at the level of translation or protein stability. Experiments to distinguish between these alternatives are in progress.

It is intriguing that two HCMV-encoded proteins that are identical to 50% or more of the essential viral protein IE2 86 and are more abundant than IE2 86 are not themselves required for productive HCMV infection of cultured fibroblasts. These factors do clearly contribute to specific events in the HCMV replicative cycle, and no

doubt there are additional events dependent on IE2 40 and IE2 60 that have not yet been identified. Further studies will aim to demonstrate how these proteins contribute to the progression of the HCMV infection by specific activation and repression of viral and cellular gene expression at late times in the virus-infected cell.

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The text of this chapter, in full, is a reprint of the material as it was accepted for publication in *Journal of Virology*, December 2006. White, E.A., C.J. Del Rosario, R.L. Sanders, and D.H. Spector. The IE2 60 kDa and 40 kDa proteins are dispensable for human cytomegalovirus replication, but are required for efficient delayed early and late gene expression and production of infectious virus. The dissertation author was the primary investigator and author of this paper.

# CHAPTER 4

# EXON 3 OF THE HUMAN CYTOMEGALOVIRUS MAJOR IMMEDIATE EARLY REGION IS REQUIRED FOR EFFICIENT VIRAL GENE EXPRESSION AND FOR CELLULAR CYCLIN MODULATION


## ABSTRACT

The human cytomegalovirus (HCMV) major immediate-early (IE) proteins share an 85-amino-acid N-terminal domain specified by exons 2 and 3 of the major IE region, UL122-123. We have constructed IE Δ30-77, a recombinant virus that lacks the majority of IE exon 3 and consequently expresses smaller forms of both IE1 72- and IE2 86-kDa proteins. The mutant virus is viable but growth impaired at both high and low multiplicities of infection and exhibits a kinetic defect that is not rescued by growth in fibroblasts expressing IE1 72-kDa protein. The kinetics of mutant IE2 protein accumulation in IE Δ30-77 virus-infected cells are approximately normal compared to wild-type virus-infected cells, but the IE Δ30-77 virus is delayed in expression of early viral genes including UL112-113 and UL44 and does not sustain expression of mutant IE1 protein as the infection progresses. Additionally, cells infected with IE Δ30-77 exhibit altered expression of cellular proteins compared to wild-type HCMV-infected cells. PML is not dispersed but is retained at ND10 sites following infection with IE Δ30-77 mutant virus. While the deletion mutant retains the ability to mediate the stabilization of cyclin B1, cdc6, and geminin in infected

cells, its capacity to up-regulate the expression of cyclin E has been reduced. These data indicate that the activity of one or both of the HCMV major IE proteins is required in vivo for the modulation of cell cycle proteins observed in cells infected with wild-type HCMV.

## INTRODUCTION

Human cytomegalovirus (HCMV), a β-herpesvirus, is a prevalent human pathogen and a major cause of virus-induced birth defects (for a review, see reference (161). After delivery of the viral genome into a permissive cell, HCMV replication begins with the expression of the immediate-early (IE) genes, which do not require de novo host or viral protein synthesis in order to be expressed (for reviews, see references (76, 147). IE gene products promote the expression of viral early genes, which mediate replication of the viral DNA and further subvert host cellular processes in favor of viral replication. Late viral genes, encoding primarily structural proteins, are expressed following viral DNA replication.

Expression of the viral IE genes begins this cascade of events, and these genes and their products have been studied extensively. The products of the major immediate-early region, the IE1 72-kDa (pUL123) and IE2 86-kDa (pUL122) proteins, are of particular interest due to their strong capacity to transactivate early promoters and to alter the expression of host cellular factors. A single transcript from the major IE region consists of five exons and is differentially spliced to produce the transcript encoding IE1 72 (exons 1 to 4) or IE2 86 (exons 1 to 3 and 5) (211-213). Translation of both RNAs begins in exon 2, resulting in proteins that share 85 amino acids at their N-termini; exon 2 contributes amino acids (aa) 1 to 23 and exon 3 contributes aa 24 to 85. Additional products of the major IE region include 60-kDa and 40-kDa proteins colinear with the C-terminus of IE2 86; these are derived from shorter, unspliced transcripts which initiate near the 5’ end of exon 5. Splice variants

of the major IE transcripts also have been characterized and are predicted to encode IE55 and IE18 from the full-length IE2 86 transcript as well as IE19, IE17.5, and IE9 from the full-length IE1 72 transcript (15, 122, 170, 196, 209, 212). Expression of the protein products of these RNAs, however, varies by cell type and is not readily observed under standard infection conditions in cultured fibroblasts.

Recent studies have extended the results of extensive characterization of the IE2 86-kDa protein in vitro and in transient transfection assays. These experiments have analyzed IE2 86 using site-directed mutagenesis in the context of the viral genome followed by characterization of viral mutants in cultured cells. Deleting the majority of the region unique to IE1 72 or IE2 86 demonstrates an important functional difference between these proteins: the IE1 72-kDa protein is not strictly required for growth of the virus, but IE2 86 is essential (79, 84, 143, 148). Importantly, growth of the IE1 72 mutant virus is multiplicity dependent, with viral early genes expressed more efficiently under high- than low-multiplicity conditions (79, 84). In contrast, eliminating the expression of IE2 86 by deleting the majority of exon 5 results in a non-viable virus (143). A bacterial artificial chromosome (BAC) clone of the UL122 deletion construct, when transfected into permissive cells, does not support the expression of early viral genes. Since constructing a cell line expressing functional IE2 86 has been difficult, complementation of this and other non-viable IE2 86 mutants has not yet been demonstrated. Smaller deletions in the UL122 open reading frame have been cloned into the viral genome, resulting in both viable and nonviable recombinant viruses (188, 230). Analysis of these viruses has

suggested functional roles in vivo for the motifs and domains mapped in previous in vitro work.

HCMV infection dramatically changes the expression of cellular proteins and results in a block in the host cell cycle (30, 69, 108, 137, 186). Altered expression of cellular cyclins in HCMV-infected cells has been described, with cyclins B1 and E up-regulated and cyclins A and D1 down-regulated relative to the levels in uninfected controls (28, 108, 145, 186, 189). The net result of these and other changes is that infected cells do not cycle and are held in a state in which cellular DNA replication does not occur, viral DNA replication is favored, and apoptosis is blocked. Many studies have aimed to elucidate the contributions of the IE proteins to these events. The antiapoptotic proteins vMIA (pUL37 exon 1) and vICA (pUL36) are expressed with IE kinetics and have been characterized using transfection assays in HeLa cells and generation of a recombinant virus, respectively (82, 144, 162, 198). The role of the IE gene TRS1 has also been investigated by mutation of the locus in the viral genome (3, 23). In contrast, the effects of IE1 72 and IE2 86 on the cell cycle and related factors have been investigated primarily using transient transfection assays (28, 45, 152, 197, 201, 231-233).

While the regions unique to the IE1 72- and IE2 86-kDa proteins have been analyzed in vivo as discussed above, the mutations in the N-terminal region shared by these proteins have not been examined in the context of the viral genome. A site of particular interest is exon 3 (aa 24 to 85) of the major IE region. Early studies indicated that an insertion of four amino acids at position 59 resulted in IE1 72 and IE2 86 proteins that retain the ability to repress transcription from the major IE

promoter but are severely impaired in activation of the HCMV DNA polymerase (UL54) promoter (210). Later work further showed that the deletion of exon 3 sequences from an IE2 86 expression vector eliminated the ability of the resulting protein to transactivate the UL112-113 early promoter (200). These findings suggested that further analysis of the function of the IE exon 3 region in infected cells might provide important information about the ability of the major IE proteins to activate the transcription of viral early genes. Exon 3 sequences have also been implicated in disruption of PML bodies by IE1 72 and appear to be required for interactions between p107 and IE1 72 (5, 7, 107, 111, 125, 151, 172, 235, 252). Based on these results, it seemed likely that both IE1 72 and IE2 86 might require these sequences for proper function in the infected cell. We therefore constructed and studied a recombinant virus in which most of IE exon 3 has been removed and observed both predicted and novel effects on viral and cellular gene expression.

## MATERIALS AND METHODS

**Cells.** Human foreskin fibroblasts (HFF) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 200 U penicillin, 200 μg streptomycin, 1.5 μg Amphotericin B, and 50 μg gentamicin per milliliter, and grown as described previously (216). ihfie1.3 cells (gift of E. Mocarski) (148) were cultured in the same medium and additionally supplemented with 400 μg G418 per milliliter.

**BAC mutagenesis.** Construction of the major IE exon 3 deletion mutant virus began with the plasmid pSP-J(Sca-RV), which contains the 800-bp fragment generated by digesting the HCMV AD169 genomic EcoRI J region (216) with ScaI and EcoRV. Mutagenic oligonucleotide primers were used in conjunction with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to create the plasmid pSP-J(Sca-RVΔ30-77), which is identical to pSP-J(Sca-RV) but lacks the 144 bp that code for aa 30 to 77 of the UL122-123 open reading frame. These constructs were sequenced to verify the presence of the correct mutations. DNA sequencing was performed by the DNA Sequencing Shared Resource, UCSD Cancer Center, which is funded in part by NCI Cancer Center Support Grant 2 P30 CA23100-18. Sequences of mutagenic primers (Integrated DNA Technologies, Coralville, IA) are as follows: sense, 5' GCCCGAGACACCCGTGGAGAAAGATGTCCTGGCAG 3'; antisense, 5' CTGCCAGGACATCTTTCTCCACGGGTGTCTCGGGC 3'.

The exon 3 (aa 30 to 77) deletion was introduced into the UL122-123 coding region using a counter-selection BAC Modification kit (Gene Bridges, Dresden,

Germany). Briefly, oligonucleotide primers were used to amplify a marker cassette containing the neomycin resistance and RpsL genes and to simultaneously introduce 50 nt of homology to the UL122-123 region onto either end of the cassette. Sequences of primers (Integrated DNA Technologies, Coralville, IA) are as follows: sense, 5' TCTCCTGTATGTGACCCATGTGCTTATGACTCTATTTCTCATGTGTTTAGGG CCTGGTGATGATGGCGGGATCG 3'; antisense, 5' CCACGTCCCATGCAGGTTAGTATACATCACATACATGTCAACAGACTTACT CAGAAGAACTCGTCAAGAAGGCG 3'. The linear product was recombined into the major IE exon 3 region contained in the wt HCMV strain AD169 BAC pHB5 (gift of M. Messerle) (26) and the resulting intermediate construct pHB5(IEexon3-RpsLneo) was selected on the basis of resistance to kanamycin. Next, pSP-J(Sca-RVΔ30-77) was used as a template in conjunction with the following primers (Integrated DNA Technologies, Coralville, IA): sense, 5' TCTCCTGTATGTGACCCATGTGCTTATGACTCTATTTCTCATGTGTTTAG 3'; antisense, 5' CCACGTCCCATGCAGGTTAGTATACATCACATACATGTCAACAGACTTAC 3' to amplify a linear fragment containing the mutated IE exon 3 region. This fragment was recombined into pHB5(IEexon3-RpsLneo), replacing the RpsLneo cassette, and the resulting IEΔexon3(aa30-77) BAC was selected on the basis of increased streptomycin resistance. The exon 3 region was amplified and sequenced to confirm that the intended deletion had been introduced into the BAC.

A rescued BAC was generated from the mutant by using the primers listed above and pSP-J(Sca-RV) as a template to produce a linear fragment containing the

wild-type IE exon 3 sequence. This fragment was inserted into the IEΔexon3(aa30-77) BAC in a single round of homologous recombination in *E. coli*. The resulting BAC pool was transfected into HFF which were monitored for development of plaques spreading with wild-type kinetics. Candidate plaques were allowed to spread and supernatant taken from these cultures was used to infect a second set of HFF. BAC DNA was isolated from these secondary cultures by the method of Hirt (99) and transformed into *E. coli* strain DH10B. A single clone was chosen, and the exon 3 region was sequenced as described above to confirm that it had been returned to wild-type. This rescued BAC was designated IEΔexon3rescue.

Wild-type, mutant, and rescued BAC DNAs were amplified and purified as described (188). Each BAC was digested with *EcoR*I and separated by field inversion gel electrophoresis to ascertain that no major alterations to the DNA were sustained during the cloning procedure.

**Reconstitution of virus.** Wild-type, IEΔexon3, and IEΔexon3rescue BACs were transfected into HFF by electroporation as previously described (230) and monitored for plaque development. When all cells in a culture exhibited cytopathic effect, supernatants were harvested and used to infect fresh cells. Stocks of wild-type, IE Δ30-77, or rescued IE Δ30-77 viruses were harvested and titered by estimating titer based on the proportion of cells in a culture reactive with IE-specific CH16.0 antibody by immunostaining 24 hours post infection (h p.i.).

**Determination of virus titers and virus growth curves.** Titers of wild-type, IE Δ30-77, and rescued IE Δ30-77 viruses were determined by diluting stocks, mixing with a known number of cells, and seeding onto coverslips. At 24 h later, cells were

fixed in 2% formaldehyde in phosphate-buffered saline (PBS) and IE1 and IE2 protein expression was detected by immunostaining with CH16.0 antibody as described below. The effective multiplicity of infection (MOI) for two or more dilutions of virus was calculated based on the percentage of cells on a coverslip expressing IE antigens. These values were used to determine a titer, expressed as $IE^+$ units/ml, for each stock of virus. Virus growth curves were repeated at least three times for the infection at a MOI of 5 in HFF; data shown are from a single representative experiment.

**Time course of virus infection.** HFF were grown to and maintained at confluence for three days prior to infection to allow synchronization in a $G_0$ state. At the time of infection, cells were released from $G_0$ by trypsinization, infected at an MOI of 0.5, 5, or 10 IE+ units/cell, and replated at a lower density. Cells were refed daily, then harvested by trypsinization and frozen at the indicated times p.i.

**Quantitative real-time PCR and RT-PCR analyses.** DNA was isolated from infected cell pellets using a blood Mini kit (Qiagen, Valencia, CA) and the concentration of each sample was determined by UV spectrophotometry. Quantitative real-time PCR was performed in an Applied Biosystems ABI Prism 7000 Sequence Detection System using TaqMan Universal PCR Master Mix (Applied Biosystems) and oligonucleotide primers and TaqMan dual-labeled (5' 6-carboxyfluorescein, 3' black hole quencher-1) probes (Integrated DNA Technologies, Coralville, IA) directed against the unspliced HCMV UL77 gene (230). PCR reactions contained 40 ng DNA each and were performed in duplicate. The DNA isolated at day postinfection from wild-type-infected cells was used to generate a standard curve, allowing comparison of the amount of DNA present in each sample to the amount present in wild-type

virus-infected cells at 1 day p.i. Real-time reverse transcriptase PCR (RT-PCR) and data analysis were performed as previously described using primers and probes directed against the HCMV genes UL112-113, UL89, and R160461 and the cellular housekeeping gene glucose-6-phosphate dehydrogenase (G6PD) (230).

**Western blotting.** Cells were lysed in reducing sample buffer (50 mM Tris [pH 6.8], 0.2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM ß-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 100 μM pepstatin A) and protein content was determined by a Bradford assay. Equal amounts of protein were separated on sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and transferred to nitrocellulose. Membranes were stained with amido black to ensure equal protein loading. After being blocked in 5 to 7.5% nonfat dried milk in TBS-T (Tris buffered saline [pH 7.4] with 0.05% Tween-20), blots were incubated with primary antibodies in 5 to 7.5% nonfat dried milk in TBS-T, diluted as follows: CH16.0 MAb, 1:15,000; UL112-113 rabbit polyclonal Ab, 1:1500; UL44 MAb, 1:10,000; pp65 MAb, 1:15,000; major capsid protein (MCP) MAb 28-4, 1:20; pp28 MAb, 1:10,000; β-actin MAb AC-15, 1:10,000; cyclin B1, 1:500; cyclin A MAb CYA06, 1:200; cyclin E sc-198, 1:400. Monoclonal antibody to MCP was the gift of Dr. William Britt (University of Alabama, Birmingham). UL112-113 antibody has been previously described (200). CH16.0, anti-UL44, anti-pp65, and anti-pp28 were purchased from the Goodwin Institute (Plantation, FL). Anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO), anti-cyclin B1 was purchased from BD Biosciences (San Jose, CA), anti-cyclin A was purchased from Lab Vision (Fremont,

CA), and anti-cyclin E was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were washed in TBS-T and incubated in horseradish peroxidase-coupled anti-mouse or anti-rabbit antibodies (Calbiochem, San Diego, CA), diluted 1:2000 to 1:10,000. After washing in TBS-T, proteins were detected using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

**Immunofluorescence analysis.** Cells were washed in PBS and fixed in 2% paraformaldehyde in PBS at the indicated times p.i. and processed as previously described (188). Briefly, cells were permeabilized in 0.2% Triton X-100, blocked in 10% normal goat serum in PBS, then incubated with primary antibody in 5% normal goat serum at the following dilutions: CH16.0 MAb (directed against IE exon 2 sequences), 1:1000; IE1 72-specific MAb p63-27, 1:1000; UL44 MAb, 1:500; pp65 MAb 28-19, 1:2; pp28 MAb 41-18, no dilution; PML MAb PG-M3, 1:50. Monoclonal antibodies p63-27, 28-19, and 41-18 were gifts from Dr. William Britt (University of Alabama, Birmingham). Anti-PML was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After three washes in PBS, coverslips were incubated with Hoechst and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1:500 (for CH16.0, pp65, and pp28 stains) or with TRITC-conjugated goat anti-mouse IgG2a and FITC-conjugated goat anti-mouse IgG1 (for IE1 and UL44 stains or IE1 and PML stains) diluted 1:75 (Southern Biotech, Birmingham, AL). Coverslips were washed and mounted onto slides with SlowFade antiphotobleaching reagent (Molecular Probes, Eugene, OR). Images were captured using a Nikon Eclipse E800

microscope and Photometrics CoolSnap fx CCD camera with Metamorph software (Universal Imaging Corp., Downington, PA) and processed in Adobe Photoshop.

## RESULTS

**Construction of a viable major IE exon 3 deletion mutant virus.** Exon 3 of the HCMV major IE region has been shown in transient assays and in vitro studies to contribute multiple functions to the products encoded by UL122-123. In the context of the IE1 72-kDa protein, amino acids specified by exon 3 contribute to an interaction between IE1 72 and the Rb family member p107 (111, 252). These residues also appear to be required for disruption of the ND10 sites by IE1 72 early in the infection (235). In contrast, exon 3 provides transactivating capacity to the IE2 86-kDa protein (200, 210). These functions have not been examined in the context of the viral genome. To better understand the contribution of exon 3 to the functions of IE1 and IE2 products and to understand the role of these products in the infected cell, we constructed a viable recombinant virus with a deletion of the majority of exon 3 of the major IE region (Figure 4.1A).

To construct this HCMV recombinant, we began with the HCMV bacterial artificial chromosome (BAC) pHB5. Using homologous recombination in *E. coli* mediated by the RecE and RecT recombinases, we first replaced the region coding for aa 30 to 77 of IE1 72 and IE2 86 with a marker cassette containing the RpsL gene, conferring increased sensitivity to streptomycin, and the neomycin resistance marker to provide kanamycin resistance. Intermediate BAC clones were isolated based on resistance to kanamycin. The integrity of these clones was checked by digestion with EcoRI and insertion of the marker cassette in the correct location was confirmed by Southern blotting (data not shown). In a second round of homologous recombination,

the entire marker cassette was replaced with a fragment containing 5' and 3' exon 3 sequences but lacking the nucleotides that encode the majority of the exon, aa 30 to 77. The altered major IE region therefore encodes smaller forms of both IE1 72- and IE2 86-kDa proteins, as the splice junctions at each end of exon 3 have been preserved. Recombinant constructs were isolated based on increased resistance to streptomycin, and the exon 2 to 3 region was amplified by PCR and sequenced to determine that the correct deletion had been inserted and that the reading frame had been preserved. Integrity of the mutant BACs was checked by digestion of the clones with EcoRI and separation and visualization of the fragments by field inversion gel electrophoresis (Figure 4.1B). A rescued BAC with wild-type exon 3 sequences replaced was generated as described (Materials and Methods) and grows essentially like the wild-type parent.

To reconstitute wild-type, IE Δ30-77, and rescued IE Δ30-77 viruses from the recombinant constructs, BACs were transfected by electroporation into confluence synchronized human foreskin fibroblasts (HFF). Plaques developed beginning 5 days postelectroporation in cultures transfected with the wild-type or rescued BACs and 12 days postelectroporation in cells which received IE Δ30-77 mutant BAC. Virus was harvested following complete infection of all cells in the culture and used to reinfect subsequent rounds of permissive cells to generate wild-type, mutant, and rescued virus stocks.

Initial attempts to titer stocks of the IE Δ30-77 mutant virus by plaque assay indicated that titers from plaque assays underestimated the number of cells which

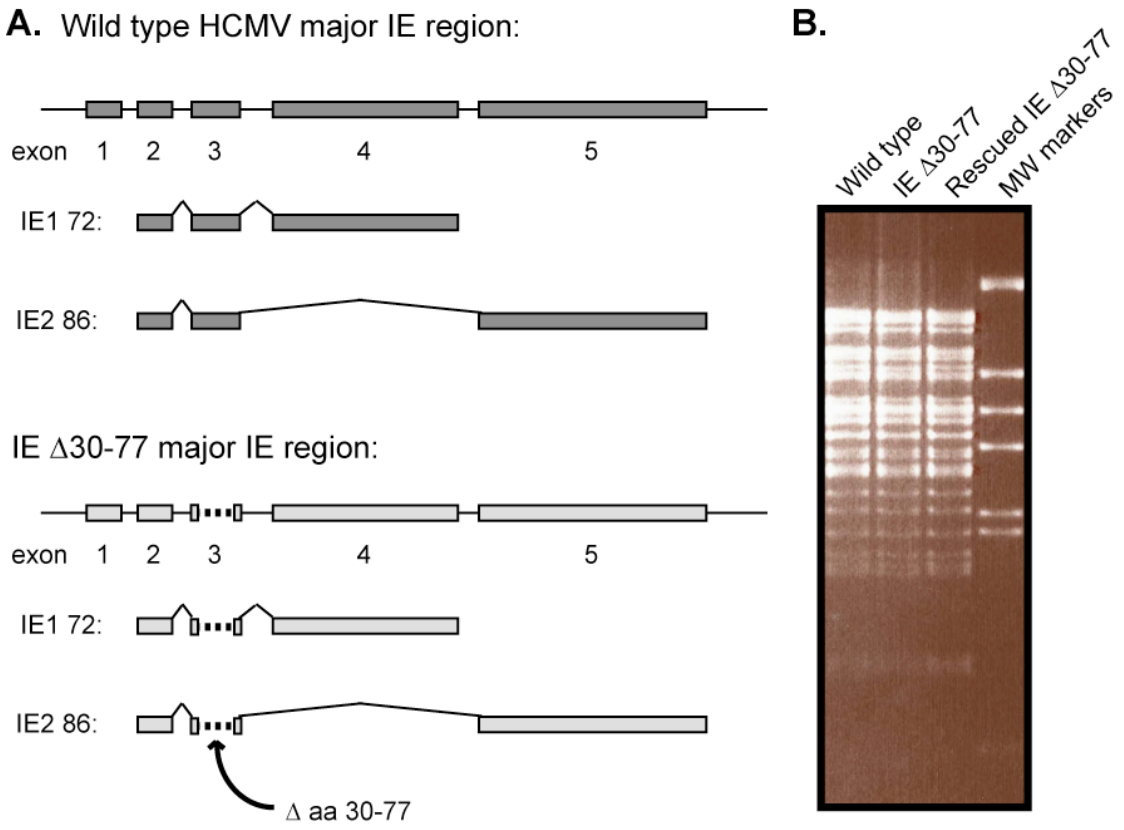



**Figure 4.1 Construction of the IE Δ30-77 mutant BAC.** (A) The HCMV major immediate early region. To construct the IE Δ30-77 BAC, nucleotides coding for amino acids 30 to 77 of IE1 72 and IE2 86 were removed from pHB5 as described in Materials and Methods. The resulting mutant BAC encodes shorter forms of both IE1 72 and IE2 86 proteins, as the nucleotides near the exon 3 splice junctions have been preserved. A rescued version of the IE Δ30-77 BAC was generated in which the altered region was replaced with wild-type sequences. (B) EcoRI digests of the recombinant BACs. A total of 1 μg wild-type (pHB5), IE Δ30-77, or rescued IE Δ30-77 BAC DNA was cut with EcoRI and the resulting digests were separated by field inversion gel electrophoresis on a 1% agarose gel in 0.5× Tris-borate-EDTA. MW, molecular weight.

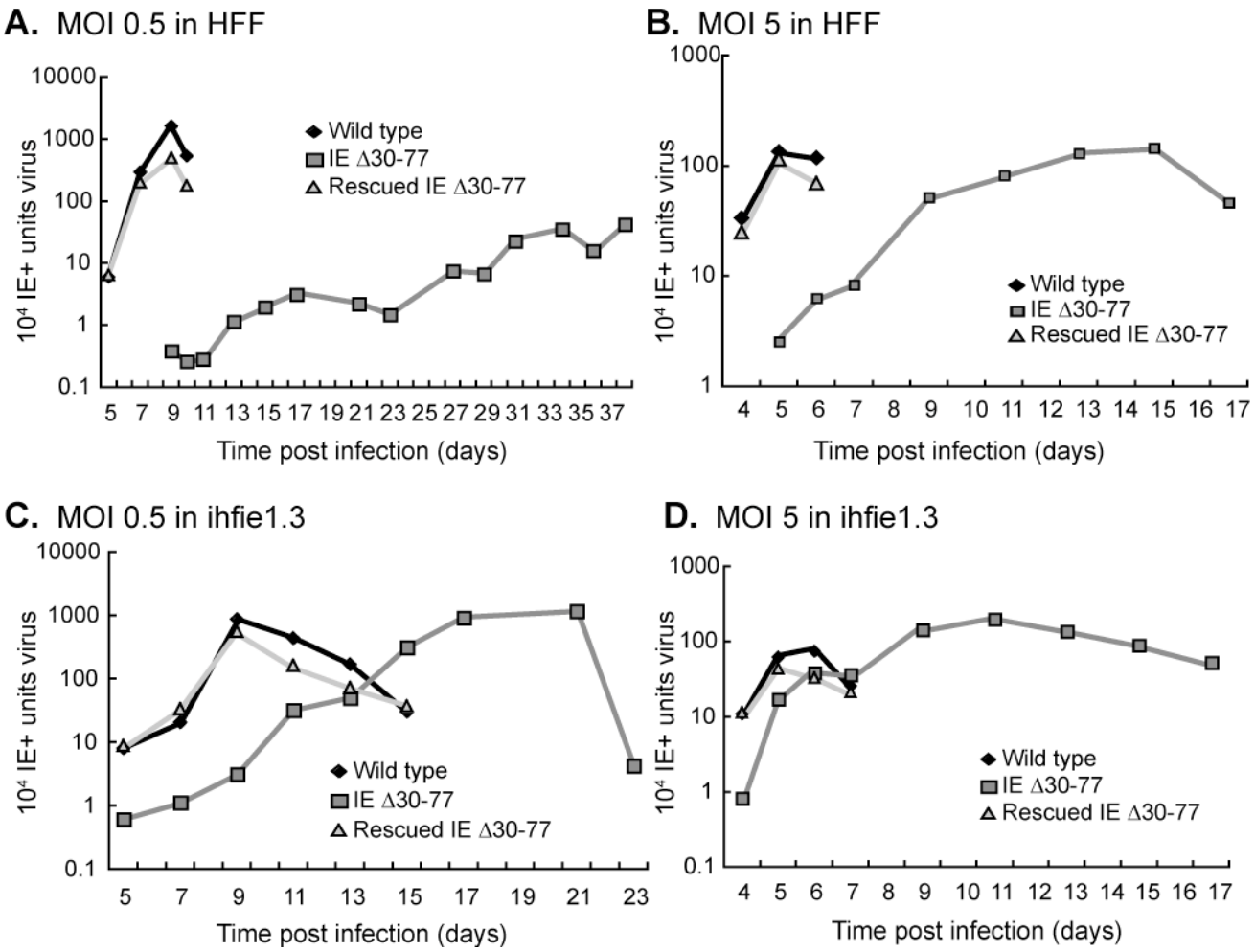
express IE proteins at 24 h p.i. (as judged by immunostaining with CH16.0 antibody) by 500 to 1,000 times. For this study, we therefore estimated titers of wild-type, IE Δ30-77, and rescued mutant viruses based on the number of IE+ cells present at 24 h p.i. (see Materials and Methods). To confirm that a high proportion of the IE+ cells in the IE Δ30-77 mutant-infected cultures were not generated by infection with defective particles, we also performed immunostaining with antibody to the tegument protein pp65 (data not shown). Since an equivalent number of cells infected with 0.5 $IE^+$ units/cell of wild-type, mutant, or rescued mutant virus exhibit staining of input pp65 protein at 6 h p.i., we conclude that the virus preparations do not contain a disproportionate number of defective vs. replication-competent particles.

**Growth of the IE Δ30-77 mutant virus is impaired at high and low multiplicities of infection.** To establish the growth characteristics of the IE Δ30-77 mutant relative to the wild-type and rescued viruses, we infected HFF at a high (5 $IE^+$ units/cell) or low (0.5 $IE^+$ units/cell) multiplicity of infection (MOI) and harvested supernatants beginning at 2 days p.i. and continuing until all cells in a culture had died. Titers of viral supernatants were determined as described above and used to generate growth curves (Figure 4.2). In a low multiplicity infection, the IE Δ30-77 mutant virus exhibits two growth defects compared to wild-type virus (Figure 4.2A). First, IE Δ30-77 replicates with delayed kinetics. Peak viral titer is observed nine days after infection with wild-type or rescued mutant virus at an MOI of 0.5 $IE^+$ units/cell, representing virus released from cells infected in the second round of infection. The peak that likely represents the corresponding second-round release of IE Δ30-77 virus occurs between days 17 and 21 p.i. IE Δ30-77 virus production under these conditions

therefore takes about twice as long as does release of the wild-type and rescued mutant strains. The second defect is in the amount of virus produced. Over 500 times more virus is produced from wild-type virus-infected cells nine days post infection than from IE Δ30-77 virus-infected cells 17 days p.i. Even 38 days p.i., IE Δ30-77 virus-infected cells still produce nearly 40-fold less virus than wild-type-infected cells did at 9 days p.i. Growth at high multiplicity rescues only one of these defects (Figure 4.2B). HFF infected at an MOI of 5 $IE^+$ units/cell still exhibit a significant kinetic defect, with peak virus production again taking over twice as long from cells infected with IE Δ30-77 virus than from cells infected with wild-type or rescued mutant viruses. IE Δ30-77 virus-infected cells, however, under these conditions produce comparable levels of infectious virus to those released from wild-type virus-infected cells. This indicates that under high multiplicity conditions, the presence of additional viral input protein or, more likely, additional copies of the mutant genome enable the IE Δ30-77 virus to replicate to wild-type levels, but not with wild-type kinetics.

We next wanted to determine whether IE Δ30-77 growth is impaired due to the absence of amino acids 30 to 77 from IE1 72 protein, IE2 86 protein, or both products. We constructed similar growth curves (Figure 4.2) following infection of ihfie1.3 cells expressing wild-type IE1 72 kDa protein (gift of E. Mocarski, (148)) with 5 or 0.5 $IE^+$ units/cell of wild-type, mutant, and rescued mutant virus. Low multiplicity (0.5 $IE^+$ units/cell) infection in ihfie1.3 cells resembles the corresponding infection in HFF, with peak virus production reached 9 days p.i. for wild-type and rescued mutant-infected cells and between 17 and 21 days p.i. for IE Δ30-77 mutant-infected cells (Figure 4.2C). While the kinetic defect is preserved, the amount of virus produced in

**Figure 4.2 IE Δ30-77 virus replicates with a kinetic defect not rescued by wild-type IE1 72 protein provided in *trans*.** HFF or ihfie1.3 cells were infected with wild-type, IE Δ30-77, or rescued IE Δ30-77 virus with 5 or 0.5 $IE^+$ units/cell, as indicated. HFF (A, B) or ihfie1.3 (C, D) were infected at a low (A, C) or high (B, D) MOI, infected cell supernatants were collected at the indicated times, and titers were determined as described in Materials and Methods. Virus aliquots were collected from a culture until all cells in the dish appeared to be dead. The MOI 5 in HFF growth curve was repeated three times, and the results of a representative experiment are shown.


A. MOI 0.5 in HFF
B. MOI 5 in HFF
C. MOI 0.5 in ihfie1.3
D. MOI 5 in ihfie1.3
10^4 IE+ units virus
Time post infection (days)
Wild type
IE Δ30-77
Rescued IE Δ30-77

these cells at the peak is comparable for wild-type, IE Δ30-77, and rescued mutant virus infections. Similarly, ihfie1.3 infected at a high multiplicity (5 $IE^+$ units/cell) produce comparable amounts of wild-type, IE Δ30-77, and rescued mutant viruses, but reaching the peak of virus production for IE Δ30-77 takes 11 to 13 days compared to 5 to 6 days for the wild-type and rescued mutant viruses (Figure 4.2D). Like high multiplicity growth in HFF, growth in ihfie1.3 cells therefore restores the ability of the IE Δ30-77 recombinant to produce wild-type levels of virus, but does not allow it to do so with wild-type kinetics.

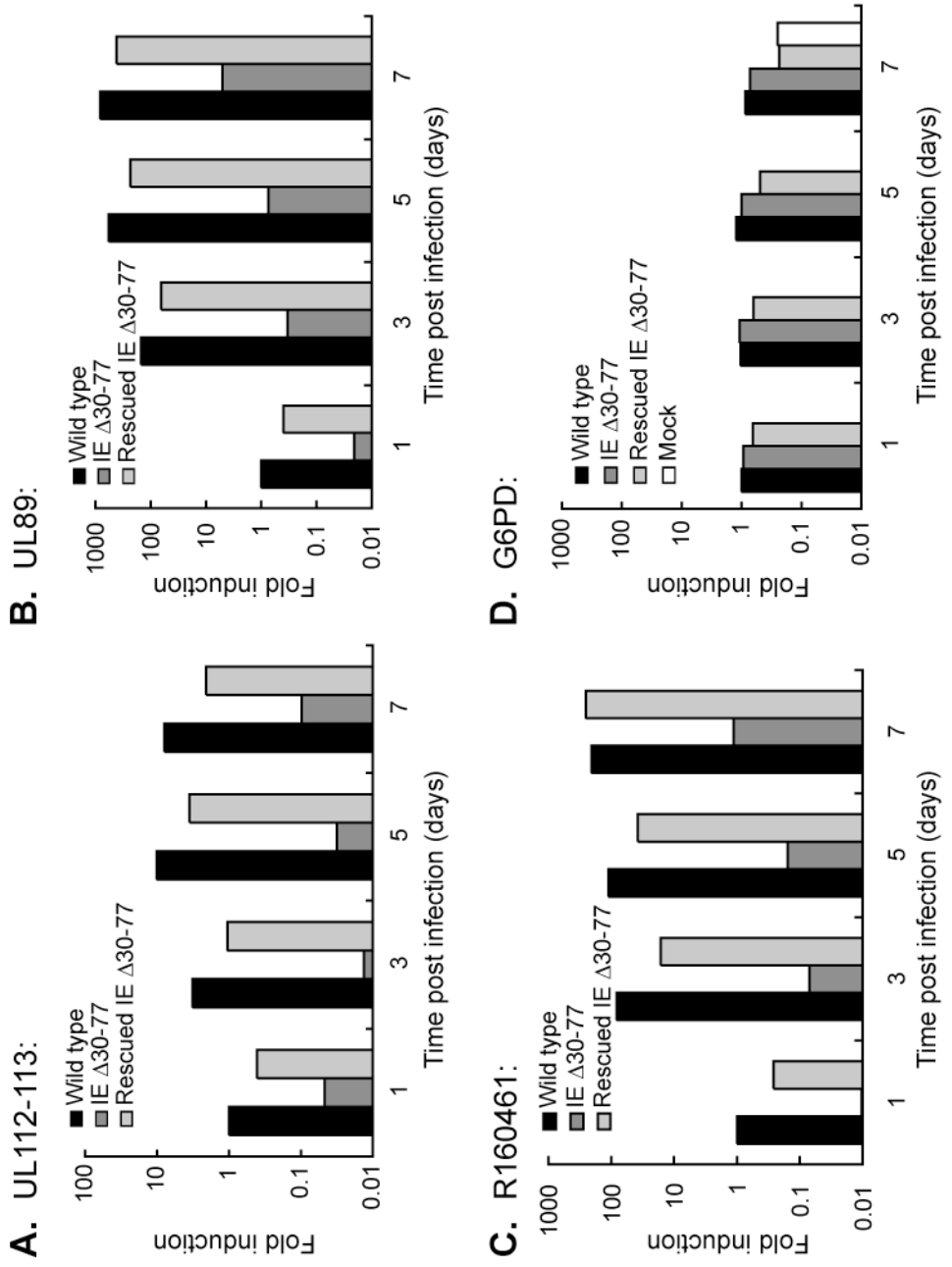
**A delay in early gene expression results in impaired replication of the IE Δ30-77 virus.** Based on this impaired growth at both high and low multiplicities of infection, we next wanted to establish the point in the viral replication cycle at which growth of the mutant is restricted. Confluence synchronized HFF were infected at a low (0.5 $IE^+$ units/cell) MOI and harvested 1, 3, 5, and 7 days post infection. Total RNA was harvested from infected cells and used as template in a series of quantitative real-time RT-PCR reactions to measure the relative amounts of UL112-113, UL89, and R160461 transcripts present in the infected cells (Figure 4.3). RNA from the cellular housekeeping gene glucose-6-phosphate dehydrogenase was quantified as a loading control (Figure 4.3D). Transcript levels for each sample are calculated and compared to the amount of RNA present in the wild-type virus-infected sample one day post infection; *n*-fold induction therefore indicates the increase or decrease in transcript level compared to the amount present in the earliest wild-type virus-infected sample.

The kinetics of accumulation of transcripts from the UL112-113 region indicate that transcription of this viral early gene is severely impaired in IE Δ30-77 virus-infected cells compared to wild-type or rescued mutant virus-infected cells (Figure 4.3A). Twenty-four hours p.i., over 20 times less UL112-113 transcript is detected in mutant virus-infected compared to wild-type virus-infected cells. At 3 days p.i., the level of detectable UL112-113 RNA in IE Δ30-77 virus-infected cells has dropped even lower relative to the wild-type and rescued mutant controls, and not until 5 days p.i. is an increase in the transcription of this representative early locus detected in mutant virus-infected cells. This increase continues through 7 days p.i., although UL112-113 expression remains significantly impaired in mutant virus-infected cultures.

The pattern of delayed early and late transcript accumulation in mutant-infected cells is similar to that of the early transcript. We examined expression of the UL89 (Figure 4.3B) and R160461 (Figure 4.3C) loci, which are transcribed with delayed early and late kinetics, respectively, as examples of each of these classes of genes. In each case, transcription of the UL89 and R160461 genes in IE Δ30-77 mutant-infected cells is severely reduced at 24 hours p.i. compared to the wild-type and rescued mutant-infected controls. In contrast to UL112-113, by 3 days p.i. transcription has begun to increase and continues to increase through the end of the time course.

Examination of viral DNA replication by real-time PCR further defines the growth restriction of the IE Δ30-77 mutant virus (Figure 4.4). Amplification of the viral genome in infected cells was detected using primers and TaqMan probe

**Figure 4.3 Early viral gene expression is delayed following low-multiplicity infection of HFF with IE Δ30-77 recombinant virus.** $G_0$-synchronized HFF were infected with 0.5 $IE^+$ units/cell of wild-type, IE Δ30-77, or rescued IE Δ30-77 viruses or mock infected and harvested at the indicated times p.i. Total cellular RNA was analyzed by quantitative real-time RT-PCR as described in Materials and Methods to measure the relative levels of transcripts expressed from the early UL112-113 (A), delayed early UL89 (B), or late R160461 (C) viral loci. Experiments were repeated at least three times, each time with duplicate reactions, and the results of a representative experiment are shown. To ensure that an equal amount of RNA was included in each reaction, samples were analyzed with G6PD-specific primers and probe (D). When mock-infected (Mock) cell RNA was analyzed with UL112-113, UL89, or R160461 probes, the result was below the limit of detection; these values are not indicated on the graphs.


A. UL112-113:
B. UL89:
C. R160461:
D. G6PD:
Wild type
IE Δ30-77
Rescued IE Δ30-77
Mock
Fold induction
Time post infection (days)
0.01
0.1
1
10
100
1000
1
3
5
7

specific to the HCMV UL77 gene, which is unspliced. The pattern of viral DNA accumulation is similar to the kinetics of UL89 and R160461 RNA expression in IE Δ30-77 infected cells, with levels of viral DNA approximately equal between days 1 and 3 p.i. and increasing beginning 5 days p.i., but still several orders of magnitude lower than those seen in wild-type or rescued mutant virus-infected cells. Taken together, these experiments examining accumulation of selected viral transcripts and replication of the viral DNA suggest that the block in replication of the IE Δ30-77 mutant virus occurs due to inefficient expression of viral early genes. Early transcripts, as represented by UL112-113 RNA, are slow to accumulate in the IE Δ30-77 infected cell, but once levels of these products begin to accumulate (between 3 and 5 days p.i.), there is no subsequent delay detected in the replication of viral DNA or in the expression of the delayed early and late genes UL89 and R160461.

**Viral protein expression is impaired in IE Δ30-77 virus-infected cells.** To further investigate the IE Δ30-77 growth defect, we analyzed the expression of selected viral proteins in cells infected at high or low multiplicities. Cells infected with 0.5 $IE^+$ units/cell of wild-type, IE Δ30-77, or rescued mutant virus were harvested 1, 3, 5, and 7 days post infection and samples were analyzed by Western blotting (Figure 4.5). Using CH16.0, an antibody directed against the shared exon 2 region of IE1 72 and IE2 86, we can detect both the wild-type and smaller mutant forms of each of these proteins. In wild-type and rescued mutant virus-infected cells, we observe a typical pattern of IE1 72 and IE2 86 protein expression in which overall expression of the two proteins increases and the relative abundance of IE1 72 compared to

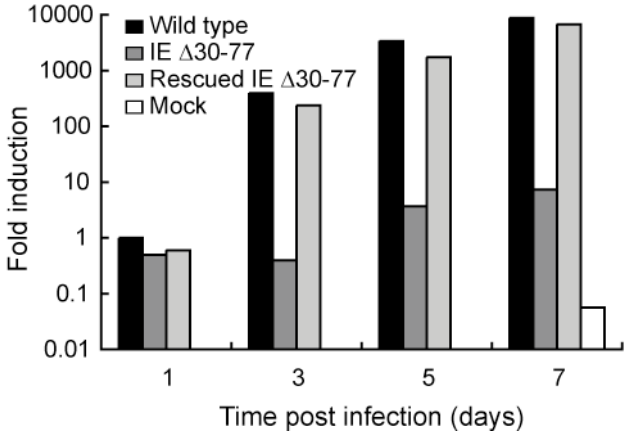



**Figure 4.4 The kinetics of viral DNA replication in IE Δ30-77 virus-infected cells follow the increase in viral early gene expression.** $G_0$-synchronized HFF cells were infected with 0.5 $IE^+$ units/cell of wild-type, IE Δ30-77, or rescued IE Δ30-77 viruses or mock infected (Mock) and harvested at the indicated times p.i. Viral DNA amplification was measured by quantitative real-time PCR as described in Materials and Methods using primers and probe directed against the unspliced viral UL77 gene.

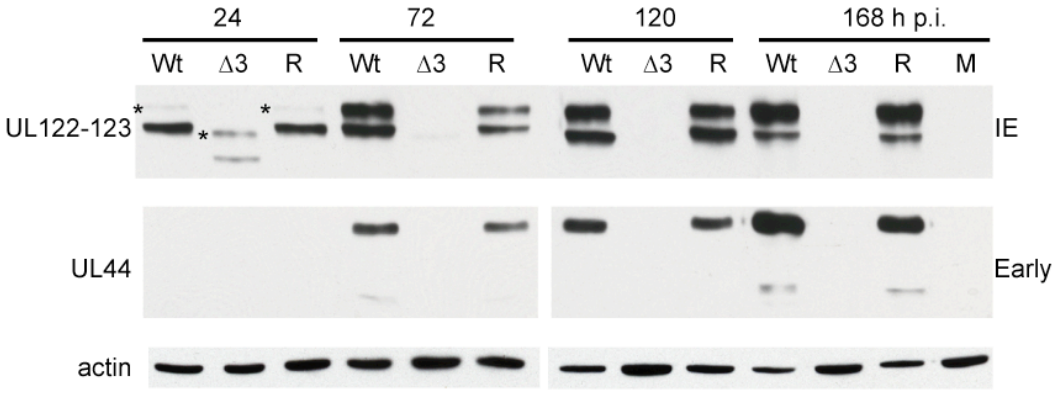



**Figure 4.5 IE and early protein expression is altered in IE Δ30-77 virus-infected cells.** $G_0$-synchronized HFF cells were infected with 0.5 $IE^+$ units/cell of wild-type (Wt), IE Δ30-77 (Δ3), or rescued IE Δ30-77 (R) viruses or mock infected (M) and harvested at the indicated times p.i. Equal amounts of cell lysates (in micrograms) were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. IE1 72, IE2 86, and UL44 protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading. Asterisks (*) indicate the position of IE2 86 or mutant IE2 86 in lanes containing 24-h p.i. samples.

IE2 86 decreases as the infection progresses. The expression of the two proteins in IE Δ30-77 virus-infected cells is significantly different. Twenty-four hours p.i., mutant forms of both IE1 72 and IE2 86 are expressed and the amount of mutant IE2 protein present is comparable to that in wild-type and rescued mutant virus-infected cells (compare the top band in each lane). In contrast to the pattern of accumulation of these proteins in wild-type virus-infected cells, the mutant forms of IE1 72 and IE2 86 are approximately equally abundant early in the infection. As the infection progresses, these proteins are no longer detectable in the IE Δ30-77 virus-infected cell, either because their expression does not continue or because the combination of spread of the wild-type and rescued viruses and growth of the uninfected cells results in much less viral protein present in a mutant virus-infected versus a wild-type virus-infected cell lysate. If the expression of IE gene products is inefficient, one would predict a corresponding defect in the expression of early gene products. Therefore, it is not surprising that while UL44 expression in wild-type and rescued mutant virus-infected cells is efficient, we are unable to detect UL44 protein by Western blot in cells infected with 0.5 $IE^+$ units/cell of IE Δ30-77 virus (Figure 4.5).

**High multiplicity growth does not completely restore viral protein expression in IE Δ30-77 virus-infected cells.** CR208, the IE1 72 deletion mutant virus, exhibits impaired growth under low multiplicity conditions but grows much like its wild-type parent when the infection is carried out at a high MOI (79, 84, 148). The growth curves shown in Figure 4.2 suggest that the IE Δ30-77 mutant virus does not share this behavior; that is, growth at a high multiplicity does not allow the IE Δ30-77 recombinant to replicate with wild-type kinetics. To confirm that viral protein

expression is reduced in cells infected with 5 $IE^+$ units/cell of IE Δ30-77 virus, we examined the expression of selected viral proteins by Western blotting (Figure 4.6). Under these high-multiplicity conditions, we are able to detect the expression of viral proteins in mutant virus-infected cells. Importantly, the expression of each of these viral proteins continues to be altered or reduced under high-multiplicity infection conditions. There is also a significant difference in the morphology of wild-type versus IE Δ30-77 virus-infected cells, with significantly less observable cytopathic effect in mutant virus-infected cells than in wild-type virus-infected cells, even when every cell in an IE Δ30-77 virus-infected culture stains positively for IE proteins detected by CH16.0 antibody (data not shown).

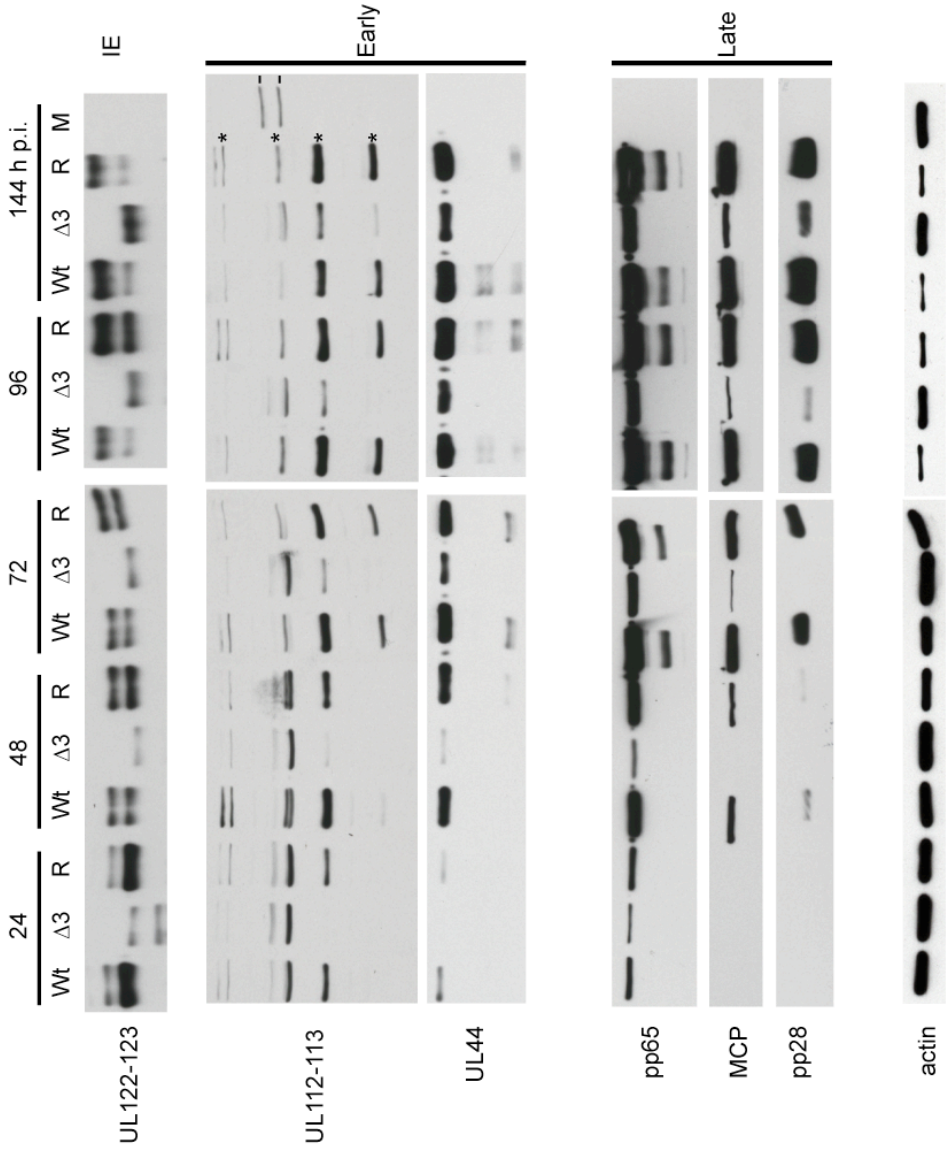
During a high-multiplicity infection, cells infected with wild-type or rescued mutant virus express the IE1 72- and IE2 86-kDa proteins beginning at early and continuing through late times p.i. (Figure 4.6). Again, we observe the characteristic shift from predominant expression of IE1 72- to IE2 86-kDa protein as the infection progresses. In contrast, IE Δ30-77-infected cells express mutant forms of both IE1 72 and IE2 86 at 24 h p.i. While the level of IE2 86 protein detected is comparable to that in wild-type-infected cells, the amount of mutant IE1 72 observed is significantly reduced compared to the wild-type control. By 48 h p.i., the mutant form of the IE1 72 protein is no longer expressed to detectable levels. Although mutant IE2 86 protein slowly accumulates as the time course continues, we cannot detect mutant IE1 72 protein in IE Δ30-77-infected cells at any subsequent time.

Western blots using antibodies directed against the early viral proteins expressed from the UL112-113 and UL44 loci indicate that early protein expression is

also delayed in the IE Δ30-77 virus-infected cell. Four related phosphoproteins are encoded by the UL112-113 gene, with 43- and 50-kDa proteins expressed first and 34- and 84-kDa forms present later (208, 240). In wild-type and rescued mutant virus-infected cells, we observe the characteristic expression and accumulation of each of these products (Figure 4.6). In cells infected with the IE Δ30-77 virus, expression of the UL112-113 protein family is substantially delayed; for example, even by 96 h p.i. there is less detectable 43-kDa protein present in mutant virus-infected cells than there was in wild-type virus-infected cells 24 h p.i. The UL44 products are also slow to appear in IE Δ30-77 virus-infected cells and lag in expression by approximately 24 to 36 hours compared to the wild-type and rescued mutant virus-infected controls. Taken together, these data indicate that as during the low-multiplicity infection, viral gene expression is slow and is blocked at the transition from IE to early gene expression when cells are infected with IE Δ30-77 virus at a high MOI. Actin levels appear to decrease in wild-type and rescued mutant-infected cells beginning 72 h p.i. due to the significantly increased contribution of viral proteins to the total starting at this time.

The altered expression of IE1 72 and delayed expression of UL44 as observed by Western blot is also easily detectable by immunostaining. Twenty-four hours p.i., all cells in a high multiplicity IE Δ30-77 virus-infected culture are infected as judged by reactivity with CH16.0 (data not shown). In the same culture, mutant virus-infected cells stained with antibody to IE1 72 or to UL44 exhibit variable staining intensity, with some cells showing bright IE1 72 staining and others expressing UL44 efficiently (Figure 4.7). Importantly, the bright IE1 72-positive cells are generally not

**Figure 4.6 IE1 72 protein expression is not maintained and viral early protein expression is delayed during high multiplicity infection with IE Δ30-77 virus.** $G_0$-synchronized HFF cells were infected with 5 $IE^+$ units/cell of wild-type (Wt), IE Δ30-77 (Δ3), or rescued IE Δ30-77 (R) viruses or mock infected (M) and harvested at the indicated times p.i. Equal amounts of cell lysates (in micrograms) were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. Immediate-early (IE1 72 and IE2 86, using antibody CH16.0), early (UL112-113 and UL44) and delayed early/late (pp65, major capsid protein [MCP], and pp28) protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading. Asterisks (*) indicate the positions of the 84, 50, 43, and 34 kDa proteins encoded by UL112-113. Dashes (-) indicate prominent non-specific proteins that cross-react with the UL112-113 antibody.


IE
Early
Late
24
48
72
96
144 h p.i.
Wt
Δ3
R
M
UL122-123
UL112-113
UL44
pp65
MCP
pp28
actin

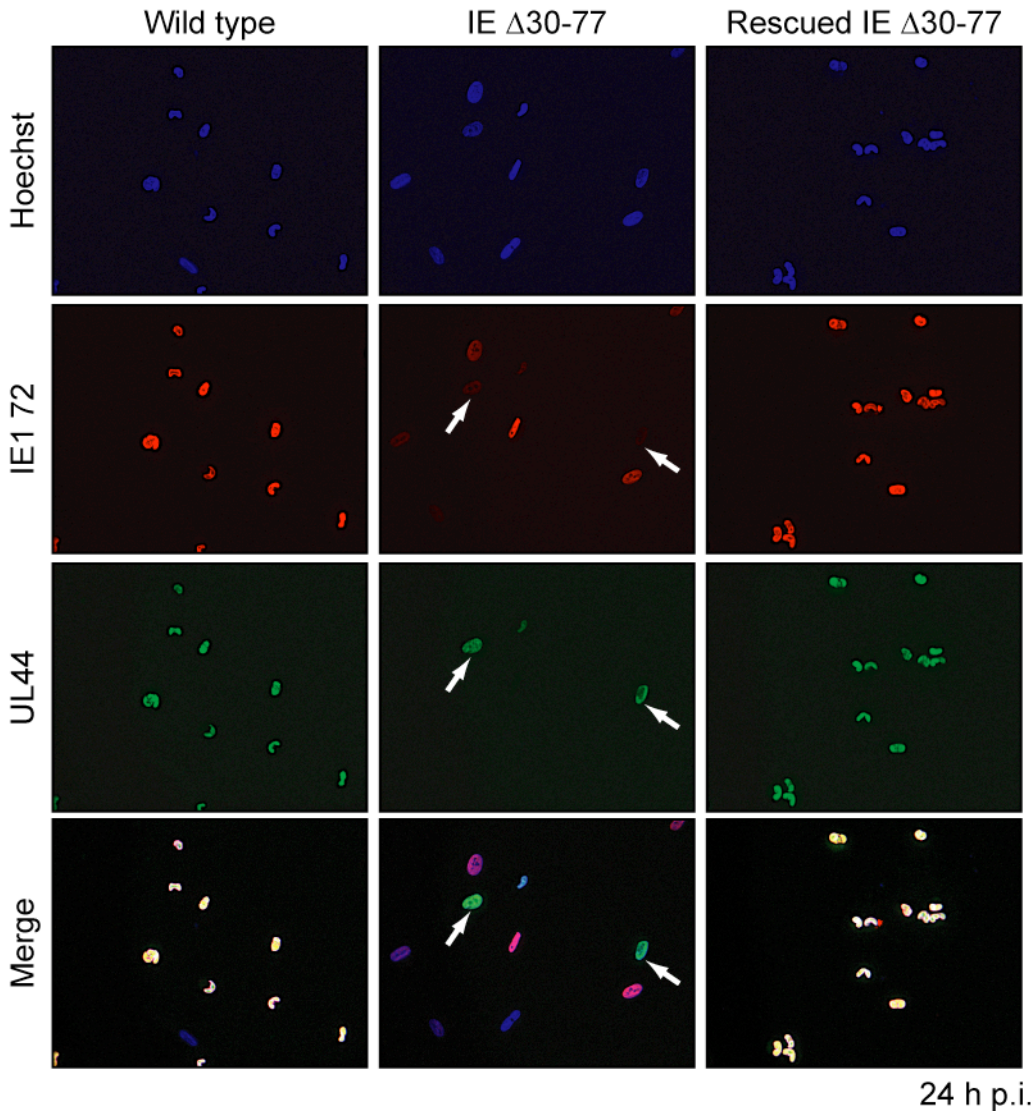



**Figure 4.7 IE1 72 and UL44 proteins are not efficiently expressed at the same time in IE Δ30-77 virus-infected cells.** $G_0$-synchronized HFF cells were infected with 5 $IE^+$ units/cell of wild-type, IE Δ30-77, or rescued IE Δ30-77 viruses and fixed in 2% paraformaldehyde 24 h p.i. Cells were stained with Hoechst dye to visualize nuclei, anti-IE1 72 monoclonal antibody p63-27, and antibody to UL44, followed by appropriate FITC- and TRITC-conjugated secondary antibodies (Materials and Methods). Arrows indicate the same cells in IE1 72 and UL44 fields. Magnification, ×400.

those that stain most intensely for UL44. This is in contrast to wild-type and rescued mutant virus-infected cells, which express IE and early antigens including IE1 72 and

UL44 simultaneously by 24 h p.i. We hypothesize that the altered progression to early gene expression during the IE Δ30-77 virus infection is being observed in single infected cells. These begin by expressing detectable IE1 72 but cannot sustain this expression when UL44 protein begins to be made. The result is a population of cells that appear to express IE1 72 or UL44, but not both. While we cannot currently determine whether the decrease in IE1 72 protein level is due to a block in expression of mutant IE1 72 or because the protein is unstable, these alternatives are discussed below.

Examining the expression of selected delayed early and late viral proteins during the high-multiplicity infection indicates that again, there is no additional defect in the progression of late events once the early proteins have been expressed. Major capsid protein, pp65, and pp28 are each present in the IE Δ30-77 virus-infected cell, but begin to be expressed following a lag comparable in length to the delay in early protein expression (Figure 4.6). Immunofluorescence analysis also indicates altered expression and localization of delayed early and late viral proteins in the IE Δ30-77 virus-infected cell. In cells infected at an MOI of 5, wild-type and rescued mutant virus-infected cells exhibit nuclear and cytoplasmic pp65 staining 72 h p.i., and all cells in the cultures express detectable pp65 protein (Figure 4.8A). A much smaller proportion of cells infected at the same multiplicity with IE Δ30-77 mutant virus express pp65 at this point, and in the majority of these cells, pp65 remains localized to the nucleus. The transition from nuclear to cytoplasmic localization of pp65 protein

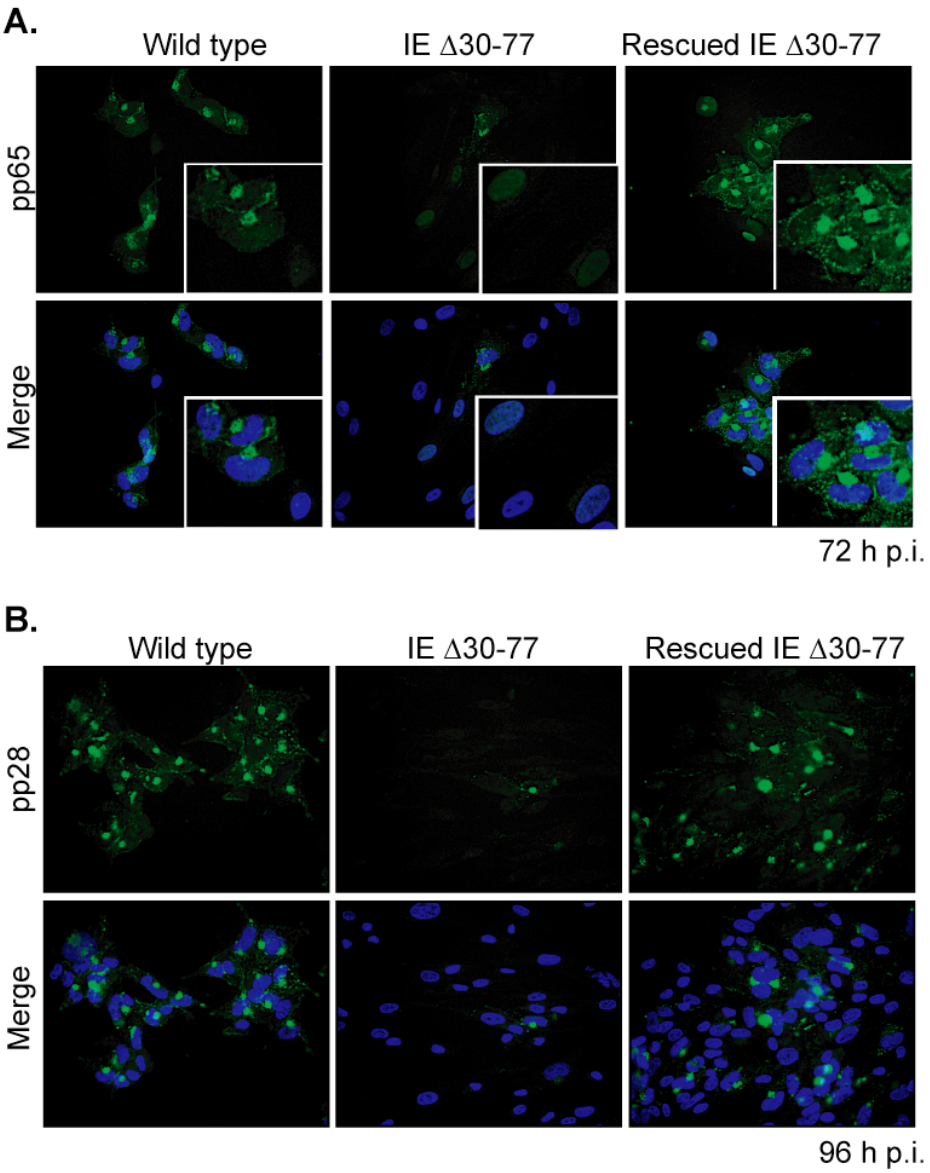



**Figure 4.8 Viral gene expression is delayed in IE Δ30-77 virus-infected cells.** $G_0$-synchronized HFF cells were infected with 5 $IE^+$ units/cell of wild-type, IE Δ30-77, or rescued IE Δ30-77 viruses and fixed in 2% paraformaldehyde at the indicated times p.i. Cells were stained with Hoechst dye to visualize nuclei and antibody to pp65 (A) or pp28 (B), followed by appropriate FITC-conjugated secondary antibodies (Materials and Methods). Magnification, ×400. Inset, 200% enlargement of pp65 expression in wild-type, IE Δ30-77, or rescued IE Δ30-77 virus-infected cells. Merge, overlaid viral antigen and Hoechst-stained images.

appears to be an indicator of the progression of late phases of the HCMV infection, and it has been observed that pp65 is retained in the nucleus of cells infected with another IE2 86 deletion mutant virus that replicates slowly (188). The expression of pp28 protein is also delayed, and few IE Δ30-77 mutant virus-infected cells in a culture contain levels of pp28 detectable by immunostaining 96 h p.i. (Figure 4.8B).

**IE Δ30-77 virus-infected cells exhibit altered expression of cellular cyclins.** Based on the differences in growth of the IE Δ30-77 mutant virus from wild-type HCMV, we wanted to investigate whether the mutant and wild-type virus infections differentially alter the host cell cycle. In wild-type HCMV-infected fibroblasts, there is a characteristic increase in cyclin B1 and cyclin E protein levels and decrease in the amount of cyclin A relative to uninfected controls following infection. To compare these effects to those occurring in cells infected with the IE Δ30-77 mutant virus, we conducted time course experiments at an MOI of 10 $IE^+$ units/cell (Figure 4.9A). These experiments were performed using media containing 1% rather than 10% serum to allow the viral effects on cellular proteins to be more easily observed against the background of serum-induced changes in protein expression. The virus used for infections was contained in media with 10% serum, so that virus was adsorbed in media with more than 1% serum.

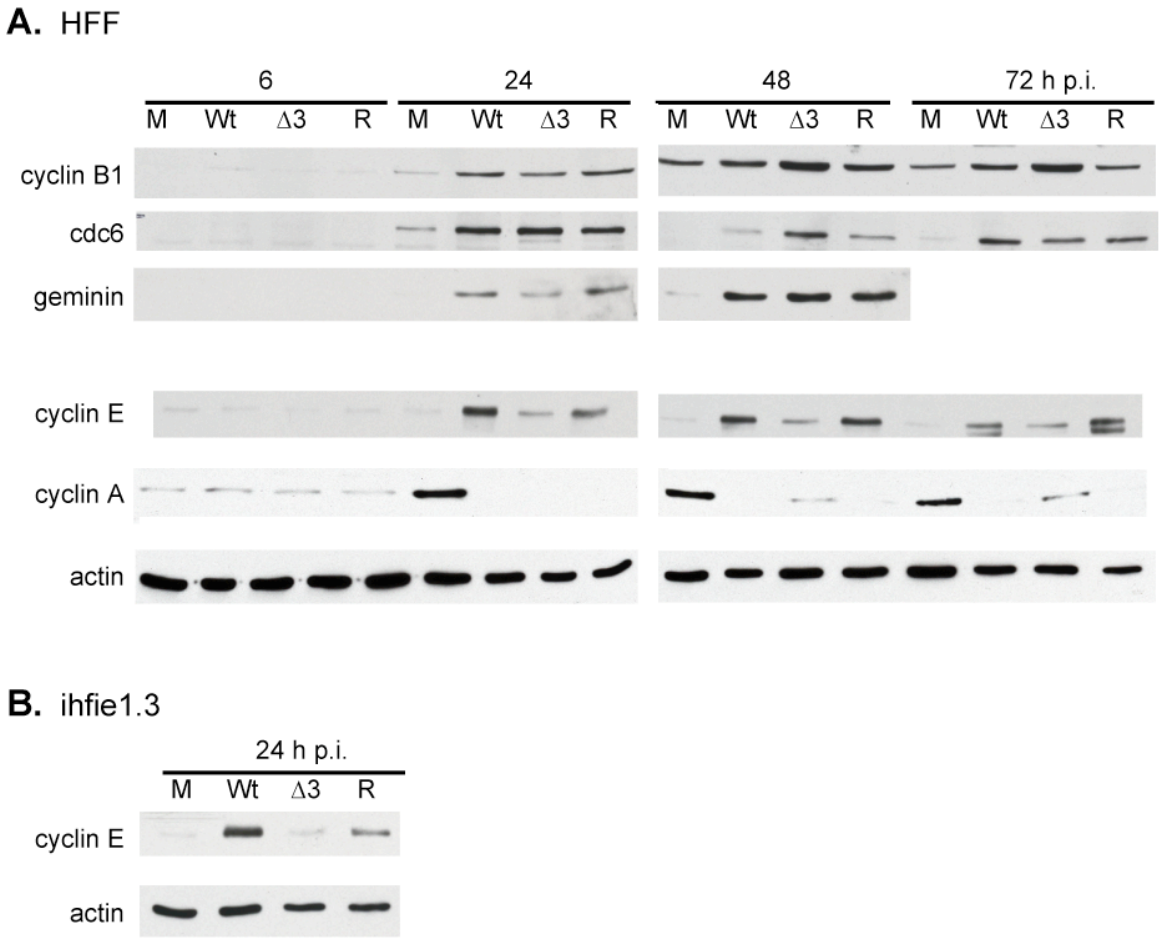
The expression of cyclin B1, cdc6, and geminin is typically altered in the HCMV-infected cell such that by 24 h p.i., these proteins are present at significantly higher levels in infected cells than in uninfected cells (22, 108, 186, 189, 234). For cyclin B1, this is the result of a combination of factors including increased synthesis of the protein and an inhibition of its degradation by the proteasome (189). Up-

regulation of cdc6 and geminin contributes to an inhibition of DNA licensing so that the replication of viral DNA is favored over that of cellular DNA in the infected cell (22, 234). In cells infected with the IE Δ30-77 mutant virus, these effects are preserved, as cyclin B1, cdc6, and geminin protein levels increase with the same kinetics as in wild-type virus-infected cells (Figure 4.9A). Lanes contain equal protein content; delayed viral gene expression in mutant virus-infected cells therefore leads to a slight overrepresentation of cyclin B1 and cdc6 in mutant virus-infected compared to wild-type virus-infected cells at 48-72 h p.i.

Cyclin E protein expression is also increased in HCMV-infected cells by 24 h p.i., although in contrast to the up-regulation of cyclin B1 its induction occurs via a transcriptional mechanism (28, 29, 145, 186). The IE Δ30-77 mutant virus appears able to support this induction only to a limited degree, as we observe cyclin E protein in mutant virus-infected cells at an intermediate level compared to the protein present in wild-type virus-infected and uninfected cells (Figure 4.9A). Cyclin A expression is down-regulated at the transcriptional level in wild-type virus-infected compared to uninfected cells and exhibits a similar effect in IE Δ30-77 virus-infected cells. Here, cyclin A protein levels are equivalent in wild-type virus-infected, IE Δ30-77 virus-infected, and uninfected cells 8 h p.i., then increase in uninfected cells and decrease in wild-type virus-infected cells by 48 h p.i. (Figure 4.9A). There appears to be a modest

increase in cyclin A protein levels in the IE Δ30-77 virus-infected cells. This is likely due to the fact that viral gene expression is significantly delayed in IE Δ30-77 virus-infected cells, resulting in a larger relative contribution of cellular versus viral proteins to IE Δ30-77 virus-infected cell lysates.

**Figure 4.9 Altered expression of cell cycle regulatory proteins in IE Δ30-77 virus-infected cells.** (A) $G_0$-synchronized HFF cells were mock infected (M) or infected with 10 $IE^+$ units/cell of wild-type (Wt), IE Δ30-77 (Δ3), or rescued IE Δ30-77 (R) viruses and harvested at the indicated times p.i. Cells were maintained in media containing 1% serum after 6 h p.i. (B) ihfie1.3 cells were mock infected (M) or infected with 5 IE+ units/cell of wild-type (Wt), IE Δ30-77 (Δ3), or rescued IE Δ30-77 (R) viruses and harvested 24 h p.i. Cells were maintained in media containing 10% serum throughout the time course. For both cell types, equal amounts of cell lysates (in micrograms) were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. Cyclin B1, cdc6, geminin, cyclin E, and cyclin A protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading.


A. HFF
6
24
48
72 h p.i.
M Wt Δ3 R M Wt Δ3 R M Wt Δ3 R M Wt Δ3 R
cyclin B1
cdc6
geminin
cyclin E
cyclin A
actin
B. ihfie1.3
24 h p.i.
M Wt Δ3 R
cyclin E
actin

To differentiate whether the effect on cyclin E was due only to the altered expression of IE1 72 rather than the deletion of aa 30 to 77 from IE2 86, we examined cyclin E protein levels in ihfie1.3 cells (Figure 4.9B). The expression of IE1 72-kDa protein in these cells does not restore the ability of the IE Δ30-77 virus to induce cyclin E. This indicates that functional IE2 86 is required for the up-regulation of cyclin E observed in HCMV-infected cells.

We have therefore observed separate effects on cellular proteins in IE Δ30-77 virus-infected cells: one in which cyclin B1 and proteins regulated by a similar mechanism behave essentially as in wild-type virus-infected cells and another in which cyclin E is expressed at levels between those observed in uninfected and wild-type virus-infected cells. The potential implications of these differences are discussed below.

**PML is not dispersed in IE Δ30-77 virus-infected cells.** A hallmark of the progression of the HCMV infection is the disruption of ND10 sites in the nuclei of infected cells. In wild-type HCMV-infected cells, these structures and their associated proteins, including PML and Sp100, become dispersed throughout the nucleus and have lost their punctate appearance by 4 h p.i. (120, 125). This dispersal is effected via an IE1 72-dependent mechanism but appears not to be required for a productive infection; even in cells infected at a high multiplicity, the IE1 72 deletion mutant virus CR208 does not disrupt ND10 but does produce infectious progeny (5, 7). We observe diffuse nuclear staining of the ND10-associated protein PML at 24 h p.i. in wild-type and rescued mutant virus-infected cells (Figure 4.10). In cells infected with

IE Δ30-77 virus, the ND10 sites remain distinct, punctate structures easily visualized by immunostaining for PML at 24 h p.i. These resemble the ND10 sites in uninfected cells. Even by 48 h p.i., PML has not been dispersed in IE Δ30-77 virus-infected cells (data not shown). In agreement with the Western blot results (Figure 4.6), IE1 72 staining to mark infected cells is much less intense in IE Δ30-77 virus-infected cells than in wild-type or rescued mutant virus-infected cells. Low levels of IE1 72 protein, however, are likely not the only reason for the lack of PML dispersal, since in transient assays transfection with a vector expressing an IE1 72 mutant lacking amino acids 25 to 85 is not sufficient to mediate dispersal of PML (235). Transient expression of wild-type IE1 72 does lead to disruption of ND10 sites.

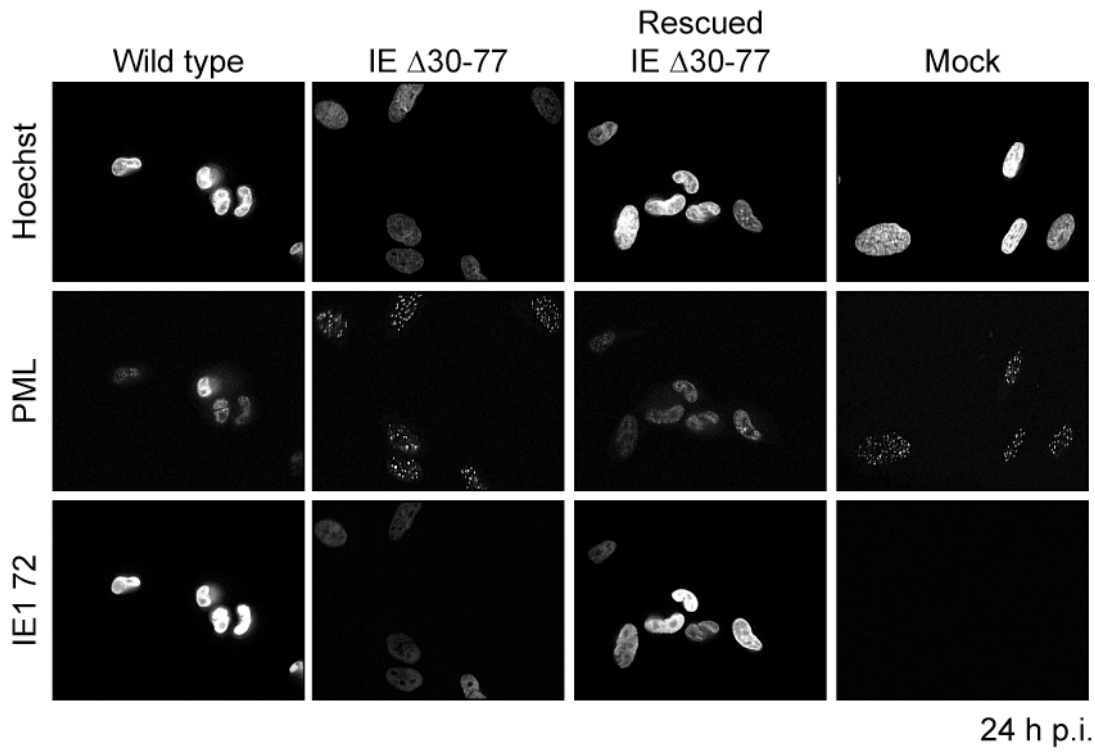



**Figure 4.10 PML is not dispersed following infection with IE Δ30-77 virus.** $G_0$-synchronized HFF cells were infected with 5 $IE^+$ units/cell of wild-type, IE Δ30-77, or rescued IE Δ30-77 viruses or mock infected (Mock) and fixed in 2% paraformaldehyde 24 h p.i. Cells were stained with Hoechst dye to visualize nuclei, anti-IE1 72 monoclonal antibody p63-27, and antibody to PML, followed by appropriate FITC- and TRITC-conjugated secondary antibodies (Materials and Methods). Magnification, ×1000.

**DISCUSSION**

Functional roles ranging from transactivation of early viral genes to control of host cell cycle progression have been assigned to the HCMV major IE proteins. Many of the functions ascribed to these factors, however, have been investigated only in transient transfection assays which do not fully reflect the cellular environment present during a permissive infection. In recent years, introducing mutations into the HCMV genome has provided important information about the activities of viral proteins in the infected cell. A pair of studies used large-scale deletional or insertional mutagenesis to remove or disrupt the majority of the viral open reading frames and to determine which are essential for viral replication in cultured cells (70, 249). The major IE region has been more carefully examined in mutational studies from a number of laboratories. Characterization of the recombinant clones and viruses resulting from these efforts has demonstrated that while IE1 72 is not essential for the progression of the infection, IE2 86 is required (79, 84, 95, 143, 148, 188, 230).

In this study, we examined a region of the major IE locus previously uncharacterized in the context of the viral genome. Exon 3 of the major immediate-early region was identified in the study of Stenberg and colleagues as important for the ability of the major IE proteins to transactivate the viral DNA polymerase promoter (210). This region is also required for an interaction between IE1 72 and the Rb-related protein p107 (111, 252). IE2 86 does not similarly interact with p107, indicating that IE exon 3 sequences are not sufficient for this interaction and that exon 4-encoded amino acids are required as well. Finally, IE exon 3 is required for IE1 72-mediated dispersal of ND10 sites upon HCMV infection (235). Based on these results

from transient and in vitro studies, we chose to introduce a deletion of the majority of IE exon 3 into the viral genome and to characterize the resulting recombinant virus.

Initial electroporation experiments indicated that this virus was slow to form plaques and to spread compared to wild-type and rescued mutant virus controls, but it was possible to generate stocks of recombinant IE Δ30-77 virus. Single- and multi-step growth curve analyses were conducted in HFF and ihfie1.3 cells; these indicate that both mutant IE1 72 and IE2 86 proteins contribute to the observed IE Δ30-77 growth defects (Figure 4.2). Under low-multiplicity conditions in HFF, IE Δ30-77 virus replicates to significantly reduced titers and with delayed kinetics compared to wild-type and rescued mutant virus controls. The peak of virus production in the second round of infection takes about twice as long to be reached in IE Δ30-77 virus-infected cells than in wild-type virus-infected cells. Complementing replication of the IE Δ30-77 mutant virus by growth in ihfie1.3 cells, which express IE1 72-kDa protein, results in a similar delay in the growth kinetics. In the presence of wild-type IE1 72, the time required for peak production of IE Δ30-77 virus is also approximately twice as long as that required for maximal release of infectious wild-type and rescued mutant viruses. The key difference is in the amount of virus produced. The IE Δ30-77 mutant is delayed but eventually produces as much virus in infected ihfie1.3 cells as does parent virus.

Growth of IE Δ30-77 virus in HFF and ihfie1.3 at high MOI is also informative. Here, the kinetic defect is maintained, with wild-type virus production peaking twice as fast as release of IE Δ30-77 virus in both cell types. Like the low multiplicity growth of the mutant in ihfie1.3 cells, IE Δ30-77 virus-infected cells

eventually produce as much virus as wild-type or rescued mutant virus-infected cells. The observation that the IE1 72 protein lacking aa 30 to 77 is detectable only transiently following infection helps to interpret these results (Figure 4.6). Notably, others have expressed IE1 72 with deletions in exon 3 from a plasmid. They observed that IE1 72 lacking aa 45 to 52 can be expressed in an in vitro translation system, but not subsequently immunoprecipitated with antibody to IE1 72, suggesting that this region is required for the stable expression of IE1 72 protein (252). Deletion of aa 70 to 77 did not have a similar effect. Whether IE Δ30-77 virus-infected cells are unable to efficiently express IE1 72 or whether it is an unstable protein, aspects of the IE Δ30-77 growth defect appear to be due to the lack of functional IE1 72 protein in mutant-infected cells. Without IE1 72, the IE Δ30-77 virus behaves like the CR208 recombinant (79, 84, 148), growing to reduced titers at low MOI but to wild-type levels during high multiplicity infection in HFF. In ihfie1.3 cells, wild-type IE1 72 protein is expressed and allows growth of the mutant to wild-type titers independent of MOI. The delay in IE Δ30-77 virus production, however, is not complemented by growth in ihfie1.3 and is likely due to a second defect resulting from the absence of the predicted exon 3 activation domain from IE2 86.

These and additional results support the idea that aa 30 to 77 of the major IE region, likely in the context of IE2 86, are required for the efficient activation of viral early gene promoters. Even under conditions of high-multiplicity growth, IE Δ30-77 virus is deficient in the expression of viral early genes including UL112-113 and UL44 (Figure 4.6). Once sufficient early gene products have accumulated, replication of the mutant virus appears to progress with kinetics comparable to those of the parent

virus. Early gene expression in mutant virus-infected cells lags at both the RNA and protein levels, but the expression of late viral genes is not additionally delayed (Figures 3.3, 3.5, 3.6). The expression of UL112-113 RNAs and proteins appears to lag even more than the appearance of UL44-encoded products, suggesting that the key restriction point in the replication of IE Δ30-77 virus occurs very early in the virus' life cycle. Only a modest accumulation of UL112-113 proteins appears to be necessary for the progression of viral replication, and lower-than-wild-type levels of these proteins allow viral DNA replication and late gene expression to proceed. This is consistent with mutational studies in which viruses with UL112-113 disrupted or deleted grow very poorly, indicating that UL112-113 proteins augment but are not strictly required for HCMV replication in cultured cells (70, 249).

These defects in IE Δ30-77 virus replication result in changes in the infected host cell which differ from those observed in wild-type virus-infected cells. Limited or slow early viral gene expression in the IE Δ30-77 virus-infected cell likely causes the mutant virus to incompletely up-regulate cyclin E expression (Figure 4.9). The result is an infected cell that expresses these proteins at intermediate levels compared to uninfected or wild-type virus-infected cells. Furthermore, the increase in cyclin E appears to require functional IE2 86, since this aspect of the IE Δ30-77 virus' phenotype is not rescued by growth in cells expressing IE1 72. In contrast, the stabilization of cyclin B1 and other substrates of anaphase-promoting complex-mediated degradation appears to require less viral early gene production, as these proteins are expressed to wild-type levels in the IE Δ30-77 virus-infected cell. Inability of IE Δ30-77 virus to disrupt ND10 sites in infected cell nuclei probably

occurs for two reasons. IE1 72 protein, demonstrated to be required for this reorganization, is not stably expressed in mutant-infected cells. Additionally, any IE1 72 protein that is expressed lacks exon 3, which is required for PML dispersal in transiently transfected cells (235).

Construction and study of a recombinant IE Δ30-77 virus has provided important information about the function of the major IE proteins in the HCMV-infected cell, both confirming predictions from in vitro studies and revealing new roles for these regulators. Experiments in progress aim to further define their functions, particularly to decipher which early genes require aa 30 to 77 of the major IE region for proper expression, how the expression of these viral genes influences the host cell cycle, and the cause of decreased expression of the mutant IE1 72 protein. These studies and the examination of additional viable recombinants with deletions in the major IE region will advance our understanding of the control of viral and cellular gene expression in HCMV-infected cells.

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The text of this chapter, in full, is a reprint of the material as it appears in *Journal of Virology*, 79:7438-52, 2005. White, E.A. and D.H. Spector. Exon 3 of the human cytomegalovirus major immediate-early region is required for efficient viral gene expression and for cellular cyclin modulation. The dissertation author was the primary investigator and author of this paper.

# CHAPTER 5

# DISCUSSION

This dissertation describes studies that have used a novel family of human cytomegalovirus (HCMV) recombinants to elucidate HCMV regulatory functions in the virus-infected cell. In particular, the viruses constructed and characterized here contain changes to the HCMV major immediate early (IE) region, a segment of the viral genome that encodes the IE1 72 and IE2 86 kDa proteins. It has been known for some time that both IE1 72 and IE2 86 function as transactivators of viral gene expression, and a large body of early work described in the introduction (Chapter 1) defined several functions for these factors by analyzing them in transient transfection assays and in *in vitro* binding studies. Key findings from these publications include that IE2 86 is a stronger transactivator than IE1 72, that it acts on the promoters of several viral genes expressed with early kinetics, and that it can also repress expression from the major IE promoter by binding to the *cis*-repression sequence there. IE2 86 was also shown to bind to a number of host cellular proteins, including many transcription factors and components of the basal transcription machinery. It was unclear from this work, however, how these functions might translate to the HCMV-infected cell, and the early studies could not predict which IE2 86-promoter or IE2 86-protein interactions would be most important in the cellular environment.

Shortly before the work described in this dissertation was begun, herpesvirus genomes were first cloned as bacterial artificial chromosomes (BACs). This

development led to a dramatic increase in researchers' ability to use herpesviruses as a forward genetic system. For the first time, it was possible to readily introduce mutations into the HCMV genome and to examine the downstream effects of these mutations. BAC-cloned HCMV recombinants form the basis of the experimental system described in this dissertation. Using one of several cloning techniques, changes to the sequence of the major IE region can be introduced into the HCMV BAC, and resulting mutant BACs are then transfected into cells permissive for HCMV infection. This system has the distinct advantage that both lethal and non-lethal mutations can be studied. A BAC either does or does not support the entire life cycle of the virus in the transfected cell, but even in cases where viable virus cannot be reconstituted, cells harboring the non-viable genome can be studied.

Several non-viable HCMV recombinants with changes to the major IE region are particularly relevant to the dissertation. One of the first HCMV BAC-cloned constructs to be cloned lacked the majority of the region unique to the IE2 gene, and therefore could not express full-length IE2 86 protein (143). This construct did not support early gene expression or produce infectious virus, demonstrating directly that full-length IE2 86 protein must be present for the HCMV infection to progress. Similarly, we have constructed an HCMV BAC that has two termination codons just after amino acid (aa) 156 of the IE2 86 gene, so that the resulting construct can express only a truncated form of the protein. Again, this recombinant is not viable and does not support early gene expression when it is transfected into cells permissive for HCMV infection (Figure 5.1). IE2 86 truncation mutant virus can be reconstituted in a limited way using cells that conditionally express IE2 86. When this virus is

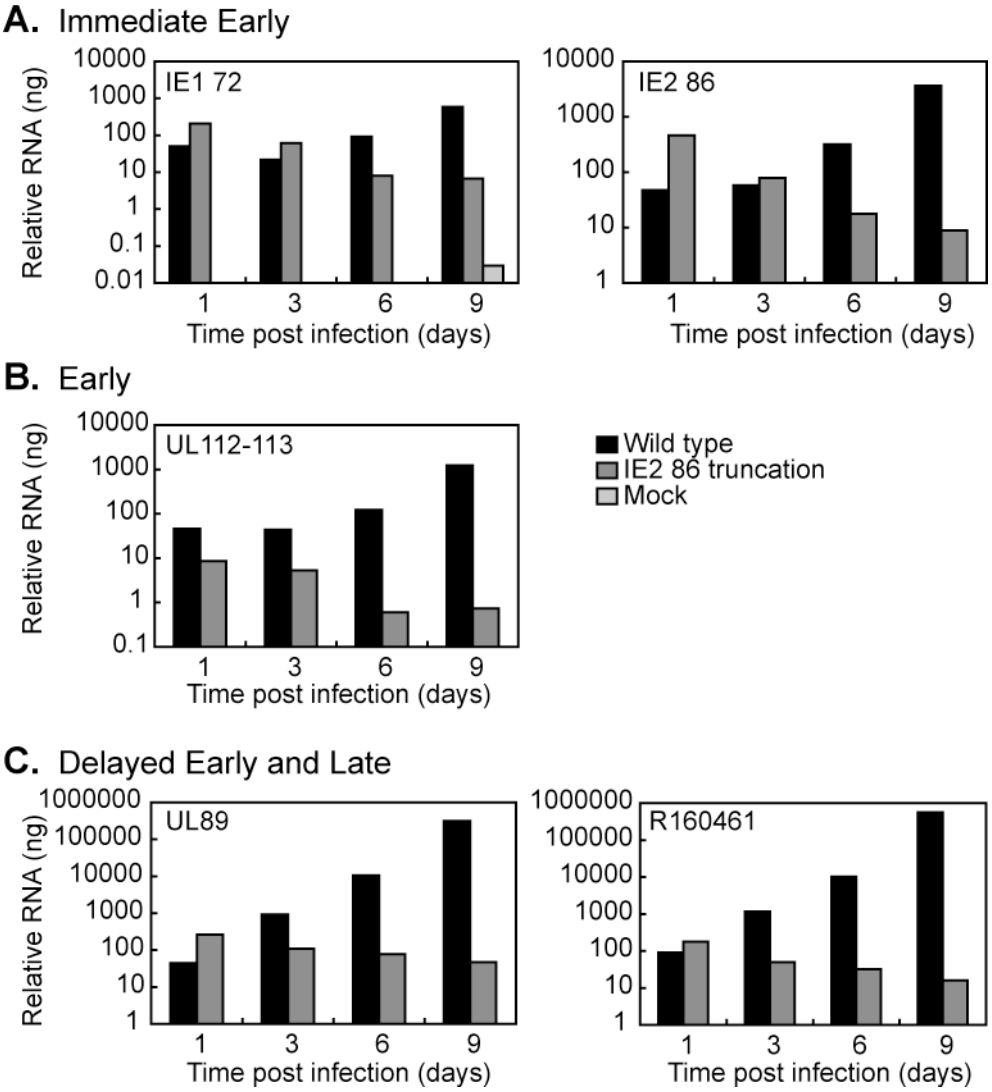


**Figure 5.1 Cells transfected with the IE2 86 truncation mutant construct express increased IE and late transcripts, but not viral early genes.** HFF cells were transfected by electroporation with wild-type or IE2 86 truncation mutant BACs, or mock transfected, and harvested at the indicated times p.i. Total cellular RNA was analyzed by quantitative real-time RT-PCR to measure the relative levels of (A) IE1 72 and IE2 86, (B) UL112-113, and (C) UL89 and R160462 transcripts. To ensure that an equal amount of RNA was included in each reaction, samples were analyzed with G6PD-specific primers and probe. Values shown in the graphs have been standardized to G6PD levels. When mock-infected cell RNA was analyzed with the IE2 86, UL112-113, UL98, or R160461 TaqMan probes, amplification was below the limit of detection.

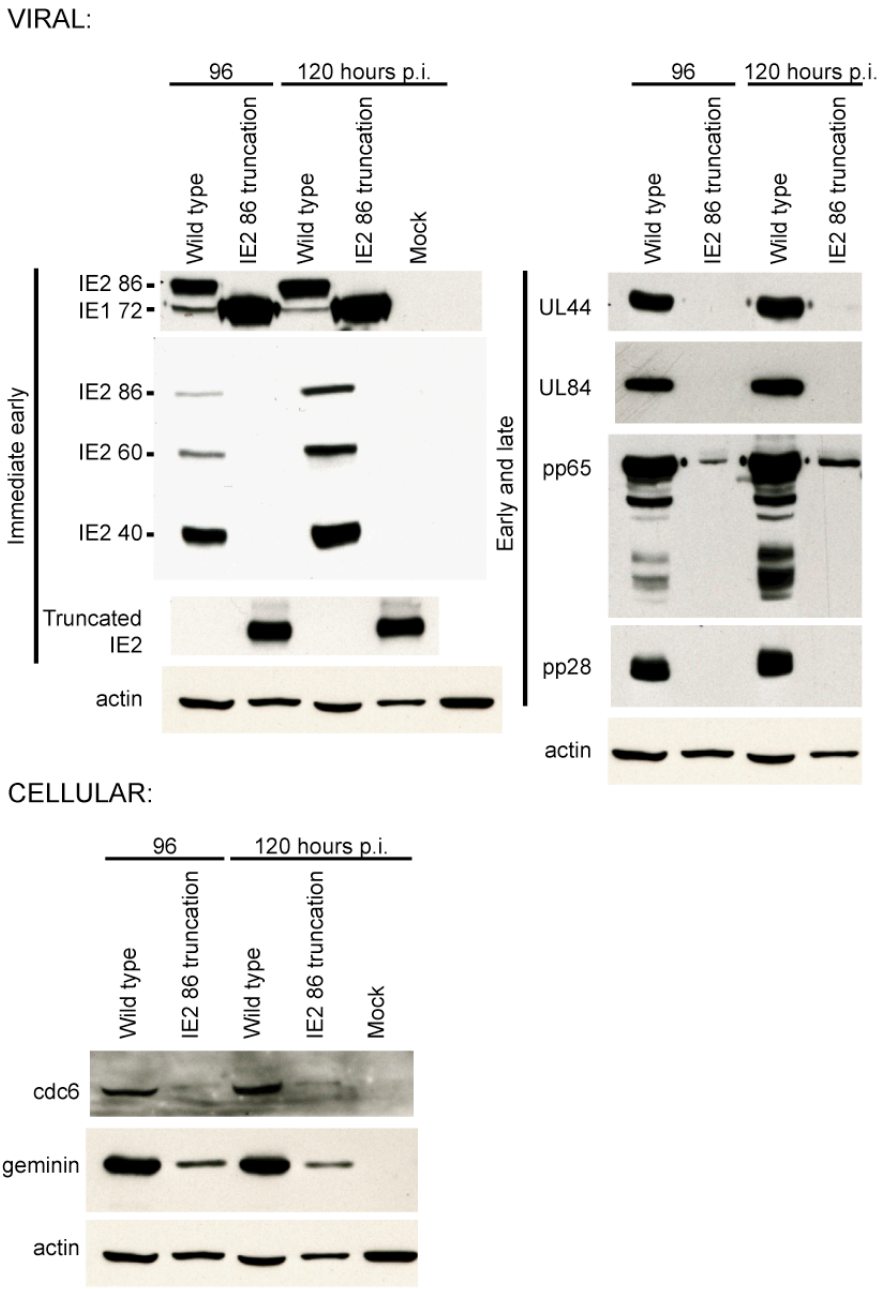


**Figure 5.2 Cells infected with the IE2 86 truncation mutant virus overexpress IE1 72 protein, but do not express early or late viral proteins or stabilize APC substrates.** $G_0$-synchronized HFF cells were infected with 15 IE+ units/cell of wild-type or IE2 86 truncation mutant virus or mock-infected and harvested at the indicated times p.i. Lysates equivalent to an equal number of cells were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. The levels of several viral (top panels) and cellular (bottom panels) proteins were analyzed by Western blotting. Cellular actin levels were analyzed as a control for protein loading.

subsequently used to infect primary fibroblasts, it is evident that the lack of full-length IE2 86 protein results in a loss of repression of the major IE promoter, resulting in elevated levels of IE1 72 protein, and a loss of the ability of the virus to express early viral genes (Figure 5.2). The IE2 86 truncation mutant virus has retained part, but not all, of its ability to stabilize substrates of the anaphase promoting complex (APC) including cdc6 and geminin.

Based on these verifications of the crucial role of IE2 86 in the control of HCMV gene expression, we continued these studies by generating recombinant BACs containing deletions of portions of the HCMV major IE region. By characterizing the various phenotypes of the resulting constructs and viruses, we have been able to discover and assign functions to many regions of IE2 86.

Chapter 2 described several HCMV BAC constructs with small deletions in predicted DNA binding and transactivation domains in the C-terminus of IE2 86. Studying these constructs resulted in several key findings. First, this work confirmed that wild-type IE2 86 represses its own promoter in cells and showed that when the DNA binding regions of the protein are disrupted, transcription from the major IE region increases. Also as predicted, deleting these crucial regions of the protein eliminates its ability to express early viral genes. What was most remarkable about this study was that it also uncovered functions of IE2 86 that were not predicted by earlier reports. In particular, IE2 86 appears to contribute to the regulation of viral gene expression at late as well as at early times post infection. When the C-terminal deletions were introduced into IE2 86, there was an increase in the expression of several late viral genes whose promoters contain elements like the *cis*-repression

sequence. This effect can also be observed in cells transfected with the IE2 86 truncation mutant BAC (Figure 5.1).

Studying viable recombinant viruses helped us to further understand the functions of IE2 in the control of late viral gene expression. Chapter 3 describes a group of viable recombinant viruses that do not express the 60 and 40 kDa forms of IE2 protein, which are expressed at late times in the HCMV-infected cell. In particular, these viruses are deficient in the regulation of major IE protein expression and in the regulation of genes expressed at late times, including UL83 and UL84. These findings are consistent with what has been described for another IE2 86 deletion mutant virus, IE2 86ΔSX-EGFP (188). The IE2 86ΔSX-EGFP virus lacks the sequence coding for aa 136 to 290 of IE2 86, which includes the initiating methionines for IE2 60 and IE2 40 proteins. Consequently, it expresses a smaller form of IE2 86 protein and does not produce either smaller protein. Several other viruses with deletions in this region of the IE2 86 gene lack aa 88 to 135, 88 to 290, and 290 to 313 of IE2 86. Each of these three viruses is viable, and IE2 86Δ290-313 virus grows comparably to wild-type virus (E.A. White and D.H. Spector, unpublished results), as predicted from a study that used transient transfection and yeast two-hybrid assays and in vitro binding studies (6). IE2 86Δ88-135 virus replication is slightly impaired and IE2 86Δ88-290 virus replication is significantly impaired compared to wild-type. The IE2 86Δ88-135 genome contains the initiating methionines and putative transcription start sites for the IE2 40 and IE2 60 proteins, but expresses much lower than wild-type levels of these proteins (Figure 5.3, top panel). IE2 86Δ88-290 lacks the initiating methionines for both IE2 40 and IE2 40 and does not express either protein. Likely

because cells infected with either virus do not express IE2 40, which functions as a repressor of major IE gene expression (Chapter 3), both IE2 86Δ88-135 and IE2 86Δ88-290 virus-infected cells over-express their respective shorter forms of IE2 protein compared to wild-type virus-infected cells (Figure 5.3, top panel). IE2 86Δ88-135 and IE2 86Δ88-290 viruses both express early (UL57) and late (pp28) viral genes (Figure 5.3, middle panels), but produce reduced levels of infectious virus (J. Shih, E.A. White, and D.H. Spector, unpublished results). Overall, deletions in a large portion of the N-terminal half of exon 5 of the major IE region reduce, but do not eliminate, the ability of the virus to replicate. This observation suggests that this highly conserved portion of the protein must have functions that are not completely discernable in cultured fibroblasts.

Further work with a viable recombinant virus, IE Δ30-77, that has a deletion in exon 3 of the major IE region began to demonstrate the role of the major IE proteins in the control of cellular as well as of viral gene expression (Chapter 4). In a wild-type HCMV-infected cell, the expression of cyclins E and B1 is up-regulated while that of cyclins A and D decreases compared to uninfected controls (29, 108, 145, 186, 189). In IE Δ30-77 virus-infected cells, these effects are not maintained. Mutant virus-infected cells contain elevated levels of cyclin B1 protein, but are not able to induce the expression of cyclin E. High-level cyclin E expression is not restored by growth in IE1 72-expressing cells, indicating that the deletion in IE2 86 is responsible for this aspect of the mutant phenotype. An additional differential effect of the mutant infection on the host cell is the inability of IE Δ30-77 virus to disrupt ND10 sites following infection, as predicted by transient expression studies.

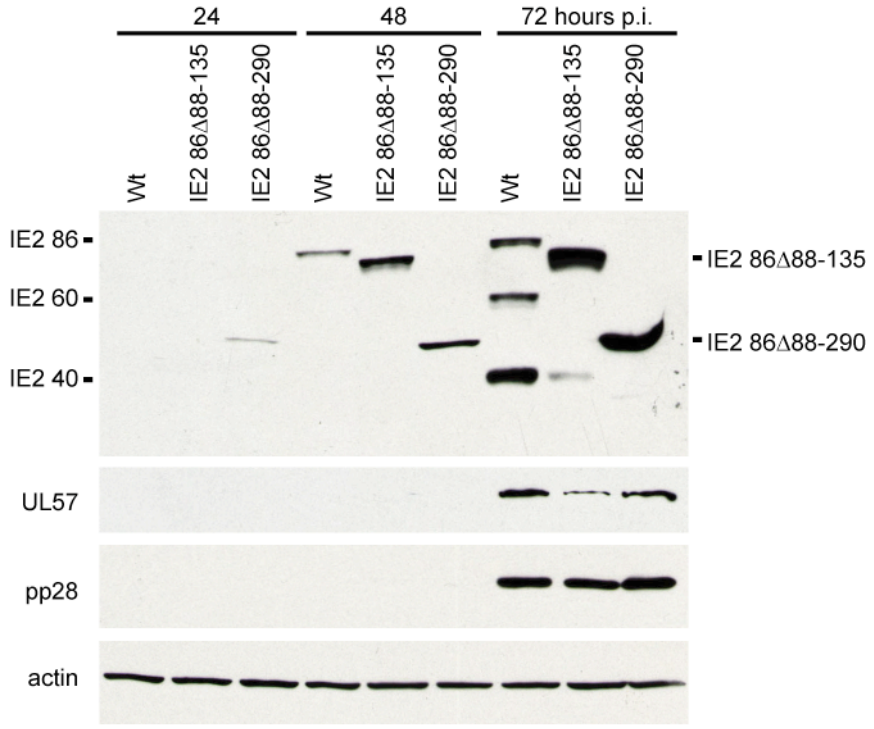


**Figure 5.3 The IE2 86Δ88-135 and IE2 86Δ88-290 viruses are viable and express early and late viral genes.** $G_0$-synchronized HFF cells were infected with 5 pfu/cell of wild-type (Wt), IE2 86Δ88-135, or IE2 86Δ88-290 viruses or mock-infected (M). Lysates equivalent to an equal number of cells were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. The levels of IE2, UL57, and pp28 proteins were analyzed by Western blotting. Cellular actin levels were analyzed as a control for protein loading.

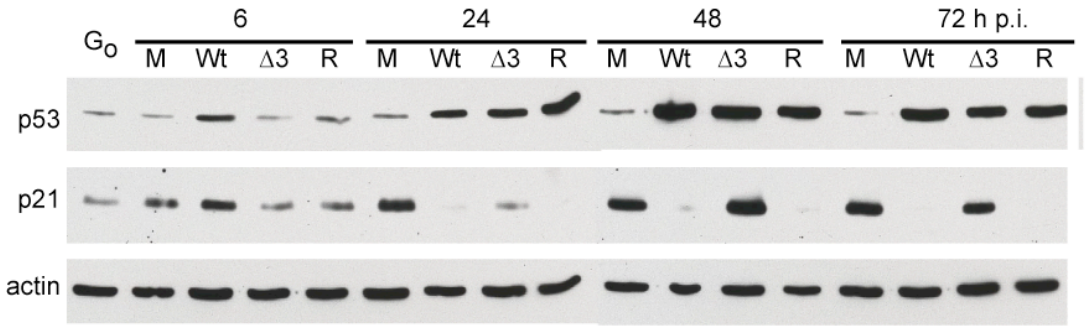


**Figure 5.4 Cells infected with the IE Δ30-77 mutant virus express elevated levels of p53 protein and continue to express p21 protein.** $G_0$-synchronized HFF cells were infected with 10 IE+ units/cell of wild-type (Wt), IE Δ30-77 (Δ3), and rescued IE Δ30-77 (R) viruses, or mock-infected (M). Lysates equivalent to an equal number of cells were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. The levels of p53 and p21 proteins were analyzed by Western blotting. Cellular actin levels were analyzed as a control for protein loading.

We examined p53 expression in wild-type versus IE Δ30-77 virus-infected cells. In wild-type HCMV-infected cells, p53 protein is expressed to higher levels than in uninfected cells, but is inactivated (Figure 5.4). The p21 gene, which is driven by a promoter that is responsive to p53, is therefore not expressed in HCMV-infected cells. In contrast, while p53 levels are also elevated in IE Δ30-77 virus-infected cells, the p53 present in these cells may have remained active. Levels of p21 protein in mutant virus-infected cells are as high as those in uninfected cells. This behavior is consistent with a model recently proposed by Hsu and colleagues (101). In a series of in vitro assays, they observe that IE2 86 inhibits the ability of acetyltransferases p300 and CBP to modify p53, and that the region of IE2 86 required for this inhibition includes exon 3 sequences. Acetylation of p53 by CBP/p300 helps to activate p53. Our results, which suggest that p53 is active on the p21 promoter only when IE2 86 exon 3 sequences are missing, are consistent with their model. It is important to note that in our experiment, DNA damage was not induced in the uninfected cells. The expression of p21 protein in those cells may not have been the result of p53 activity, and the comparable induction of p21 in IE Δ30-77 virus-infected cells might also result from a p53-independent pathway.

While these studies have answered some questions about the functions of IE2 86 in the HCMV-infected cell, they have raised many more. Studies on the IE2 Δ60 virus and related recombinants have provided data consistent with the idea that an HCMV-encoded microRNA, UL112-1, could help to regulate the expression of IE1 72. These experiments are presented in the Appendix and provide the starting point for a more detailed analysis of the interactions between UL112-1 and its targets in the

HCMV genome. The p53 and cyclin E results mentioned above, along with work from other laboratories (36, 101, 105, 153, 157, 177, 178, 239), led to the hypothesis that IE2 86 affects gene expression in part through interactions with chromatin modifying enzymes: histone acetyltransferases and histone deacetylases. The recombinant viruses described in this dissertation, or others like them, could be used to investigate which regions of IE2 86 are important for interactions with these enzymes. Examining viral and cellular gene expression on a genome-wide level in cells infected with the recombinant viruses also has the potential to provide much information about which regions of IE2 86 participate in the control of families of related genes.

This work has shown that many of the predicted functions of IE2 86, such as autorepression and early promoter activation, are relevant in the HCMV-infected cell as well as in other systems. More importantly, these studies have revealed that IE2 86 provides several more functions, including a contribution to host cell cycle dysregulation and to the control of late viral gene expression, in the virus-infected cell. The ability to introduce mutations into the HCMV genome with ease and to study viable and non-viable recombinant viruses has allowed us to develop a system that will allow many more questions about the regulation of viral and cellular gene expression in HCMV-infected cells to be investigated.

# APPENDIX

# ALTERATION OF THE IE1 72 3' UTR LEADS TO UP-REGULATION OF RNA AND PROTEIN EXPRESSION CONSISTENT WITH A DEFECTIVE MICRORNA-TARGET INTERACTION

## ABSTRACT

We have constructed and characterized several human cytomegalovirus (HCMV) recombinants in which sequences between exons 4 and 5 of the major immediate early region have been altered. While these were initially constructed to disrupt the expression of several transcripts expressed from this region at late times post infection, they exhibit unexpected behaviors in addition to the predicted decrease in the expression of the late forms of IE2 protein. In particular, cells infected with the recombinant viruses express higher than wild-type levels of the IE1 72 kDa protein and its corresponding transcript. This effect is consistent with the observation that an HCMV-encoded microRNA (miRNA), UL112-1, has a target site in the 3' untranslated region of IE1 72, and that this sequence was fortuitously altered by the introduction of the late IE2 promoter mutation. Although a number of studies have documented the expression of virus-encoded miRNAs, the targets and function of these miRNAs are relatively unknown, and virus-encoded miRNAs that act on multiple sites within a viral genome have not yet been documented. Separate observations suggest that the HCMV UL114 (uracil DNA glycosylase) locus is also

regulated by UL112-1. UL112-1 is completely complementary to a portion of UL114, and the products that would result from a cleavage event at the complementary site can be detected in HCMV-infected cells. This data is consistent with the hypothesis that the HCMV UL112-1 miRNA regulates the expression of multiple viral genes and suggests that UL112-1 is not conserved among or between herpesviruses because it contributes regulatory functions unique to HCMV.

## INTRODUCTION

MicroRNAs (miRNAs) are small single stranded RNAs of approximately 20-24 bases in length that generally regulate gene expression at the post-transcriptional level (for a review, see (17). MiRNA precursors are expressed in the nucleus where their primary transcripts (pri-miRNAs) form hairpin loop structures. There, the hairpin loop sequence containing the miRNA is recognized and cleaved by the RNase III Drosha complex, then transported to the cytoplasm where the complex is processed further by a second endonuclease, Dicer, to produce a double stranded RNA. One strand of the dsRNA is incorporated into an RNA induced silencing complex (RISC). Transcripts are targeted by RISC depending on the complementarity between the miRNA and the target transcript. A perfect match between the 'seed' sequence of the miRNA, which is nucleotides 2-8 of the fully processed species, and the 3' untranslated region (UTR) of the target mRNA appears to be particularly important for a functional interaction to occur (110, 132). This interaction can lead to degradation of the target transcript or inhibition of its translation (13, 103, 246). When the miRNA is a perfect antisense match for a portion of the mRNA, the target transcript can be cleaved. In either case, the interaction between the miRNA and its target leads to decreased expression of the target gene(s). For the vast majority of identified miRNAs, validated target transcripts and regulatory functions are unknown.

Many viruses have recently been recognized to encode miRNAs but in most cases, the functional relevance of these genes with respect to viral replication is unknown (39, 41-43, 65, 66, 71, 85, 86, 154, 156, 159, 164-166, 187, 191, 192, 214).

Both computational predictions and direct cloning experiments have led to the identification of over 70 novel miRNAs from DNA viruses, many of which are conserved in closely related species, and it seems likely that not all virus-encoded miRNAs have been identified. Most of the viral miRNAs identified to date have been found in herpesviruses, although SV40 and adenovirus also encode miRNAs.

Herpesviruses comprise a large family of enveloped, double-stranded DNA viruses that is divided into three groups: alpha, beta, and gamma; and members of all three groups encode miRNAs. The gammaherpesvirus miRNAs are encoded within regions of the genome associated with latency genes and can be detected during latent viral infection (41-43, 164, 187). Similarly, the alphaherpesviruses herpes simplex virus type 1 (HSV-1) and Marek's disease virus (MDV) encode miRNAs located adjacent to and within regions expressed during latency (39, 65, 87). In contrast, miRNAs encoded by human cytomegalovirus (HCMV), a betaherpesvirus, are located throughout the genome and are expressed during lytic infection (71, 85, 165). The lytic life cycle of herpesviruses has been introduced previously (see Chapter 1). Most of the nine expressed HCMV miRNAs accumulate during the early phase of replication (85). Four of these miRNAs are encoded antisense to open reading frames, while the others are encoded within intergenic regions. UL36-1 is located within an intron.

Although most targets of virus-encoded miRNAs have not yet been identified, a few have recently been reported. The small DNA papovavirus SV40 encodes a miRNA that is directly antisense to the large T antigen transcript (215). This miRNA regulates the production of large T protein during acute infection through direct

cleavage of the messenger RNA. Similarly, mouse polyomavirus encodes a miRNA that regulates large T antigen through direct cleavage of the target transcript. However, the miRNA of mouse polyomavirus is encoded in a different genomic region and does not share sequence homology with the SV40 miRNA. This observation suggests that these miRNAs do not target related cellular transcripts, but instead that their primary role is in the regulation of viral gene expression. In contrast, it has been proposed that an miRNA encoded by HSV-1 within the minor LAT transcript inhibits the expression of TGF-β1 and SMAD3 messages, protecting cells against apoptosis (87). Here, the primary function of the viral miRNA is to subvert cellular processes to favor viral maintenance in the latent state. Despite these two examples and many fortuitous deletions of miRNA-encoding regions, the functions of the many other miRNAs present in virus genomes remain elusive. It is clear that interactions between host cellular miRNAs and viruses will prove to be crucial as well. A remarkable example of the human liver-specific miR-122 interacting with and promoting the replication of the hepatitis C virus (HCV) genome has already been described (114).

Here, we describe several observations that are consistent with the prediction that UL112-1, an HCMV-encoded miRNA, regulates the expression of the UL123 (IE1 72) locus in the viral genome. Using recombinant viruses with deletions in and near a predicted UL112-1 target site in the 3' UTR of UL123, we show that disruption of this site results in increased IE1 72 RNA and protein expression and decreased production of infectious virus. In addition, we show that although disruption of this site also leads to the decreased expression of IE2 60 protein at late times post infection

(p.i.), completely eliminating the expression of IE2 60 protein using the point mutation IE2 M170L does not alter IE1 72 production. UL112-1 is encoded antisense to the HCMV UL114 gene, and there is also evidence consistent with the prediction that UL112-1 directs cleavage of UL114 message in the HCMV-infected cell. If both of these miRNA-target interactions can be validated, this will be the first documented example of a virus-encoded miRNA that regulates the expression of two different viral genes and influences the production of infectious virus in tissue culture.

## MATERIALS AND METHODS

**Cells.** Human foreskin fibroblasts (HFF) were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 200 U penicillin, 200 μg streptomycin, 1.5 μg Amphotericin B, and 50 μg gentamicin per milliliter, and grown as described (216).

**BAC mutagenesis.** The construction of the IE1 72 3' UTR sequence change mutant was previously described in this dissertation as the construction of the IE2 Δ60 virus (Chapter 3). The construction of the IE1 72 3' UTR deletion mutant virus began with the plasmid pSP-J(BglII-StuI), which contains the approximately 2000 base-pair (bp) fragment generated by digesting the HCMV AD169 genomic *EcoR*I J region (216) with *Bgl*II and *Stu*I. Mutagenic oligonucleotide primers were used in conjunction with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to create the plasmid pSP-J(BglII-StuI 3'UTRΔ) which is identical to pSP-J(BglII-StuI) but contains a deletion of part of the 3' UTR of the UL123 gene. This construct was sequenced to verify the presence of the correct mutations. Sequences of mutagenic primers (Integrated DNA Technologies, Coralville, IA) for the 3' UTR deletion are sense: 5' CAGTTACTGTTATGGATCCTACTCTGTAATCCTACTC 3', antisense: 5' GAGTAGGATTACAGAGTAGGATCCATAACAGTAACTG 3'.

The IE1 72 3' UTR deletion was introduced into the UL122-123 coding region using the Counter Selection BAC Modification kit (Gene Bridges, Dresden, Germany). Briefly, oligonucleotide primers were used to amplify a marker cassette containing the neomycin resistance and RpsL genes and to simultaneously introduce

50 nt of homology to the UL122-123 region onto either end of the cassette. Sequences of primers (Integrated DNA Technologies, Coralville, IA) are sense: 5' GATAGAGGAAGTTGCCCCAGAGGAAGAGGAGGATGGTGCTGAGGAACCC AGGCCTGGTGATGATGGCGGGATCG 3', antisense: 5' CCTTCTCGTTGTCCAACTCGGAGATGCGTTTGCTCTTCTTCTTGCGGGGTTC AGAAGAACTCGTCAAGAAGGCG 3'. The linear product was recombined into the UL122-123 region contained in the wt HCMV strain AD169 BAC pHB5 (gift of M. Messerle (26)) and the resulting intermediate construct pHB5(IEexon5-RpsLneo) was selected on the basis of resistance to kanamycin. Next, pSP-J(BglII-StuI 3'UTRΔ) was used as the PCR template in conjunction with the following primers (Integrated DNA Technologies, Coralville, IA): sense: 5' CAGGAAGAAAGTGAGCAGAGTGATG 3', antisense: 5' AGCGATTGGTGTTGCGGAAC 3' to amplify a linear fragment containing the mutated UL122-123 region. This fragment was recombined into pHB5(IEexon5-RpsLneo), replacing the RpsLneo cassette, and the resulting IE1 72 3' UTR deletion BAC was selected on the basis of increased streptomycin resistance. The altered region was amplified and sequenced to confirm that the intended deletion had been introduced into the BAC.

A rescued BAC for the 3' UTR sequence change mutant BAC has been described (Chapter 3). A rescued BAC was generated from the deletion mutant BAC by the same procedure. The RpsL-neomycin marker cassette was introduced into the IE1 72 3' UTR deletion mutant BAC using the procedure and primers described above. pSP-J(BglII-StuI) was then used as a template in conjunction with the primers

described above to produce a linear fragment containing the wild-type HCMV sequence. This fragment was inserted into the RpsL-neomycin containing intermediate BAC by homologous recombination and designated IE1 72 3' UTR deletion mutant rescue.

Wild-type, mutant, and rescued BAC DNAs were amplified and purified as described (188). Each BAC was digested with *Hind*III and separated by field inversion gel electrophoresis to ensure that no major alterations to the DNA were sustained during the cloning procedure.

**Reconstitution of virus, determination of virus titers, and growth curves.** Wild-type, IE1 72 3' UTR deletion, IE1 72 3' UTR sequence change, or rescued BACs were transfected into HFF by electroporation as previously described (230) and monitored for plaque development. When all cells in a culture exhibited cytopathic effect (CPE), supernatants were harvested and used to infect fresh cells. Stocks of wild-type, mutant, or rescued mutant viruses were harvested and titered by plaque assay. To analyze the kinetics of virus replication, confluence-synchronized HFF were infected at an MOI of 3 pfu/cell. Supernatant from infected cells was harvested daily, replaced with fresh media, and titered by plaque assay.

**Time course of virus infection.** HFF were grown to and maintained at confluence for three days prior to infection to allow synchronization in a $G_0$ state. At the time of infection, cells were released from $G_0$ by trypsinization, infected at an MOI of 3 pfu/cell, and re-plated at a lower density. Cells were re-fed daily, then harvested by trypsinization and frozen at the indicated times p.i.

**Quantitative real-time RT-PCR analysis.** RNA was isolated with the NucleoSpin II kit (Clontech, Mountain View, CA) and subsequently treated with DNase using the Turbo DNA-free kit (Ambion, Austin, TX) to remove any residual DNA contamination and to allow the analysis of unspliced viral transcripts. Real time RT-PCR and data analysis was performed as previously described using primers and probes directed against the HCMV genes IE1 72 (UL123) and IE2 86 (UL122) and the cellular housekeeping gene glucose-6-phosphate dehydrogenase (G6PD) (230).

**Western blotting.** Cells were lysed in reducing sample buffer (50 mM Tris [pH 6.8], 0.2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM ß-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, and 100 μM pepstatin A) and protein content was determined by Bradford assay. Equal amounts of protein were separated on SDS-PAGE gels and transferred to nitrocellulose. Membranes were stained with amido black to ensure equal protein loading. After blocking in 5% nonfat dried milk in TBS-T (Tris buffered saline [pH 7.4] with 0.05% Tween-20), blots were incubated with primary antibodies in 5% nonfat dried milk in TBS-T, diluted as follows: CH16.0 MAb, 1:10,000; IE2 MAb 8140, 1:1,000 to 1:1,500; pp65 MAb, 1:10,000; UL84 MAb, 1:2000; β-actin MAb AC-15, 1:10,000. CH16.0 was purchased from Virusys (Sykesville, MD) and recognizes both IE1 72 and IE2 86. Anti IE2 was purchased from Clontech (Mountain View, CA). Anti-pp65 was purchased from the Goodwin Institute (Plantation, FL). Anti-β-actin was purchased from Sigma-Aldrich (St. Louis, MO). Anti-UL84 was the gift of Dr. Greg Pari (University of Nevada, Reno). Membranes were washed in TBS-T and incubated in horseradish peroxidase (HRP)-

coupled anti-mouse antibody (Calbiochem, San Diego, CA), diluted 1:2000. After washing in TBS-T, proteins were detected using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

## RESULTS

**The HCMV-encoded UL112-1 miRNA directs cleavage of the viral uracil DNA glycosylase mRNA.** The HMCV miRNA UL112-1 is encoded within the open reading frame of the viral uracil DNA glycosylase gene (UL114) in an antisense orientation (Finn Grey and Jay Nelson, unpublished results. Presented as abstract 1.03 at the International Herpesvirus Workshop, Seattle, Washington, July 2006). UL112-1 can be detected beginning 24 hours post infection (h p.i.) in HCMV-infected cells, and it increases in abundance as the infection progresses. Since both UL112-1 and UL114 transcripts are detected following during lytic infection, it has been predicted that UL112-1 directs the cleavage of the uracil DNA glycosylase mRNA, in HCMV-infected cells, leading to down-regulation of protein production. Consistent with this prediction, an RNase protection assay (RPA) using RNA from HCMV-infected cells detected at least one product of a size that would be predicted to result from such a cleavage event. A co-transfection assay showed that transfection of a vector expressing the UL112-1 miRNA, but not a negative control miRNA, is sufficient to down-regulate the expression of UL114 protein encoded by a second plasmid. Together, these results suggest that UL112-1 can regulate the expression of UL114 both in vitro and in vivo, and these observations served as the starting point for the study of the recombinant viruses described below.

**The HCMV genome contains additional predicted target sites for UL112-1.** UL112-1 does not possess complete homology to any part of the HCMV genome other than a segment of the UL114 ORF. Having shown that UL112-1 could

potentially act as a functional miRNA, however, the authors of the study described above wanted to look for targets with partial homology that could also be subject to regulation by UL112-1 (Finn Grey and Jay Nelson, unpublished results. Presented as abstract 1.03 at the International Herpesvirus Workshop, Seattle, Washington, July 2006). Using the miRNA target prediction algorithm RNAhybrid, they searched a database containing the 3' UTRs of HCMV ORFs for potential UL112-1 target sites. They identified thirty-eight unique target sites in 33 different HCMV ORFs. Since the sequence of UL112-1 is completely conserved between HCMV and chimpanzee cytomegalovirus (CCMV), they also analyzed the corresponding 3' UTRs of CCMV for potential UL112-1 target sites. The finding most relevant to this dissertation was that there is a cluster of potential target sites within the 3' UTRs of ORFs in the HCMV major immediate early region. One site was identified within the 3' UTR of UL123, the last coding exon of IE1 72, and two overlapping sites are predicted within the 3' UTRs of ORFs UL120 and UL121. The predicted interaction between the 3' UTR of UL123 and the miRNA UL112-1 is shown (Figure A.1A). CCMV sites corresponding to those identified in the HCMV genome were identified in all three 3' UTRs. The site within CCMV UL123 maintained less homology to UL112-1 than the others, and would presumably form a less effective binding site. Further upstream a second site, not found in the HCMV counterpart, was identified, but it is not clear whether in CCMV, these could function as two less effective binding sites in place of the more homologous single site found in HCMV.

**The UL112-1 miRNA could target the 3' UTR of IE 72 to reduce HCMV gene expression.** The HCMV major immediate early region, UL122-123, consists of

five exons that are transcribed, differentially spliced, and translated to give rise to several viral regulatory proteins (147). The full-length product of the UL123 gene is the IE1 72 kDa protein (IE72, IE1 72), which contains sequences encoded by exons 2, 3 and 4. UL122 encodes the IE2 86 kDa protein (IE2 86, IE86), consisting of exon 2, 3, and 5 sequences. IE1 72 and IE2 86 proteins are expressed with immediate early kinetics and contribute regulatory functions to the HCMV life cycle. Smaller proteins expressed from UL122 are 60 and 40 kDa in size, are identical to the C-terminus of IE2 86, and are expressed with late viral kinetics. The expression of additional forms of both IE1 72 and IE2 86 has also been reported (12, 122, 170, 174, 196, 209).

The intron between exons 4 and 5 of the major IE region contains several key sequence features, including the 3' UTR of the UL123 ORF and the sequence that is predicted to function as a promoter for the ~1.75 kb transcript(s) that are expressed from the UL122 region at late times p.i. (Figure 1.2, Figure 3.1). To construct viruses that do not express these late UL122 RNAs, we introduced sequence changes into this region in two separate HCMV recombinants. These viruses were generated using the HCMV strain AD169 BAC pHB5, which contains a viral genome that is replication-competent when transfected into permissive cells (Materials and Methods). One of these viruses was previously described in the dissertation as the IE2 Δ60 virus (Chapter 3), in which the sequence of a 24-nucleotide region in the late promoter/3'UTR region has been significantly altered. Here, this virus will be referred to as the 3' UTR sequence change mutant (3' UTR mut) virus (Figure A.1B). The second virus has not been previously described and lacks 29 base pairs in the late promoter/3'UTR region of the HCMV genome. This virus is the 3' UTR deletion

mutant (3'UTR Δ). For both constructs, the altered sequences were returned to wild-type by the reverse cloning procedure. The resulting rescued mutant BACs were used in subsequent studies to ensure that the effects observed were not due to accidental changes introduced during the cloning procedure. To reconstitute infectious virus, the wild-type, 3' UTR deletion mutant, 3' UTR sequence change, and rescued mutant BACs were transfected by electroporation into primary human foreskin fibroblasts (HFF). Virus was harvested and titered as described (Materials and Methods).

These changes in the 3' UTR of UL123 and their relationship to the predicted UL112-1 target site in that 3' UTR are indicated (Figure A.1B). In the 3' UTR deletion mutant virus, 10 bases have been deleted from the predicted target site, as has some adjacent sequence. The bases deleted here appear to be particularly important to a potential miRNA-target interaction, since they are the part of the target site that would base pair with the miRNA seed sequence. In the 3' UTR sequence change mutant virus, the predicted target site has not been altered directly, but adjacent sequence has been significantly changed. To understand how these changes in and around the UL112-1 target site might affect the regulation of the major IE region, we proceeded to characterize gene expression from this region in wild-type, mutant, and rescued mutant virus-infected cells.

Mutation of the IE1 72 3' UTR results in increased expression of IE1 72 protein, but not IE2 86 protein, in HCMV-infected cells. To understand the importance of the UL112-1—target site interaction in the regulation of IE1 72 protein levels in the HCMV-infected cell, we examined the expression of IE1 72 and IE2 86

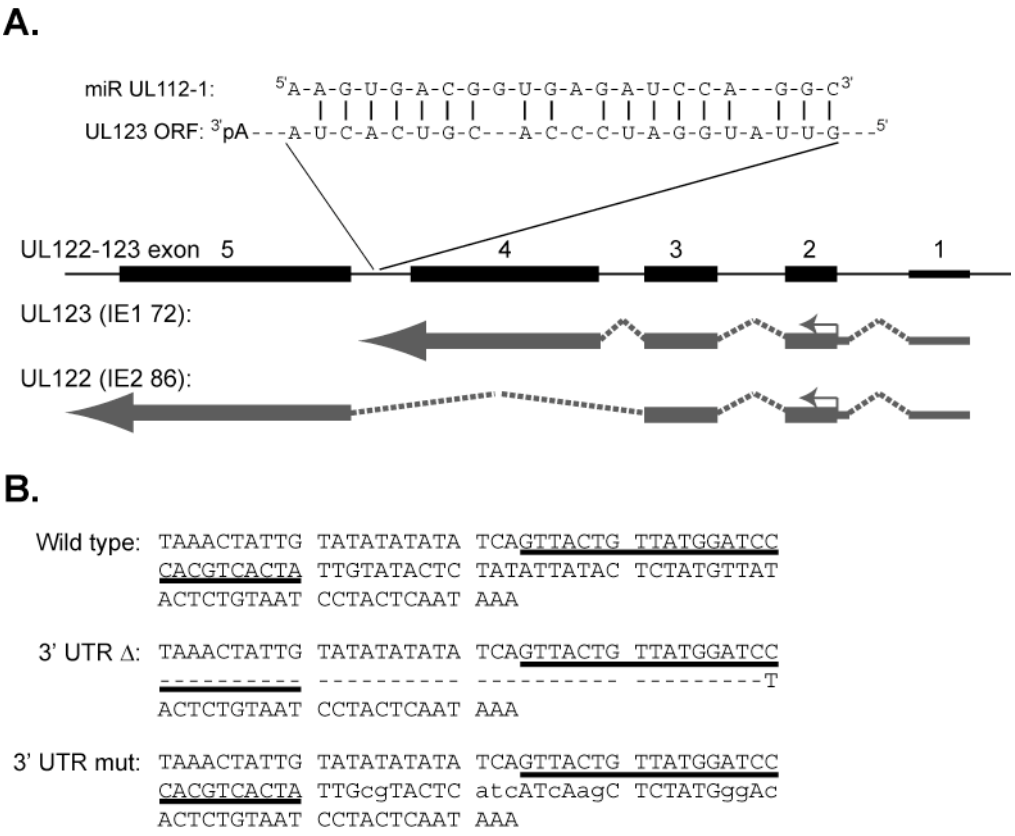


**Figure A.1 The HCMV major IE region contains a predicted target site for the HCMV miRNA UL112-1.** (A) The HCMV major IE region, including predominant UL123 (IE1 72) and UL122 (IE2 86) transcripts are indicated. The sequence of the fully processed HCMV miR UL112-1 and its putative target site are indicated. In the HCMV genome, the target site is located in the intron between major IE exons 4 and 5, so that the sequence is present in the 3' UTR of the UL123 transcript. (B) Sequences of the wild-type, deletion mutant, and sequence change mutant UL123 3' UTRs, from stop codon (TAA) to canonical polyadenylation site (AATAAA), are indicated. The predicted UL112-1 binding site is underlined. Nucleotides that were deleted in the recombinant viruses are indicated by –, and nucleotides that were changed from the wild-type sequence are in lower case.

proteins following a time course of virus infection. Following high multiplicity (MOI = 3) infection of HFF with wild-type, mutant, or rescued mutant virus, cells were harvested at 8, 24, 48, and 72 hours post infection (h p.i.) and processed for Western blots. First, we examined protein levels in the infected cell lysates using an antibody directed against the exon 2 region common to IE1 72 and IE2 86 (Figure A.2, top panel). Both 3' UTR deletion mutant and 3' UTR sequence change mutant virus-infected cells exhibited elevated levels of IE1 72 protein relative to wild-type and rescued mutant control virus-infected cells. The increase in IE1 72 protein followed the kinetics of UL112-1 expression, beginning 24 h p.i. and continuing through the time course. In contrast, IE2 86 protein levels increased overall as the infection progresses, but levels of this protein in cells infected with either mutant virus were indistinguishable from those in control virus-infected cells.

To further verify that IE2 86 protein levels were not altered by changes in the UL123 3' UTR, we examined the expression of IE2 86, as well as the smaller IE2 60 and IE2 40 kDa proteins, using an antibody directed against the C-terminal region shared by these factors (Figure A.2, middle panel). Again, IE2 86 protein levels were not increased by alterations in the UL123 3' UTR, and by 72 h p.i. these levels were identical in wild-type, mutant, and rescued mutant virus-infected cells. It should be noted that we detected reduced expression of IE2 60 protein when either mutation has been introduced into the 3' UTR of UL123. This is likely because the promoter driving the expression of the ~1.75 kb transcript that specifies some IE2 60 protein (Chapter 3) is located in the intron between exons 4 and 5 of the major immediate early region and has also been altered by introduction of the 3' UTR mutations. The

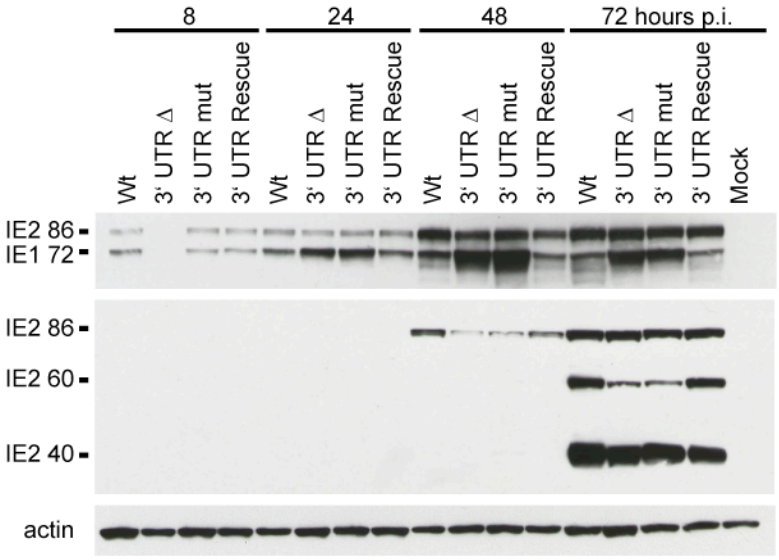


**Figure A.2 Changes to the UL123 3' UTR result in increased IE1 72 protein expression, but not increased IE2 86 protein expression.** $G_0$-synchronized HFF cells were infected with 3 pfu/cell of wild-type (Wt), UL123 3' UTR deletion mutant (3' UTR Δ), UL123 3' UTR sequence change mutant (3' UTR mut), and rescued sequence change mutant (3' UTR Rescue) virus or mock-infected (M) and harvested at the indicated times p.i. Lysates equivalent to an equal number of cells were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. IE1 72 and IE2 protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading.

expression of IE2 60 protein is not required for HCMV replication in cultured cells (Chapter 3). Disruption of the UL123 3' UTR therefore resulted in the predicted increase in IE1 72 protein levels and no change in IE2 86 protein levels, suggesting that the UL112-1—target site interaction was functional and specifically regulated the expression of IE1 72 protein in HCMV-infected cells.

**UL123 transcript levels are specifically increased in UL123 3' UTR mutant virus-infected cells.** Given that protein levels are affected as predicted by the UL123 3' UTR mutations, we next examined the levels of the full-length UL122 and UL123 transcripts expressed in the virus-infected cells. Following a time course of infection of HFF, cells were harvested at 8, 24, 48, and 72 h p.i. RNA was harvested from uninfected cells or from cells infected with wild-type, 3' UTR deletion mutant, 3' UTR sequence change mutant, or rescued mutant viruses. UL122 and UL123 transcript levels were quantified by real-time RT-PCR (Materials and Methods). In wild-type HCMV-infected cells, UL123 transcripts were abundantly expressed 8 h p.i., but expression decreased rapidly as the infection progressed (Figure A.3). When the 3' UTR of UL123 was disrupted by either mutation, however, levels of UL123 transcript remained several-fold higher in mutant-virus infected cells than in wild-type virus-infected cells. Again, this effect occurred with kinetics consistent with the expression pattern of UL112-1. In contrast, UL122 transcript levels in wild-type virus-infected cells remained relatively constant from early to late times p.i. The levels of UL122 RNA in mutant virus-infected cells were comparable to those in wild-type virus-infected cells, and were not changed by the introduction of either mutation into the 3' UTR of UL123. This result suggests that in wild-type HCMV-infected

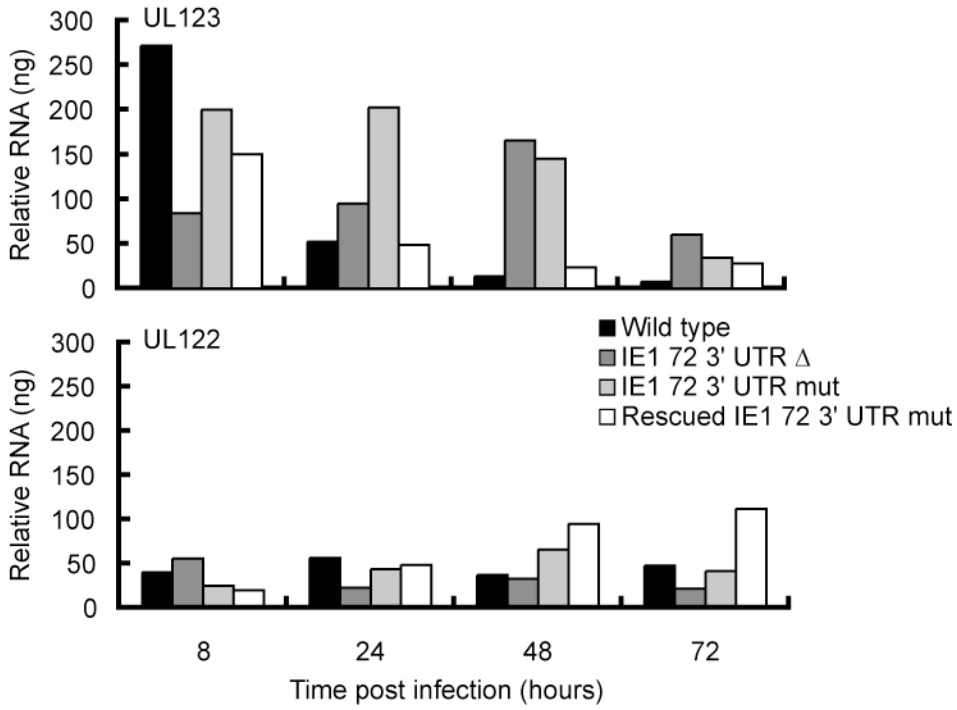


**Figure A.3 Changes to the UL123 3' UTR result in increased UL123 transcript levels.** $G_0$-synchronized HFF cells were infected with 3 pfu/cell of wild-type (Wt), UL123 3' UTR deletion mutant (3' UTR Δ), UL123 3' UTR sequence change mutant (3' UTR mut), and rescued sequence change mutant (3' UTR Rescue) virus or mock-infected (M) and harvested at the indicated times p.i. Total cellular RNA was analyzed by quantitative real-time RT-PCR as described in Materials and Methods to measure the relative levels of UL123 (IE1 72) or UL122 (IE2 86) transcripts. To ensure that an equal amount of RNA was included in each reaction, samples were analyzed with G6PD-specific primers and probe. Values shown in the graphs have been standardized to G6PD levels. When mock-infected cell RNA was analyzed with the UL123 TaqMan probe, amplification was below the limit of detection, and when it was analyzed with the UL122 TaqMan probe, the amount of RNA detected was less than 0.5% of what was detected in virus-infected cells.

cells, the interaction of the Ul112-1 miRNA with its target site in the UL123 3' UTR results in the degradation of UL123 transcript, rather than just a block in IE1 72 translation.

**Mutation of the IE72 3' UTR reduces the production of infectious virus.** Finally, to begin to understand how the interaction of UL112-1 with its UL123 target site affected the production of infectious virus, we monitored the release of infectious virus following high multiplicity (MOI = 3) infection of HFF with wild-type, 3' UTR deletion mutant, 3' UTR sequence change mutant, or rescued mutant virus. Virus was collected from infected cells every 24 h p.i. and titered by plaque assay. The replication of wild-type and rescued mutant viruses peaked at 5 days p.i. and began to decrease subsequently (Figure A.4). Cells infected with either 3' UTR mutant virus also maximally produced infectious virus at 5 days p.i., but the peak amount of virus released was approximately 10-fold lower in mutant virus-infected cells than in cells infected with either wild-type or rescued mutant virus. This suggests that the down-regulation of IE1 72 transcript and protein expression mediated by UL112-1 contributes to the full production of infectious virus. It is important to note, however, that this decrease in titer could be at least partly due to the decreased expression of IE2 60 protein also observed in cells infected with either mutant virus.

**Changes in IE1 72 protein levels do not result from the deletion of IE2 60.** When the 3' UTR of the UL123 transcript is disrupted by either mutation discussed in this study, we observe a decrease in IE2 60 protein levels as well as an increase in IE1 72 protein levels (Figure A.2). From that result, it is not possible to rule out the possibility that the increase in IE1 72 levels is related to the decreased expression of

IE2 60, and not to the change in the 3' UTR of the IE1 72 RNA. To distinguish between these alternatives, we constructed a recombinant virus in which the UL123 3' UTR sequences are wild-type, but the initiating methionine (M170 of IE2 86) for the IE2 60 protein has been changed to a leucine. We also created a rescued version of this virus in which the mutated sequence has been returned to wild-type. We analyzed the growth of the IE2 M170L mutant and rescued mutant viruses compared to wild-type and 3' UTR deletion mutant viruses. We detected wild-type levels of IE2 86 and IE2 40 proteins in the cells infected with the IE2 M170L mutant virus, and the expression of IE2 60 was restored in the rescued virus-infected cells (Figure A.5, middle panel). In contrast, we detect the increased expression of IE1 72 protein only when the 3' UTR of its transcript has been altered, and not when IE2 60 expression has been eliminated by introducing the point mutation (Figure A.5, top panel). While this result does not prove that an interaction between the UL112-1 miRNA and the UL123 3' UTR is occurring in the infected cell, it does indicate that the increase in IE1 72 protein levels we observe is not due to changes in the levels of IE2 60.

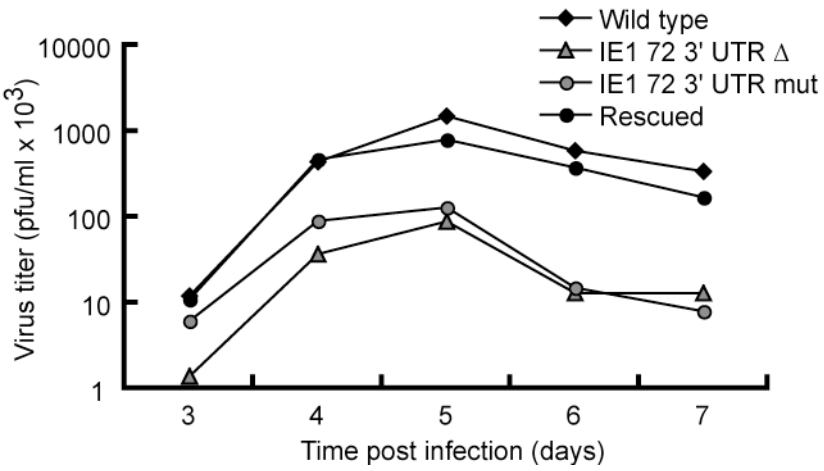


**Figure A.4 Changes to the UL123 3' UTR result in decreased production of infectious virus.** $G_0$-synchronized HFF cells were infected with 3 pfu/cell of wild-type (Wild type), UL123 3' UTR deletion mutant (IE1 72 3' UTR Δ), UL123 3' UTR sequence change mutant (IE1 72 3' UTR mut), and rescued sequence change mutant (Rescued) virus. Infected cell supernatants were collected at the indicated times, and titers were determined by plaque assay as described in Materials and Methods.

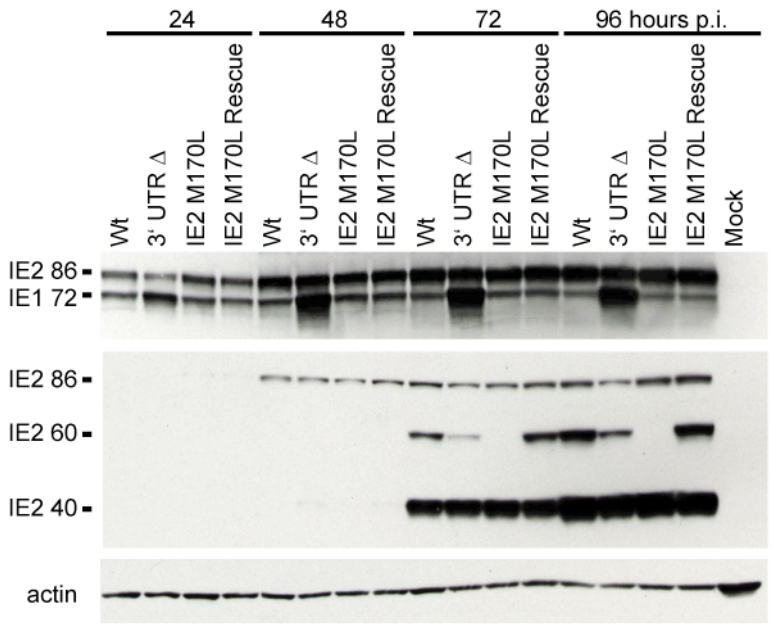


**Figure A.5 Eliminating the expression of IE2 60 does not alter levels of IE1 72 protein.** $G_0$-synchronized HFF cells were infected with 3 pfu/cell of wild-type (Wt), UL123 3' UTR deletion mutant (3' UTR Δ), IE2 M170L mutant, and rescued IE2 M170L viruses or mock-infected (M) and harvested at the indicated times p.i. Lysates equivalent to an equal number of cells were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose. IE1 72 and IE2 protein levels were analyzed by Western blotting as described in Materials and Methods. Cellular actin levels were analyzed as a control for protein loading.

## DISCUSSION

While many virus-encoded microRNAs have been identified to date and many more surely remain to be discovered (39, 41, 42, 65, 71, 85, 86, 159, 165, 166), studies demonstrating that these species are functional in virus-infected cells are scarce. Human cytomegalovirus (HCMV), a betaherpesvirus, has been predicted to encode 13 miRNAs, and the expression of several of these has been verified experimentally (71, 85). Here we provide evidence consistent with the idea that the HCMV-encoded miRNA UL112-1 is functional in the lytically-infected cell. The results of an RNase protection assay suggest that an interaction between UL112-1 (which is encoded by a transcript oriented antisense to the UL114 gene) and the UL114 RNA could mediate the cleavage of this RNA at the site where the two interact. Expression of the UL112-1 miRNA also leads to reduced expression of the viral uracil DNA glycosylase product of the UL114 gene in transfected cells. What is unique about UL112-1 among viral miRNAs characterized so far is that there is evidence to suggest that it might act on heterologous targets within the HCMV genome as well as on its antisense target. One potential target site is located in the 3' UTR of the UL123 transcript. Cells infected with target site mutant viruses produce more of the IE1 72 protein product of UL123 and less infectious virus than do wild-type virus-infected cells, which suggests that the UL112-1 miRNA – UL123 target site interaction could be functional in the HCMV-infected cell. IE1 72 is one of the immunodominant antigens presented by infected cells in the HCMV-infected host (176), and miRNA-directed down-regulation

of IE1 72 expression would be particularly interesting if it does contribute to efficient immune evasion by the virus.

Several families of viruses express small RNAs predicted to function as miRNAs. Herpesviruses in particular have been the source of many of these recently predicted miRNAs. To date, the only herpesvirus-encoded miRNA for which targets have been identified is the latency associated transcript (LAT)-associated miRNA expressed in cells latently infected with HSV-1. The LAT miRNA targets cellular genes, including TGF-β and SMAD3, for down-regulation (87), although it should be noted that the proposed target sites in these messages do not contain perfect matches to the HSV LAT miRNA seed sequence. This result was interpreted to indicate a mechanism by which latently-infected cells can avoid apoptosis. In an elegant example of a virus-encoded miRNA acting on the viral genome, Sullivan and colleagues identified an SV40 miRNA that targets an early viral transcript to down-regulate T antigen expression (215). It is important to note, however, that only one target site within the viral genome has so far been identified for both the SV40 miRNA and for a related mouse polyomavirus miRNA. UL112-1 is so far unique in that it has many potential target sites within the HCMV genome. One of these target sites is located within the UL114 gene, and a second is in the 3' UTR of the UL123 gene. Showing that both of these target sites are functional would indicate that as for cellular miRNAs (132), virus-encoded miRNAs can act simultaneously on multiple viral transcripts. In both of the cases described here, the miRNA-mediated decrease in gene expression appears to result from a cleavage or degradation of the target transcript, although an effect on translation cannot be excluded. Furthermore, UL112-

1 is expressed and is functional during the lytic phase of human cytomegalovirus replication, a key difference from the pattern of expression and predicted role of the HSV-1-encoded LAT miRNA. This study begins to extend these results, suggesting only the second example of a virus-encoded miRNA acting on viral targets. It also appears that UL112-1 can act on multiple viral targets and that herpesvirus-encoded miRNAs are functional during the productive infection.

Neither the UL114 nor the UL123 targets of the UL112-1 miRNA described here are strictly required for HCMV replication (70, 249). However, the expression of both genes contributes to efficient virus growth (63, 79, 84, 148, 173). One might envision that it is advantageous for the virus to down-regulate the expression of non-essential genes like these as the infection progresses, allowing the virus to direct its limited resources most efficiently at any point in the viral life cycle. For herpesviruses, with their particularly long and complex replicative cycles, miRNAs could provide a way to down-regulate large numbers of target genes at once with only a very small corresponding increase in genome size. It has been calculated that only 200 bp of the viral genome would be required to provide the regulatory signals and sequence for a miRNA. A previously described HCMV recombinant has been constructed in which the UL114 ORF, including the region encoding the UL112-1 miRNA, is replaced with a *gpt* cassette (63, 173). This virus is viable, but delayed in the initiation of viral DNA synthesis and in virus production. Although these aspects of the mutant phenotype were attributed to the deletion of UL114, we must now recognize that the lack of UL112-1 expression and associated regulatory functions could also be contributing to the virus' altered growth. Notably, UL114 deletion

mutant virus-infected cells express elevated levels of IE1 72 protein beginning 24 h p.i. relative to wild-type controls. This result is complementary to our finding and although the study did not examine the role of the UL112-1 miRNA directly, it suggests that the loss of the miRNA-target interaction resulting from the deletion of either binding partner has the same effect.

The major immediate early region of HCMV is a complex segment of the genome that encodes several regulatory factors. Two of these are the IE1 72 and IE2 86 kDa products of the UL123 and UL122 genes, respectively. Although these two proteins are generated from the alternative splicing of a single, five-exon primary transcript, one paradox has been that the levels of IE1 72 protein drop dramatically relative to IE2 86 protein levels as the infection proceeds. This study demonstrates that this differential drop in protein levels could be mediated by the UL112-1 miRNA, as our data is consistent with UL112-1 acting specifically on the 3' UTR of UL123, but not on the 3' UTR of UL122. Since IE1 72 is one of the two viral proteins that provides most of the immunogenic peptides presented on the surface of HCMV-infected cells (176), the virus has a vested interest in downregulating the expression of this protein at late times in the infection. The prediction from this result would be that HCMV lacking the UL112-1 target site in the 3' UTR of UL123 would be less able to avoid detection by the immune system in an infected host. Interestingly, even in cultured cells, alteration of this 3' UTR leads to a drop in the level of virus produced, suggesting that reduction of IE1 72 levels is needed for viral replication.

Several caveats must be considered in the interpretation of this data. First, the results obtained with the 3' UTR mutant viruses were initially complicated by the fact

that the mutations affected the expression of IE2 60 as well as the 3' UTR of UL123. As a control, we constructed a virus that expresses no IE2 60 and has a wild-type UL123 3' UTR, and cells infected with this virus (IE2 M170L) do not over-express IE1 72. This eliminates the possibility that the increase in IE1 72 protein levels is due to the change in IE2 60 expression and confirms that in some way, the change in the 3' UTR has led to the altered expression of IE1 72.

Second, two different alterations of the UL123 3' UTR altered replication of the HCMV recombinants in the same way: one in which part of the miRNA target site was deleted from the viral genome, and one in which adjacent sequence was changed. While these viruses behave identically in cultured cells, only one contains a deletion of part of the target site. It is not clear whether introducing a mutation into the region adjacent to the target site would be enough to disrupt the interaction of the miRNA with its target. One possible explanation for this result would be that local RNA structure around the target site might influence miRNA binding. Computationally predicting the secondary structures of the wild-type and sequence change mutant UL123 3' UTRs (data not shown) shows that introducing the nucleotide changes into the 3' UTR of UL123 results in a mutant transcript in which the miRNA target site forms a tight hairpin structure in the middle of an almost completely base paired region of the RNA. In contrast, the miRNA binding site in the context of the wild-type transcript is predicted to form a large open loop that is significantly more accessible to target binding by UL112-1. It is possible that local structure around the binding site, not only its sequence, contributes to allow an efficient interaction between the miRNA and its target, although these secondary structure predictions

might vary widely based on the algorithm used to generate them. We cannot exclude possible alternatives including the idea that the adjacent sequence is important not for the binding of the miRNA to its target, but for the binding of a viral or cellular protein, perhaps a component of the RISC complex. In this case, disruption of the adjacent sequence might prohibit the functional processing complex from assembling on the RNA.

Third, in vitro experiments have given conflicting results about the importance of the interaction of UL112-1 with the 3' UTR of UL123. In a transient transfection assay, a vector encoding the firefly luciferase gene fused to the 3' UTR of UL123 was cotransfected with either a UL112-1 expression vector, the same vector expressing a negative hairpin RNA, or a vector expressing the HMCV miRNA UL22A-1, which is not predicted to target the UL123 3' UTR sequence. Expression of UL112-1 led to an approximate 60% reduction in relative luciferase activity compared to the negative hairpin RNA control (Finn Grey and Jay Nelson, unpublished results. Presented as abstract 1.03 at the International Herpesvirus Workshop, Seattle, Washington, July 2006). Luciferase activity was not reduced when the reporter construct contained the 3' UTR from UL122, which is not predicted to contain target sites for UL112-1. This result was interpreted to mean that the miRNA-mediated reduction in luciferase activity is specific to the 3' UTR of UL123. Next, the deletion or sequence change mutations that were used in the recombinant viruses were included in the UL123 3' UTR in this assay. Based on the behavior of the recombinant viruses, one would expect that introducing either mutation would eliminate the ability of the transfected UL112-1 vector to reduce luciferase activity. While luciferase activity was not

reduced when the UL123 3' UTR deletion was included, introducing the sequence change mutation led to approximately wild-type level of reduction in luciferase activity. Again, it is not clear whether the luciferase assay results differ from those obtained in infected cells because the luciferase assay does not include all the components present in the infected cell, because the structure of the 3' UTR is different in the context of the luciferase transcript than when it is part of its native, full-length IE1 72 transcript, or for another reason.

Viruses must use a diverse set of mechanisms to enable their establishment, replication, and maintenance in their natural hosts. Regulation of gene expression by miRNAs could be particularly useful for viruses as they perform these functions, and many of the HCMV-encoded miRNAs not studied here could participate in such events either in lytically or latently infected cells. Down-regulation of multiple host cellular genes by a virus-encoded miRNA has been shown to help infected cells evade apoptosis, and one might envision that down-regulation of cellular genes involved in antigen presentation could also help infected cells avoid clearance by their host's immune system. Down-regulation of many cellular targets by DNA virus miRNAs may also be important for the virus' ability to limit cellular DNA replication and favor the replication of its own DNA. In contrast, SV40 approaches the immune evasion question from the opposite direction, downregulating the expression of large T antigen, a viral product, to limit its presentation on the surface of infected cells. The results described here suggest that limiting the expression of immunodominant antigens using a miRNA could be a strategy employed by several viruses. In addition, the down-regulation of multiple viral targets by a single miRNA would suggest that

miRNAs function not only in latently infected cells, but can regulate the viral life cycle as well. These miRNAs could potentially limit the expression of multiple genes or classes of genes as the virus proceeds through its infectious cycle. Finally, the finding that not all predicted miRNA target sites are conserved between human and chimpanzee cytomegaloviruses suggests that these miRNA-target interactions must have arisen quite late in the evolution of these viruses. Only the further identification of functional target sites for virus-encoded miRNAs can fully elucidate what the many roles of this novel regulatory mechanism might be in virus-infected cells.

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