UNIVERSITY OF CALIFORNIA, SAN DIEGO

Analysis of Key Regions, Roles, and Interactions of the IE2 Family of Proteins throughout the Human Cytomegalovirus Infection Process

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

Doctor of Philosophy

in

Biological Sciences

by

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This dissertation of Rebecca L. Sanders is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2009

DEDICATION

To my friends and family who have guided me through each step in life; you have made all of my accomplishments possible, and taught me to appreciate every challenge in life.

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Sanders R.L., C.J. Del Rosario, E.A. White, and D.H. Spector. (2008) Internal Deletions of IE2 86 and Loss of the Late IE2 60 and IE2 40 Proteins Encoded by Human Cytomegalovirus Affect the Levels of UL84 Protein but Not the Amount of UL84 mRNA or the Loading and Distribution of the mRNA on Polysomes. J. Virol. 82:11383-11397.

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Professor Deborah H. Spector

ABSTRACT OF THE DISSERTATION

Analysis of Key Regions, Roles, and Interactions of the IE2 Family of Proteins throughout the Human Cytomegalovirus Infection Process

by

Rebecca L. Sanders

Doctor of Philosophy in Biological Sciences

University of California, San Diego, 2009

Professor Deborah H. Spector, Chair
Professor Michael David, Co-Chair

Studies of the human cytomegalovirus (HCMV) major immediate early (MIE) protein, IE2 86 have yielded significant knowledge as to how this virus regulates many aspects of the viral infection. IE2 86 has been shown to be an essential regulatory factor during HCMV infection, and is thought to play many different roles throughout the different stages of the infection process. Two other proteins that are 3 prime co-terminal

to IE2 86, IE2 60 and IE2 40, have also been found to be important at the later stages of the infection.

Here, we have developed a system to overcome these limitations by using a translationally induced complementation system. Viruses expressing Cre and FLP recombinases have been constructed, and are used in conjunction with cell lines containing an inducible lenti-viral based vector to facilitate expression of IE2 86, IE2 60 or IE2 40. These cell lines are selected by a drug resistance marker placed upstream of IE2, and following a recombination event governed by Cre of FLP, the selectable marker is removed and allows expression of the 2nd open reading frame (ORF) encoding IE2 86, IE2 60 or IE2 40.

These studies have also defined new roles of IE2 86, IE2 60, and IE2 40. We have found that expression of another early-late protein, UL84, is regulated post-transcriptionally by each of the IE2 proteins. This regulation can occur without any other viral proteins, but requires some cellular processes. We find that IE2 40 plays a major role in the expression of UL84 at late times post infection (p.i.), and that IE2 60 may also play a similar role, albeit to a lesser degree. IE2 86, IE2 60, and IE2 40 can interact with UL84 through the C-terminal region common to all three IE2 proteins.

We have also identified that the first 105 aa of UL84 is responsible for an interaction with IE2 86 and IE2 40. Further, this domain is necessary and sufficient for the post-transcriptional regulation of UL84 governed by the IE2 proteins, and a protein-protein interaction is likely necessary for the regulation.

CHAPTER 1

INTRODUCTION

HUMAN CYTOMEGALOVIRUS

The diseases associated with human cytomegalovirus (HCMV) infection have underscored the need for increased understanding of the regulatory pathways governing a productive infection. Much has been learned regarding the pathogenicity of the virus, although a significant need for understanding how this virus persists, and then subsequently becomes reactivated and causes disease exists. HCMV infection was first observed in the first half of the 20th century; however the causative agent was not isolated until the mid-1950's (46, 145, 163, 187, 221). As much as 50-90% of the world's population is infected by the age of 30, and HCMV is the leading viral cause of birth defects. Congenital infection with HCMV has been shown to lead to hearing and vision loss, mental disabilities, corioretinitis, and even death (17, 142, 145, 149). Further, while HCMV is primarily asymptomatic in healthy individuals, the virus has been shown to pose a severe threat to immunocompromised and transplant patients, and has been linked to atherosclerosis and restinosis following coronary angioplasty (244). HCMV is readily transmissible and can infect many different cell types, including skin fibroblasts, lung fibroblasts, endothelial cells, epithelial cells, hepatocytes, muscle cells, leukocytes, dendritic cells, and to some degree monocytes and macrophages (181). However, HCMV is extremely species specific, with only some homology to its other species counterparts, for example mouse CMV (MCMV), rhesus CMV (RhCMV), chimp CMV

(CCMV), and guinea pig CMV (GpCMV). Recent clinical isolates of HCMV have been shown to infect a wider variety of human cells, including endothelial cells and cells of the macrophage/monocytic lineage, compared to the common laboratory passaged strains (57-58, 206).

HCMV is a member of the betaherpesvirus family of viruses. Common to all herpesvirus, it contains a double stranded DNA genome and enveloped particle. However, unique to betaherpesviruses, the replicative cycle is slow and the host range is restricted. There are three types of herpesviruses, including the alpha, beta, and gamma subfamilies. The alphaherpesviruses have the smallest genomes, while gamma are intermediate in size, and beta-viruses are the largest (181). Betaherpesvirus genomes are linear when isolated from virions, and have been shown to have a single unpaired base at each end (204).

Many genes have been conserved throughout all isolated strains of HCMV; however fewer have been conserved amongst the different types of CMV. CMVs represent the principle members of the betaherpesvirus subgroup, and while they share many common characteristics such as virion structure and the ability to establish a persistent infection, they are also divergent in many ways. CMVs exhibit a tropism for salivary glands, strict species specificity, and grow very slowly in culture (reviewed in 89, 154, 193). Mammals have distinct versions of CMV, and based on genome nucleotide sequence comparisons of HCMV, MCMV, and human betaherpesvirus HHV-6, the divergence is very prominent (36, 72, 159). A core set of betaherpesvirus-common open reading frames (70-80 common ORFs) does show predicted amino acid homology,

and allows these viruses to be classified in the same subfamily (36, 72, 139, 159, 211). These standard sequence comparisons, along with other more advanced comparison strategies have allowed CMVs to be classified together (103), and show that they are more closely associated with each other than with other herpesviruses.

The genome is organized into what is known as “long” and “short” segments, and each domain is comprised of a central unique region (U_L and U_S) and is flanked by repeated segments that are at either end of the complete genome (TR_L and TR_S). This genome encodes for over 200 open reading frames, giving way to three classes of genes, including the immediate early (IE), early (E), and late (L) genes, which fall into these categories based on their temporal expression patterns and functions. IE genes do not require any *de novo* host or viral protein synthesis for expression and serve primarily to activate early gene expression and shut down host defenses. E genes are mostly associated with DNA replication of the virus. L genes are expressed following E gene expression and primarily serve in structure and assembly of the virus (reviewed in (60). A significant subset of the U_L region likely encodes for genes that have roles *in vivo* but are dispensable for infection in cell culture (33).

The HCMV life cycle begins with adsorption of the virus to a permissive cell, followed by uncoating of the virus particle and delivery of the viral genome to the nucleus (Figure 1.1). Viral gene expression begins without delay following transport of the viral genome, and immediate early gene expression can be detected as soon as 2-3 hours post infection. Early gene expression has been shown to be expressed as soon as 8 hours post infection, however many early genes do not arise or increase in abundance

until much later in the infection (approximately 48 hours post infection). DNA replication occurs between 48 and 72 hours post infection and is mediated by several identified early genes, including UL44 and UL84, two essential DNA replication factors that have been readily studied. HCMV DNA is replicated by a rolling circle mechanism which creates multiple tandemly linked copies of the viral genome. When packaged, the tandemly linked genome copies are cleaved and packaged as single copies. The unpaired bases serve to begin the next round of replication. During the last stage of the infection, late genes are expressed (approximately 72-96 hours post infection), and encode for many tegument and capsid proteins. The structure of the virus has been partially elucidated. The HCMV DNA is contained within a capsid which is surrounded by the tegument. This tegument is surrounded by an envelope comprised of mainly glycoproteins (56).

Depending on the study conducted, many different predictions have been made regarding the number of functional ORFs actually encoded by HCMV. The first estimate of the number of ORF's came from studies analyzing a strain of the virus called AD169, which predicted that AD169 had the ability to encode 208 ORFs. This study deemed a coding region an ORF if it encoded a polypeptide of at least 100 amino acids, and this region did not overlap a lager ORF across more than 60% of its length (36). Another study using a different strain (Toldedo) identified an additional 19 ORFS, while others have identified many additional ORFs (69, 107, 134). The most recent predictions for the number of HCMV ORFs utilize many different strain isolates (47, 51, 135), and currently

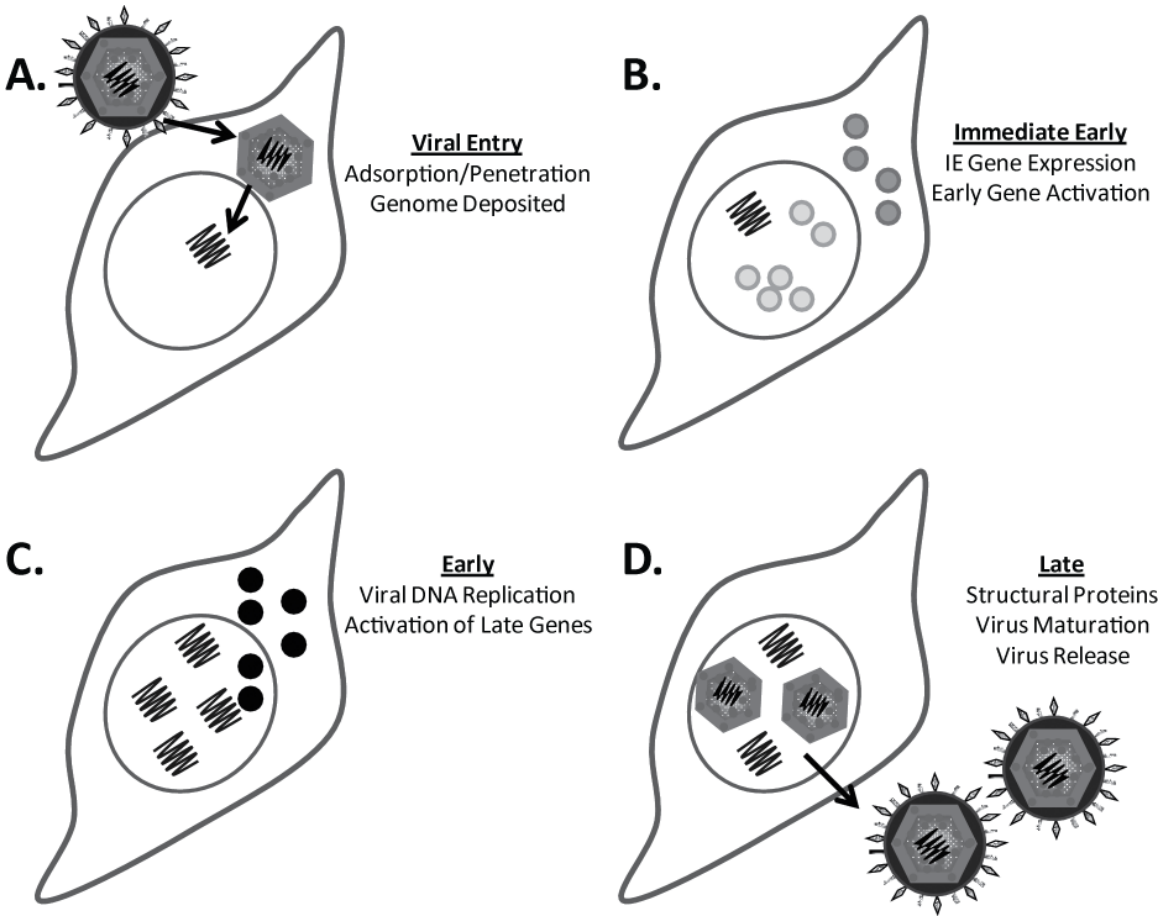


Figure 1.1. HCMV infection cycle. (A) The HCMV life cycle begins with adsorption and penetration, followed by the genome being delivered to the nucleus. (B) Immediate early gene expression begins and does not require any *de novo* protein synthesis. Early genes become activated. (C) Early gene expression commences followed by viral DNA replication. During this stage, late genes are activated which serve primarily in structure and assembly of the virus. (D) During late stages of the infection cycle, structural proteins are produced, the virus matures, and is released following appropriate packaging and assembly.

predict the number of ORFs to be similar to the original prediction, falling somewhere between 165-252 (56). Of these, approximately only 30% have been shown to be absolutely essential to the infection in cell culture, while close to half the genes are dispensable in these studies and 20% contribute to the efficiency of the infection, but are not required to produce infectious virus (52, 238).

Many different strains of HCMV exist, both in the laboratory setting, as well as throughout the population. Two of the most commonly used laboratory strains, Towne and AD169, were derived from a candidate vaccine strain from the urine of an infected child, and from the adenoids of an infected child, respectively (153, 163). Each of these strains have been widely examined, and have been found to be missing key genes thought to be required for infection outside of fibroblasts. For instance, the AD169 strain is missing a DNA segment that encodes for 19 potential ORFs (UL133-UL151), while Towne has been shown to encode three additional ORFS (UL152-154), but lacks many additional genes encoded by other clinical strains (33). For laboratory study, these strains are best propagated in primary human foreskin fibroblasts (cells used for the majority of the infection studies in this dissertation). The improved replication in fibroblasts has been shown to be in part due to the disruption of a glycoprotein complex (gH-gL-pUL128-pUL130-pUL131) present in the viral envelope (217). Many other strains exist for both laboratory settings as well as clinical settings, and all have been found to have minor differences in their genome.

Much has been studied regarding the role of IE, E, and L genes throughout the HCMV infection. IE gene expression has been characterized as being important for

transactivating early genes, as well as shutting down host defenses. The most prominently studied IE genes are the major-IE genes (MIE), UL122-123, which encode for proteins known as IE1 72 and IE2 86. These proteins have been shown to be important for the transactivation of many early gene promoters, and have also been shown to have a role in autorepression of the MIE gene promoter. Further, many studies have shown that these proteins, while sharing some similar functions, have different roles as well, and have been shown to alter the expression of many host cellular genes in order to make the environment favorable for viral replication (for reviews, see 63, 132). Early genes have also been extensively studied. For instance, the product of many E genes have been shown to be essential for DNA replication, including UL44 (DNA prcessivity factor), UL54 (DNA polymerase), UL70 (primase), UL105 (helicase), UL102 (primase-associated factor), UL57 (single-stranded DNA-binding protein), and UL84 (DNA replication factor). Late gene expression is widely characterized by expression of packaging and structural proteins. The structure of the HCMV virion particle has been divided into three regions: the capsid, tegument, and envelope (for a review, see 25). The capsid is the innermost icosahedral structure that contains the viral DNA and other viral proteins. It is primarily encoded by UL86 (major capsid protein), UL48-49 (small capsid protein), UL85 (minor capsid protein), UL46 (minor capsid binding protein), and UL80 (assembly protein) (37, 69, 220). The tegument is composed of many proteins, and is an unstructured coating of the capsid that links the capsid to the viral envelope. The most common and well understood tegument proteins are UL99, UL69, UL53, UL83, and UL32 (169). Much about the roles of the tegument proteins and how it is formed remain

to be elucidated, but several of the proteins have been shown to regulate both host and viral gene expression (9, 68, 70, 98-99, 101, 116, 194, 229). The viral envelope is composed primarily of glycoproteins (24, 74-76, 102, 120), although the exact composition and mechanism of assembly is still being investigated.

HCMV and the Host Cell

Since HCMV has the ability to infect many types of cells, it is clear that the virus utilizes multiple strategies to overcome host defenses and requires that a significant portion of its genome be dedicated to manipulating the host cell. Evolutionarily, it is advantageous for the virus to infect many cell types, allowing infection of multiple organs and dissemination of the virus throughout the body for easy transmission and infection (132). Given the vast cellular signaling that normally occurs between many of these target cells, the virus must possess clever strategies to circumvent this possible problem. Further, the virus may be able to utilize the natural cellular communication strategies in place to make the environment favorable for viral infection. There are multiple ways in which the virus is able to exploit these systems and promote persistence and survival. Many groups have identified viral ligand-mediated signaling as being important for viral attachment, fusion, and entry (14-15, 19, 23, 45, 55, 104, 184, 217-218, 239, 241).

Attachment of HCMV to the host cell initiates the multitude of changes that the cell begins to undergo. The envelope glycoproteins play an essential role in viral attachment and entry (23, 132) and these molecules are also involved in beginning the manipulation of the host cell. HCMV encodes a number of envelope glycoproteins (23,

132), although the exact role of each of the glycoproteins and how essential they are to the viral infection is a point of debate. Many believe that both gB and gH stimulate the activation of cellular transcription factors, such as NF-κB and Sp1 (14-15, 19, 184, 217-218, 239-240). Further studies revealed that HCMV glycoproteins are also important for interacting with the epidermal growth factor receptor, integrins, and toll-like receptor 2 (TLR2) (14, 45, 55, 217-218). These changes have been demonstrated in fibroblasts, monocytes, and endothelial cells, among many others, underlying the hypothesis that this aspect of the viral infection represents a major way that HCMV is able to undergo a productive infection across cell types.

HCMV infection leads to a multitude of cellular changes including alterations in calcium flux and lipid metabolism, activation of kinase signaling cascades, cytoskeletal changes, activation of cellular transcription factors, and induction of proto-oncogenes (4, 22, 49, 54). For instance, it has been demonstrated that pathways involving calcium/calmodulin-dependent protein kinases, cell cycle-regulated kinases, epidermal growth factor receptors (EGFRs), IκB kinase cascade, and various aspects of the mitogen activated protein kinase (MAPK) pathway as well as the phosphatidylinositol 3-kinase (PI(3)K) pathway are affected. Cellular changes following infection are usually grouped into one of two categories: those that occur prior to the initiation of viral gene expression, and those that occur temporally after the production of viral gene products. The virion itself can act as a potent regulator of cellular gene expression (reviewed in 49, 54). Further, the virus is able to capture cellular proteins during packaging, and following entry and release of these proteins upon infection, these cellular proteins can facilitate

cellular modulation (65, 129, 140). The tegument proteins, which are packaged with the virus, have also been shown to manipulate the host cell upon infection (132).

After viral gene expression begins, a number of viral proteins have also been shown to influence the cell (reviewed in 5, 30). For example, some immediate early proteins have been shown to alter the cell cycle and regulate apoptosis. Interestingly, the virus has also mimicked many cellular proteins, encoding for a protein similar to cellular cytokine/chemokine signaling receptors (US28) (reviewed in 201-202, 210), as well as proteins that mimic G protein-coupled receptor (GPCR), and a tumor necrosis factor-like receptor (12, 156). IE1 72 and IE2 86 have been shown to be important for regulating transcription factors to increase transcription of both viral and cellular genes necessary for the infection, as well as for interacting with cell cycle regulators p53, pRB, p107, and others (reviewed in 30, 49, 100, 132). Two other IE genes, UL36 and UL37, have been shown to be involved in regulating caspase activation and apoptosis, respectively (71, 125-126, 160, 186). Other important HCMV encoded cellular regulators include the viral kinase UL97 (157), four GPCRs that modulate smooth muscle cell migration (reviewed in 201-202, 210), and a TNF-like receptor that activates NFκ-B (156).

HCMV modulation of the cell cycle has been identified as a key component of a productive viral infection. Cells that become infected with HCMV have been shown to halt their cell cycle at a pseudo G_1/S phase of the cycle that has been shown to have G_1-phase, S-phase, and M-phase characteristics (34, 50, 88, 115, 166, 226-227). Cyclins E and B1 accumulate in infected cells, while the levels of cyclin A and cyclin D1 have been shown to be downregulated (21, 94, 166, 170, 227). The manipulation of these cell cycle

regulators is both at the level of transcription as well as protein stabilization (166, 170). DNA damage pathways have also been thought to be important for HCMV infection (59).

Another major feat of the virus is to shut down cellular DNA replication. This happens through several different mechanisms during the licensing of the origins-for example, the loading of the MCM proteins (2 to 7) onto chromatin is inhibited in infected cells (228). Interestingly, HCMV requires that cells be in G_0 or G_1 upon infection, allowing optimal onset of virus mediated events (61, 166). Many viral proteins have been shown to be important for these cell cycle modifications, including UL69, UL82 (52, 83, 98-99, 116), IE1 72 and IE2 86 (28, 31, 81, 124, 136, 141, 155, 190, 225, 227, 230, 243).

IE2 86 FUNCTION AND REGULATION

The MIE proteins, IE1 72 and IE2 86, have been extensively studied throughout the course of examination of HCMV gene expression (for a review, see 127, 199). Many groups have focused on understanding the roles and regulations governing the expression of IE1 72 and IE2 86, as well as the consequential roles the proteins play throughout the infection process. Both IE1 72 and IE2 86 arise from the same transcript that is alternatively spliced to include exons 1-4 for IE1 72, and exons 1-3 and 5 for IE2 86. Translation initiation begins in exon 2 for both proteins and while the two proteins share similar expression patterns at the beginning of the infection, they differ in both levels and function later in the infection. Further, each encodes several additional minor transcripts (95, 105, 158, 182, 196). Studies from our laboratory and others have shown that while

IE2 86 is essential to the infection, IE1 72 is non-essential (52, 67, 73, 85-86, 123, 131, 162, 168, 222, 224, 238). Two other proteins arise from exon 5 of IE2 86, termed IE2 60 and IE2 40 (60 and 40 kDa, respectively) which exhibit different expression kinetics, accumulating only at the early-late stages of the infection (95, 151, 158, 172, 195, 223). IE2 60 initiates at amino acid 170 of IE2 86, with a putative TATAA region for its mRNA upstream of UL122 exon 5, while IE2 40 initiates at methionine 242 of IE2 86, with a putative TATAA region just upstream of the IE2 60 translation initiation site. The functions of IE2 60 and IE2 40 have not been well characterized, and are a main focus of this dissertation (See Chapters 2, 3 and 4).

IE2 86 has been shown to interact with a number of proteins during the infection process and *in vitro.* For instance, like IE1 72, IE2 86 has been shown to interact with p53 and a member of the RB family of proteins, pRb (16, 41, 62, 77, 188, 192, 208). The functions of these interactions have begun to be elucidated, furthering the understanding of the host cell regulation governed by IE1 72 and IE2 86. IE2 86 has been shown to bind to p53 via its N-terminus, and this interaction inhibits some of the transactivation functions of p53 (208). Further this interaction was shown to inhibit the ability of p53 to transactivate both a p53-responsive promoter as well as some p53 target genes (192). Other proteins that IE2 86 has been suggested to interact with include TBP, TFIIB, TBP-associated factors (TAFs), HDAC2, Sp1, TEF-1, c-Jun, JunB, ATF-2, NF-κB, p300, CBP, and Egr-1, among others (16, 26-27, 32, 41, 62, 64, 68, 77-78, 96, 108, 118-119, 144, 176-178, 188, 191-192, 219, 232, 237). IE2 86 has also been shown to be important for regulating cell cycle progression in many cells, as mentioned above. For instance,

expression of IE2 86 in the human cell line, U373, blocked cell cycle progression in the G_1-phase, which mimics the effect of the HCMV infection in these (and other) human cells (226). Others have attributed IE2 86 as being important for the transition from G1 to S-phase through transactivation of the cyclin E promoter (227). Many other cell cycle regulators have been shown to be modulated by IE2 86, including B-myb, cyclin E, cdk-2, E2F-1, ribonucleotide reductase 1 and 2, thymidine synthetase, MCM3, and MCM7 (189). Although IE2 86 has been reported to be important for regulation of and interaction with many cellular proteins, it should be noted that most of the activities have not been confirmed in the context of the infection process, and have subsequently been questioned as to their functional relevance and actual role during viral replication.

Many important domains of IE2 86 have been elucidated. For instance, the regions involved in the majority of the identified protein binding domains lie in the C-terminal portion of the protein, and are largely mapped to regions not shared with IE1 72 (39, 188). The DNA binding domain has been mapped between the regions of 290-579 of IE2 86, which is also shared with IE2 60 and IE2 40 (39, 109, 177). Identification of binding domains has been complicated however, given that deletion of certain domains likely removes inhibitory or positive regions that may contribute to a domain being important for binding. The dimerization domain of IE2 86 has been mapped to amino acid 388-542, also contained in IE2 60 and IE2 40 (2, 39, 64).

To overcome the limitations in understanding the role of IE2 86 outside the infection process, many techniques have been developed to further understand the functional domains of IE2 86, and the roles and interactions that IE2 86 can have during

the infection process. One such technique has been the advent of the bacterial artificial chromosome (BAC). This strategy has allowed BACs to be used as vectors for cloning of the herpesvirus genomes since the majority of the viral genome is present in the BAC. Specific mutations can be made throughout the genome in bacteria, and then this construct can be transfected into permissive cells with another viral protein (pp71, UL82) allowing reconstitution of the virus containing the specific mutation (9). MCMV was the first herpesvirus to be cloned as a BAC, and this technique has now allowed analysis of specific domains in HCMV, herpes simplex virus type 1, Epstein-Barr virus, and others (1, 18, 48, 79-80, 128, 135, 165, 213). Many groups have utilized this technique to construct HCMV BACs containing mutations in IE2 86 and have characterized the resulting viruses if viable (85-86, 123, 168, 172, 222-224). Using this technique, several domains have been mapped within IE2 86.

BAC cloning has allowed elucidation of the roles of IE1 72, IE2 86, IE2 60, and IE2 40 in the context of the infection, some of which was completed for this dissertation. Most C-terminal mutants of IE2 86 have been shown to be non-viable (123, 222). Further, the functional roles of IE2 86 and IE1 72 have begun to be distinguished in this system. While IE2 86 has been shown to be essential for growth of the virus, IE1 72 has been shown to be non-essential, although multiplicity of infection appears to be important for the roles governed by IE1 72 (67, 73, 123, 131). Deleting the majority of exon 5 of IE2 86 results in a non-viable virus (123, 172), while other deletions throughout the protein have resulted in both viable and non-viable viruses (168, 222). We have further characterized the roles of the IE2 60 and IE2 40 proteins, and have shown that while

these proteins are non-essential for virus replication, they are important for the proper expression of many late genes and optimal virus production (223).

One obstacle with the above technique is that many mutations in essential genes lead to non-viable viruses, making identification and characterization of some domains impossible. Attempts to create complementing cell lines and other ways to circumvent this problem have been to a large degree unsuccessful. One reason this has proven to be a difficult task is that IE2 86 is deleterious to the cell when expressed constitutively (53). One fibroblast complementing cell line that was reported to successfully express IE2 86 was later shown to have mutations in important regions of the protein (16, 136). Further, our laboratory has tried multiple strategies to create a complementing cell line that will allow for analysis of IE2 86 during the infection (Deborah Spector, unpublished data). One key focus of this dissertation has been to develop a successful strategy for complementation, and is discussed in Chapter 2.

In understanding the functional roles of IE2 86, IE2 60, and IE2 40 it has been imperative to ascertain the function of the interaction of these proteins with the only other viral protein IE2 86 has been shown to interact with, UL84 (164, 191). UL84 is present at low levels during the beginning of the infection, and then accumulates throughout the infection, with peak protein production occurring at after the onset of viral DNA replication (84). This interaction has been proposed to downregulate the ability of IE2 86 to activate early promoters in transient assays (68). Work from our lab has determined that, along with IE2 86, IE2 60 and IE2 40 play a key role in regulating the expression of UL84 at late times post infection (223). Further, two groups have attempted to identify

the binding domains for IE2 86 and UL84, although there is conflicting data regarding the domains responsible for the interaction of these proteins (43, 68). These studies have led to one of the key focuses of this dissertation, that is understanding the role of IE2 86, IE2 60, and IE2 40 in regard to UL84 regulation.

Much remains to be understood regarding the role of UL84, along with its associated binding partners. One likely role for UL84 involves its role during HCMV DNA replication as it has been shown to be required for initiating viral lytic DNA replication in transient assays (143, 174, 233-234). It appears that an interaction with IE2 86 mediates the activation of the bidirectional promoter within *ori-lyt* (43, 233). It has also been demonstrated that the interaction between IE2 86 and UL84 downregulates the ability of IE2 86 to activate some early promoters in these assays (68). UL84 has been shown to interact with the stem-loop structure of the RNA/DNA hybrid region of *ori-lyt*, and has been suggested to have UTPase activity (42, 44). UL84 is a unique protein that has very little similarity (and no known homolog) to any other viral or cellular protein, and thus it is important to understand the complexities of the regulation governed by this protein, both alone, and in the context of an interaction with the IE2 proteins.

Goal of the Work

Given the need for increased understanding of the roles and functions of IE2 86, IE2 60, and IE2 40, the goals presented in this dissertation include elucidating the role of IE2 86 in the context of the infection as well as identifying minimal necessary cellular components mediating IE2 86 function. Further, given that very little was known about

the functions of the late IE2 60 and IE2 40 proteins, these studies have continued to identify both similar and novel functions as those identified for IE2 86.

Previous experimental limitations in understanding the functions of IE2 86, IE2 60, and IE2 40 led to the development of a novel approach to create a complementing cell line by which we are able to study the effects of creating mutations in essential regions of the IE2 proteins. This approach has broad applicability, and will be an asset to not only the field of HCMV research, but to any research that requires a tightly controlled inducible system for expression. This system utilizes a lenti-viral based vector encoding for IE2 86 or IE2 40 that can be induced upon expression of either Cre or FLP recombinases. Importantly this lenti-based system makes use of a bi-cistronic RNA, allowing expression of only the first ORF in the vector. The lenti-virus is transduced into cells and contains a selection marker for selecting drug-resistant cells. Following a DNA recombination event mediated by Cre or FLP, the selection marker is removed and expression of the IE2 proteins is observed.

This system has allowed extensive analysis of the contribution of the individual IE2 proteins, and has allowed characterization of both debilitated and non-viable IE2 mutant viruses. For instance, one mutant virus that is severely debilitated at late times post infection, called IE2 86ΔSX, was shown to be complemented to a great degree in regard to the spread and amount of the virus released from the cells. Expression of IE2 86 alone aided in the spread of the virus from cell to cell, while expression of both IE2 86 and IE2 40 together allowed for increased spread and release of the previously debilitated virus. In regard to viral protein expression during this infection, many late protein

expression patterns were similar to the WT virus when grown on the complementing cell lines expressing both IE2 86 and IE2 40. These studies aided in the understanding of the individual contributions of each of the IE2 proteins during the infection process, and allowed for ease of preparation of a previously highly debilitated virus, overcoming one major hurdle in studying these mutant viruses.

Further, these studies were the first to show a system that was able to complement a previously non-viable IE2 mutant virus. Expression of wt IE2 86 alone was able to complement a virus missing all of exon 5 of IE2 86 to some degree. Grown on this cell line, expression of immediate-early, early, and late proteins could be identified, although progression of the virus was slow when compared to wt virus. Growth and expression of all gene types was enhanced significantly on cells that were expressing both IE2 86 and IE2 40, again indicating that both proteins provide the optimal environment for growth and progression of the virus. The results of these data are described in Chapter 2.

Further analyses of the IE2 86ΔSX virus have allowed increased understanding of the role of IE2 86, as well as the contribution of IE2 60 and IE2 40 to the infection. Deletion of amino acids 136-290 of IE2 86 (which also deletes expression of IE2 60 and IE2 40) resulted in significant loss of both UL83 and UL84 protein expression. Both of these proteins have been shown to be key players in the infection, and understanding their regulation is important for identifying mechanisms utilized by the virus to allow for a productive infection. Interestingly, expression of UL84 was shown to be regulated by IE2 86, IE2 60, and IE2 40 in a post-transcriptional manner, which is a novel function for the IE2 proteins (Chapter 3). Post-transcriptional and translational mediated regulation is

a poorly understood area of HCMV research, and has intriguing possibilities as a novel type of regulation utilized by HCMV to govern a productive viral infection.

Mutant viruses with selective loss of IE2 60 and IE2 40 expression led to the discovery that IE2 60 and IE2 40 are able to interact individually with UL84, independent of IE2 86. Further we were able to show that IE2 40 is a major player in regard to UL84 protein expression (Chapters 2, 3 and 4). Analyses of each of these proteins outside the context of the infection indicate that the regulation and these interactions can occur outside of the viral infection, but cannot be recapitulated *in vitro* outside of cellular processes (Chapter 4). We were also able to determine that this aspect of regulation of UL84 protein expression during the viral infection is both proteasome and calpain independent, and that the IE2 proteins are governing UL84 protein expression down-stream of RNA export into the cytoplasm, as well as RNA loading onto polyribosomes. Although the entire mechanism of regulation was not discerned, much about this novel form of regulation has been identified. Given that this is the first instance of this type of regulation during HCMV infection, continued studies will undoubtedly reveal important aspects of the contribution of these proteins to the infection.

Final conclusions and implications drawn from these studies are discussed in the last chapter of this dissertation (Chapter 5). We have identified many novel types of regulation governed by the IE2 proteins, and have continued to elucidate the roles of each of these proteins to the viral infection.

CHAPTER 2

DEVELOPMENT OF CELL LINES THAT PROVIDE TIGHTLY CONTROLLED TEMPORAL TRANSLATION OF THE HUMAN CYTOMEGALOVIRUS IE2 PROTEINS FOR COMPLEMENTATION AND FUNCTIONAL ANALYSES OF GROWTH-IMPAIRED AND NON-VIABLE IE2 MUTANT VIRUSES

ABSTRACT

The human cytomegalovirus IE2 86 protein is essential for viral replication. Two other proteins, IE2 60 and IE2 40, which arise from the C-terminal half of IE2 86, are important for later stages of the infection. Functional analyses of IE2 86 in the context of the infection have utilized bacterial artificial chromosomes as vectors to generate mutant viruses. One limitation is that many mutations result in debilitated or non-viable viruses. Here, we describe a novel system that allows tightly controlled temporal expression of the IE2 proteins, and provides complementation of both growth-impaired and non-viable IE2 mutant viruses. The strategy involves creation of cell lines with separate lentiviruses expressing a bicistronic RNA with a selectable marker as the first ORF, and IE2 86, IE2 60, or IE2 40 as the second ORF. Induction of expression of the IE2 proteins occurs only following DNA recombination events mediated by Cre and FLP recombinases that delete the first ORF. HCMV encodes Cre and FLP, which are expressed at immediate-early (for IE2 86) and early-late times (for IE2 40 and IE2 60), respectively. We show that the presence of full-length IE2 86 alone provides some complementation for virus

production, but the correct temporal expression of IE2 86 and IE2 40 together has the most beneficial effect for early-late gene expression and synthesis of infectious virus. This approach for inducible protein translation can be used for complementation of other mutations as well as controlled expression of toxic cellular and microbial proteins.

INTRODUCTION

The diseases associated with HCMV infections have contributed to a major drive for understanding the regulatory pathways governing its replication and interactions with the host. The DNA genome is approximately 240 kbp and has the potential to encode more than 160 proteins. However, the function of most of these proteins in viral replication and pathogenesis remains unknown. HCMV gene expression is divided into three major phases - immediate-early (IE), early, and late (for review, see 132). The IE genes are transcribed immediately following viral entry and rely on host factors as well as input virion proteins for their expression. The IE proteins have been shown to be important for activating early gene promoters, inhibiting apoptosis, and countering host defenses. Some of the early gene products are directly involved in viral DNA synthesis while others function to create a cellular environment that is optimal for viral gene expression and DNA replication, either by modulating factors involved in the control of cellular DNA synthesis or by altering the host's immune response to the virus. Late genes, which are transcribed after the onset of viral DNA replication, primarily encode structural proteins that function in assembly and maturation of the virus.

A region of IE transcription that includes the two genetic units, IE1 and IE2, has been the focus of many studies (for review, see 127). The IE1 RNA consists of 4 exons; a single ORF (UL123) initiates in exon 2 and specifies a 72-kDa nuclear protein designated IE1 72. The IE2 gene product, IE2 86 (ORF UL122), is an 86-kDa protein that is encoded by an alternatively spliced RNA that contains the first 3 exons of IE1 and a different terminal exon. Two other proteins corresponding to the 3′ end of IE2 86, termed IE2 60

and IE2 40, arise later in the infection (95, 151, 158, 195). The IE2 60 protein has an initiator methionine (Met) at aa 170 of IE2 86, with the putative TATAA box for its mRNA upstream of UL122 exon 5. The IE2 40 protein initiates translation at Met 242 of IE2 86. This latter protein is expressed from a 1.5 kb RNA, and has a putative TATAA box just upstream of the IE2 60 translation initiation site. Studies from our laboratory and others have demonstrated with mutant viruses that IE1 72 is required at a low but not high MOI, while IE2 86 is essential (52, 67, 73, 85, 123, 131, 162, 168, 222, 238).

Multiple functions have been ascribed to IE2 86. It has been implicated in playing a role in cell cycle control, mutagenesis, countering apoptosis, and limiting expression of host innate antiviral gene products and proinflammatory cytokines (40, 117-118, 136, 141, 147, 179-180, 205, 207, 225, 227, 245). The function that has received the most attention, however, is its ability to transactivate a large number of heterologous promoters and HCMV early promoters in transient expression assays with regions spanning the length of the protein (between aa 1-98 and 170-579) appearing to be important (87, 110, 122, 151, 178, 188, 196, 214, 236). IE2 86 can also negatively regulate its own transcription by binding to a 14 bp cis-repression signal (CRS) between the TATAA box and RNA start site in the major IE promoter (MIEP) (38, 93, 110, 114, 121, 150). Other 14 bp sites of DNA binding are found upstream of the TATAA box in promoters for viral early genes including UL112-113, TRL7, and UL4 (8, 35, 93, 177-178). The region of IE2 86 involved in binding to DNA overlaps the transactivation domain in the carboxy-terminal half of the protein (aa 313-579).

Based on the very large number of proteins that IE2 86 has been found to bind to, it is suspected that protein-protein interactions likely underlie most of its functions. IE2 86 binds to itself, to the viral UL84 protein, and to a still expanding list of cellular proteins, including: TBP; TFIIB; TBP-associated factors (TAFs); pRb; p53; p21; mdm2; CHD-1; histone acetylases (HAT) CBP, p300, and P/CAF; histone deacetylase1 (HDAC1), HDAC2 and HDAC3; histone methyltransferases G9a and Suvar(3-9)H1; SUMO-1 and Ubc9: PIAS1; Sp1; Tef-1; c-Jun; JunB; ATF-2; NF-kB; Nil-2A; and Egr-1 (3, 16, 27, 32, 39, 41, 62, 64, 68, 77-78, 97, 108, 113, 118-119, 138, 144, 161, 176-178, 185, 188, 191-192, 219, 231-232, 237, 242). The ability of IE2 86 to interact with the majority of these proteins maps broadly to the region not shared with IE1 72, aa 86-542, although binding to the amino-terminal region of IE2 86 has been reported for p53 and the histone acetylase (HAT) domain of CBP/p300 (aa 737-1626) (2, 39, 64, 91, 188, 208). A major concern with the above findings, however, is that most of the interactions were observed either in cells where the proteins were overexpressed or in *in vitro* binding assays.

To date, there has only been limited evidence that IE2 86 has any of these functions in the context of the infection. Elucidating the role of IE2 86 in viral replication and pathogenesis and understanding its mechanism of action requires that mutants be analyzed in the context of the viral infection. One obstacle to accomplishing this goal was the difficulty of generating HCMV recombinants. The advent of bacterial artificial chromosomes (BACs) as vectors for cloning herpesvirus genomes, however, has largely solved this problem (1, 18, 80, 135). The great advantage is that any mutation is easily

introduced into the viral genome, and the mutagenized genome is physically characterized as a BAC. The mutated BAC, along with a plasmid encoding HCMV UL82, is electroporated into cells (9), generating mutant viruses that are free of the wild-type (wt) virus.

With the above strategy, several growth-impaired viruses with mutations in IE2 86 have been studied. For example, using the AD169 BAC, we constructed a recombinant virus with a mutant IE2 86 gene that has an internal deletion of aa 136 to 290 (IE2 86ΔSX) (168). The deletion also removes the promoter and initiator Met for IE2 40 (aa 242) and the initiator Met for IE2 60 (aa 170), and thus these late gene products are not expressed. When cells are infected at a low MOI, there is a marked delay in the production of virus and slower cell-to-cell spread as compared to a wt infection. By immunofluorescence assay (IFA) and Western blot analysis, it appeared that the expression of viral early genes was comparable to that of the wt virus. However, in mutant infected cells, there was no longer a block in the expression of cellular HMGA2 and cyclin A (183) and viral mediated transcriptional induction of cellular antiviral gene products and proinflammatory cytokines was not suppressed (207). The most notable molecular defects were observed at late times. The levels of the IE2 protein, pp65 (UL83) matrix protein, and UL84 protein were greatly reduced, but surprisingly, only in the case of UL83 was there a corresponding decrease in the mRNA. Subsequently, we showed that the loss of the IE2 60 and IE2 40 early-late proteins played a major role in the observed effects at late times (223).

Another viable mutant virus is IEΔ30-77, which lacks the majority of IE exon 3 (aa 30-77) and thus expresses smaller forms of both IE1 72 and IE2 86 (224). This virus is growth impaired at both high and low MOI and exhibits a kinetic defect that is not rescued by growth in cells expressing IE1 72. The kinetics of mutant IE2 protein accumulation in IE Δ30-77 infected cells are comparable to that in wt virus-infected cells, but the mutant shows delayed expression of early viral genes and only low levels of IE1 72. Its capacity to upregulate the expression of cellular cyclin E has also been reduced. Interestingly, a mutation in exon 5 of IE2 that substitutes arginine for glutamine at aa 548 also alters the inhibitory effects of the virus on cellular DNA synthesis and the cell cycle, but the MIE promoter is still autoregulated and viral early genes are activated (147).

There are many examples of mutations in IE2 86 that do not yield virus when the mutant BACs are electroporated into fibroblasts, including: deletion of the entire exon 5; deletion of aa 356-359, aa 427-435, or aa 505-511; substitution of alanine for arginine at aa 356, 357, and 359; substitution of alanine for the proline at aa 535 and the tyrosine at aa 537; and substitution of alanine for the histidines at aa 446 and aa 452 (146, 222). Studies on these recombinants have been limited to the characterization of viral protein and mRNA expression following electroporation of the BACs into fibroblasts and have required immunostaining with antibodies to representative IE, early, and late proteins and quantitative real-time RT-PCR to determine the block in the replication cycle. Most of the mutant viruses do not activate early genes and are defective in repression of the major IE promoter. Surprisingly, in cells electroporated with two of the mutant IE2 86 BACs that have a deletion of aa 427-435 or aa 505-511, there is significant induction of selected

delayed early (UL89) and late genes at early times in the infection, suggesting that IE2 86 may play a role in inhibiting some delayed early or late promoters at early times in the infection.

Although the above studies on cells electroporated with BACs have provided some insight into the functions of IE2 86, an understanding of the molecular mechanisms governing its functions throughout the infection requires the generation of recombinant viruses. To this end, our laboratory and others have attempted to create cell lines that can complement some of these defects and will allow for the production of infectious virus. This has proven to be a very challenging endeavor, as constitutive expression of wt IE2 86 appears to be deleterious to the cell (53). The one fibroblast cell line that was reported to express IE2 86 (16) was later found to have mutations in critical regions of the protein (136). Over the years, our laboratory has tried to generate complementing cell lines with multiple inducible eukaryotic gene expression systems, with no success.

Here, we describe a system that allows complementation of IE2 86 mutant viruses. With a lentiviral based vector encoding IE2 86, we have devised a strategy in which expression of Cre recombinase allows specific induction of IE2 86 following a DNA recombination event during the IE stages of the infection. In a similar manner, we have created cell lines with lentiviral transductions that can be induced to translate IE2 60 and IE2 40 during the early-late stages of the infection following exogenous expression of FLP recombinase. This system allows appropriate temporal translation and expression of the IE2 proteins. Finally, we have constructed a cell line that can be induced to express both IE2 86 and IE2 40 proteins with appropriate kinetics during the infection. By

placing the genes encoding Cre and FLP in the mutant virus under the control of a viral IE and early-late gene promoter, respectively, the temporal induction of the IE2 86 and IE2 40 proteins occurs only in cells that have been infected with the virus.

We have utilized this system to complement the growth of the previously characterized and debilitated IE2 86ΔSX virus, as well as a non-viable virus that is lacking the entire region encoding exon 5 of IE2 86 (IE2 86ΔExon5 C-F). Many of the altered expression patterns of the IE2 86ΔSX virus were restored to varying degrees as compared to the wt phenotypes when assessed on these cell lines, especially with respect to late gene expression. A greater level of complementation was seen on the cell line that could be induced to express both IE2 86 and IE2 40. Notably, electroporation of the non-viable IE2 86ΔExon5 C-F BAC into cell lines that can be induced to express IE2 86 alone or both IE2 86 and IE2 40 leads to the production of infectious virus. On both cell lines, this virus exhibits delayed kinetics and grows to reduced titers relative to wt. Plaque size and spread is slightly impaired, but less so on the cell line that expresses both IE2 86 and IE2 40, indicating that both proteins are necessary for wt viral growth.

MATERIALS AND METHODS

Molecular cloning of lentiviral vectors.

The lentiviral vectors pLV:CMV-*Bam*HI-*Swa*I and p156RRLsinPPThEF1-αGFPPRE were kind gifts from Dr. Inder Verma. The pLV:CMV-*Bam*HI-*Swa*I vector utilizes the HCMV IE promoter as its internal promoter followed by unique *Bam*HI and *Swa*I cloning sites. The p156RRLsinPPThEF1-αGFPPRE vector utilizes the human elongation factor 1-alpha (EF1-α) promoter driving the expression of green fluorescent protein (GFP). The other relevant features of these vectors have been described elsewhere (148). The original pLV:CMV-*Bam*HI-*Swa*I was modified prior to using it to construct our IE2-expressing lentivectors. The vector was first digested with *Sma*I which removed a 35-bp G-C rich region, as well as the unique *Swa*I cloning site downstream of the HCMV IE promoter. The vector was then re-ligated to form pLV:CMV-*Bam*HI-*Sma*I. The unique cloning sites downstream of the promoter were now *Bam*HI and *Sma*I. Because of HCMV's ability to down-regulate expression from the IE promoter, we replaced it with the human EF1-α promoter. The HCMV IE promoter was removed by digestion with *Hpa*I and *Bam*HI and replaced with a 1.4-kb *Hpa*I-*Bam*HI fragment containing the human EF1-α promoter that had been removed from the p156RRLsinPPThEF1-αGFPPRE vector. The modified vector was referred to as pLV:EF1-α-*Bam*HI-*Sma*I.

The Towne strain 1.7-kb IE2 86 cDNA used in our vectors was originally constructed by and obtained from R. Stenberg. This cDNA was found to contain several mutations (136). We partially corrected the cDNA by replacing the *Sma*I-*Stu*I fragment

(corresponding to aa 136-543) with the corresponding fragment from pHCMV *Eco*RI J (203), which is from strain AD169. The corrected cDNA, a hybrid containing both Towne and AD169 sequences, was then subcloned into the vector pcDNA3 (Invitrogen) to form pcDNA3:IE2 86. To be able to isolate the IE2 86 cDNA for cloning into the lentiviral vector, *Swa*I sites were introduced both upstream and downstream of the cDNA in pcDNA3:IE2 86 simultaneously using the Quikchange Multi-site Directed Mutagenesis Kit (Stratagene). Finally, an in frame FLAG tag (D-Y-K-D-D-D-D-K) was introduced at the C-terminus of and in frame with the IE2 86 cDNA by Quikchange mutagenesis. The resulting vector was called pcDNA3:IE2 86-FLAG.

Both the 1.2-kb IE2 60 and 1-kb IE2 40 cDNAs were PCR amplified from pcDNA3:IE2 86. The sense primers used in both PCRs were designed to introduce *Swa*I sites just upstream of the AUG for both cDNAs. The antisense primers used for both reactions introduced a 9 aa HA tag (Y-D-V-P-D-Y-A-S-L) in frame with the carboxy terminus of each cDNA, followed by another *Swa*I site. Both PCR products were then cleaved with *SwaI* and cloned as *Swa*I fragments into pcDNA3, forming pcDNA3:IE2 60-HA and pcDNA3:IE2 40-HA, respectively. Finally, the 5' ends of pcDNA3:IE2 86-FLAG, pcDNA3:IE2 60-HA, and pcDNA3:IE2 40-HA were modified by Quikchange mutagenesis (Stratagene) to introduce 51-bp of IE sequence between the upstream *Swa*I site and the initiator Met in all 3 vectors. The 51-bp is comprised of 34-bp of the 3' end of IE exon 1 (nt 173610-173641) plus 17-bp at the 5' end of IE exon 2 just upstream of the initiator Met located in exon 2 (nt 172766-172782). As a result of this insertion, the upstream untranslated regions for each cloned cDNA are identical to what they would be

in the HCMV mRNA, putting the AUG for each in a more accurate context for translation. All 3 vectors were tested for expression by transfection into cos-7 cells. The cos-7 cell lysates were analyzed by Western blots using antibodies to FLAG or HA. The size and expression of each of the 3 tagged cDNAs were as expected (data not shown). All 3 tagged cDNAs were then removed from their vectors by digestion with *Swa*I and subcloned into the *Sma*I site of the lentiviral vector pLV:EF1-α-*Sma*I to form pLV:EF1-α-*Bam*HI-IE2 86-FLAG, pLV:EF1-α-*Bam*HI-IE2 60-HA, and pLV:EF1-α-*Bam*HI-IE2 40-HA, respectively.

Several shuttle vectors were constructed in order to introduce selectable marker genes between loxP or FRT recombination sites into our lentiviral vectors. The plasmid phr:*Swa*I-loxP/GFP/loxP-*Swa*I was made using successive Quikchange site directed mutagenesis reactions. The first introduced a *Swa*I and a 34-bp loxP site just downstream of the HCMV IE promoter in the vector phrGFP (Stratagene). A second loxP site adjacent to a second *Swa*I was then introduced 89-bp downstream of the GFP stop codon in a subsequent reaction. A third Quikchange reaction was used to introduce a *Kpn*I site between the upstream loxP site and the initiation codon for GFP. A *Kpn*I digest was then used to remove the GFP ORF, and the plasmid was re-ligated to form phr:*Swa*I-loxP/*Kpn*I/loxP-*Swa*I. Next, this vector was digested with *Swa*I and the insert ligated to *Bam*HI linkers. Following digestion with *Bam*HI, the cassette was subcloned into the *Bam*HI site of pGEM-1 (Promega) to form pGEM:loxP-*Kpn*I-loxP. The Neomycin (Neo) resistance marker, without its promoter, was PCR amplified from pcDNA3. *Kpn*I recognition sites were designed into the 5' end of each primer. The PCR

product was then digested with *Kpn*I and subcloned into pGEM:loxP-*Kpn*I-loxP to form pGEM:loxP-Neo-loxP.

Two successive Quikchange reactions were used to replace the upstream and downstream loxP sites in the vector pGEM:loxP-*Kpn*I-LoxP with 48-bp FRT sites. The resulting plasmid was designated as pGEM:FRT-*Kpn*I-FRT. The puromycin (PURO) ORF was then PCR amplified from the vector pPUR (Clontech). Again, *Kpn*I recognition sites were designed into the 5' ends of each primer. Following a *Kpn*I digest of the PCR product, the PURO ORF was subcloned into pGEM:FRT-*Kpn*I-FRT to form pGEM:FRT-PURO-FRT.

The final step in the construction of the lentiviral vectors was the addition of the marker genes. The loxP-Neo-loxP cassette was isolated from pGEM:loxP-Neo-loxP by digestion with *Bam*HI and subcloned into the *Bam*HI site of pLV:EF1-α-*Bam*HI-IE2 86-FLAG to form pLV:EF1-α-loxP-Neo-loxP-IE2 86-FLAG. The FRT-PURO-FRT *Bam*HI cassette was then isolated from pGEM:FRT-PURO-FRT and subcloned into both pLV:EF1-α-*Bam*HI-IE2 60-HA and pLV:EF1-α-*Bam*HI-IE2 40-HA to form pLV:EF1-α-FRT-PURO-FRT-IE2 60-HA and pLV:EF1-α-FRT-PURO-FRT-IE2 40-HA, respectively.

IE2 cell line creation via lentiviral transduction.

All three IE2 lentiviral vectors were packaged using the reagents and protocol of the ViraPower Lentiviral Expression System (Invitrogen). Briefly, 3µg of lentiviral vector was co-transfected with 9µg of the ViraPower Packaging mix into actively growing 293FT cells using the Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM

media (GIBCO). The ViraPower Packaging mix consists of 3 plasmids: pLP1, which provides gag/pol function as well as the RRE; pLP2, which provides rev function; and pLP/VSVG, which provides the VSV-G glycoprotein. The media was changed at 24 h post transfection and the lentivirus containing supernatants was harvested at 48 and 72 h p.i. Fresh supernatants were used to transduce low-passage human foreskin fibroblasts (HFFs) for two consecutive days. Prior to use, each supernatant was clarified by low-speed centrifugation and filtered through 0.45μM PVDF filters (Millipore). Transduction of HFFs was done in the presence of 3μg/ml polybrene (Sigma). Individual supernatants, representing each of the 3 lentiviral vectors were used to make the FS2:86F, FS2:IE2 60HA and FS2:IE2 40HA cell lines. HFFs were co-transduced with supernatants prepared from the pLV:EF1-α-loxP-Neo-loxP-IE2 86-FLAG and pLV:EF1-α-FRT-PURO-FRT-IE2 40-HA vectors to make the FS2:86F/40HA double cell line.

The transduced HFFs were passed 1:2 48 h after the second transduction, and put under selection with either G418 (400μg/ml), PURO (1μg/ml), or both, the next day. The resulting polyclonal populations of resistant cells were frozen down and used in the experiments described in the results section. Each of the integrated coding regions was analyzed by sequencing following transduction to ensure that there were no major mutations or deletions in the expressed proteins. The cell lines were tested for expression by infection with HCMV viruses that expressed both Cre and FLP (see below), or those that did not. Induction of IE2 86-FLAG, IE2 60-HA, and IE2 40-HA protein expression was determined by both IFA and Western blot analysis. For the purposes of this paper,

these cell lines are referred to as 86F, 60HA, 40HA and 86F/40HA. The proteins expressed from these cell lines are referred to as IE2 86F, IE2 60HA, and IE2 40HA.

Construction of recombinant BACs and reconstitution of recombinant viruses.

All recombinant BACs were prepared from the wild-type HCMV strain AD169 BAC (pHB5), obtained from M. Messerle (18). The construction of the IE2 86 deletion mutant BAC IE2ΔSX BAC has been previously described (168). Both BACs were introduced by electroporation into the recombination strain DY380 (a kind gift from Dr. William J. Britt used with permission from D. Court). This strain harbors a defective lambda prophage that provides the recombination genes *exo*, *bet*, and *gam* under the control of a temperature-sensitive α cI-repressor.

In order to facilitate the recombination of the IE-Cre/kan/1.2kb-FLP cassette into both of the above BACs between the viral genes US11 and US12, a plasmid was first constructed to serve as a PCR template. To begin, a 4.2-kb *Pml*I fragment was subcloned from pHCMV *Eco*RI B (203) into the *Eco*RV site of the cloning vector pACYC184 to form pACYC:US11-US14. Quikchange mutagenesis was then used to introduce *Spe*I and *Bgl*II sites just downstream of a unique *Xba*I site already present within the intergenic region between US11 and US12. The Cre ORF was removed from the vector pTurbo-Cre and subcloned into the vector pFastBac1 (Invitrogen), just downstream of the HCMV IE promoter, resulting in the vector pFB1:IE-Cre. The entire Cre cassette, including the promoter, was then subcloned into the *Xba*I site of pACYC:US11-US14, forming the vector pACYC:US11-IE-Cre-US14. Next, the Kanamycin (kan) resistance marker with its promoter was PCR amplified from the vector pACYC177 and subcloned

into the *Spe*I of pACYC:US11-IE-Cre-US14, yielding the vector pACYC:US11-IE-Cre/kan-US14. Finally, the 1.2-kb early-late RNA promoter was removed from the vector p456-OCAT (212) and subcloned into pFastBac1, creating the vector pFB1:1.2. The FLP ORF was then removed from the plasmid pOG44 (Invitrogen) and subcloned just downstream of the 1.2-kb promoter. The resulting plasmid was referred to as pFB1:1.2-FLP. The entire 1.2-FLP cassette was then subcloned into the *Bgl*II site of pACYC:US11-IE-Cre/kan-US14, creating the final vector pACYC:US11-IE-Cre/kan/1.2-FLP-US14.

A pair of 21-mer primers, US11 E-T (nt 199869-199889 of the AD169 virus) and US12 E-T (nt 200878-200898) were used to amplify a 6.4-kb linear cassette from pACYC:US11-IE-Cre/kan/1.2-FLP-US14 which included the IE-Cre, kan, and 1.2-FLP genes flanked by approximately 500-bp homology arms to US11 and US12. This linear fragment was used for recombination into the HB5 and IE2 86ΔSX BACs in the DY380 cells as described previously (111), and BACs were selected on the basis of kanamycin and chloramphenicol resistance. The recombinant BACs were analyzed extensively by restriction endonuclease digestion and field inversion gel electrophoresis (FIGE). BACs showing the expected restriction pattern and no other deletions or rearrangements were transformed into DH10B cells and BAC DNAs were amplified and purified as previously described (168). These preps were also analyzed by restriction endonuclease digestion and FIGE to confirm that they were correct (data not shown).

The IE2 86ΔExon5 Cre/FLP virus was prepared using the HB5 Cre/FLP BAC. First, the ampicillin (amp) gene was amplified by PCR from the vector pcDNA3. This

cassette contained the promoter, downstream sequences necessary for expression of the amp gene, and homology arms to the desired recombination site within the IE2 86 coding region (outside of exon 5) in the HB5 Cre/FLP BAC. This recombination was facilitated in DY380 cells and selected on the basis of resistance to chloramphenicol, kanamycin, and ampicillin. Resistant clones were prepared as described earlier and screened extensively by restriction endonuclease digestion and FIGE.

Reconstitution of the HB5 Cre/FLP, IE2 86ΔSX Cre/FLP, IE2 86ΔExon5 Cre/FLP viruses was done by electroporating 6.25µg of the appropriate BAC along with 3.75µg of pCDNA:pp71-tag into HFFs or 86F/40HA cells using a BTX630 generator set at 300V/ 75 Ohms/ 2500µF as previously described (222, 224). Viral supernatants were harvested when the cultures reached 100% cytopathic effect (CPE) and titered by plaque assay and expression of IE/early proteins by IFA. In the paper, the HB5 Cre/FLP, IE2 86ΔSX Cre/FLP, and IE2 86ΔExon5 Cre/FLP viruses are referred to as HB5 C-F, IE2 86ΔSX C-F, and IE2 86ΔExon5 C-F, respectively.

A rescued virus was derived from the IE2 86ΔExon5-Cre/FLP BAC by homologous recombination in HFFs between the mutant BAC with pHCMV EcoRI J (203), which contains the MIE region of HCMV strain AD169, followed by the outgrowth of replication competent virus. To generate the rescued virus, HFFs were electroporated with IE2 86ΔExon5-Cre/FLP BAC DNA (6.25 µg), EcoRI-digested pHCMV EcoRI J DNA (5 µg), and pCDNApp71tag (3.75 µg) as previously described (222). Supernatant was harvested from the infected cells and a limiting dilution assay was set up. The virus clones from wells that contained a single plaque were further

propagated. HCMV-infected cell DNA was isolated (QIAmp DNA Blood Mini Kit, Qiagen) from viral clones and the IE2 exon 5 region was analyzed by PCR and DNA sequencing to verify that the wt IE2 exon 5 sequence had replaced the Amp^R cassette in the mutant. Also, the resulting BAC was obtained from the infected cells using a modified HIRT protocol, retransformed into bacteria, and assessed a second time to make sure that no large deletions or rearrangements had occurred during the growth of virus in HFFs.

Cell Culture and Infections

HFFs were obtained from the University of California, San Diego Medical Center and cultured in Earle's minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1.5 μg/ml amphotericin B (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37°C at 7% CO_2 and maintained at confluence for 3 days before infection. HFFs or the cell lines described above were infected at the MOI indicated. Mock-infected cells were treated with media and DMSO as a control. At various times p.i., cells were washed with phosphate-buffered saline (PBS), trypsinized, and processed for the appropriate assay. 86F cells were kept under constant selection with G418 (Invitrogen) until infection, while the 60HA and 40HA were kept under selection with PURO (Mediatech). The 86F/40HA cells were kept under selection with both G418 and PURO.

Growth of the viruses was assessed by infecting cells at an approximate MOI of 0.05-0.1 pfu/cell and then allowing cells to become confluent, or by following the spread

of the virus after electroporation. The virus was allowed to spread until all cells in the flask showed CPE. At this time, supernatants were harvested and titered by both plaque assay and IFA for both IE and early protein expression.

Western Blotting

Infected or mock-infected cells were harvested at various times p.i. Cells were lysed and processed for Western blot analyses as previously described (223). The following antibodies and dilutions were used: CH16.0 (1:10,000), UL84 (1:5,000-10,000), monoclonal IE2 86 mAB8140 (1:1000), UL57 mAB (1:5,000), UL99 mAB (1:10,000), UL83 mAB (1:10,000), UL44 mAB (1:10,000), β-actin mAB Ac-15 (1:5,000-1:10,000), Flag mAB (1:2,500), HA mAB (1:100), IE1 72 mAB (1:2000-10,000) and horseradish peroxidase (HRP)-coupled anti-mouse antibody (1:2,500-1:10,000). CH16.0, anti-UL99, anti-UL83, anti-UL44 and anti-UL57 were purchased from the Goodwin Institute and Virusys. Anti-UL84 antibodies were kind gifts from G. Pari and E.S. Huang. Anti-IE2 was purchased from Chemicon, while anti-β-actin and anti-Flag were purchased from Sigma-Aldrich. Anti-HA was from Santa Cruz Biotechnology. Anti-IE1 72 was obtained from Virusys and B. Britt (University of Alabama, Birmingham). HRP-coupled anti-mouse antibody was obtained from Calbiochem. After incubation and washes, proteins were detected with SuperSignal chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

Immunofluorescence

Cells were seeded onto coverslips and fixed at various times p.i. using 2% paraformaldehyde and then processed as previously described (168). Coverslips were

then stained with the following primary antibodies: CH16.0 (1:1,000), IE1 72 mAB (1:50-1:500), UL44 mAB (1:1,000), UL83 mAB (1:1000), Flag mAB (1:250), and HA mAB (1:50). All suppliers and manufacturers are described above. After 3 washes with PBS, cells were stained with a secondary antibody (FITC or TRITC, 1:1000 or 1:100 respectively, and Hoechst, 1:500). Pictures were taken with a Photometrics CoolSNAP camera mounted on a Nikon Eclipse E800 Microscope (20X and 40X objective).

Quantitative real-time PCR and RT-PCR analyses.

For the quantitative real-time PCR analyses, an aliquot of cells used for the Western blot analysis or separately infected cells (for the second experiment) were harvested at 24 h p.i., and then DNA was prepared with the Qiagen DNA Blood Mini kit and analyzed for input viral DNA as previously described (224). For the quantitative real-time reverse transcription PCR (RT-PCR) analyses, cells were harvested and RNA was prepared using the Qiagen RNA extraction kit as recommended by manufacturer. RNA samples were then rigorously treated with DNase and diluted to 12.5 ng/μl. A portion of the RNA sample was treated with RNase as a negative control for amplification. Real-time RT-PCR and analysis were performed as previously described (223) using primers and probes directed against the HCMV IE1 72 gene, and the cellular housekeeping gene glucose-6-phosphate dehydrogenase (G6PD).

RESULTS

Generation of IE2 86-Flag, IE2 60-HA, and IE2 40-HA Cell Lines and Construction of HB5 C-F, IE2 86ΔSX C-F, and IE2 86ΔExon5 C-F Recombinant BACs

Many attempts to create cell lines that can complement the growth of non-viable and debilitated viruses with mutations in IE2 86 have led to the method presented here. The strategy that was successful utilized lentiviral vectors to create cell lines that can be induced to undergo a DNA recombination event, allowing proper temporal translation of the IE2 proteins. This approach was based on the principle that only the first open reading frame (ORF) on a bicistronic eukaryotic mRNA is translated. Thus, by placing the ORF for the IE2 protein as the second ORF on the lentiviral mRNA, it should not be translated until the first ORF was removed.

One lentiviral vector, termed LV/EF1a:loxP-Neo-loxP:IE2 86-FLAG, was constructed such that the ORF for a protein conferring Neo resistance was flanked by loxP sites and placed just upstream of the AUG for IE2 86 (Figure 2.1A). A FLAG tag was placed at the C-terminus of the IE2 86 gene in order to discriminate between the IE2 86 provided by the cell line versus the IE2 86 provided by the virus. The EF1-α promoter was used to drive expression of the lentiviral mRNA (Figure 2.1A) because we had found that it was not affected by the HCMV infection (unpublished results). Low passage HFFs were transduced with the lentivirus, and the cells were selected for drug resistance. These cells were designated 86F. Since the lentiviral mRNA produced is bicistronic, only the AUG for the Neo ORF could be translated. The expression of Cre recombinase then could be used to initiate recombination events through loxP sites,

resulting in the deletion of the Neo ORF. Following the recombination, the AUG for the IE2 gene became the first ORF, allowing translation of the protein from the lentiviral mRNA.

Similarly, cell lines were constructed to express the IE2 60 and IE2 40 early-late proteins. Construction of these cell lines was similar to that of the 86F cell line, except that the resistance marker used was PURO, and the tag placed at the C-terminus of the IE2 proteins was HA instead of FLAG (Figure 2.1A). In this case, the PURO ORF was flanked with FRT sites, and placed just upstream of the AUG for the early-late IE2 60 and IE2 40 proteins. These vectors were termed LV/EF1a:FRT-PURO-FRT:IE2 40 (or 60)-HA (Figure 2.1A). Low passage HFFs were transduced with the either the IE2 40HA or IE2 60HA lentivirus, the cells were selected for resistance to PURO, and these cells were designated 40HA and 60HA, respectively. The expression of FLP recombinase then could be used to initiate recombination events through the FRT sites, resulting in the deletion of the PURO ORF and translation of the IE2 60 or IE2 40 protein from the lentiviral mRNA.

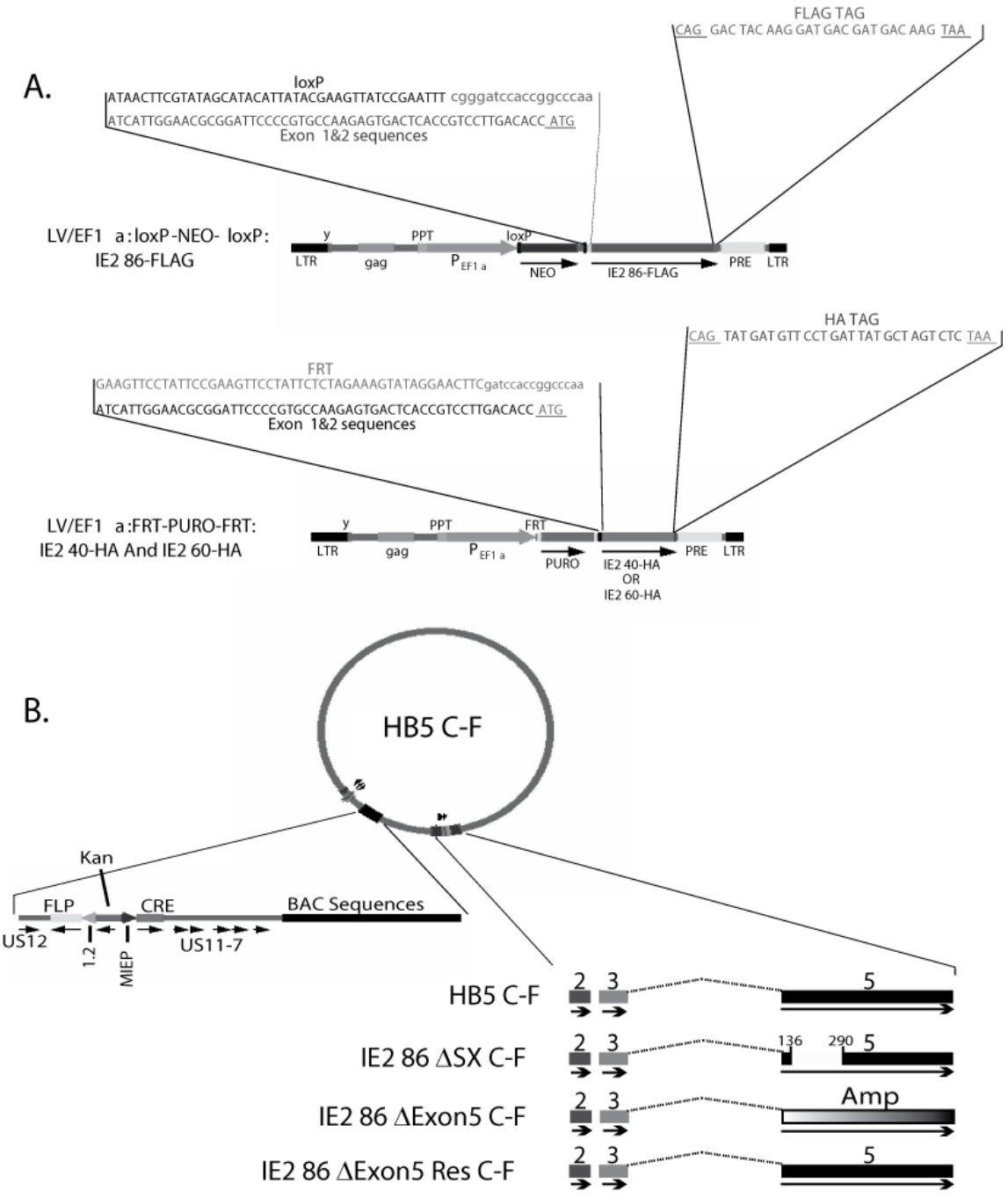
A cell line that could be induced to express both IE2 86F and IE2 40HA was also constructed by co-transducing HFFs with the two lentiviruses encoding these proteins and selecting for cells that were resistant to both drugs. This cell line was designated 86F/40HA.

Since prolonged expression of IE2 86 is toxic for the cell and propagation of the recombinant virus would require multiple rounds of replication following electroporation of the BAC into the cells, it was necessary to devise a means for induction of the IE2

proteins only when the cell became infected. We also wanted to maintain the normal temporal pattern of expression of the IE2 proteins, with IE2 86 expressed at IE times and the IE2 60 and IE2 40 proteins expressed at early-late times. This was accomplished by placing the Cre coding sequence under the direction of the HCMV major IE promoter between the US12 and US11 ORFs in BACs that encoded wt IE2 86 or mutant IE2 86 (Figure 2.1B). Thus, after the electroporation of the BAC into the appropriate cell line, the Cre expressed from the IE promoter would allow the deletion of the first ORF from the lentivirus and expression of IE2 86. This process would be repeated each time the virus infected a new cell. In order for the IE2 60 and IE2 40 proteins to be expressed later in the infection, we placed the FLP recombinase under the control of the viral 1.2 kb promoter (212), which is not expressed until the early-late stages of the infection. This construct was placed in the BACs adjacent to the cassette containing the Cre driven by the HCMV IE promoter. This BAC, as well as the virus propagated from this BAC, is referred to as HB5 C-F.

Figure 2.1B shows the recombinant BACs (expressing Cre and FLP) that were used in the studies reported here. All recombinant BACs were prepared from the wt HCMV strain AD169 BAC (pHB5). One mutant recombinant, termed IE2 86ΔSX C-F, was derived from the previously described IE2 86ΔSX BAC that contains an in-frame deletion of amino acids 136-290 of IE2 86 (168). The second mutant is a newly constructed BAC that is missing all of exon 5 of the IE2 86 coding region, termed IE2 86ΔExon5 C-F.

Figure 2.1. IE2 cell lines and recombinant BACs. (A) Lentiviral vectors used to express IE2 86F, IE2 60HA or IE2 40HA are shown. Sequences of one loxP site, the FLAG tag, and a portion of the IE2 coding region are displayed in the first vector. Coding regions are indicated as capital leters, and non-coding regions are represented in lower-case letters. The initiator Met for IE2 86 (underlined) is shown immediately after the Exon 1 and 2 sequences. The end of the IE2 coding sequences are also underlined, as well as the stop codons following the FLAG and HA tags. The lentiviral vectors used to create the 60HA and 40HA cell lines have FRT sites that are targeted by the FLP recombinase. The IE2 60 and IE2 40 proteins are tagged with HA. Sequences of one FRT site, the HA tag, and a portion of the IE2 coding region are shown in the second vector. Important regions within the vectors are noted, including the long terminal repeats (LTR), the *gag* gene, the promoter used to drive expression of the selectable marker (P_{EF1a}), and the gene to be expressed (IE2 86-Flag, IE2 40-HA, or IE2 60-HA). (B) Schematics of the BACs used to create the HB5 C-F, IE2 86ΔSX C-F, IE2 86ΔExon5 C-F, and IE2 86ΔExon5 Res C-F viruses expressing Cre and FLP recombinases are shown. The Cre and FLP coding regions, the 1.2kb (1.2) and MIE promoters (MIEP), the selectable marker (Kan), and the HCMV ORFs in this region of the BAC (US12 and US11-7) are noted. The region of the viral IE2 gene that is present in HB5 C-F, IE2 86ΔSX C-F, IE2 86ΔExon5 C-F, and IE2 86ΔExon5 Res C-F are illustrated. The IE2 86 coding regions in exons 2, 3, and 5 and the deletions within exon 5 are indicated. In the case of the IE2 86ΔExon5 C-F BAC, the entire exon 5 coding region has been replaced by an Ampicillin resistance gene (Amp).


A.
loxP
ATAACTTCGTATAGCATACATTATACGAAGTTATCCGAATTT cgggatccaccggcccaa
ATCATTGGAACGCGGATTCCCCGTGCCAAGAGTGACTCACCGTCCTTGACACC ATG
Exon 1&2 sequences
FLAG TAG
CAG GAC TAC AAG GAT GAC GAT GAC AAG TAA
LV/EF1 a:loxP-NEO- loxP:
IE2 86-FLAG
y
PPT
loxP
LTR
gag
P EF1 a
NEO
IE2 86-FLAG
PRE
LTR
FRT
GAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCgatccaccggcccaa
ATCATTGGAACGCGGATTCCCCGTGCCAAGAGTGACTCACCGTCCTTGACACC ATG
Exon 1&2 sequences
HA TAG
CAG TAT GAT GTT CCT GAT TAT GCT AGT CTC TAA
LV/EF1 a:FRT-PURO-FRT:
IE2 40-HA And IE2 60-HA
y
PPT
FRT
LTR
gag
P EF1 a
PURO
IE2 40-HA
OR
IE2 60-HA
PRE
LTR
B.
HB5 C-F
Kan
FLP
CRE
BAC Sequences
US12
1.2
MIEP
US11-7
HB5 C-F
2 3 5
IE2 86 ΔSX C-F
2 3 136 290 5
IE2 86 ΔExon5 C-F
2 3 Amp
IE2 86 ΔExon5 Res C-F
2 3 5

The IE2 86ΔExon5 C-F BAC contains an ampicillin resistance marker (Amp) in place of exon 5 of IE2 86. A rescued BAC was derived for the IE2 86ΔExon5 C-F BAC to ensure that all phenotypes observed in the mutant were due to the loss of exon 5, and not due to any extraneous mutations throughout the rest of the BAC coding sequence. This virus was termed IE2 86ΔExon5 Res C-F. Rescued forms of the wt HB5 C-F and IE2 86ΔSX C-F viruses were not constructed, since infection with these viruses minus the Cre/FLP has already been described (168), and as described below, there were no notable differences in the replication of the Cre/FLP versions of these viruses. All of the recombinant BACs were analyzed by digestion with restriction endonucleases and FIGE to ensure that no major rearrangements of the genome had occurred during mutagenesis (data not shown). The BACs were reconstituted as viruses following electroporation into HFFs or the designated cell line.

Growth of the wt HB5 Virus is not Affected by the Presence of the Cre/FLP Cassette

To ensure that the addition of the Cre/FLP cassette did not have any deleterious effects on the viral life cycle or virus production, HFFs were infected with either the HB5 virus or the HB5 C-F virus at a MOI of 1 pfu/cell. At various times p.i., cells were harvested and protein expression was analyzed by Western blot (Figure 2.2). IE gene expression was detected with both the CH16.0 antibody, which is directed against a sequence common to IE1 72 and IE2 86, as well as the mIE2 86 antibody, which is specific for IE2 86, IE2 60, and IE2 40. The levels of IE1 72, IE2 86, IE2 60, and IE2 40 were comparable at all time points in cells infected with the two viruses. Similarly, the two viruses showed no differences in early, early-late, and late gene expression, as

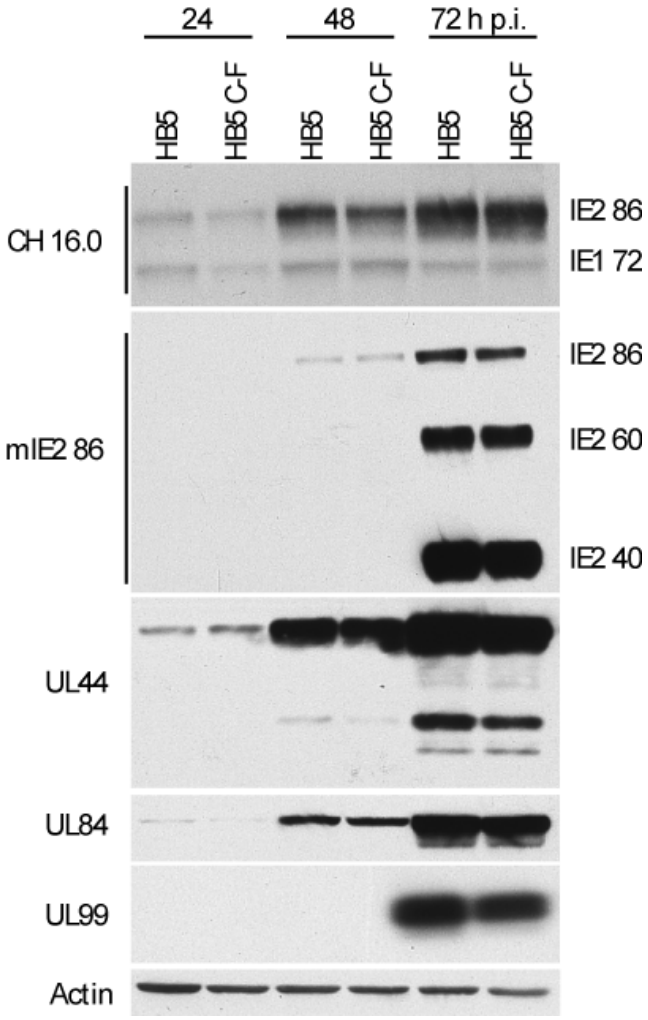


Figure 2.2. Comparison of HB5 and HB5 C-F growth in HFFs following infection. HFFs were infected at an MOI of 1 pfu/cell with either the wt virus without the Cre/FLP cassette (HB5), or the wt virus with the Cre/FLP cassette (HB5 C-F), and then harvested at 24, 48, and 72 h p.i. to assess viral protein expression. Protein from an equal number of cells was loaded in each lane and separated by SDS-PAGE. The gels were analyzed by Western blot for IE protein expression (CH16.0 Ab to detect IE2 86 and IE1 72, and mIE2 86 antibody to detect IE2 86, IE2 60, and IE2 40), early, and early-late protein expression (UL44 and UL84). Late protein expression was analyzed with an antibody to UL99. Actin serves as a loading control.

indicated by the kinetics of appearance and levels of UL44, UL84, and UL99, respectively. Many stocks of the HB5 C-F and HB5 virus were prepared and analyzed on each of the different cell lines (including HFFs, 86F, 60HA, 40HA, and 86F/40HA) during the course of our studies. Based on plaque size, it appeared that there were no significant differences in spread of either virus on any of the cell lines. Both viruses also appeared to replicate with similar kinetics at both high and low MOI, and comparable levels of infectious virus were harvested from the cell lines after they showed full CPE. The only effect that we noticed was that when the same stock of HB5 C-F virus was titered on either the 86F or 86F/40HA cell lines, the number of plaques observed at the higher dilutions was consistently slightly lower (less than 2-fold) relative to those observed on the HFFs (data not shown). When all factors are considered, however, there were no significant differences in the replication of the HB5 and HB5 C-F viruses.

The Kinetics of Induction of the IE2 86, IE2 60, and IE2 40 Proteins from the Cell Lines Parallels Their Expression from the Virus

To assess the pattern of induction of the tagged IE2 proteins in the 86F, 60HA, 40HA, and 86F/40HA cell lines, the cells were infected with the HB5 C-F virus at a MOI of 3 pfu/cell and analyzed by Western blot at 24 and 72 h p.i. (Figure 2.3). Expression of IE2 86F could be detected with an antibody to the FLAG tag at both 24 and 72 h p.i (FLAG/HA). This indicated that IE2 86F was being expressed during all stages of the infection. To ensure that the protein being made by the cell line was migrating at the appropriate size, samples were run on the same gel, and parallel Western blots were performed with the mIE2 86 antibody (mIE2 86). As can be seen in Figure 2.3, at 24 h

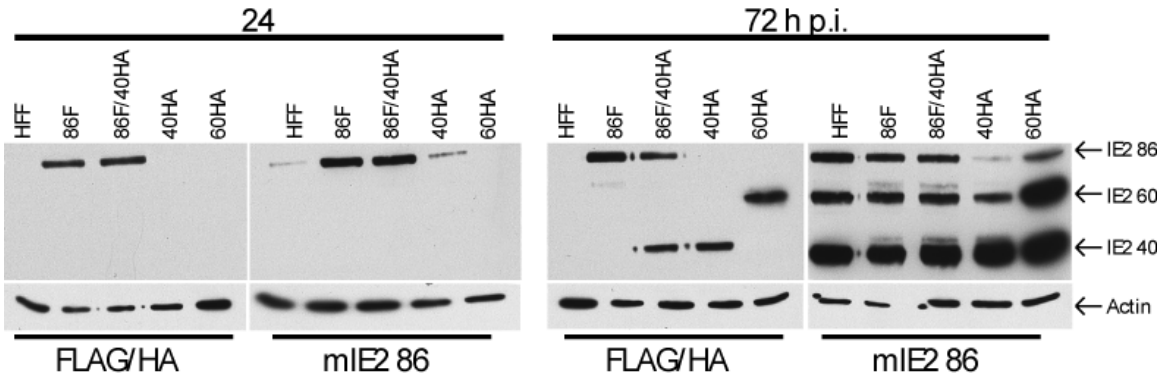


Figure 2.3. Kinetics of induction of IE2 86F, IE2 60HA, and IE2 40HA in the cell lines. Cells (HFFs, 86F, 86F/40HA, 40HA, and 60HA) were infected at an MOI of 3 pfu/cell with the HB5 C-F virus and then harvested at 24 and 72 h p.i. Equal amounts of protein were separated by SDS-PAGE, and then analyzed by Western blot for expression of the FLAG- and HA-tagged proteins and for IE2 86, IE2 60, and IE2 40 proteins. The FLAG/HA and the mIE2 86 blots at 24 h p.i. are from the same gel to compare migration of the induced proteins with the proteins expressed by the virus. The 72 h p.i. blots are from a different gel. Membranes were cut in half and stained with antibodies to FLAG and HA (FLAG/HA) or with the mIE2 86 antibody (mIE2 86) that recognizes IE2 86, IE2 60 and IE2 40. Actin serves as a loading control.

p.i., both the FLAG-tagged version of IE2 86 and the protein being expressed from the virus migrated at the same size. In this experiment, the only difference between the cell lines that we noted was that the viral IE2 86 was present at lower levels in the 40HA cells at 72 h p.i. Also, expression of the IE2 86F was slightly higher than expression of IE2 86 from the HB5 C-F virus. However, this difference was minimal and did not cause any deleterious effects on the infection process. Similarly, expression of the IE2 60HA and IE2 40HA proteins was assessed at both times p.i. Expression of these proteins could not be detected at 24 h p.i. using an antibody directed against the HA tag (FLAG/HA blot at 24 h p.i). However, by 72 h p.i., IE2 60HA and IE2 40HA were readily detected, and both migrated at the appropriate size (compare FLAG/HA blot with mIE2 86 blot at 72 h p.i.). This was in accord with the known expression pattern of early-late genes.

As a complement to the above analysis, we used IFA with antibodies to both FLAG and HA to confirm that the majority of the cells could be induced to express the tagged IE2 proteins. Cells were infected at an MOI of 1 pfu/cell with the HB5 C-F virus, and then fixed onto coverslips at either 24 or 72 h p.i. As expected at both 24 and 72 h p.i., expression of IE2 86F from the 86F and 86F/40HA cell lines could be detected with antibody to the FLAG tag, athough only the IFAs from 72 h p.i. are shown (Figure 2.4A). This was congruent with the expression pattern seen in the Western blot analyses. In contrast, the expression of IE2 60HA and IE2 40HA was only detected at 72 h p.i. by IFA with an antibody to the HA tag, confirming that the 1.2 kb viral promoter was directing expression of these proteins with the kinetics of an early-late gene (Figure 2.4A, HA column). The cell line that was co-transduced, 86F/40HA, showed expression of both

IE2 86F and IE2 40HA by 72 h p.i. We did note, however, that on a cell-to-cell level, not all of the 86F/40HA cells showed the same relative levels of IE2 86F and IE2 40HA. This can be explained by the fact that in a polyclonal population, it is unlikely that each cell contains the same number of integrated lentiviruses or that the different sites of integration allow the same level of transcription. Despite these minor differences, the majority of cells that expressed IE2 86F also expressed IE2 40HA in the double cell line, indicating that there was correct induction of the tagged proteins.

Expression of IE2 86F and IE2 40HA Aid the IE2 86ΔSX C-F Virus in Both Cell-to-Cell Spread and Titer.

The replication of the pHB5 and IE2 86ΔSX viruses has been extensively analyzed (168, 223). In the following studies, we evaluated the growth of the mutant virus containing the Cre/FLP cassette in the different cell lines with respect to the ability of the IE2 family of proteins to aid in viral gene expression, production of infectious virus, and cell-to-cell spread. Since we did not observe any significant complementation by the cells that expressed only IE2 40 (40HA) or IE2 60 (60HA), the remainder of the studies in this paper show the experiments with the HFFs, 86F, and 86F/40HA.

To confirm that the IE2 86ΔSX C-F virus could induce the synthesis of the exogenously expressed IE2 proteins in the same manner as HB5 C-F virus, we first used IFA to assess their expression in the 86F and 86F/40HA cells following infection at a MOI of 1 pfu/cell. At 24 and 72 h p.i., cells infected with the IE2 86ΔSX C-F virus were fixed onto coverslips and co-stained for FLAG and HA. As a representative example of the induction of the FLAG and HA tagged proteins by this virus, Figure 2.4B shows that

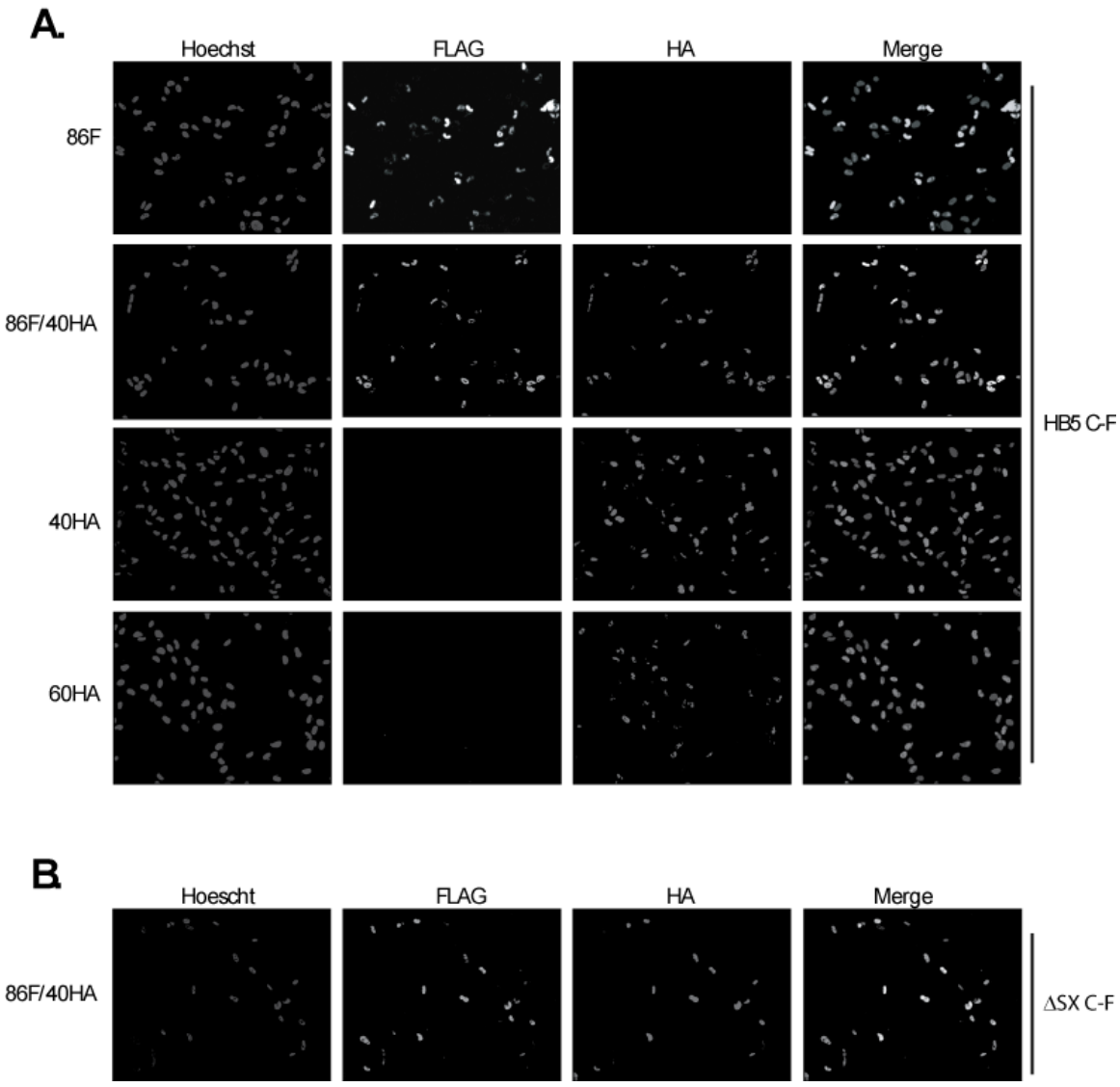


Figure 2.4. Expression of inducible proteins with HB5 C-F and ΔSX C-F viruses. Detection by IFA of the induction of IE2 86F, IE2 60HA, and IE2 40HA proteins in individual 86F, 86F/40HA, 60HA, and 40HA cells that were infected with the HB5 C-F virus (A) or the mutant IE2 86ΔSX C-F virus (B) at an MOI of 1 pfu/cell. Cells on coverslips were fixed at 72 h p.i. and co-stained with FLAG and HA antibodies and Hoechst dye (nuclear stain). Panels are shown individually (Hoechst, FLAG, HA) or combined (Merge).

IE2 86F and IE2 40HA could be detected at 72 h p.i in the 86F/40HA cell line. The 86F and 86F/40HA cells also showed appropriate expression of the IE2 86F protein at 24 h p.i. (data not shown). Analogous to what was observed in the HB5 C-F virus-infected cells, some cells expressed either IE2 86F or IE2 40HA to a greater degree in the double cell line.

The IE2 86ΔSX virus has been previously shown to grow with slower kinetics and to reduced titers in HFFs when compared to HB5 virus (168) and unpublished data, R. L. Sanders and D. H. Spector). As expected, this was also true for the IE2 86ΔSX C-F virus. When the IE2 86ΔSX C-F BAC was electroporated into HFFs, plaque formation was slow, and it took an average of 60 days to grow until all cells exhibited substantial CPE (Table 2.1). The titer of the extracellular virus generated was also very low following electroporation of the BAC into HFFs (Table 2.1). This is in contrast to observed growth kinetics of the HB5 virus, which often produces titers of 10^6-10^7 pfu/ml and spreads throughout the culture in approximately 9-11 days (unpublished results). Table 2.2 shows that a similar pattern of viral replication was observed following infection of the HFFs with the IE2 86ΔSX C-F virus at a MOI of 0.05 pfu/cell. It took an average of 44 days for the HFFs to reach full CPE, and the titers of extracellular virus were only 2.2 X 10^2 pfu/ml. This is consistent with previous observations regarding the growth of the IE2 86ΔSX virus, indicating that the addition of the Cre/FLP cassette did not change the growth of the mutant virus in HFFs (168).

In contrast, when the IE2 86ΔSX C-F virus was grown in 86F cells, cell-to-cell spread as assessed by plaque formation was significantly enhanced compared to the virus

grown in HFFs (data not shown). This was also reflected in the reduced number of days for all cells to exhibit CPE (Tables 2.1 and 2.2). However, the titers of the extracellular IE2 86ΔSX C-F virus that was released from the 86F cells after reaching full CPE following electroporation were only slightly increased compared to those obtained on the HFFs (Table 2.1). Likewise, there was only a modest increase in titer (~6-fold) when 86F cells were infected at a low MOI (Table 2.2).

To further elucidate the role of IE2 60 and IE2 40 in restoring the spread and titer of the IE2 86ΔSX C-F virus, replication in the 60HA, 40HA, and 86F/40HA cells was also assessed. The 60HA and 40HA cell lines alone were comparable to HFFs and did not facilitate the growth of the virus (data not shown). However, when the combination of IE2 86F and IE2 40HA were expressed (86F/40HA cell line), both the spread and the titer of the mutant virus increased substantially. The more rapid cell-to-cell spread was illustrated by the significant reduction in the days p.i. required to reach full CPE. Moreover, following the initial electroporation, the IE2 86ΔSX C-F progressed nearly twice as fast in the 86F/40HA cell line and the average titer obtained from these harvests of virus was increased by more than 10^3 fold (Table 2.1). These cells infected with the IE2 86ΔSX C-F virus at a low MOI also showed full CPE earlier and produced greatly increased titers (Table 2.2). Taken together, these results show that both IE2 86 and IE2 40 are important for cell to cell spread, as well as overall progression of the viral infection.

Table 2.1. Growth and titer of the IE2 86ΔSX C-F virus following the initial electroporation of the BAC in HFFs, 86F cells, or 86F/40HA cells. The number of days that it took for all cells to exhibit CPE and the titer of the extracellular virus harvested at this time (as assessed by plaque assay) are given.

Growth and Titer Following Electroporation-ΔSX C-F

Cell Line	Days To Harvest	Titer (pfu/ml)
HFF	60	2.5×10^2
86F	42	2.7×10^2
86F/40HA	33	3.0×10^5

Table 2.2. Growth and titer of the IE2 86ΔSX C-F virus following infection of HFFs, 86F cells, or 86F/40HA cells at an approximate MOI of 0.05 pfu/cell. The number of days that it took for all cells to exhibit CPE and the titer of the extracellular virus harvested at this time (as assessed by plaque assay) are given. Standard deviation is shown and is representative of at least 4 experiments, with the exception of the virus propagated on the HFFs, in which case only 2 experiments were performed.

Growth and Titer Following Propagation-ΔSX C-F

Cell Line	Days To Harvest	Titer (pfu/ml)
HFF	44.0 ± 1.4	$2.2 \times 10^{2} \pm 2.1 \times 10^{1}$
86F	26.0 ± 1.8	$1.3 \times 10^{3} \pm 1.8 \times 10^{3}$
86F/40HA	13.8 ± 1.0	$2.7 \times 10^{5} \pm 2.8 \times 10^{5}$

Induction of wt IE2 86 and IE2 40 During the IE2 86ΔSX C-F Infection Allows Partial Recovery of the Expression of UL84, UL83, and UL99.

The IFA experiments in Figure 2.4B showed that the IE2 86ΔSX C-F virus was able to induce the IE2 86F and IE2 40HA in the same temporal fashion as the HB5 C-F virus. Western blot analysis with antibodies to HA and FLAG was then used to compare the levels of the induced IE2 proteins in the IE2 86ΔSX C-F infected cells compared to those infected with HB5 C-F (Figure 2.5). The HFFs, 86F and 86F/40HA cell lines were infected or mock-infected at a comparable multiplicity (based on pfu/ml), and then harvested at 48 or 96 h p.i. At 48 h p.i., a significant amount of the IE2 86F and IE2 40HA proteins was expressed in the HB5 C-F virus-infected cells, and as expected for a gene induced with early-late kinetics, the level of IE2 40HA increased markedly by 96 h p.i (Figure 2.5, FLAG and HA, HB5 C-F). The level of the IE2 86F protein increased only slightly between 48 and 96 h p.i. Expression of IE2 86F could be detected with similar kinetics and to similar levels in the two cell lines infected with the IE2 86ΔSX C-F virus as with the HB5 C-F virus at both 48 and 96 h p.i, although the HB5 C-F virus-infected cells showed slightly increased levels of IE2 86F at 96 h p.i. when compared to the IE2 86ΔSX C-F virus-infected cells (Figure 2.5, FLAG and HA, ΔSX C-F). However, although the level of induced IE2 40HA in the 86F/40HA cells infected with the IE2 86ΔSX C-F virus increased between 48 and 96 h p.i., the levels were still significantly lower than that in the HB5 C-F virus-infected cells at both times (Figure 2.5, HA, ΔSX C-F virus)**.**

The expression of IE and early proteins was also assessed. As seen in Figure 2.5, with an antibody that detects both IE2 86 and IE1 72 (CH16.0), IE1 72 levels at both 48 and 96 h p.i. were comparable in all cell lines infected with the HB5 C-F virus. The level of total IE2 86 detected at both 48 and 96 h p.i. was slightly higher in the 86F and 86F/40HA cells, which was due to the combined expression of FLAG-tagged IE2 86 and viral-encoded IE2 86. This could also be seen using an antibody that recognizes all forms of the IE2 proteins (mIE2 86), including IE2 86, IE2 60, and IE2 40. In addition, it appeared that both the IE2 60 and IE2 40 levels were higher at 48 h p.i. in these cells. This may be due in part to increased IE2 40 in the 86F/40HA cells and slightly faster progression of the infection in both cell lines, as the levels were comparable at 96 h p.i. Early gene expression remained unaffected in the HB5 C-F virus-infected cells as judged by expression of UL57.

The same proteins were assessed by Western blot in the cells infected with the IE2 86ΔSX C-F virus. Expression of IE2 86 from both the IE2 86ΔSX C-F virus and the cell lines was assessed with the CH16.0 (IE1 72 and IE2 86) and mIE2 86 antibodies (IE2 86, IE2 60, and IE2 40) (Figure 2.5). As previously described (168), the IE2 86ΔSX C-F virus showed a delay in the expression of mutant IE2 86 (IE2 ΔSX at 48 p.i.), while IE1 72 remained generally unaffected, although the level was slightly lower in the HFFs at 96 h p.i. As expected, no IE2 60 was detected in any of the infected cells, full length IE2 86 was only seen in the infected 86F and 86F/40HA cells, and IE2 40 was only present in the infected 86F/40HA cells. It should be noted that although the level of full-length IE2 86 in the IE2 86ΔSX C-F infected 86F and 86F/40HA cells (which only contained the

induced protein) was slightly lower than in the same cells infected with the wt HB5 C-F virus (which contained both induced and viral encoded IE2 86), the 86F and 86F/40HA cells infected with the mutant virus expressed comparable levels of IE2 86F as the HFFs infected with the wt virus. However, a greater amount of IE2 40 was present in all of the cells infected with the wt virus than in the 86F/40HA cells infected with the mutant. As expected, there was no significant difference in the expression of the early protein UL57 in cell lines infected with IE2 86ΔSX C-F virus. This confirmed that the infections with the HB5 C-F and IE2 86ΔSX C-F viruses were properly matched and showed that the presence of the induced IE2 86F, IE2 60HA, or IE2 40HA proteins had no significant effect on the expression of UL57.

Since we previously found that the levels of many early-late and late proteins, particularly UL83, UL84, and UL99, were substantially lower in IE2 86ΔSX virus - infected cells (168, 223), we were interested in whether any of the IE2 family of proteins could aid in recovery of expression of these proteins. In general, the kinetics of appearance and levels of UL84 and UL83 (early-late proteins) and UL99 (a late protein) were comparable in the three HB5 C-F virus-infected cell lines (Figure 2.5). However, differences were detected in the recovery of expression of UL84, UL83, and UL99 in the cell lines infected with the IE2 86ΔSX C-F virus. Interestingly, the induction of IE2 86F alone was sufficient for a partial increase in the levels of UL84 and UL83 by 48 h p.i. compared to their respective levels in HFFs, and the levels increased further by 96 h p.i over those in HFFs. Enhancement of the UL84 and UL83 levels, however, was most notable in the IE2 86ΔSX C-F-infected 86F/40HA cells that expressed both IE2 86F and

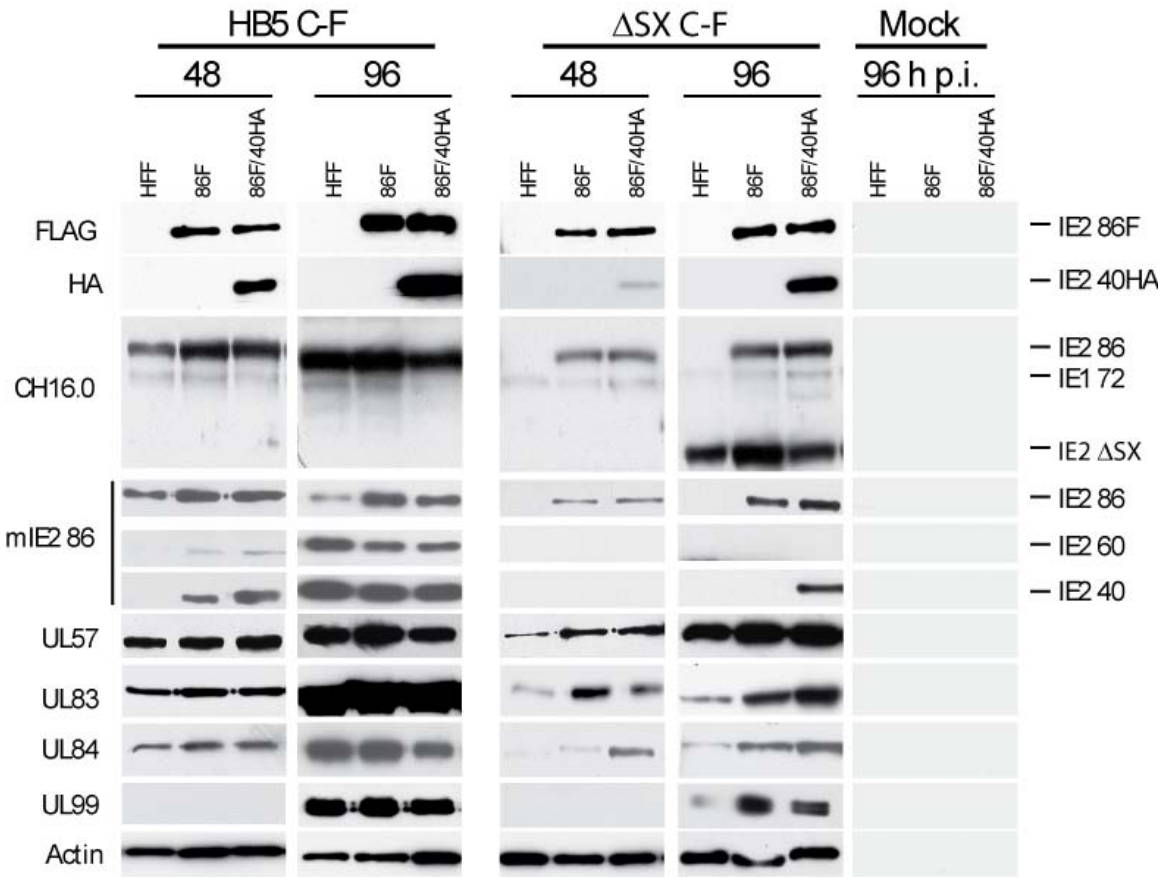


Figure 2.5. Expression of viral proteins in the HB5 C-F and IE2 86ΔSX C-F virus-infected cells. Cells were infected or mock-infected at an MOI of 3 pfu/cell with either the HB5 C-F or IE2 86ΔSX C-F (ΔSX C-F) virus and then analyzed at 48 and 96 h p.i. by Western blot for expression of the indicated viral proteins. Equal amounts of protein were loaded in each lane. Expression of IE2 86, IE2 86F and IE1 72 was analyzed with the CH16.0 antibody (CH16.0). Expression of IE2 86, IE2 86F, IE2 60, IE2 40, and IE2 40HA was analyzed with a monoclonal IE2 86 antibody (mIE2 86). The levels of an early protein (UL57), early-late proteins (UL84 and UL83), and a late protein (UL99) were also assessed with their corresponding antibodies. Induction of IE2 86F and IE2 40HA in the 86F and 86F/40HA cell lines was visualized with antibodies to FLAG and HA. Actin serves as a loading control. Mock-infected samples are also shown (Mock).

IE2 40HA. The late protein UL99 also exhibited slightly reduced levels of the protein in HFFs at 96 h p.i. when infected with the mutant virus. However, in either the 86F or 86F/40HA cells, the levels of the protein were comparable to that of the HB5 C-F virus at 96 h p.i.

Expression of IE2 86 Alone or in Combination with IE2 40 Complements a Non-Viable IE2 Mutant Virus

Since a major obstacle in studying IE2 86 function in the context of the infection has been the lack of a means to propagate a non-viable mutant virus, an important test of our system was whether it would complement the growth of a virus with a null mutation in the IE2 gene. To this end, we used a mutant virus that is missing exon 5 of IE2 86, IE2 86ΔExon5 C-F (see schematic, Figure 2.1). HFFs, 86F, and 86F/40HA cells were electroporated simultaneously and initially analyzed for viral expression and plaque formation (data not shown). By day 6 post electroporation, IE gene expression was detectable, and early gene expression (UL44) was visible in a few of the 86F/40HA cells. By day 9 post electroporation, early gene expression had progressed to a great degree, and some small plaques were visible. This was also seen in the 86F cell line. In contrast, only expression of IE1 72 could be detected with HFFs infected with this virus, and no plaques were visualized at either time post electroporation. In general, the progression of the plaques on the 86F cells following electroporation with IE2 86ΔExon5 C-F BAC was slower than those on the 86F/40HA cells, although some plaques on the 86F cells were comparable in size to those on the 86F/40HA cells at the same time post electroporation. Overall, more plaques were observed on the 86F/40HA cell line following

electroporation and infection, and many of these plaques spread more rapidly relative to those on the 86F cells. Virus grown in 86F cells following electroporation took approximately 57 days to spread completely through the culture, while the 86F/40HA cell line reached 100% CPE in approximately 48 days. This is in contrast to the known growth characteristics of the wt HB5 virus following electroporation, which forms plaques much more quickly and spreads throughout the culture by day 9 post electroporation (data not shown).

The titer of the IE2 86ΔExon5 C-F virus that was grown in the 86F/40HA cell line (approximately $3.5X10^4$ pfu/ml) was also consistently higher than that grown in the 86F cell line, although it was still significantly lower than the titer of wt HB5 C-F virus grown in these cell lines (data not shown). For these reasons, only the 86F/40HA cells were used to prepare stocks of the mutant virus. Taken together, these data indicate that IE2 86 alone is necessary for formation of productive virus, and that expression of IE2 40 provides additional benefits for complementing a non-viable virus.

To verify that the IE2 86ΔExon5 C-F virus (as well as the rescued version of the mutant virus) was able to induce proper expression of the IE2 86F and IE2 40HA proteins, the 86F/40HA cells were infected at an MOI of 1 pfu/cell with the IE2 86ΔExon5 C-F or IE2 86ΔExon5 Res C-F virus, fixed at 72 h p.i., and then assayed by IFA with antibodies specific for the FLAG and HA tags (Figure 2.6). Expression of both IE2 86F and IE2 40HA could be readily detected in both infections, and no differences in expression were noted as compared to what was seen with the HB5 C-F virus-infected cells.

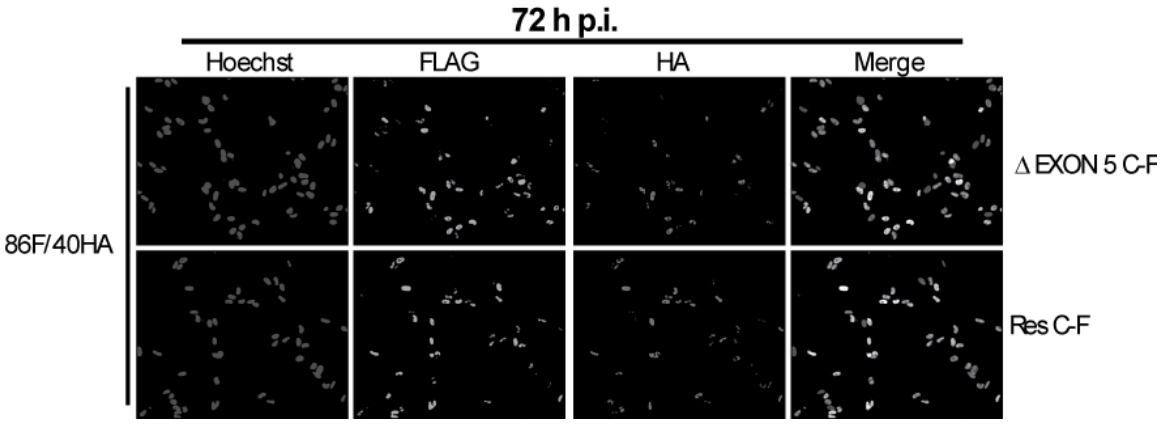
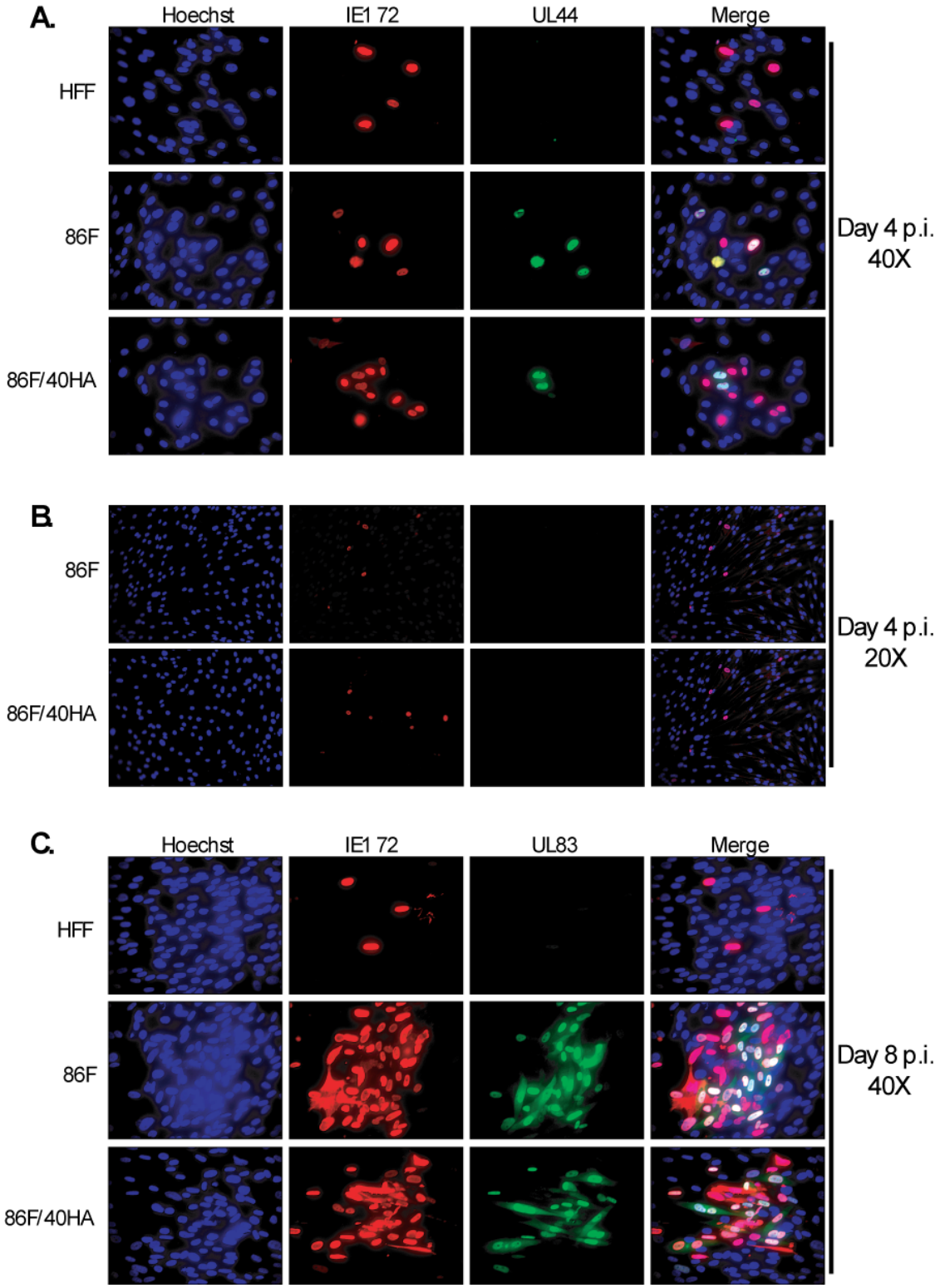


Figure 2.6. Expression of IE2 86F and IE2 40HA in 86F/40HA cells infected with either the IE2 86ΔExon5 C-F (ΔExon5 C-F) and IE2 86ΔExon5 Res C-F (Res C-F) virus. 86F/40HA cells were infected at an MOI of 1 pfu/cell with either the IE2 86ΔExon5 C-F or IE2 86ΔExon5 Res C-F virus and were seeded onto coverslips. At 72 h p.i., cells were fixed with 2% paraformaldehyde and then analyzed for expression of FLAG and HA by IFA. The nucleus is indicated by Hoechst staining and the Merge panels show all three stains combined.

At Low MOI, Expression of the IE2 86ΔExon5 C-F Viral Early Genes is Still Partially Blocked in the Complementing Cells

The finding that the IE2 86ΔExon5 C-F virus plaques developed slowly, even when grown on the 86F/40HA cells, and that titers were reduced relative to HB5 C-F virus, suggested that the mutant still had some growth defect at a low MOI. To determine the nature of this defect, HFF, 86F, and 86F/40HA cells were infected at a MOI of 0.01 pfu/cell with the IE2 86ΔExon5 C-F virus that was prepared on the 86F/40HA cells, and then seeded onto coverslips. The coverslips were fixed at day 4 and day 8 p.i. and analyzed by IFA with antibodies specific for the different temporal classes of viral proteins (Figure 2.7). As expected, only IE1 72 protein expression could be detected in the HFFs at day 4 or day 8 p.i. (Figure 2.7A and C). These data indicated that no viral replication was occurring at either time p.i., and that the IE2 86ΔExon5 C-F virus was in fact non-viable on normal HFFs without the expression of IE2 86F or IE2 40HA. In the 86F and 86F/40HA cells, expression of the early gene product UL44 was visible in some cells by day 4 p.i. (Figure 2.7A), but most cells that were positive for IE1 72 were negative for the UL44 early protein. This is reflected in the lower magnification of the same coverslips in panel 7B. Based on analysis of multiple fields in the coverslips, we determined that approximately 10-15% of IE1 72 positive cells also expressed UL44. In contrast, our many analyses of cells infected with wt virus at low MOI have shown that most cells that express IE1 72 also express UL44 by day 4 p.i. The percentage of IE2 86ΔExon5 C-F virus-infected cells that were positive for both IE1 72 and UL44 also correlated with the relative number of small plaques observed at day 8 p.i., indicating that

Figure 2.7. IE, early, and early-late gene expression following infection with the IE2 86ΔExon5 C-F virus at low MOI. HFFs, 86F, and 86F/40HA cells were infected at a low MOI (0.01 pfu/cell), and seeded onto coverslips. At day 4 p.i. (A and B) coverslips were fixed and then stained with antibodies directed against IE1 72 and UL44. (A) Pictures were taken of cells observed with a 40X objective. The field in the figure was specifically selected to show cells that expressed both proteins. (B) Pictures were taken of cells on the same coverslips as in (A) observed with a 20X objective to show that a significant number of the cells only expressed IE1 72 and had not begun to form plaques. (C) At day 8 p.i., cells were fixed and coverslips were stained with an antibody directed against IE1 72 and UL83. The nucleus is indicated by Hoechst staining. Individual panels and the Merge of all panels are shown.


A.
Hoechst
IE1 72
UL44
Merge
HFF
86F
86F/40HA
Day 4 p.i.
40X
B.
86F
86F/40HA
Day 4 p.i.
20X
C.
Hoechst
IE1 72
UL83
Merge
HFF
86F
86F/40HA
Day 8 p.i.
40X

only the cells that were able to support early gene expression produced infectious virus that spread to adjacent cells. Panel 9C shows that in the plaques, almost all cells that expressed IE1 72 also expressed the early-late protein UL83 by this time, although there was a small percentage that only expressed IE1 72. We could not determine, however, whether these were newly infected cells, or abortively infected cells that were not able to progress to early-late gene expression. Based on these results, it is likely that at low MOI, viable IE2 86ΔExon5 C-F virus can be produced on the complementing cells, but the block to early gene expression is only partially overcome. Furthermore, early-late gene expression seems to progress in the same percentage of cells that overcome the block to early gene expression, indicating that once viral replication commences, early-late gene expression is efficient.

Kinetics of Viral Gene Expression Following High MOI Infection of 86F and 86F/40HA Cells With IE2 86ΔExon5 C-F

To more carefully characterize the pattern of viral gene expression in the IE2 86ΔExon5 C-F virus-infected cells, and to determine the patterns of expression at a higher MOI, Western blot analyses were used. HFF, 86F, and 86F/40HA cells were infected or mock-infected at an MOI of 1 pfu/cell with the IE2 86ΔExon5 C-F or the HB5 C-F virus, and then harvested at 24, 72, and 120 h p.i. (Figure 2.8, panels A, B, and C, respectively). Levels of representative viral proteins from each stage of the IE2 86ΔExon5 C-F virus infection were assessed and compared to those in the three cell lines infected with HB5 C-F virus.

At 24 h p.i., there were slightly higher levels of IE2 86 in the HB5 C-F virus-infected 86F and 86F/40HA cells due to the presence of the induced IE2 86F from the lentivirus. All of the detectable IE2 86 from the IE2 86ΔExon5 C-F virus-infected cells was expressed from the induced protein, and the levels were comparable to that present in the HB5 C-F virus-infected cells. As expected, no IE2 86 could be detected in the HFFs infected with the IE2 deletion virus. Interestingly, all three cell lines infected with the IE2 86ΔExon5 C-F virus exhibited greatly increased levels of IE1 72 expression by 24 h p.i., and this expression continued throughout the time-course (Figure 2.8A-C). Expression of the IE2 86F provided by the cell lines was also assessed with the FLAG antibody. At 24 h p.i., an exposure comparable to that at the later times does not indicate any expression of IE2 86F, however upon longer exposure, expression of IE2 86F can be detected to a comparable degree in the IE2 86F and IE2 86F/IE2 40HA cell lines (Figure 2.8A, only the longer exposure of the FLAG blot is shown). As expected, no expression of IE2 40HA could be detected at 24 h p.i., even upon long exposure (Figure 2.8A).

As representative early genes, we analyzed the expression of the viral replication proteins UL44 and UL57. At 24 h p.i., expression of both UL44 and UL57 in the IE2 86ΔExon5 C-F virus-infected cells was greater than in the HB5 C-F virus-infected cells, most likely due to the entry of a larger number of IE2 86ΔExon5 C-F virions into the cells, relative to the measured pfu. This is also reflected in the higher levels of early-late and late virion matrix proteins UL83 and UL99 in the IE2 86ΔExon5 C-F virus-infected cells at 24 h p.i. in all cell lines tested. In particular, the presence of these virion proteins in the HFFs at this time p.i. indicated that they were input proteins, since in these cells the

IE2 86ΔExon5 C-F infection does not proceed beyond the IE phase. Although we cannot exclude the possibility that there was some late gene expression from the mutant viral genomes at early times, the experiments described below support the conclusion that the input particle to PFU ratio of the IE2 86ΔExon5 C-F virus was significantly higher than that of the HB5 C-F virus. Furthermore, given that the expression of these proteins did not continue throughout the time-course (see Figure 2.8B and C), this further supported the idea that the expression seen at 24 h p.i. was due to the increased number of input virions.

At 72 h p.i., expression of IE2 86, IE2 60, and IE2 40 could be detected in the HB5 C-F virus-infected cells (Figure 2.8B). IE2 86F was still present in the IE2 86ΔExon5 C-F virus-infected cells, although no IE2 60 or IE2 40 could be seen with the mIE2 86 antibody. However, staining with the HA antibody indicated that some IE2 40HA was present at this time in the 86F/40HA cells infected with either virus. The levels of IE2 86F, as detected with the FLAG antibody, were higher in the 86F cells infected with HB5 C-F than with IE2 86ΔExon5 C-F, but there was comparable expression in the 86F/40HA cell line infected with either virus. Similar to what was observed at 24 h p.i., the amount of IE1 72 continued to be much higher in the mutant virus-infected cells than the HB5 C-F virus-infected cells. This could be seen with both the CH16.0 and IE1 72 specific antibodies. At this time, early gene expression was comparable for the two viruses, as indicated by UL44 and UL57. No expression of these proteins could be detected in the HFFs infected with IE2 86ΔExon5 C-F, providing

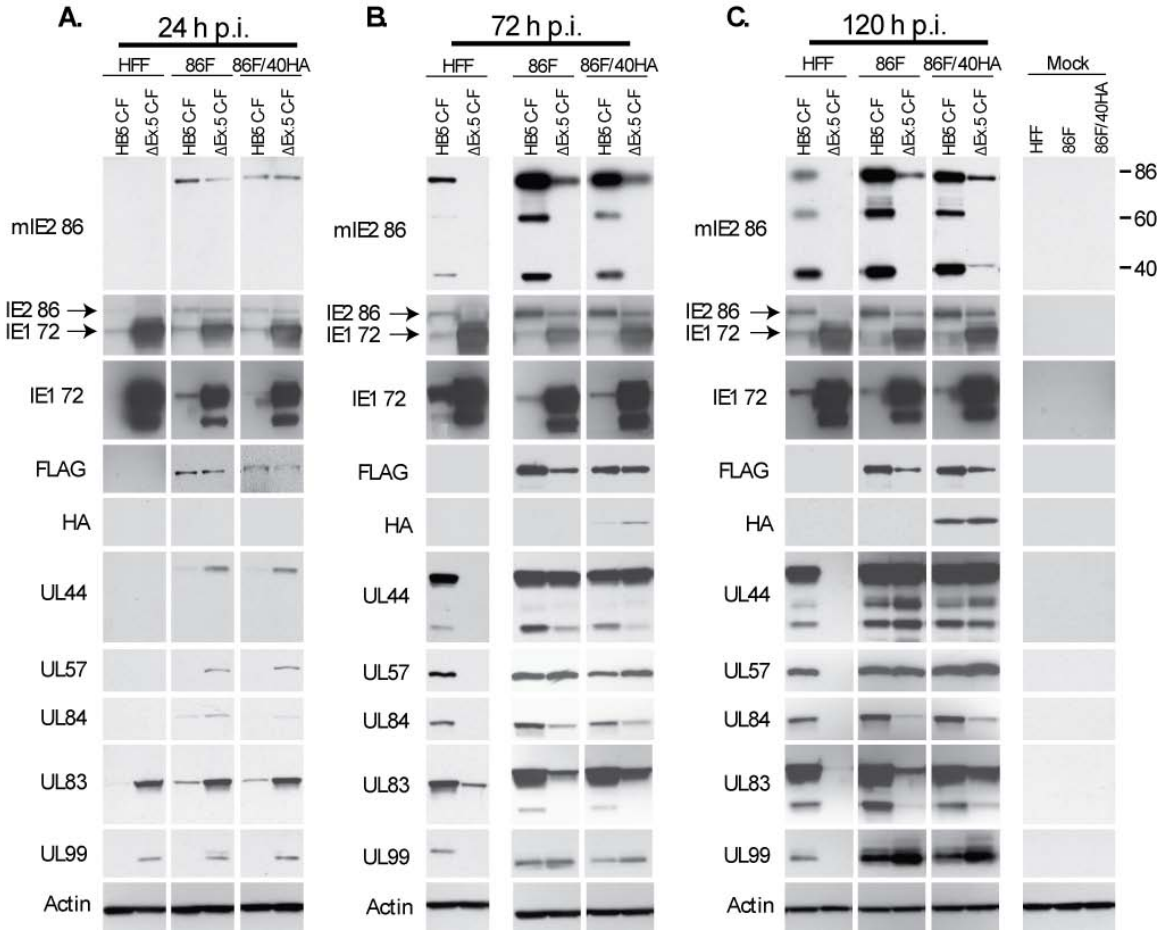


Figure 2.8. Pattern of protein expression in the HFFs (HFF), 86F, and 86F/40HA cells infected with the IE2 86ΔExon5 C-F (ΔEx.5 C-F) and HB5 C-F viruses. The cells were mock-infected or infected or at an MOI of 1 pfu/cell and then harvested at 24 (A), 72 (B), or 120 h p.i. (C) for Western blot analyses. In all cases (A, B, and C), IE, early, early-late, and late protein expression were assessed. IE protein expression is represented by IE2 86 and IE1 72. All forms of the IE2 gene products-IE2 86, IE2 60, and IE2 40 are visualized with the mIE2 86 antibody (mIE2 86) and are indicated as 86, 60, and 40 (respectively). Early protein expression is represented by UL44 and UL57. The levels of representative early-late and late protein expression were assessed with antibodies to UL84, UL83, and UL99. The induced proteins, IE2 86F and IE2 40HA, were analyzed with antibodies against the FLAG tag (FLAG) and the HA tag (HA), respectively. Actin serves as a loading control.

additional evidence that the virus cannot progress to the early stages of the infection without IE2 86. Early-late and late gene expression was complemented to various degrees in the 86F and 86F/40HA cell lines at 72 h p.i., as indicated by the levels of UL84, UL83, and UL99. Interestingly, although the levels of UL99 were comparable for both viruses, the levels of UL84 and UL83 were lower in the cells infected with the IE2 86ΔExon5 C-F virus than with the HB5 C-F virus. Our previous studies with other mutant viruses have indicated that a very dynamic relationship between these two proteins and IE2 86 and IE2 40 exists, which might account for the lack of total complementation. As can be seen with the mIE2 86 antibody in Figure 2.8 (B and C), the amount of the IE2 40HA protein induced in the 86F/40HA cell lines was significantly lower than the levels that are present during the normal wt infection at the later times. Given these results, it is likely that more IE2 40 protein would be needed to see full complementation of this virus.

As shown in Figure 2.8C, IE1 72 levels in all three cell lines infected with IE2 86ΔExon5 C-F virus remained very high at 120 h p.i. IE2 86F and IE2 40HA could be detected with the FLAG and HA antibodies in the 86F and 86F/40HA cells, and the levels of the viral early proteins in these cells remained comparable between the two viruses. Interestingly, at this point, the amount of UL99 was slightly higher in the IE2 86ΔExon5 C-F virus-infected cells than in the HB5 C-F virus-infected cells. UL84 and UL83 levels, however, were still lower in the mutant-infected 86F and 86F/40HA cells than HB5 C-F virus-infected cells. The levels of UL83, however, seemed to be higher in the IE2 86ΔExon5 C-F-infected 86F/40HA cell line than in the 86F cell line, indicating

that UL83 expression is dependent on the presence of IE2 40. This confirms previous data that the loss of IE2 60 and IE2 40 results in decreased expression of UL83 protein and RNA (223).

As noted above, there were very high levels of IE1 72 throughout the infection and increased levels of the virion matrix proteins UL83 and UL99 at early times in all three cell lines infected with the IE2 86ΔExon5 C-F virus. In these infections, the multiplicity was based on the number of pfu in the viral inoculum, and thus it was possible that the actual number of input virions (that did not progress to form plaques) was significantly higher for the mutant virus. To determine if the increased input viral proteins were due to an increase in input viral genomes, quantitative PCR was performed to analyze the comparative amount of input genomes when normalized to the same number of pfu. Cells were infected with the same pfu/cell, and at 24 h p.i., the levels of intracellular viral DNA were measured by quantitative real-time PCR (Figure 2.9A). Interestingly, the IE2 86ΔExon5 C-F virus-infected cells showed approximately 40 fold higher levels of viral DNA, indicating that many more genomes were entering the cell than were forming plaques. Furthermore, in order to determine if IE gene expression was affected at the RNA level, quantitative real-time RT-PCR assays were performed at 24 h p.i. (Figure 2.9B). Analogous to what was observed for the IE1 72 protein, the levels of IE1 72 RNA were markedly higher (~100 fold) in the IE2 86ΔExon5 C-F virus-infected cells as compared to the HB5 C-F virus-infected cells.

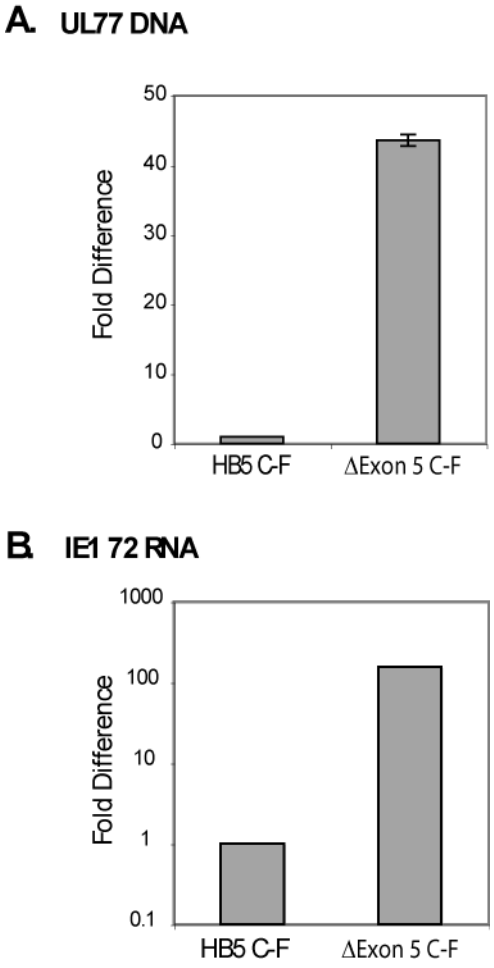


Figure 2.9. Input viral DNA and expression of IE1 72 RNA during the IE stages of the infection. At 24 h p.i., 86F/40HA cells that were infected with the HB5 C-F (HB5 C-F) and IE2 86ΔExon5 C-F (ΔExon 5 C-F) viruses (from the same infection shown in Figure 8) were harvested for analysis of viral DNA (A) or RNA (B). (A) Quantitative real-time PCR was used with primers and probe for the viral UL77 gene to measure the amount of total input viral DNA. A separate experiment was also set up with another preparation of the IE2 86ΔExon5 C-F virus in order to ensure that these data were not specific to a single viral preparation. Fold increase relative to the HB5 C-F infected cells (value set to 1) is shown and is an average of these two experiments (represented by error bars). Duplicate samples were assayed for each experiment. (B) The RNA from the 86F/40HA cells infected with both viruses was analyzed using quantitative real-time RT-PCR analyses with primers and probe to IE1 72. Only RNA from the experiment in Figure 8 is shown. Fold increase is based on the HB5 C-F virus-infected cells (value set to 1), and duplicate reactions were assessed and averaged for each sample. For both (A) and (B), a standard curve was generated in order to assess the relative quantities of either DNA or RNA in each sample.

In view of the very high levels of IE1 72 produced in cells infected with the IE2 86ΔExon5 C-F virus and the large particle to pfu ratio, it was important to ensure that all defects seen in the IE2 86ΔExon5 C-F virus were due to the loss of exon 5. A rescued virus, IE2 86 ΔExon5 Res C-F (RES C-F), was created and the patterns of protein expression were compared to that of the HB5 C-F. HFFs, 86F, and 86F/40HA cells were infected at an MOI of 1 pfu/cell, and then analyzed by Western blot at 24 and 72 h p.i. (Figure 2.10). At 24 h p.i., expression of IE2 86 and IE1 72 could be detected, and the levels were comparable for the two viruses. Furthermore, similar levels of the UL44 early gene were present in the 86F and 86F/40HA cell lines infected with either virus, and induction of the FLAG tagged IE2 86 could be detected. At 72 h p.i., there also were no differences in the expression of the viral proteins for the two viruses in any of the cell lines. Likewise, the particle to pfu ratio was comparable to the HB5 C-F virus, indicating that this was also a result of the loss of exon 5 (data not shown). These results showed that all of the defects seen in the mutant virus-infected cells were likely due to the deletion of exon 5 of IE2 86.

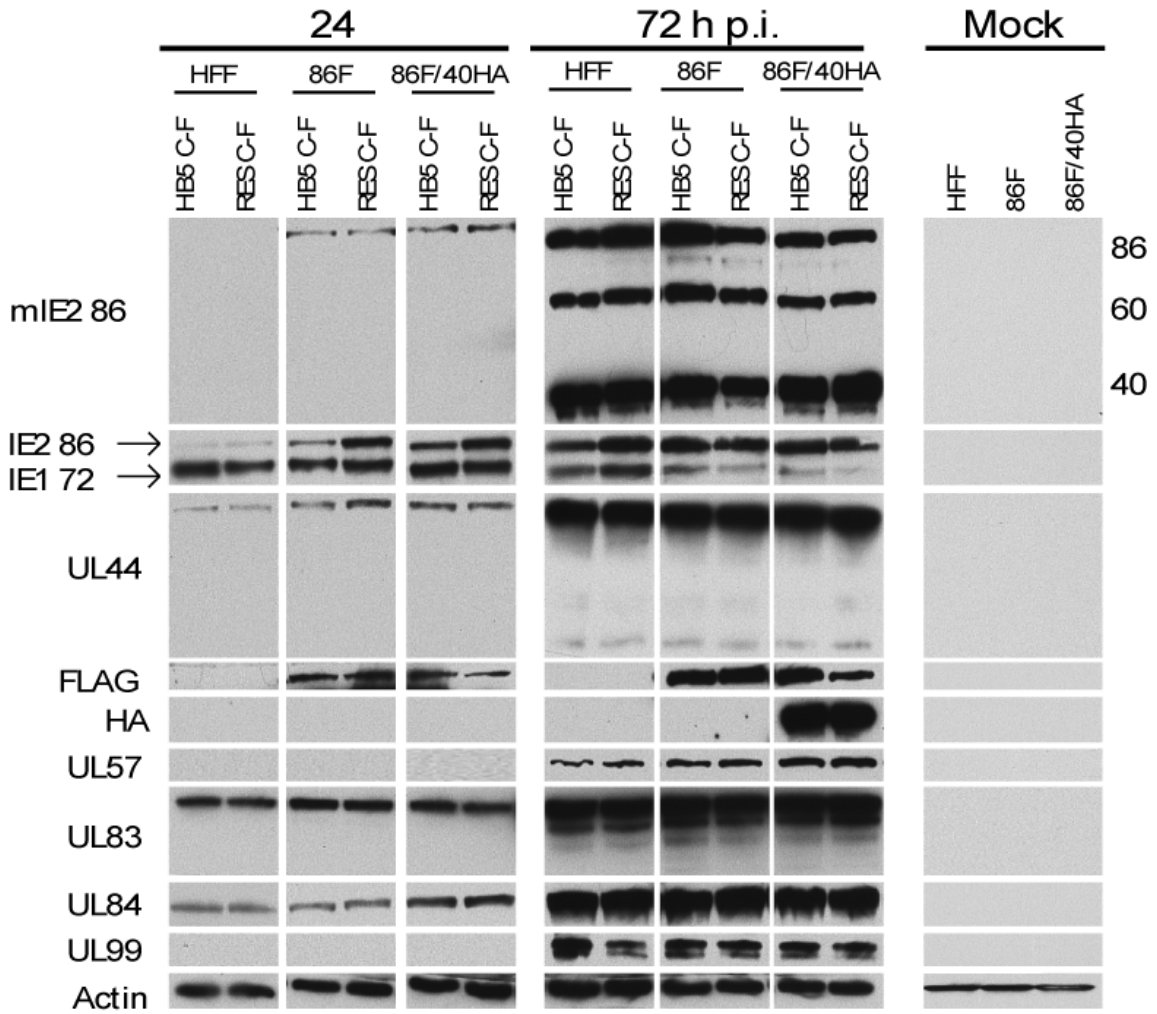


Figure 2.10. Pattern of protein expression in the HFFs (HFF), 86F, and 86F/40HA cells infected with the HB5 C-F and IE2 86ΔExon5 Res C-F (RES C-F) viruses. HFFs, 86F, and 86F/40HA cells that were mock-infected or infected at an MOI of 1 pfu/cell with the HB5 C-F and IE2 86ΔExon5 Res C-F viruses were harvested at 24 and 72 h p.i. Lysates were prepared and assayed by Western blot with the mIE2 86 antibody that detects IE2 86, IE2 40, and IE2 60 and with the CH16.0 antibody that detects IE2 86 and IE1 72. Early proteins analyzed were UL44 and UL57. As representative early-late and late protein expression, the blots were analyzed with antibodies to UL84 and UL83 and to UL99, respectively. The exogenously expressed proteins from the 86F and 86F/40HA cell lines were also assayed with antibodies against the FLAG tag (FLAG) and HA tag (HA). Actin serves as a loading control. Mock-infected lysates are also shown (Mock).

DISCUSSION

The IE2 86 protein is multifunctional and is essential for productive HCMV infection. Most studies on this protein, however, have been in an isolated context due to the difficulty of constructing and propagating recombinant viruses with selected mutations in its coding region. A major goal of our laboratory for many years has been to use mutant viruses to elucidate the functional domains of this protein and the underlying mechanisms governing its activities in the context of the infection, with the long-term objective of applying this information towards understanding the role of the protein in *in vivo* pathogenesis and developing strategies for treatment or prevention of disease.

The use of the BAC recombination system has greatly facilitated the introduction of mutations into the viral genome. However, the lack of a complementing cell line for IE2 86 has made it impossible to propagate a mutant virus that is non-viable and to study the consequences of the mutation at a more mechanistic level. Moreover, even in the case of debilitated but viable mutant viruses, there is the concern that propagation of the virus in the absence of complementation will lead to the selection of viruses that have sustained second site mutations that enhance the replication of the mutant virus.

Many attempts have been made to create a system that has the ability to complement non-viable viruses with mutations in the MIE coding region. Although a cell line has been constructed to complement mutations in the IE1 region (131), there have been no reports of a successful strategy for complementing mutations in the IE2 region. The advantages to the complementation system that we have developed include tightly controlled expression using an inducible recombination system, the ability to provide

exogenous expression of the IE2 family of proteins in the same temporal pattern as the infection, as well as the decreased likelihood of second-site mutations or rearrangements occurring during the propagation of the virus. This system provides added benefits in that the IE2 86 is expressed in the cell line only when necessary for infection, and therefore IE2 86-mediated cell toxicity in uninfected cells is circumvented. The synthesis of the IE2 86 protein in the cell line is induced with a protein (Cre recombinase) that is expressed from the virus under the control of the MIE promoter. Thus, the IE2 86 is expressed with the same kinetics as the protein normally expressed by a wt virus. Similarly, the IE2 60 and IE2 40 proteins in the cell lines are induced with a protein (FLP recombinase) that is expressed from the virus under the control of the 1.2 kb viral promoter (early-late promoter), which allows proper temporal expression of these proteins as well. To this end, we have created cell lines that allow the assessment of IE2 86, IE2 60, and IE2 40 alone, as well as a double cell line expressing IE2 86 and IE2 40. These studies have helped define the individual roles of these proteins, and have provided a means to propagate both a growth impaired virus, as well as a completely non-viable virus.

The deletion of aa 136-290 from the C-terminal exon of IE2 86 results in a viable virus, IE2 86ΔSX, that expresses a smaller IE2 86 protein but is unable to express the early-late IE2 60 and IE2 40 proteins (168, 223). Prior studies with this mutant showed that early gene expression remains comparable to the wt HB5 virus, as evidenced by the levels of the UL44 and UL57 proteins (168). In contrast, there is delayed and decreased expression of the mutant IE2 86 throughout the infection. In addition, the accumulation

of the early-late proteins UL83 and UL84 is significantly inhibited. However, the mechanisms governing these effects appear to differ, as RNA expression remains normal for the mutant IE2 86 and UL84, but is greatly reduced for UL83 (168, 223).

To determine which of the IE2 proteins was responsible for the defects observed in the IE2 86ΔSX-infected cells, each of the cell lines was infected with the mutant IE2 86ΔSX C-F virus, and gene expression as well as virus production was assessed. The cells that could be induced to express either the IE2 60 protein (60HA) or IE2 40 protein (40HA) alone were unable to provide any notable complementation. Interestingly, expression of full-length IE2 86 alone was able to counter some of the defects. However, the presence of IE2 86 and IE2 40 in combination appeared to have the most beneficial effect on expression of UL83 and UL84 and virus production. It seems likely that IE2 86 plays an important role in regulation of both of these gene products, and that IE2 40 is involved in much of this regulation at the later stages of the infection.

It was notable that even in the 86F/40HA cells, the levels of UL83 and UL84 in the IE2 86ΔSX-infected cells were still significantly lower than the levels in the wt virus-infected cells. It seems unlikely that there was insufficient full-length IE2 86 induced in the complementing cells, as the levels in the mutant infected cells were comparable to that observed in HFFs infected with wt virus. Rather, we suspect that the problem is due, at least in part, to the relative amount of the IE2 40 protein induced in the complementing cells, which was significantly lower than the levels of the IE2 40 protein expressed by the wt virus at late times in the infection. In accord with this, we previously found that the levels of UL83 and UL84 were also substantially less in HFFs infected with an IE2

mutant virus (IE2 Δ40+60) that expressed full-length wt IE2 86 but no IE2 40 or IE2 60 (223). We hypothesize that this may be due to a very tight regulatory system by which proper levels of IE2 86 and IE2 40 are necessary for UL84 protein expression. To date, the mechanism underlying this phenomenon is unclear, but studies are underway to address this. The defect in UL83 expression appears to be at the level of RNA synthesis, which more clearly fits into the known roles of the IE2 proteins. Based on our observation that the combination of IE2 86 and IE2 40 is required for optimal cell-to-cell spread and production of the mutant virus, it is tempting to consider that IE2 40 may also play a role in packaging and release of the viral particle, or in regulation of other genes important for this aspect of the infection.

Assessment of the replication of the IE2 86ΔExon5 C-F virus has led to several interesting findings. By IFA and Western blot analysis, it appears that both the 86F and the 86F/40HA cell lines allow expression of all temporal classes of viral proteins, but only IE expression can commence on HFFs. However, despite the complementation of the non-viable IE2 86ΔExon5 C-F virus, the growth and titers of the virus obtained were still impaired relative to the wt HB5 C-F virus. When cells were infected at a relatively high MOI (MOI of 1 pfu/cell), the expression of the early proteins in the complementing cells infected with the mutant virus was comparable to that observed with the wt virus at a comparable MOI, but the levels of the newly synthesized UL83 and UL84 proteins were lower. A striking result was that expression of IE1 72 is significantly upregulated in the complementing cell lines when infected at this apparent MOI based on pfu. This appears to be due to a requirement for a large number of input virions for the infection to

progress beyond the IE phase even in the complementing cells, and is consistent with the observation that many mutant viruses replicate at high MOI but show a significant growth defect at low MOI. In accord with this, we found by IFA that at low MOI only 10 to 15% of the complementing cells that expressed viral IE proteins also expressed early proteins and went on to form plaques. At this point, we do not know whether this is due to some defect in the viral DNA or the composition of the input virions. Although these defective viral genomes likely contributed to the observation that many more cells expressed IE1 72 than UL44 following low MOI infection (Figure 2.7), it is not likely that this is the major reason for the lack of early gene expression in these mutant virus-infected cells.

One question raised by these results is what would be required to achieve full complementation. The temporal expression and levels of full-length IE2 86 induced in the complementing cells are comparable to the endogenous IE2 86 produced by the wt virus in HFFs, but the amount of induced IE2 40 is still much lower than the endogenous IE2 40 produced by the wt virus at late times. Yet, if insufficient levels of IE2 40 were solely responsible for the growth defect of the progeny IE2 86ΔExon5 C-F virus during the next round of infection at low MOI, we might expect that IE2 86ΔSX C-F progeny virus would show a similar phenotype with inefficient progression from IE to early gene expression. As previously reported and shown here, early gene expression and viral DNA replication appear to be normal in IE2 86ΔSX infected cells, and the primary effects of the mutation appears to be at late stages of the infection. The major difference between IE2 86ΔExon5 and IE2 86ΔSX viruses is that the mutant IE2 86 produced by the IE2

86ΔSX still includes much of exon 5 (aa 85-135 and 291-579). It is possible that in the cells infected with IE2 86ΔSX virus there are alternative gene products that contain sequences from exon 5. We also cannot exclude the possibility that the absence of exon 5 has indirect effects on the expression of other genes that are not related to the IE2 protein itself. Studies to address these questions are currently in progress.

The approach for deriving complementing cell lines that is described here can be applied to other HCMV genes, particularly in the case where even very low levels of the viral protein may be toxic for the cell and where correct temporal expression is critical for the replication of the virus. There is a slight delay in production of the induced protein using this system, which may provide some restrictions for its use; however, clearly the tightly controlled expression provides an advantage over many other expression systems. Our experience with multiple approaches that are inducible at the transcriptional level has been that it is difficult to completely eliminate expression of the gene. In contrast, translation in eukaryotic cells of the second ORF on a multicistronic RNA, in the absence of an internal ribosome entry site (IRES), is very uncommon, and control at this level is therefore much tighter. Thus, this strategy could also be generally useful for controlled expression of other highly toxic cellular or microbial proteins.

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CHAPTER 3

INTERNAL DELETIONS OF IE2 86 AND LOSS OF BOTH OF THE LATE IE2 60 AND IE2 40 PROTEINS ENCODED BY HUMAN CYTOMEGALOVIRUS AFFECT THE LEVELS OF THE UL84 PROTEIN BUT NOT THE AMOUNT OF UL84 mRNA OR THE LOADING AND DISTRIBUTION OF THE mRNA ON POLYSOMES

ABSTRACT

The major immediate-early (MIE) region of human cytomegalovirus (HCMV) encodes two IE proteins, IE1 72 and IE2 86, that are translated from alternatively spliced transcripts that differ in their 3′ end. Two other proteins that correspond to the C-terminal region of IE2 86, IE2 60 and IE2 40, are expressed at late times. In this study, we used IE2 mutant viruses to examine the mechanism by which IE2 86, IE2 60 and IE2 40 affect the expression of a viral DNA replication factor, UL84. Deletion of aa 136 to 290 of IE2 86 results in a significant decrease in UL84 protein during the infection. This loss of UL84 is both proteasome- and calpain-independent, and the stability of the protein in the context of the mutant infection remains unaffected. The RNA for UL84 is expressed to normal levels in the mutant virus-infected cells, as are the RNAs for two other proteins encoded by this region, UL85 and UL86. Moreover, nuclear to cytoplasmic transport and the distribution of the UL84 mRNA on polysomes are unaffected. A region between aa 290-369 of IE2 86 contributes to the UL84-IE2 86 interaction *in vivo* and *in*

vitro. IE2 86, IE2 60, and IE2 40 are each able to interact with UL84 in the mutant-infected cells, suggesting that these interactions may be important for the roles of UL84 and the IE2 proteins. Thus, these data have defined the contribution of IE2 86, IE2 60, and IE2 40 to the efficient expression of UL84 throughout the infection.

INTRODUCTION

Human cytomegalovirus (HCMV) is the major viral cause of birth defects and poses a severe threat to immunocompromised and transplant patients (for review, see 132). Gene expression has been classified into three major groups, referred to as the immediate-early (IE), early, and late genes, which are temporally regulated throughout the infection. The two major IE (MIE) genes, IE1 72 and IE2 86 (encoded by UL122-123) are of particular interest for understanding the various regulatory mechanisms that govern a productive viral infection. They can transactivate viral early promoters, serve in viral promoter repression, and alter the expression of many host cellular genes in order to make the environment favorable for viral replication (for reviews, see 63, 132). Both MIE proteins arise from a single transcript that consists of five exons that are differentially spliced to produce IE1 72 (exons 1-4), and IE2 86 (exons 1-3, and 5) (197-198, 200). While IE1 72 is dispensable for infection at high multiplicity of infection (MOI), IE2 86 is essential (67, 73, 123, 131, 146-147, 222).

At late times in the infection, transcripts that arise from within exon 5 of the UL122 gene encode the late IE2 60 and IE2 40 proteins, which correspond to the C-terminal region of IE2 86 (158). The IE2 60 protein is expressed from an initiator methionine at aa 170, with the putative TATAA region occurring in the intron between exons 4 and 5. The IE2 40 protein is expressed from a 1.5 kb RNA, with translation initiating at methionine 242, and a putative TATAA box just upstream of the IE2 60 translation initiation site. It has been proposed that a small amount of IE2 60 protein is also expressed from the RNA encoding IE2 40 (223). These proteins have been shown to

have a role in transactivation, as well as repression of both MIE genes (95, 152, 158, 195). Our laboratory has determined that the IE2 60 and IE2 40 proteins also have a role in the expression of the two early-late viral proteins pp65 (UL83) and UL84; UL83 is a tegument protein, while UL84 is an essential DNA replication factor. Although IE2 60 and IE2 40 are dispensable for the infection, they are required for efficient replication at later stages in the viral life cycle (223).

Many of the functions of both IE1 72 and IE2 86 have been studied in transient transfection assays and, more recently, in the context of the infection using bacterial artificial chromosomes (BACs) with defined mutations in the genes (123, 146-147, 162, 168, 222-224). One viral mutant of particular interest lacks the region between aa 136-290 of IE2 86 (termed IE2 86ΔSX) (168, 223). Since the initiating methionines for IE2 60 and IE2 40 (aa 170 and 242, respectively) are deleted, this virus also does not express IE2 60 and IE2 40. Previous studies of the IE2 86ΔSX virus revealed that it grows slowly and is significantly debilitated in its ability to produce infectious virus. Furthermore, there is a severe lag in IE2 86 protein expression, although IE1 72 expression remains normal. Early genes do not seem to be affected, while expression of many late proteins is greatly reduced. In particular, UL83 and UL84 show significant decreases in protein expression during the later stages of the infection process, and these defects can be partially overcome in complementing cell lines that express IE2 86 and IE2 40 (168, 171, 223).

The only viral protein that IE2 86 has been shown to interact with in the infection is UL84 (167, 191). UL84 is present in low levels at early times in the infection and

accumulates to high levels after the onset of viral DNA replication (84). In transient assays, it is required for *ori-Lyt*-dependent replication (143, 174, 234, 238), and through its interaction with IE2 86 appears to be important for the activation of the bidirectional promoter within *ori-Lyt* (43, 233). However, it also down-regulates the ability of IE2 86 to activate some early promoters in transient assays (68). Other properties of UL84 are that it interacts with a RNA stem-loop sequence within the RNA/DNA hybrid region of ori-Lyt, displays UTPase activity, and shows some homology to the DExD/H box family of helicases (42, 44). However, many of its specific functions during the course of the infection remain to be determined.

Our previous work showing that the IE2 86ΔSX virus exhibits a severe dysregulation of UL84 at late times post infection (p.i.) (223), prompted us to examine the interactions and regulatory mechanisms that govern the expression of the UL84 and IE2 proteins at both early and late times p.i. Here, we show that infection with the IE2 86ΔSX virus results in a marked decrease in the protein expression of UL84 throughout the infection. In contrast, the transcription of UL84 and the mRNAs encoding UL85 and UL86, which are 3' co-terminal with the UL84 RNA (164), remains normal in this mutant infection. The defect in accumulation of the UL84 protein is after the initiation of translation, as nuclear export of the UL84 RNA, as well as the distribution of the mRNA on polyribosomes (polysomes), is unaffected in the IE2 86ΔSX infection. Immunoprecipitation analyses of lysates from cells infected with IE2 mutant viruses and *in vitro* GST binding assays reveal that a domain that plays a prominent role in the formation of a complex of IE2 86 and UL84 lies between aa 290-369 of IE2 86, although

there may be some contribution from other regions within the protein. Consistent with these results is the finding that UL84 is found in a complex with IE2 60 and IE2 40 in virus-infected cells. Taken together, these data suggest that an interaction of UL84 with IE2 86 alone may play a role in the expression of UL84, but this interaction is not sufficient for accumulation of normal levels of the UL84 and IE2 86 proteins. Moreover, an interaction of IE2 60 and IE2 40 with UL84 is likely necessary for proper expression of the UL84 protein and may help regulate the overall progress of the viral infection at late times.

MATERIALS AND METHODS

Construction of viruses.

The WT-EGFP, IE2 86ΔSX-EGFP, and Rev-EGFP viruses have been previously described (168). Other versions of the WT and IE2 86ΔSX virus are previously described in the paper by Sanders et al. (171), or were produced from the AD169 BAC in the same fashion as the enhanced-GFP (EGFP) viruses, except that the EGFP was not present in the recombinant BAC. Briefly, the IE2 86ΔSX C-F virus was constructed from the IE2 86ΔSX BAC. This virus contains the Cre and FLP recombinases which can be used on inducible cell lines to produce either IE2 86 using the Cre induction, or IE2 40 or IE2 60 using the FLP induction. These inductions are temporally regulated in that IE2 86 is expressed at the IE times of the infection (driven by the MIE promoter), while IE2 60 and IE2 40 are expressed at early-late times in the infection (driven by the HCMV 1.2 Kb promoter). A WT version of this virus was also constructed (HB5 C-F) for comparison (171). All versions of the IE2 86ΔSX virus have been extensively studied to ensure that each behaves identically to the original IE2 86ΔSX-EGFP virus. The IE2 86Δ88-290 and IE2 86Δ88-135 mutants were created using the Quik-change (Stratagene) protocol as described by manufacturer's instructions, except that separate PCR reactions were carried out for the forward and reverse reactions initially (5 cycles). The forward and reverse reactions were then pooled, and the Quik-change PCR was allowed to continue for 18 cycles. The following primers were used to create the mutations within the IE2 86 cDNA in the pSG5-J(BglII-StuI)WT vector:

IE2 86Δ88-290:

sense, 5′ CACCATCAGGTGACAGCCACCATGGGCGC 3′,

antisense, 5′ GCGCCCATGGTGGCTGTCACCTGATGGTG 3′;

IE2 86Δ88-135:

sense, 5′ CACCATCAGGTGACGGGGCATCCGCTACTCC 3′,

antisense, 5′ GGAGTAGCGGATGCCCCGTCACCTGATGGTG 3′.

Following mutagenesis, these mutations were placed into the UL122-123 coding region contained in the WT HCMV AD169 BAC pHB5 (gift of M. Messerle) (18) using a counter-selection BAC modification kit (Gene Bridges) to create a HCMV BAC containing the desired mutations as previously described (224).

The IE2 Δ40 mutant has been previously described (223). The M170L mutant virus was constructed as described above. The primers used to create the mutation at the start site of IE2 60 were:

sense, 5′ CTCCCGCGCCTATCCTCCTGCCCCTCATCA 3′,

antisense, 5′ TGATGAGGGGCAGGAGGATAGGCGCGGGAG 3′.

All mutations were confirmed by restriction digest and sequencing (Eton Biosciences). The recombinant BACs were digested with restriction endonucleases and subjected to field inversion gel electrophoresis (FIGE) to ensure that there were no rearrangements of the genome. All viruses were reconstituted, titered, and propagated from the BACs as previously described (222, 224).

Cell culture and infections.

Human foreskin fibroblasts (HFFs) were obtained from the University of California, San Diego Medical Center and cultured in Earle's minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 1.5 μg/ml amphotericin B (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). Cells were incubated at 37°C with 7% CO_2 and allowed to grow 3 days past confluency before infection for G_0-synchronization. At the time of infection, the HFFs (passage number 12-23) were trypsinized, re-plated, and infected at the MOI indicated. At various times p.i., cells were washed with phosphate-buffered saline (PBS), trypsinized, and processed accordingly.

Western Blotting.

Infected or mock-infected cells were harvested at various times p.i. Cells were lysed in reducing sample buffer containing 50 mM Tris [pH6.8], 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 50 mM leupeptin, 100 mM pepstatin A, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate, 5 mM β-glycerolphosphate. Samples then were sonicated to shear DNA and aid cellular lysis, and then assayed for protein content. Samples were heated to 95°C for 5 minutes and equivalent protein amounts were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Membranes were stained with amido black to assess protein loading, blocked in 5% non-fat milk in tris-buffered saline including 0.05% Tween-20, and then incubated with the following antibodies: CH16.0 mouse antibody (mAb) (1:10,000), UL84 mAb (1:2,000-

1:10,000), UL85 mAb (1:200), UL86 mAb (1:50), monoclonal IE2 86 mAb 8140 (1:1000), or β-actin mAb Ac-15 (1:5,000-1:10,000), washed, then incubated with horseradish IgG peroxidase (HRP)-coupled anti-mouse antibody (1:5000-1:10,000). CH16.0 was purchased from Virusys. Anti-UL84 antibodies used were kind gifts from G. Pari and E.S. Huang. Anti-UL85 and UL86 were kind gifts of B. Britt. Anti-IE2 86 was purchased from Chemicon, and anti-β-actin was purchased from Sigma-Aldrich. HRP-coupled anti-mouse IgG antibody was obtained from Calbiochem. After incubation and washes, proteins were detected with SuperSignal chemiluminescent substrate (Pierce Biotechnology) according to manufacturer's instructions.

Northern blot analyses.

HFFs were infected at an MOI of 5 pfu/cell with WT-EGFP, IE2 86ΔSX-EGFP, or rescued IE2 86ΔSX-EGFP (Rev-EGFP) viruses. Approximately $1.5X10^7$ cells were harvested at either 24 or 96 h p.i. and mRNA was prepared using the FastTrack 2.0 kit (Invitrogen). Northern blot assays were carried out using the NorthernMax kit (Ambion) according to manufacturer's instructions. Briefly, one microgram of mRNA per lane was resolved by agarose gel electrophoresis on a 1% formaldehyde gel, and then transferred to a nylon membrane. ^{32}P-labeled probes were synthesized by random priming using the StripEZ DNA kit (Ambion). A 650-bp UL84 probe was generated by restriction digest of the UL84 coding sequence using the vector PTARGET-UL84-HA (gift of G. Pari) with the Eco0901 enzyme. A UL83 probe was generated using the vector pEGFP-pp65 which was digested with BamH1 to remove the entire 1.7 Kb UL83 coding region. A β-actin

probe was also used as a control for loading. Membranes were hybridized overnight at 42°C, and following washes, were exposed to film for autoradiography.

Immunoprecipitations.

HFFs (3×10^5-5×10^5) were used per immunoprecipitation (IP) reaction. Briefly, cells were lysed in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl pH 7.6, 1 % NP-40, 1% Sodium deoxycholate, 0.1% SDS) for 30 minutes and then spun down to remove cellular debris. Lysate was allowed to incubate at 4°C with rotation overnight with ProteinG-PLUS agarose beads (Santa Cruz Biotechnology) coupled with the appropriate antibody. The next day, beads were washed twice in lysis buffer, heated to 95°C for 5 minutes and then collected for analysis by SDS-PAGE. An aliquot of the lysate was taken before addition to the beads (pre), as well as after incubation with the beads (post) to assess protein concentration before and after the IP had been carried out. For the sequential IPs, samples were immunodepleted of all IE1 72 and IE2 86 using the CH16.0 antibody as described above. Following this first IP, the post-binding supernatant was collected and used for a subsequent IP with another antibody that recognizes IE2 86, IE2 60, and IE2 40 (mAb 8140, Chemicon). The second IP therefore contained only IE2 60 and IE2 40 bound to the antibody/bead complex.

Molecular cloning of IE2 86-GST mutants and GST binding assays

The individual cDNAs of IE2 86 and all of the corresponding deletion mutants provided in the pGEX2TK vector have been previously described (188). Expression and purification of GST fusion proteins were carried out as previously described (106, 188) with the exception that GST fusion protein-bead complexes were resuspended in NETN

buffer (20mM Tris [pH 8.0], 1 mM benzamidine, 1 mM sodium metabisulfate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1mM EDTA [pH 8.0], 0.5 mg of bovine serum albumin per ml) containing 200 mM NaCl. Following normalized binding of the GST IE2 proteins, the *in vitro* translated protein was added to the mixture and allowed to incubate at room temperature for 1 h with constant rotation. Samples were extensively washed and bound protein was eluted in reducing sample buffer. Protein was then separated by SDS-PAGE. Following staining (GelCode Blue, Pierce Biotechnology) and drying, gels were analyzed for UL84 binding using autoradiography.

In vitro transcription/translation reactions.

In vitro transcription/translation experiments were conducted using the TNT Coupled Reticulocyte Lysate System (Promega) according to manufacturer's instructions. The PTARGET-UL84-HA vector (gift of G. Pari) containing the coding region for UL84 under control of the T7 promoter was used to obtain *in vitro* translated protein. Briefly, DNA was incubated with ^{35}S labeled methionine with the TNT reaction mixture for 90 minutes. *In vitro* translated protein was then either used immediately in the binding reaction, or was stored at -80°C for later use.

Drug treatments.

Approximately $1X10^6$ cells per time point were infected at an MOI of 5 pfu/cell. Calpeptin (Calbiochem) was added at either 18 h p.i. or 90 h p.i. at a concentration of 50 μM, and cells were incubated for 6 h, or treated with DMSO for control-treated samples. At 24 and 96 h p.i., cells were washed twice with PBS, and then harvested, snap frozen in

liquid nitrogen, and placed at -80°C. Whole cell lysates were prepared and protein was analyzed by Western blot. Lactacystin (Sigma-Aldrich) was added as above to a final concentration of 10 μM.

For the cycloheximide experiments, cells were infected at an MOI of 1 pfu/cell, and then at 36 h p.i. were either harvested to assess protein concentration, or were washed twice with media containing 100 μg/ml cycloheximide (Sigma-Aldrich) (or regular media for mock-treated samples), and, following washes, were control-treated or treated with cycloheximide (100 μg/ml) for 6 h. Cycloheximide was replenished 1 h after treatment. Following drug treatment, cells were harvested for Western blot analysis. Cells were snap frozen in liquid nitrogen and subsequently prepared for Western blot analysis. Approximately 5 X 10^5 cells were harvested per time point per virus.

Nuclear/Cytoplasmic Fractionation

Following infection at an MOI of 1-3 pfu/cell, cells were harvested at 24 h p.i., and the nuclear and cytoplasmic fractions were prepared using the PARIS kit (Ambion) according to the manufacturer's instructions. Briefly, cells were resuspended in ice cold cell fractionation buffer. Lysate was incubated for 10 minutes on ice, and then centrifuged for 5 minutes at 500×*g*. Supernatant was removed and saved as the cytoplasmic fraction. The pellet following centrifugation was washed in ice cold cell fractionation buffer, centrifuged again at 500×g. The pellet was then resuspended in cell disruption buffer, and mixed vigorously by pipetting. RNA was then isolated from both fractions using the Nucleospin RNAII kit (Clontech), and was also treated with turbo

DNase I (Ambion). RNA was then analyzed by real-time RT-PCR as described previously (223).

Polysome Fractionation

HFFs (3×10^7 cells per sample) were infected at an MOI of 1 pfu/cell with either the HB5 C-F or IE286ΔSX C-F viruses (171), and then at 48 h p.i., cells were incubated with cycloheximide (Sigma) (100μg/ml) in HFF media for 30 minutes. Cells were then washed with PBS containing cycloheximide (100μg/ml), trypsinized, and resuspended in polysome extraction buffer (140 mM KCl, 5mM $MgCl_2$, 20 mM Tris-HCl, 0.5 mM Dithiothreitol, and 0.1 mg/ml cycloheximide) with 1% TritonX-100 on ice. Lysates were incubated for 30 minutes on ice and then the lysate was centrifuged for 10 minutes at 10,000×G at 4°C. The supernatant was then either control-treated or treated with EDTA (50mM) for 20 minutes on ice. Sucrose gradients (10-50%) were poured using a Hoefer SB15 Gradient Maker (Amersham Biosciences). Supernatants were then layered gently onto the gradient and centrifuged in a Beckman L7 Ultra-centrifuge in a SW-55 swing bucket rotor at 39,000 RPM for 90 minutes at 4°C. Samples were then fractioned from the heaviest to the lightest fractions (16 fractions total) and dripped into SDS to achieve a final concentration of 1%. Absorbance was measured with a UV spectrophotometer throughout the fractionation. Samples were treated with Proteinase K (0.2 mg/ml) and incubated at 37°C for 90 minutes, and then extracted by phenol:chloroform treatment, followed by an ethanol precipitation at -20°C overnight. Samples were spun down the next day at 14,000 RPM for 30 minutes at 4°C, washed with 70% ethanol, and then spun again for 15 minutes. The supernatant was removed and pellets were allowed to dry.

RNA was then resuspended in TE. The appropriate fractions were pooled according to the polysome (absorbance) profile (6 pooled fractions total), and then each sample was DNased using the Turbo DNase I kit according to manufacturers instructions (Ambion). RNA was then analyzed by real-time RT-PCR as described previously (223). A cellular housekeeping gene G6PD was also assessed. The primers and probe for UL84, IE2 86, and G6PD have been previously described (223). The primers and probe for the UL44 gene are: FWD: 5'-GCTTTCGCGCACAATGTCT -3'

REV: 5'- GCCCGATTTCAATATGGAGTTC-3'

PROBE: 5'-56-FAM/CGT GCA CGC AGG CCG AGC/3BHQ1-3'

The quantified RNA from the real-time RT-PCR was then normalized to the total amount of RNA collected in each respective fraction, and then normalized again so that the value of the RNA collected in Fraction 6 from the WT virus-infected cells was equal to 1 to give relative quantities of RNA in each fraction.

RESULTS

UL84 protein expression is reduced in IE2 86ΔSX virus-infected cells.

The IE2 86 gene is encoded by the UL122 transcript and is alternatively spliced to include exons 1, 2, 3, and 5, while IE1 72 includes exons 1, 2, 3 and 4. Exon 5 also encodes both the IE2 60 and the IE2 40 proteins at late times p.i (152, 158, 195, 223). These proteins share the C-terminal portion of IE2 86, but they do not have any sequence in common with IE1 72 (Figure 3.1A). Figure 3.1B represents a schematic of the deletions within IE2 86 that were created and subsequently used throughout the course of our studies. The WT-, IE2 86ΔSX-, and Rev-EGFP viruses have been previously characterized (168). A version of the IE2 86ΔSX virus lacking EGFP expression (IE2 86ΔSX) was also constructed to ensure that the defects seen in the mutant virus infection were not due to the addition of EGFP to IE2 86. Another version of the IE2 86ΔSX which encodes the Cre and FLP recombinases (IE2 86ΔSX C-F) was also used in these studies, and has been previously described (171). This virus can be grown on complementary cell lines expressing IE2 86 and IE2 40 in order to obtain higher titer virus, thus allowing analysis of this virus in large scale experiments. The presence of the Cre and FLP recombinases does not affect the growth of the virus on HFFs however, and only allows generation of higher titer virus following preparation on complementing cell lines (171). Two other recombinant viruses, IE2 86Δ88-290 and IE2 86Δ88-135, were constructed using the AD169 pHB5 BAC that does not contain EGFP (Figure 3.1B) to further assess the role of the N-terminal portion of exon 5 of IE2 86. In addition, mutants lacking expression of either IE2 60 (M170L) or IE2 40 (IE2 Δ40) were analyzed to

further define the functional roles of IE2 60 and IE2 40. These viruses contain mutations to disrupt either the putatative TATAA box for IE2 Δ40 or the initiator methionine for the M170L virus (Figure 3.1B, and 223).

Previous characterization of the IE2 86ΔSX mutant virus revealed that the kinetics of the infection are slower and the virus grows to reduced titers (168). The mutant virus also expresses markedly lower levels of IE2 86, as well as two early-late proteins, UL83 and UL84. IE1 72 and early gene expression remain normal (168, 223). This mutant is missing the coding region of IE2 86 between aa 136-290 of exon 5, which includes the initiator methionines for two smaller IE2 proteins (IE2 60 and IE2 40) that appear later in the infection, the sumoylation sites (aa 175 and 180), and the domain that interacts with SUMO-1 (aa 200-208) (3, 90, 95, 112-113, 158, 168, 223). Furthermore, expression of IE2 86 and IE2 40 from inducible cell lines allows recovery of some of these phenotypes, especially at the later stages of the infection (171).

Since IE2 86 has an effect on the regulation of other early-late proteins throughout the infection but is known to interact with only one other viral protein, UL84, we compared the expression of UL84 during the IE2 86ΔSX-EGFP virus infection to that during the WT- and IE2 86ΔSX Revertant-EGFP (Rev) virus infections. To assess the levels of UL84 protein, HFFs were infected at an MOI of 5 pfu/cell and then harvested 12-96 h p.i. Lysates were prepared and then analyzed by Western blotting with a monoclonal antibody specific for UL84. Here, we show that throughout the entire infection, there is a significant reduction in the production of the UL84 protein in HFFs infected with the IE2 86ΔSX-EGFP virus, with the effect more pronounced at late times

p.i. (Figure 3.2A). In the WT-EGFP infection, the level of UL84 steadily increases between 24 and 96 h, and can be detected as early as 12 h p.i., whereas in the IE2 86ΔSX-EGFP infection, the protein shows a severe decrease in expression (Figure 3.2A). The Rev-EGFP virus fully recovers expression of UL84, indicating that this loss of UL84 expression is due to the deletion within IE2 86, and not due to any other mutation created during mutagenesis of the BAC (Figures 3.2A). Furthermore, when assessed by immunofluorescence assays, UL84 in the IE2 86ΔSX infection is still present in the replication centers where IE2 86 is known to accumulate at late times p.i., indicating that UL84 may also be functioning in replication processes in the mutant infection (data not shown). All of the above data were confirmed with the IE2 86ΔSX and IE2 86ΔSX C-F viruses to rule out any possibility that the virus was impaired due to the presence of EGFP on IE2 86 (data not shown). In order to ensure that the WT-, IE2 86ΔSX- and Rev-EGFP infections were properly matched, other viral proteins that are known to be expressed to normal levels in the IE2 86ΔSX mutant virus-infected cells were assessed and shown to be comparable (data not shown).

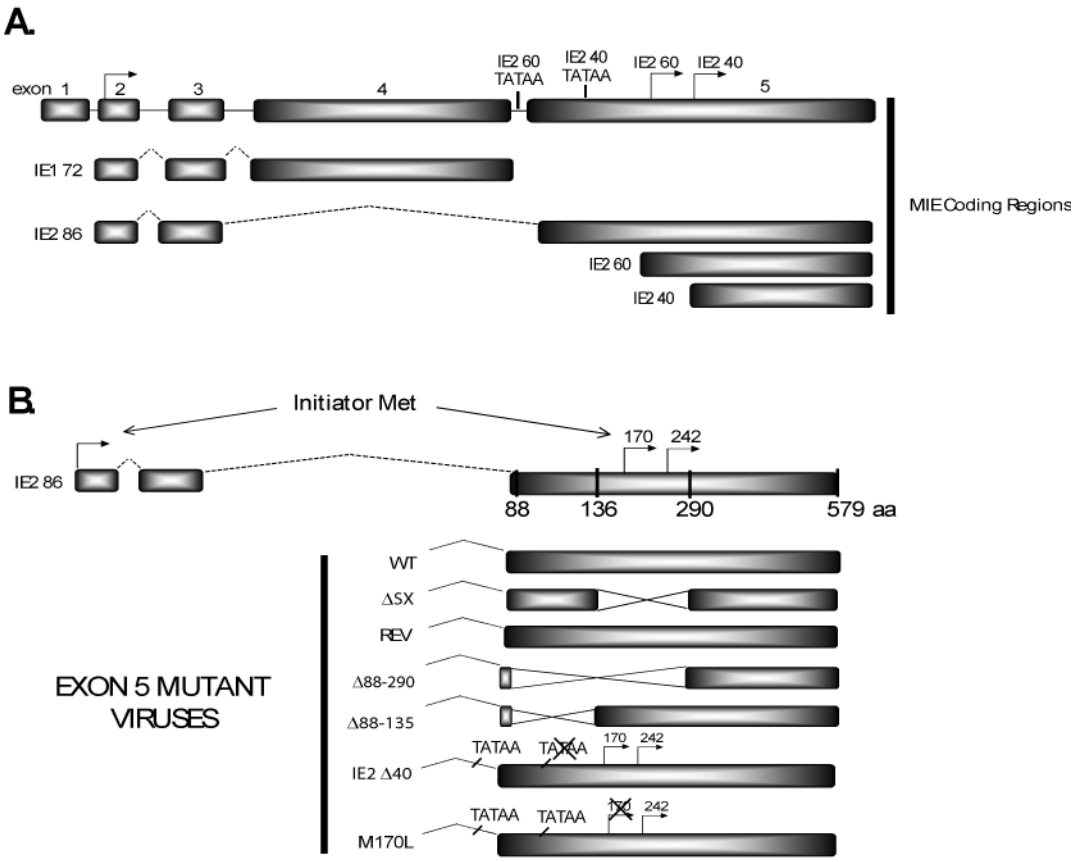


Figure 3.1. Schematic representation of the UL122-123 transcripts and deletions created within the IE2 86 coding regions of mutant viruses. (A) UL122-123 transcripts and representation of the MIE genes, IE1 72 and IE2 86, in the wild-type HCMV coding region. Exons 1, 2, 3, and 4 specify IE1 72 mRNA, while exons 1, 2, 3, and 5 specify IE2 86 mRNA. Translation begins at exon 2 for both IE1 72 and IE2 86, while translation of IE2 60 and IE2 40 begins at aa 170 and 242, respectively. (B) Schematic of the mutant viruses used in these analyses to identify the dysregulation of UL84, as well as the important binding domain within IE2 86 for UL84. The WT, IE2 86ΔSX (ΔSX), and a revertant form of the IE2 86ΔSX (REV) are represented in the schematic. Other deletion mutants created, including Δ88-290 and Δ88-135, IE2 Δ40 mutants are also shown. An additional mutant virus, M170L, was constructed that contains a mutation in the initiator methionine for IE2 60. Diagrams are not to scale.

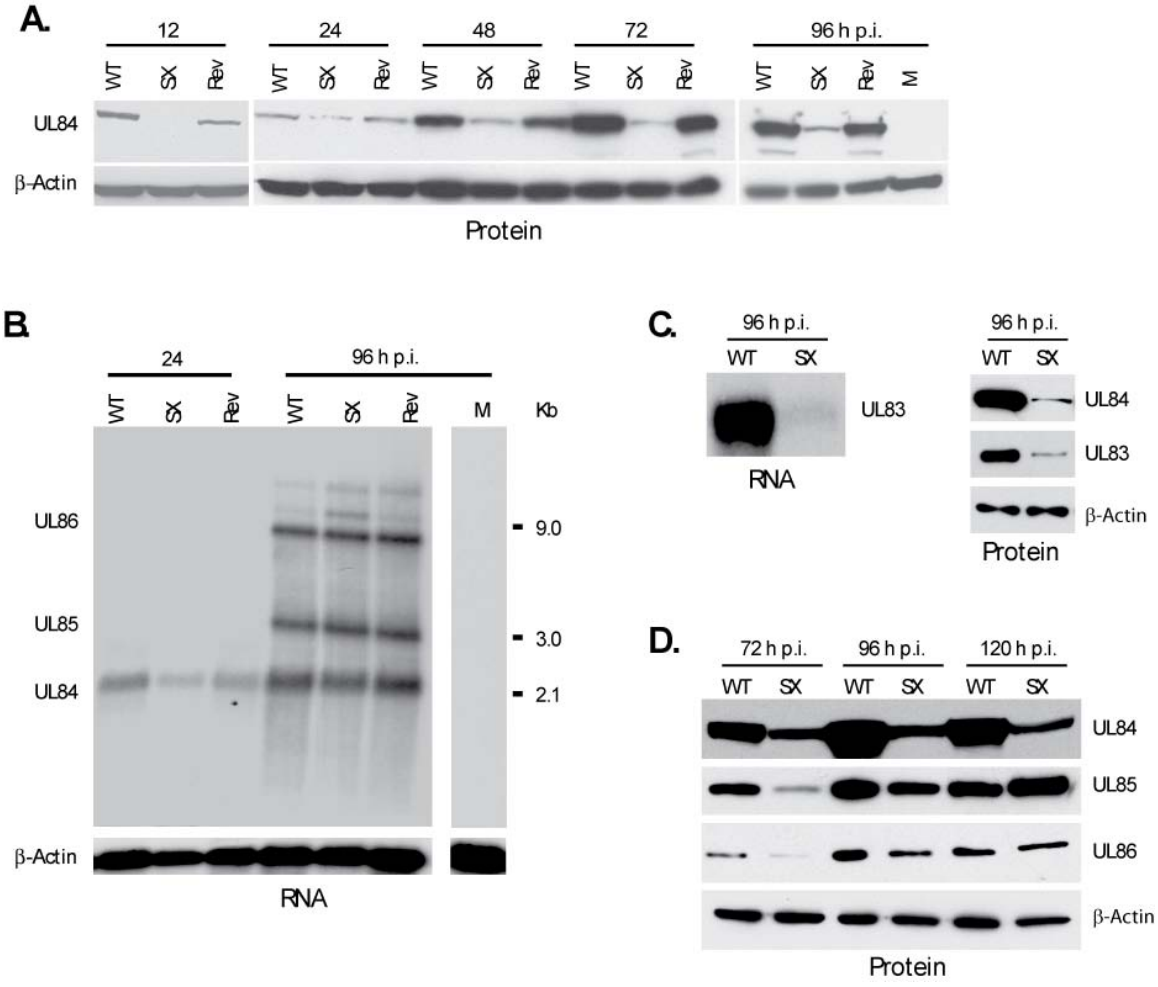
Transcription of UL84, UL85 and UL86 is not affected in IE2 86ΔSX-EGFP infected cells.

Our lab has recently shown that although protein expression is debilitated at late times p.i., UL84 RNA remains unaltered as analyzed by quantitative real-time RT-PCR. Furthermore, a mutant that does not express either IE2 60 or IE2 40 (IE2 Δ40+60) also shows no loss of the UL84 transcript, even though protein levels are significantly decreased, similar to that of the IE2 86ΔSX infection by 96 h p.i. (223). Since overlapping transcripts are encoded by the UL84 coding region at late times (84, 164), we used Northern blot analyses to assess each of the transcript levels at both early and late times. Briefly, HFFs were infected at an MOI of 5 pfu/cell, and then harvested at either 24 or 96 h p.i. mRNA was isolated and then separated by denaturing agarose gel electrophoresis. Levels of mRNA were then analyzed using a probe that recognizes UL84, UL85, and UL86, as well as a probe that recognizes β-actin (as a loading control). Figure 3.2B shows that at 24 h p.i., a 2.1 kb transcript, which encodes UL84, was expressed in WT-, IE2 86ΔSX-, and Rev-EGFP infected cells. At 96 h p.i., the 2.1 kb transcript increased in abundance, and transcripts of approximately 3.0 and 9.0 kb, encoding UL85 and UL86, respectively, were also expressed in cells infected with all three viruses. Low levels of other high molecular weight transcripts, which likely are read-through RNAs, also appear at late times. At both 24 and 96 h p.i., the levels of each of the transcripts were not significantly affected in cells infected with the WT-, IE2 86ΔSX-, and Rev-EGFP viruses. There was a slight decrease in the levels of the UL84 mRNA, but this difference was minimal, and likely does not account for the dramatic loss

of UL84 at the protein level. These data confirmed the previous quantitative real-time RT-PCR data (223), providing additional evidence that the reduced levels of UL84 protein in the IE2 86ΔSX infection was not due to reduced levels of RNA. Real-time RT-PCR assays of the samples used for the Northern analyses were also performed in parallel to confirm these results and no differences were seen between any of the three viruses at either 24 or 96 h p.i. (data not shown). To confirm that a previously characterized decrease in the UL83 mRNA was occurring, samples from the same infection as in Figure 3.2B were analyzed by Northern analyses. At 96 h p.i., the IE2 86ΔSX-EGFP exhibited a severe defect in the expression of UL83 mRNA (Figure 3.2C), which is consistent with previous reports (168). The UL83 protein exhibits a comparable decrease to that of UL84 at this time p.i. (Figure 3.2C), further emphasizing the differences in the type of regulation of these two transcripts. These studies led our investigation of the potential role of IE2 86, IE2 60 and IE2 40 in UL84 expression at the post-transcriptional level.

We also analyzed the protein expression of UL85 and UL86 in these studies to determine if they were being regulated in a similar fashion as UL84. Cells were infected at an MOI of 5, and then, at late times p.i, cells were analyzed by Western blot for the expression of these proteins. At 72 h p.i., the UL84, UL85, and UL86 proteins exhibited similar decreases in the IE2 86ΔSX virus-infected cells as compared to the WT virus-infected cells in all cases (Figure 3.2D). However, by 96 and 120 h p.i., the UL85 and UL86 protein expression had recovered, while the UL84 protein level was still lower than that in the WT virus-infected cells. The decrease in the late UL85 and UL86 proteins is

Figure 3.2. Analysis of UL84, UL85, UL86 and UL83 RNA and protein at late times p.i. (A) G_0-synchronized HFFs were infected at an MOI of 5 pfu/cell with the WT- (WT), IE2 86ΔSX- (SX), and Rev-EGFP (Rev) viruses, or were mock-infected (M) and harvested at times indicated p.i. (12-96 h). The mock lane was added to indicate that the UL84 antibody was specific for the viral protein. An equal amount of protein from the cell lysates was analyzed by Western blot analyses. β-Actin was used as a loading control. (B) Analysis of the UL84, UL85, and UL86 transcripts in the IE2 86ΔSX-EGFP infection. HFFs were infected or mock infected (M) at an MOI of 5 pfu/cell with the WT-, IE2 86ΔSX-, or Rev-EGFP viruses, and then harvested at either 24 or 96 h p.i. mRNA was oligo-dT selected, resolved by agarose gel electrophoresis, and then transferred to a nitrocellulose membrane. ^{32}P-labeled probes were synthesized that recognize UL84, UL85, and UL86 or β-actin. Following hybridization of the probe, membranes were exposed to film for autoradiography. The 3 transcripts occurring at 2.1, 3.0, and 9.0 Kb encode the UL84, UL85, and UL86 RNA transcripts, respectively. Cellular β-Actin mRNA served as a loading control. A representative example of 3 experiments is shown. (C) The same mRNA as in (B) was analyzed by Northern blot for the expression of the UL83 mRNA transcript at 96 h p.i., when UL83 is most abundant. Both the WT-EGFP and IE2 86ΔSX-EGFP viruses-infected cells are shown. The UL84 and UL83 protein from this infection is also shown for comparison at 96 h p.i for the 2 viruses. (D) Western blot of the UL84, UL85, and UL86 proteins at 72, 96, and 120 h p.i. are shown for the WT- and IE2 86ΔSX-EGFP viruses. Actin serves as a loading control.


A.
12
24
48
72
96 h p.i.
WT
SX
Rev
M
UL84
β-Actin
Protein
B.
24
96 h p.i.
Kb
9.0
3.0
2.1
UL86
UL85
UL84
β-Actin
RNA
C.
96 h p.i.
UL83
RNA
UL84
UL83
β-Actin
Protein
D.
72 h p.i.
96 h p.i.
120 h p.i.
UL84
UL85
UL86
β-Actin
Protein

consistent with the growth kinetics of the mutant virus, and confirms previous observations that there is a lag in the expression of many late genes (168). These data indicate that UL84 is also regulated in a different fashion than UL85 and UL86.

UL84 RNA in the IE2 86ΔSX virus-infected cells does not show a defect in nuclear export compared to WT.

Given that our analyses of the UL84 RNA revealed no dramatic differences in transcript expression, we next determined whether the RNA was being exported to the cytoplasm appropriately. Briefly, cells were infected at an MOI of 1-3 pfu/cell with either the WT or IE2 86ΔSX viruses, harvested at 24 h p.i., and then the nuclear and cytoplasmic fractions were separated by centrifugation. RNA was prepared from each fraction and then analyzed by quantitative real-time RT-PCR. Interestingly, these analyses showed that in the IE2 86ΔSX mutant-infected cells, the RNA was distributed between the two fractions in a comparable fashion as the WT virus-infected cells (Figure 3.3). At this time p.i., the distribution of the UL84 RNA was more cytoplasmic than nuclear in cells infected with both viruses. Distribution of the GAPDH RNA and protein was assessed in order to confirm that the fractionation had occurred appropriately (data not shown). These data indicated that the RNA is being appropriately exported out of the nucleus in order to be translated.

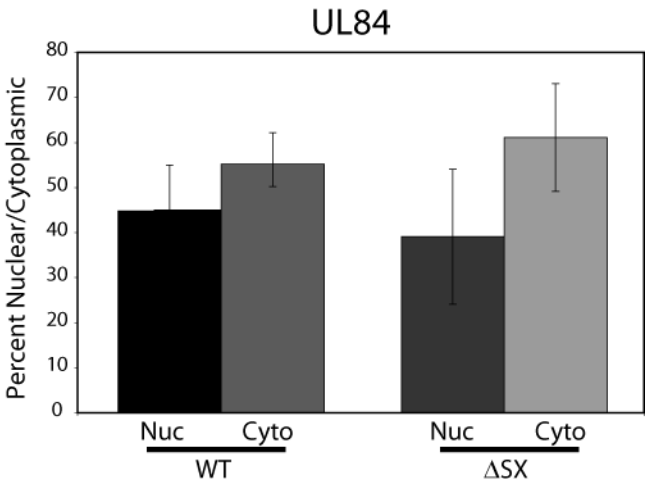


Figure 3.3. **Nuclear-cytoplasmic distribution of the UL84 RNA in the WT and IE2 86ΔSX infection.** At 24 h p.i., cells were harvested and the nuclear and cytoplasmic fractions were separated by centrifugation. RNA was prepared from each fraction, and quantitated by quantitative real-time RT-PCR. The percent nuclear versus cytoplasmic was calculated for each sample set. The nuclear fraction (Nuc) and cytoplasmic fraction (Cyto) are shown for both the WT and IE2 86ΔSX (ΔSX) virus-infected cells. Each fraction was normalized to a cellular house keeping gene (G6PD) to account for the amount of RNA in each reaction. An average of 3 experiments is shown, with error bars representing the margin of error between experiments.

There is no defect in the loading or distribution of UL84 or IE2 86 mRNA on polysomes in the IE2 86ΔSX virus-infected cells.

To determine whether the UL84 RNA was being appropriately loaded onto polysomes in the IE2 86ΔSX infection, we next assayed the distribution of the RNA in a polysome fractionation assay. Due to the large number of cells necessary for these analyses, we used a stock of both the WT and IE2 86ΔSX viruses that had been prepared on complementary cell lines that expressed both IE2 86 and IE2 40. The characterization of these recombinant viruses (termed HB5 C-F and IE2 86ΔSX C-F) and the complementary cell lines are described in the recent paper by Sanders et al. (171). These viruses have the same growth properties as the WT and IE2 86ΔSX viruses lacking the Cre and FLP recombinases (171). Cells were infected at an MOI of 1 pfu/cell with either the WT C-F or the IE2 86ΔSX C-F viruses, and then harvested at 48 h p.i. Immediately following harvest, the cells were lysed and the cytoplasmic fraction was separated from the nuclear fraction. This cytoplasmic fraction was either control-treated or treated with EDTA, separated on a sucrose gradient by ultra-centrifugation and then fractioned from the heaviest to the lightest fractions. Treatment of polysome extracts with EDTA has been shown to promote free ribosomes to disassemble into their 40S and 60S subunits, causing a release of the bound mRNA. This results in a shift of the mRNAs into the lighter fractions. The mRNAs bound to large ribonucleoproteins (RNPs), however, should be unaffected by this treatment (13), allowing confirmation that the mRNA measured in the samples lacking EDTA are actually loaded onto polysomes. Absorbance of the polysomes (A_{254}) was measured throughout collection (Figure 3.4A). RNA was

then prepared from each fraction, and analyzed by real-time RT-PCR analyses. At this point in the infection, only the UL84 RNA is present and thus the 3′ co-terminal RNAs expressing UL85 and UL86 do not complicate the real-time RT-PCR analyses (data not shown).

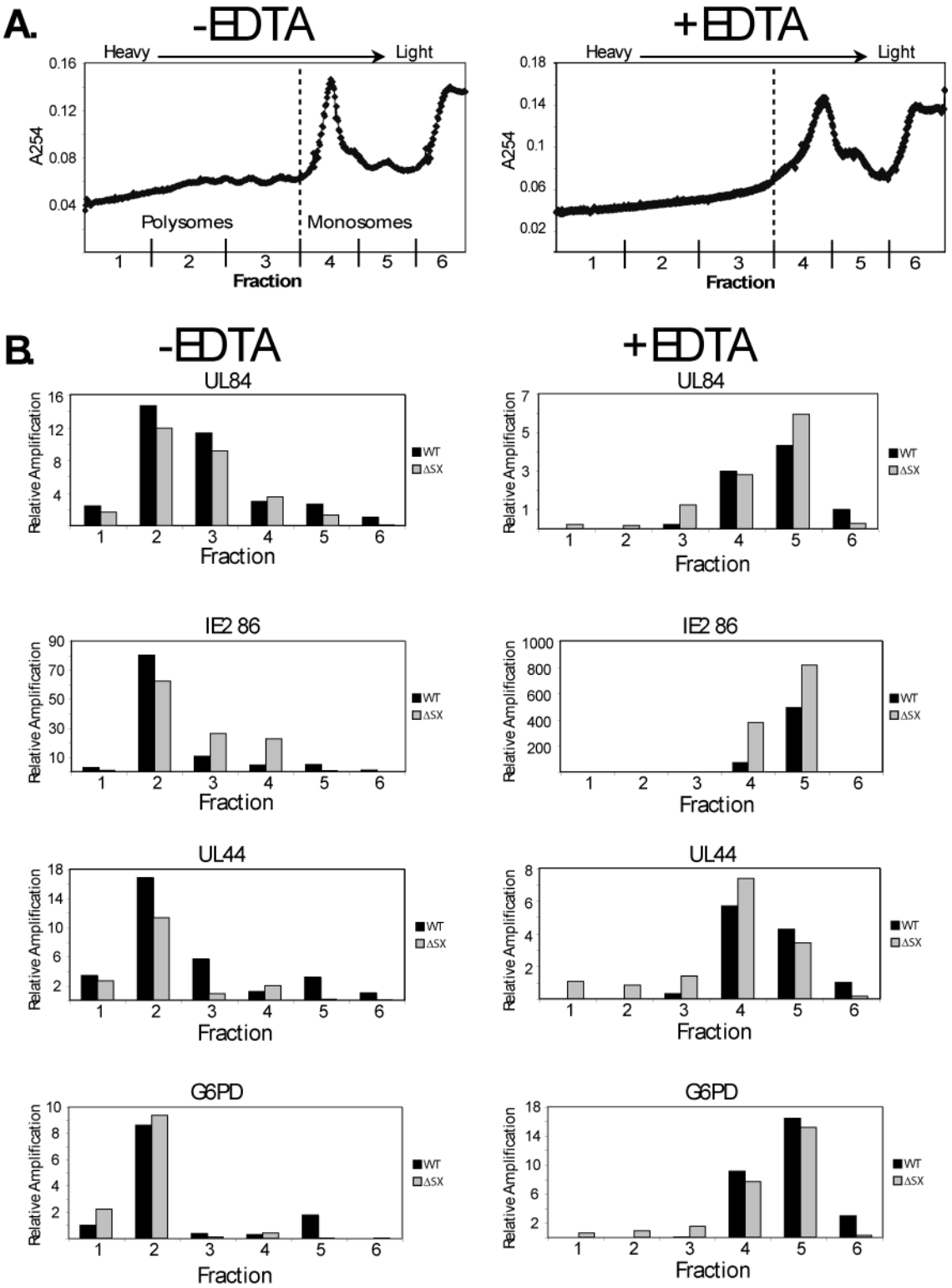
Figure 3.4A shows a representative polysome distribution following fractionation. In the samples without EDTA treatment, the polysome fractions are separated into Fractions 1, 2, and 3. The monosome fractions are represented by Fraction 4 and 5. Fraction 6 represents the lightest fraction containing free RNP RNA. In the EDTA treated (+EDTA) samples, the polysome peaks are no longer visible, indicating that the EDTA treatment was efficient in disrupting the polysomes before fractionation. All quantities were normalized to the WT Fraction 6 (value equal to 1) to compare the relative amounts of RNA from the two viruses present in each fraction. A representative of three different experiments is shown for Figure 3.4A and 4B.

At 48 h p.i., the distribution of the UL84 RNA was measured for both the WT and IE2 86ΔSX virus-infected cells (Figure 3.4B). Interestingly, no major differences were found between the two viruses in regard to the loading or distribution of the UL84 RNA on polysomes. Fractions 2 and 3 (-EDTA) contained the most RNA, were comparable between the two infections, and no major shift in the distribution of the RNA was detected. Treating the samples with EDTA shifted the RNA most predominantly to Fractions 4 and 5, indicating that the RNA measured in the untreated samples was in fact loaded onto the polysomes. These data indicated that the UL84 RNA is appropriately

loaded onto the polysomes in the IE2 86ΔSX virus-infected cells when compared to the WT virus-infected cells.

The distribution of the IE2 86, UL44, and G6PD RNAs was also analyzed (Figure 3.4B). It is known that the levels of the RNA and protein for UL44 (early protein) remain unaffected in the IE2 86ΔSX virus-infected cells (168) and unpublished data). IE2 86 RNA is unaffected in these virus-infected cells, but the IE2 86 protein exhibits decreased expression (168). In each case, the distribution of each of the RNAs was comparable between the two virus-infected cells. Small differences in amount of RNA in each fraction were noted between experiments, but the difference was not more than 2-fold. The slight shift in the distribution of the IE2 86 RNA on the polysomes in the IE2 86ΔSX infected cells is likely due to the smaller size of the transcript as a result of the deletion in exon 5. This result was consistent between experiments. In the untreated samples, the IE2 86 RNA was present most predominantly in the 2nd fraction, as was the UL44 RNA, and each fraction was comparable between the two viruses in both cases. The distribution of G6PD (cellular housekeeping gene) was also assessed and showed no difference in distribution. All samples treated with EDTA also showed that the RNA shifted into the lighter fractions (4 and 5), indicating that the measurements of RNA in the untreated samples were loaded onto the polysomes, and that treatment with EDTA shifted the RNA into the monosomal fractions as expected.

Figure 3.4. Polysome distribution of the UL84, IE2 86, UL44, and G6PD RNAs in the WT and IE2 86ΔSX infections. (A) Absorbance measured during fractionation from the heaviest to the lightest fractions. Fractions were pooled into 6 separate samples (Fraction 1-6), and are represented in the figure. Absorbance (A254) was measured throughout the collection. The polysomal fractions are represented in fractions 1, 2, and 3. The monosomal fractions are represented in fractions 4 and 5. Fraction 6 represents the free RNP RNA. (B) The distribution of the RNA and quantification of each fraction is shown for UL84, IE2 86, UL44, and G6PD. Samples were either treated with EDTA (+EDTA) or mock-treated (-EDTA). Quantified RNA was measured by real-time RT-PCR and then normalized to the total amount of RNA collected in that fraction. Relative amounts of RNA are shown compared to the total WT RNA present in Fraction 6 for each gene, which is set to a value of 1. The RNA from the WT virus-infected cells are represented by the black bars, while the RNA from the IE2 86ΔSX virus-infected cells are represented by the grey bars.


A.
-EDTA
+EDTA
Heavy
Light
A254
Polysomes
Monosomes
Fraction
B.
UL84
IE2 86
UL44
G6PD
Relative Amplification
WT
ΔSX

Loss of UL84 expression is not due to increased proteasome or calpain-dependent degradation, and the UL84 protein in the IE2 86ΔSX virus-infected cells is stable.

To determine whether UL84 is being degraded in a proteasome-dependent manner in the IE2 86ΔSX infection, a proteasome inhibitor, lactacystin (10 μM), was added at either 18 or 90 h p.i., and then cells were harvested 6 h later at either 24 or 96 h p.i., respectively. The addition of this inhibitor did not result in increased levels of UL84 expression at either early or late times of the infection (Figure 3.5A). Furthermore, addition of the calpain inhibitor, calpeptin (50 μM), which is known to inhibit the Ca^{2+}-stimulated cleavage of p35 to p25 by calpain (209), during the same period was unable to rescue the levels of UL84 (Figure 3.5B). As seen at both 24 and 96 h p.i. in the lanes treated with either drug, the levels of UL84 are still much lower than that of the WT-EGFP infection and remain comparable to that of the untreated samples (Figure 3.5A and B). These results demonstrate that the low levels of UL84 are not due to enhanced degradation of the protein by either the proteasome or calpain.

To assess whether the translation or stability of UL84 was impaired in the IE2 86ΔSX mutant virus, protein levels were analyzed following treatment with cycloheximide. HFFs were infected at an MOI of 1 pfu/cell with the WT and IE2 86ΔSX viruses and at 36 h p.i., cells were either harvested to analyze the amount of protein present prior to treatment, or were control-treated or treated with 100 μg/ml cycloheximide. The drug treatment was stopped 6 h later (42 h p.i.), and following washes with PBS, cells were harvested immediately. Our results show that there was a decrease in the amount of UL84 present in the WT virus-infected cells immediately

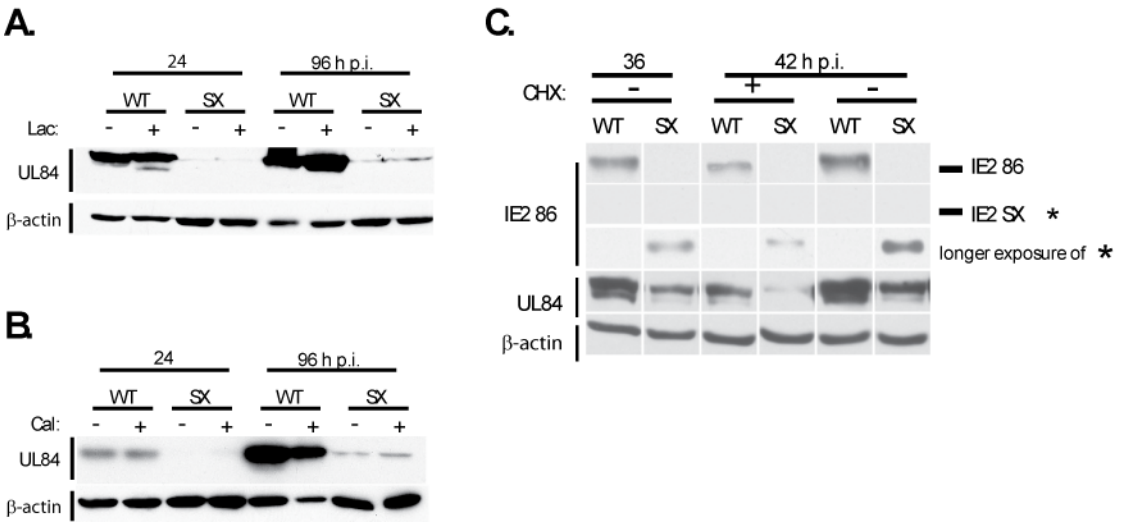


Figure 3.5. Analysis of proteasome and calpain dependent degradation in the IE2 86ΔSX and WT-infected cells and assessment of the stability of UL84 and IE2 86 protein in these infections. (A) HFFs were infected at an MOI of 5 pfu/cell with either the WT- or IE2 86ΔSX-EGFP (SX). Lactacystin (10 μM) (Lac, +), or DMSO for mock-treatment (-), was added at either 18 or 90 h p.i., and the cells were incubated for 6 h. Cells were harvested at 24 and 96 h p.i. and then analyzed for UL84 protein expression by Western blot analyses. (B) HFFs were infected as above, and then treated with Calpeptin (50 μM) (Cal, +) for 6 h, or mock-treated with DMSO (-). Cells were harvested and then lysates were analyzed by Western blotting for UL84 protein expression. (C) Cells were infected at an MOI of 1 pfu/cell with the WT or IE2 86ΔSX (SX), and at 36 h p.i. were either harvested to assess protein concentration before drug treatment, or were treated with cycloheximide (100 μg/ml) (CHX, +) or DMSO (-) as a control for 6 h. Following treatment, cells were harvested at 42 h p.i. to analyze the loss of UL84 and IE2 86 during that time. Two exposures of the IE2 86 blot are shown in order to visualize the small amount of IE2 86 in the IE2 86ΔSX infection (∗). β-Actin was analyzed in all of the above experiments in order to assess protein loading.

following drug treatment as compared to the untreated sample, however a portion of the protein still remained (Figure 3.5C). There was also a comparable decrease in the amount of UL84 present in the IE2 86ΔSX infection following treatment, indicating that the protein was not turning over more rapidly than in the WT infection (Figure 3.5C). These data reveal that the stability of UL84 that is measured by the assay remains the same compared to the WT infection. However, it has not been determined whether the stability of free UL84 is the same as UL84 complexed with other proteins such as IE2 86.

Since we had previously observed that in the IE2 86ΔSX infected cell, IE2 86 levels were severely diminished, but RNA levels remained unaffected (168), we also assessed the stability of the IE2 86 protein. IE2 86 appears to be slightly more stable than UL84, but the difference between the cycloheximide-treated and untreated samples is comparable for both the WT virus and the mutant virus (Figure 3.5C). As expected, the amount of IE2 86 remains much lower in the IE2 86ΔSX virus-infected cells than that in the WT virus-infected cells. A longer exposure of the IE2 86ΔSX is shown in order to more readily assess the levels of IE2 86 present in the IE2 86ΔSX infection.

IE2 86, IE2 60 and IE2 40 are able to interact with UL84 throughout the infection.

The interaction between UL84 and IE2 86 has been shown to be important for inhibiting IE2 86-mediated transactivation of the early and late genes as judged by co-transfection assays (43, 68, 235). However, this interaction has not been extensively characterized in the context of the HCMV infection. To further illustrate the contribution of IE2 86, IE2 60 and IE2 40 to UL84 expression, we assessed whether these proteins had the ability to interact with UL84 throughout the entire infection, which might further

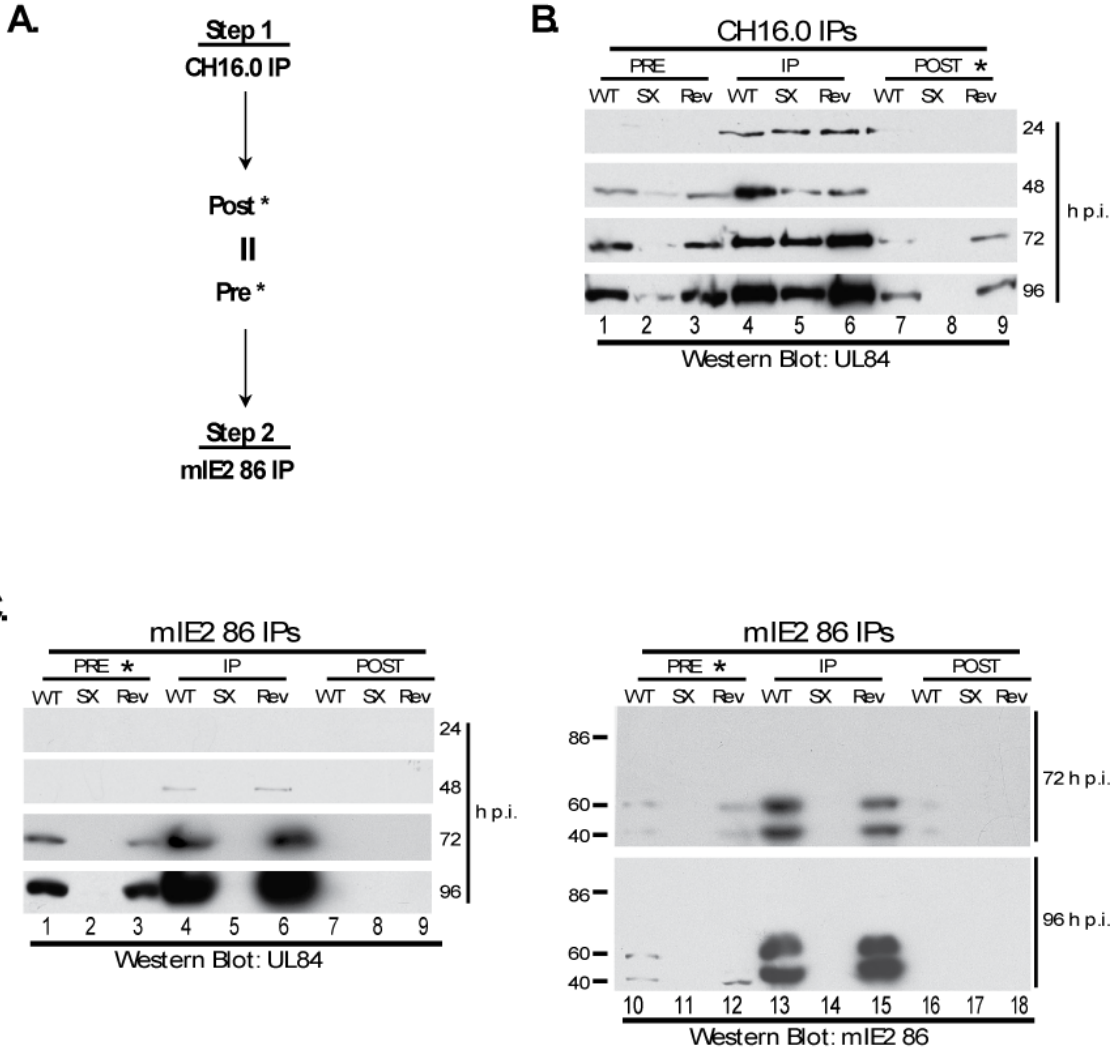
allude to their involvement in UL84 function. Furthermore, we wanted to address whether IE2 86ΔSX could interact with UL84. We first performed immunoprecipitations (IPs) that selectively immunodepleted the lysates of full-length IE2 86 and IE1 72 (using the CH16.0 mAb). This antibody would not immunoprecipitate IE2 40 or IE2 60 unless they had formed heterodimers with IE2 86. In addition since it is known that UL84 does not interact with IE1 72, any interaction seen in the IP is due to the interaction between IE2 86 and UL84 (68). Subsequent IPs with the monoclonal IE2 86 antibody (mIE2 86) were then used to assess whether IE2 60 and IE2 40 were in complex with UL84 in the absence of the full-length protein (For schematic, see Figure 3.6A).

Briefly, HFFs were infected at an MOI of 1 pfu/cell and then harvested at various times p.i. Cells were first immunoprecipitated with the CH16.0 antibody that recognizes only full-length IE2 86 and IE1 72 (both the IE2 86ΔSX and WT versions), but not IE2 60 or IE2 40 (Figure 3.6A, Step 1). However, as note above, IE2 60 and IE2 40 that is present as a heterodimer with IE2 86 should be immunoprecipitated in Step 1, as the dimerization domain is common to all three proteins (2, 39, 64). Following incubation, the bound proteins were eluted from the agarose beads and analyzed by Western blot for the presence of the UL84 protein. A significant amount of the UL84 in the cells infected with WT-, IE2 86ΔSX-, and Rev-EGFP virus was in a complex with IE2 86, as judged by the protein present in the IP lanes (Figure 3.6B, lanes 4-6). However, a substantial amount of the UL84 protein was not immunoprecipitated in the WT- and Rev-EGFP infections at late times and was present in the Post-IP samples (Figure 3.6B, lanes 7-9). These blots were also probed with the CH16.0 antibody to confirm that IE2 86 had been

immunodepleted during the IP (data not shown). Further confirming this, Western blot analysis with the mIE2 86 antibody showed that the full-length protein was efficiently removed from the post-IP supernatant (see below). The pre- and post-binding lanes correspond to approximately 10% of the sample used for the IP (Figure 3.6B, Lanes 1-3 and 7-9, respectively). These data show that IE2 86 and IE2 86ΔSX are interacting with UL84 throughout the entire infection, but that there is UL84 that is not in complex with IE2 86 in the WT infection.

To further determine whether the UL84 that was not in complex with IE2 86 was bound to the IE2 60 and IE2 40 proteins, the post-IP (Figure 3.6B, POST*, lanes 7-9) samples from the previous experiment were subjected to an IP with a monoclonal IE2 86 antibody (mIE2 86) that recognizes the C-terminal region included in the full-length IE2 86, IE2 60 and IE2 40 (indicated as Step 2 in the schematic shown in Figure 3.6A). We confirmed that IE2 60 and IE2 40, but not IE2 86 were present in this IP by Western blotting with the monoclonal IE2 86 antibody (Figure 3.6C, lanes 10-18), as well as the CH16.0 antibody (data not shown). Western blot analysis with the antibody to UL84 showed that the protein is very abundant in the IP lanes corresponding to the WT and Rev-EGFP infections, but not in the IE2 86ΔSX-EGFP infection (Figure 3.6C, lanes 4-6). This is as expected, given that IE2 60 and IE2 40 are not produced in the IE2 86ΔSX infection. Furthermore, since no UL84 protein could be detected in the binding reaction for the IE2 86ΔSX-EGFP infection (Figure 3.6C, lane 5), these results also indicate that all full-length IE2 86 was removed in the first binding experiment with the CH16.0 antibody, and that any interaction seen would be due to the IE2 60 and IE2 40 proteins.

Figure 3.6. UL84 interacts with IE2 60 and IE2 40 without the contribution of IE2 86. (A) A schematic representation of the experiment is shown, indicating that the Post of the CH16.0 IPs (Step 1) is the same sample as that used for the Pre in the mIE2 86 IPs (Step 2). The corresponding Post and Pre samples are denoted with a * in all subsequent parts of the figure. (B) IPs were carried out exactly as in described in Figure 6A, and cells were harvested 24-96 h p.i. The pre (PRE, lanes 1-3) and post (POST*, lanes 7-9) equal approximately 10% of the IP (lanes 4-6). (C) The Post-binding supernatant (in Panel A, this corresponds to POST*, lanes 7-9) was subjected to a following IP with an antibody that recognizes IE2 86, IE2 60 and IE2 40 (mIE2 86) to determine if UL84 interacts with IE2 60 and IE2 40 in combination, without the presence of IE2 86. UL84 was analyzed again by Western blotting (lanes 1-9). PRE* indicates that this corresponds to the POST* supernatant of the first IP in (B). The second set of IPs (mIE2 86 IPs) were also analyzed by Western blot for the presence IE2 86, IE2 60, and IE2 40 using the mIE2 86 antibody (lanes 10-18). Only the 72 and 96 h p.i. of the Western blot with the mIE2 86 antibody are shown, as IE2 86, IE2 60 and IE2 40 are most abundant at these times.


A.
Step 1
CH16.0 IP
Post *
II
Pre *
Step 2
mIE2 86 IP
B.
CH16.0 IPs
PRE
IP
POST *
WT SX Rev WT SX Rev WT SX Rev
24
48
72
96
h p.i.
1 2 3 4 5 6 7 8 9
Western Blot: UL84
C.
mIE2 86 IPs
PRE *
IP
POST
WT SX Rev WT SX Rev WT SX Rev
24
48
72
96
h p.i.
1 2 3 4 5 6 7 8 9
Western Blot: UL84
mIE2 86 IPs
PRE *
IP
POST
WT SX Rev WT SX Rev WT SX Rev
86
60
40
72 h p.i.
86
60
40
96 h p.i.
10 11 12 13 14 15 16 17 18
Western Blot: mIE2 86

Representative Western blots with the mIE2 86 antibody are shown at both 72 and 96 h p.i. in Figure 3.6C, lanes 10-18.

To address whether both IE2 60 and IE2 40 are necessary for a proper interaction, or whether each of the proteins could interact with UL84 individually, the same sequential IP reactions were carried out using recombinant viruses that express either the IE2 60 or the IE2 40 protein, but not both. We have recently characterized the viruses that lack expression of either IE2 40 or IE2 40 and IE2 60 combined. Without these two proteins, the levels of UL84 are significantly decreased at 96 h p.i., similar to the decrease in UL84 levels in the IE2 86ΔSX virus-infected cell. In addition, the loss of IE2 40 alone results in some loss of UL84 (223).

Since we did not have a mutant that exhibited a total loss of IE2 60, we constructed a new mutant virus that lacks any expression of IE2 60 (Figure 3.7A). This mutant, termed M170L, has a mutated initiator methionine (M170) that has been changed to a leucine, thus preventing the expression of IE2 60 (Figure 3.7A and B). IE2 40 is still expressed since its initiator methionine is the next methionine in the sequence. When assessed by Western blot, expression of UL84, IE2 86, and IE2 40 in the M170L mutant virus-infected cells remains comparable to that in the WT-infected cell (Figure 3.7B). Furthermore, a revertant virus of this mutant fully restores the expression of IE2 60, indicating that the mutant virus created did not have any other defects or mutations (Figure 3.7B). These data indicated that IE2 60 might play a less important role than IE2 40 in regard to UL84 expression. However, we have not ruled out that IE2 60 has some ability to aid in the expression of UL84 when IE2 40 is not present.

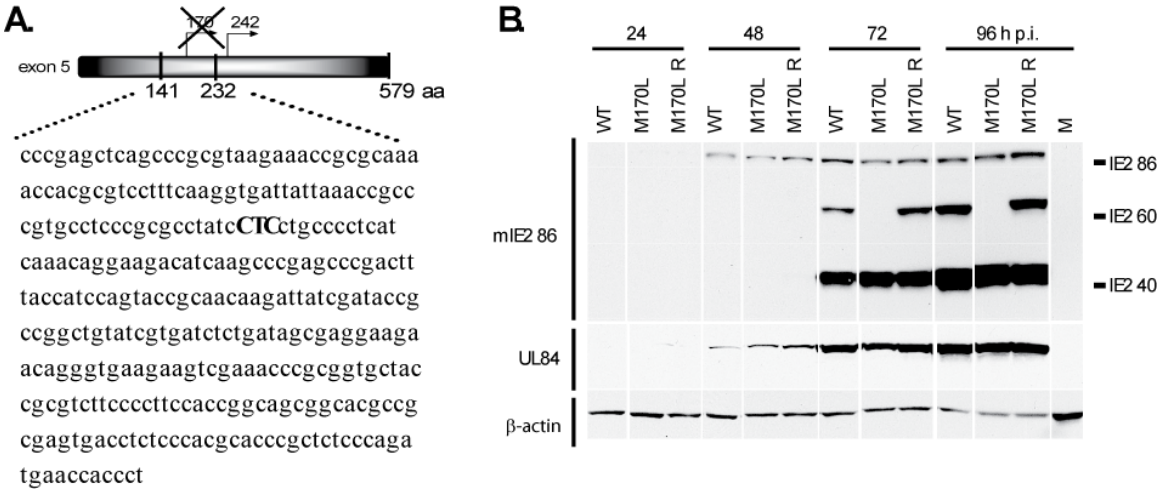


Figure 3.7. Sequence and characterization of the M170L mutant. (A) A portion of the IE2 86 coding region is shown. The mutated initiator methionine (M170, originally ATG) for IE2 60 was changed to a leucine (**CTC**) to create this mutant virus (M170L). (B) Expression of IE2 86, IE2 60 and IE2 40 in the M170L mutant as compared to the WT and the M170L revertant (M170L R) at 24-96 h p.i. in HFFs infected at an MOI of 3 pfu/cell. A mock-infected sample (M) is also shown. Western blot analyses were used to examine expression of UL84 as well. Cellular β-actin was used as a control for protein loading.

Since the above IP studies showed that UL84 was able to interact with IE2 60 and IE2 40 in the absence of full-length IE2 86, we next wanted to identify the individual contribution of IE2 60 and IE2 40 to the interaction with UL84. Sequential IPs were conducted as described previously. Cells were infected with either the WT, IE2 Δ40, or M170L mutant viruses at an MOI of 1 pfu/cell, and then harvested at 96 h p.i. when IE2 60 and IE2 40 are most abundant. Cells were lysed, and IPs were carried out using the CH16.0 antibody. As described previously, the post-IP supernatant was then subjected to a subsequent IP with the C-terminal mIE2 86 antibody, and the presence of UL84, IE2 86, IE2 60, and IE2 40 were analyzed by Western blot. As expected, UL84 is present in all of the IP lanes corresponding to the CH16.0 IP (Figure 3.8, lanes 2, 5, and 8). Interestingly, UL84 is able to interact with both IE2 60 and IE2 40 alone, as judged by the IP lanes in the mIE2 86 IP corresponding to either IE2 Δ40 or the M170L viruses (Figure 3.8, lanes 14 and 17). The majority of UL84 that is not in complex with full-length IE2 86 appears to be in complex with either IE2 60 or IE2 40, as indicated by the fact that very little UL84 is detected in the supernatant following the subsequent mIE2 86 IPs (Figure 3.8, lanes 12, 15, and 18); UL84 is only observed upon long exposure of the Western blots (data not shown). This was also seen in the previous IPs (Figure 3.6B). It was noteworthy that there was a direct relationship between the amount of UL84 and IE2 proteins in the IP lanes for all viruses. This is most apparent in comparing lane 14 with lanes 11 and 17. These results suggest that UL84 must be in a complex with the various IE2 proteins for proper UL84 function and that these interactions may play a key role in the regulatory functions of each of the IE2 proteins.

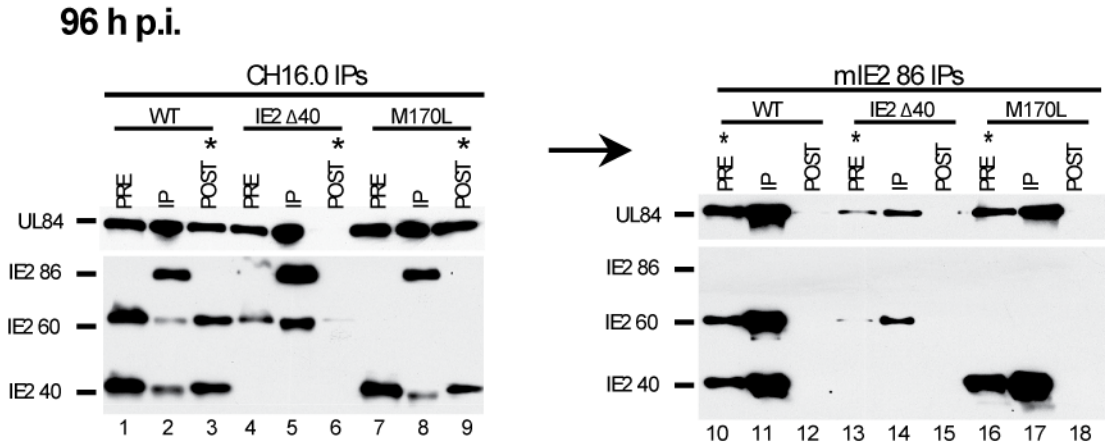


Figure 3.8. IE2 60 and IE2 40 interact individually with UL84 in the absence of IE2 86. As in Figure 6, sequential IPs were performed, with the exception of the mutant viruses used, which were the IE2 Δ40 and M170L mutants, and the time points taken (only 96 h p.i. is shown here). At 96 h p.i., cells were harvested and the first IP (lanes 2, 5, and 8) was carried out using the CH16.0 antibody. Western blot analyses were performed to assess the interactions occurring and expression patterns of UL84, IE2 86, IE2 60 and IE2 40. The supernatants following IP (POST*, lanes 3, 6, and 9) were used for the next immunoprecipitation (mIE2 86 IPs, lanes 10-18). POST* (lanes 3, 6, and 9) and PRE* (lanes 10, 13, and 16) indicate that the post binding supernatant for the first IP was used for the pre-binding sample for the second IP. The pre and post samples represent approximately 10% of the IP. The interaction of UL84 and IE2 86 were assessed in lanes 2, 5, and 8, while the interactions between UL84, IE2 60, and IE2 40 were assessed in lanes 11, 14, and 17. Lanes 14 and 17 represent the individual interactions with UL84 and IE2 60 or IE2 40, respectively. The arrow indicates the sequential nature of the IPs from the initial CH16.0 IPs to the mIE2 86 IPs.

Our finding that IE2 60 and IE2 40 were present in the IP lane corresponding to the CH16.0 IP with full-length IE2 86 and UL84 also confirms that IE2 60 and IE2 40 can interact with IE2 86 (Figure 3.8, lanes 2, 5, and 8). Immunodepletion of the full-length IE2 86 protein was again confirmed by Western blotting with both the monoclonal IE2 86 antibody at 96 h p.i. (Figure 3.8, lanes 10-18), as well as the CH16.0 antibody (data not shown).

A C-terminal portion of IE2 86 contributes to the interaction between IE2 86 and UL84.

The above results with the IE2 40 protein showed that a domain C-terminal to aa 242 of IE2 86 was sufficient to interact with UL84. The finding that IE2 86ΔSX, which lacks aa 136 to 290, was also able to form a complex with UL84 suggested that either the region sufficient for an interaction with UL84 resided C-terminal to aa 290 or that a second domain in exon 5 between aa 85 and 135 could contribute to the interaction with UL84 in the absence of aa 136-290.

To address these possibilities, two additional mutant viruses were tested for this interaction - one containing a deletion of aa 88-135 (Δ88-135), and the second with a deletion of aa 88-290 (Δ88-290). Cells were infected at an MOI of 3 pfu/cell, and then harvested at 72 and 96 h p.i. when UL84 is most abundant. The lysates were incubated with beads conjugated to an antibody that recognizes the amino terminal region of IE2 86 and IE1 72 (CH16.0). Following IP, the proportion of UL84 pulled down by the assay was analyzed by Western blot with a monoclonal antibody specific for UL84 (Figure 3.9A). Deletion of either of these regions from IE2 86 still did not result in a loss of

interaction with UL84, indicating that the region between aa 88 and 290 was not required for this complex to form (Figure 3.9A). It was notable, however, that significantly less UL84 accumulates in cells infected with the IE2 86Δ88-290 virus. Since less UL84 was present in the cells infected with this mutant, the amount of UL84 present in the Pre lane was not visible at this exposure of the blot. These results, coupled with the newly identified interaction between UL84 and IE2 60 and IE2 40, differ from those previously reported that identified the first 290 aa as the only region that binds UL84 (43). However, these experiments were conducted as co-transfection assays, which may behave differently than our experiments conducted in the virus-infected cell. It is possible that both regions of IE2 86 have the ability to contribute to the interactions we observe.

To further define the region within IE2 86 necessary for interaction with UL84, we conducted *in vitro* GST-binding assays. Here, IE2 86 and several mutant forms of the protein were expressed as GST fusion proteins in bacteria. Following induction and lysis of bacteria, the GST protein was bound to glutathione agarose beads. Figure 3.9B shows the mutants tested.

To test UL84 binding to IE2 86, ^{35}S-labeled UL84 was synthesized in an *in vitro* transcription/translation reaction, and then analyzed for binding following incubation with bound GST-IE2 86. The UL84 and IE2 86 complex was then analyzed by gel electrophoresis and autoradiography. The amount of GST protein bound to the beads was normalized based on visualization of the GST protein in the gel following staining and to BSA as a standard (data not shown). UL84 bound efficiently to WT GST-IE2 86, as well

as to a number of the mutants (Figure 3.9C). The minimal domain involved in the interaction with UL84 was localized to aa 290-369, based on the finding that the binding of UL84 to a GST-IE2 86 mutant containing only this region plus the N-terminal 85 aa (MX364) was comparable to that observed for the WT GST-IE2 86 construct. As expected, a mutant containing only the N-terminal 85 aa (IE2 86-Stop) was unable to bind to UL84, since IE1 72, which shares the amino terminal 85 aa with IE2 86, does not interact with UL84 (68). Interestingly, loss of the region between aa 290 and 369 (XN) did not abolish binding, indicating that there is some contribution from another region that is able to aid in the binding of UL84 and IE2 86 *in vitro* (Figure 3.9C). However, deletion of aa 290-579 (TM 293) resulted in a loss of binding comparable to that of the mutant that is missing the region between aa 85-369 (MN), indicating that the C-terminal domain (290-369) is the primary region that is important for binding to UL84. Loss of aa 136-290 (SX) or 85-290 (MX) also did not significantly disrupt binding, confirming the interaction studies seen in the mutant virus infections (Figure 3.9C). It should be noted that the domain defined here (aa 290-369) is also contained within IE2 60 and IE2 40, further implicating these proteins and this region of IE2 86 as being important for this interaction.

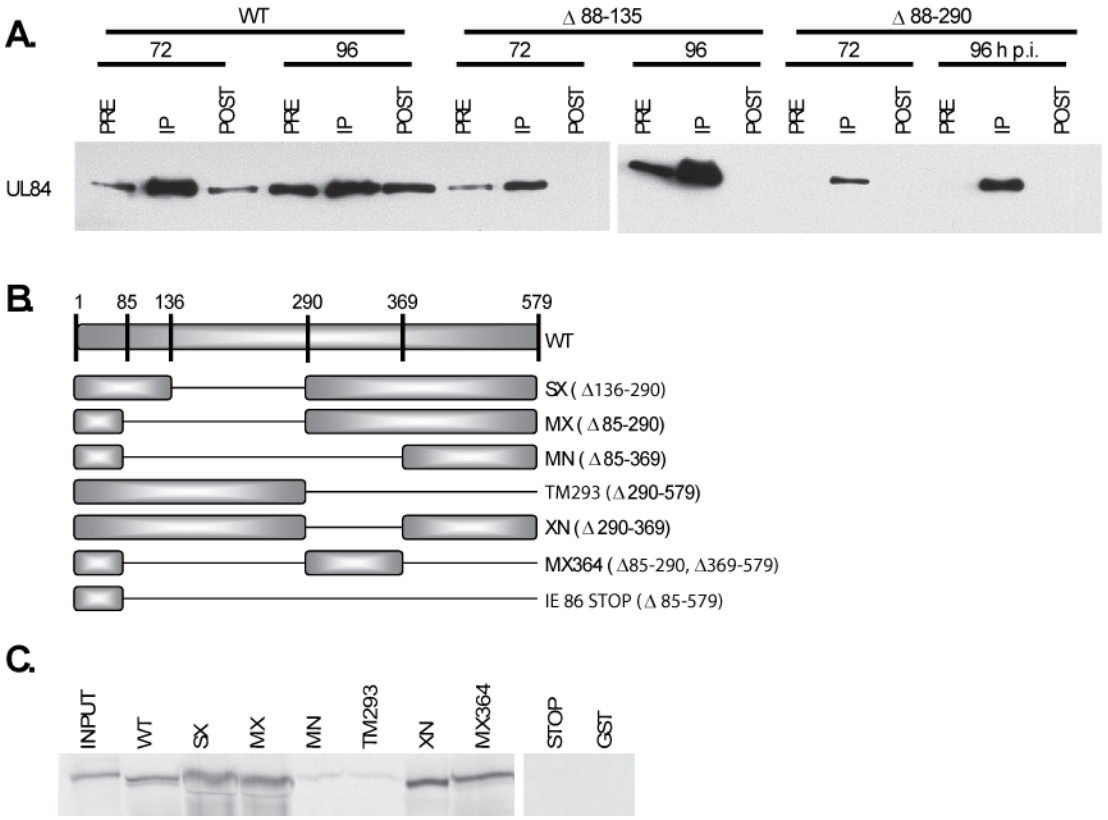


Figure 3.9. Identification of a C-terminal domain involved in the interaction between IE2 86 and UL84. (A) HFFs were infected at an MOI of 3 pfu/cell with the WT, Δ88-135, or Δ88-290 viruses and harvested at either 72 or 96 h p.i. Lysates were prepared, a small aliquot was removed (PRE), and the remainder was incubated with beads conjugated with the CH16.0 antibody (recognizes IE1 72 and IE2 86). Following incubation, an aliqout of the supernatant was removed (POST). The beads were then washed and IPs were eluted from the beads (IP). The PRE and POST lanes represent 10% of the protein loaded in the IP lanes. UL84 was analyzed by Western blotting as described in Materials and Methods. (B) Schematic of the IE2 86 coding region and all of the mutated forms assayed. Amino acid deletions: SX=Δ136-290, MN=Δ85-369, MX=Δ85-290, TM293=Δ290-579, XN=Δ290=369, MX364=Δ85-290 and Δ369-579, STOP=Δ85-579. (C) UL84 and IE2 86 binding was assessed as described in Materials and Methods. Eluted protein was analyzed by agarose gel electrophoresis, and the amount of UL84 bound was analyzed by autoradiography. Input=10% of bound reactions. All mutants are described above. GST only is used as a negative control for non-specific binding. A representative of 4 experiments is shown.

DISCUSSION

Multiple functions have been ascribed to IE2 86. the majority of which converge on its effects on both viral and cellular transcription (38, 68, 93, 106, 110, 114, 121-122, 150-151, 177-178, 188, 196, 214, 236). However, the involvement of IE2 86 in regulating the steady-state levels of many of the proteins expressed at early-late times p.i. has not been investigated. Our previous characterization of a mutant virus that is missing aa 136-290 of IE2 86, termed IE2 86ΔSX, showed that this region was important for potential functions of IE2 86 during the late stages of the infection. Since this mutant is also missing the initiator methionines for IE2 60 and IE2 40, these proteins are not expressed in the infected cells. The IE2 86ΔSX virus displays delayed expression of IE2 86, shows severe dysregulation of proteins important for late stages of the infection, and grows to reduced titers (168, 223). To further identify the relative contribution due to the loss of the IE2 60 and IE2 40 proteins versus the loss of the region between aa 136-290 of full-length IE2 86, we previously constructed a virus that does not express any IE2 60 or IE2 40, but expresses full-length IE2 86. This virus displayed very similar characteristics to the IE2 86ΔSX virus at late times p.i. with respect to expression of UL84 (223). This observation pointed to a role for both full-length IE2 86 and the late IE2 60 and IE2 40 with respect to UL84 expression.

Previous studies showed that the levels of UL84 protein were markedly decreased throughout the infection in the IE2 86ΔSX virus-infected cells, but there appeared to be no decrease in the UL84 RNA as measured by real-time RT-PCR (223). Here, we have confirmed by Northern blot analysis that the amount of UL84 mRNA is not affected at

either early or late times in the IE2 86ΔSX infection. Importantly, these data also documented that there is no disruption of the other transcripts (UL85 and UL86) that are 3' co-terminal with the UL84. The UL85 and UL86 transcripts encode two capsid proteins (minor and major, respectively), and do not seem to share any of the same functions as UL84. We show that, similar to other late viral proteins in the mutant infected cells, there is a delay in the accumulation of the UL85 and UL86 proteins at 72 h p.i. (168). However, in contrast to the UL84 protein levels, which remain low throughout the IE2 86ΔSX infection, the levels of the UL85 and UL86 proteins in the WT and mutant-infected cells are comparable by 96 h p.i.

Addition of a proteasome or calpain inhibitor did not increase the levels of the UL84 protein; thus, this loss in the IE2 86ΔSX infection appears to be mediated through a proteasome and calpain independent pathway. Moreover, studies with cycloheximide addressing the stability of UL84 indicate that the protein is not degraded more rapidly in the IE2 86ΔSX infection than that in the WT infection. However, we cannot exclude the possibility that the IE2 86ΔSX and UL84 proteins measured in these assays were representative of only those proteins in complex with one another. It is possible that the interaction with any of the IE2 proteins aids in the stability of UL84, and that without this interaction, the protein is very rapidly degraded via some proteasome and calpain independent mechanism.

To further elucidate the mechanisms of regulation between this family of IE2 proteins and UL84, we also assessed the nuclear to cytoplasmic export and distribution and loading of the UL84 mRNA onto polysomes. Surprisingly, these analyses indicated

that both processes were functioning in a comparable fashion to that in the WT virus-infected cells. Similarly, the IE2 86 mRNA did not exhibit any defects in loading and distribution on polysomes in IE2 86ΔSX-infected cells. These data further indicate that these proteins are regulated in a post-transcriptional fashion. Interestingly, the distribution of the UL84 RNA was different than other transcripts assessed of the same size, which could indicate a difference in the translation of this transcript. It is also possible that IE2 86, IE2 40, and/or IE2 60 function co-translationally to assure the synthesis and release of full length UL84 and IE2 86 protein. Alternatively, the IE2 proteins may be important for some type of post-translational regulation. Although the simplest explanation is that a rapid interaction of UL84 and IE2 proteins during or immediately after protein synthesis is necessary for the accumulation of both proteins, there is no a priori reason that the IE2 proteins might not affect UL84 expression indirectly through some other viral or cellular protein. Studies are underway to assess these possibilities.

To further understand which regions of the IE2 proteins might be involved in the regulation of UL84 protein levels throughout the infection, we conducted IP and GST binding studies to determine the domains that contributed to the IE2-UL84 complex formation. Interestingly, IE2 86, IE2 60 and IE2 40 are each able to interact individually with UL84, as shown by the IP studies. These data also reveal that IE2 60 and IE2 40 are able to form heterodimers with IE2 86, which is not surprising given that the dimerization domain of IE2 86 is present in these two smaller proteins (2, 39, 64).

Further evidence for the above interactions observed *in vivo* is provided by the *in vitro* experiments that indicate that the region between aa 290 and 369, which is also contained in the IE2 60 and IE2 40 proteins, contributes to the interaction with UL84. Although, at least *in vitro*, this region is not essential for binding and there appears to be a contribution from other regions, a construct containing this domain alone was able to interact with UL84. The data from the IE2-UL84 interaction studies were surprising since it had previously been proposed that the N-terminus (aa 85-290) was important for UL84 binding (43). Using our viral mutants and GST constructs that removed either aa 136-290 (IE2 86ΔSX), 88-135, or 88-290, a strong interaction still occurred between UL84 and IE2 86 in the context of the actual infection and in the *in vitro* assays. It is possible that these differences in experimental procedure- transfection vs. infection- may explain some of the discrepancies between these results. Although the region between aa 85-290 of IE2 86 may have the ability to interact with UL84 in some *in vitro* studies, the data clearly show that loss of this region does not prevent formation of the IE2 86-UL84 complex during the infection. Further confirming the *in vivo* data presented here, we show that a GST mutant containing only the first 290 aa of IE2 86 displays greatly reduced binding to UL84, which also indicates that a site outside of this region may be important for the IE2 86-UL84 interaction. Finally, IE2 60 and IE2 40, which do not contain the amino terminal region of IE2 86, are able to interact with UL84.

The possibility exists that any of the three versions of IE2 can contribute to efficient expression of the UL84 protein, and that it is the levels of the IE2 proteins present in the infection that are important for proper UL84 regulation. Although we

previously showed that the loss of IE2 40 resulted in a partial loss of UL84 protein expression (223), the data presented here with the M170L virus revealed that total loss of the IE2 60 protein did not have an effect on UL84 expression, indicating that IE2 40 and IE2 86 likely play a more important role in UL84 expression. It is possible, however, that IE2 60 can partially compensate for the loss of IE2 40, or vice versa.

There are many potential sites within the region of both aa 136-290 and 290-369 of IE2 86 that may be important for proper expression of and interaction with UL84. For example, there are serine rich regions and sites of sumoylation and phosphorylation within these domains that are important for efficient viral replication (6, 10-11, 29, 44, 68, 82, 90, 137). Furthermore, mutations of the serines within IE2 86 aa 258-275 have been previously shown to influence the expression of some late proteins in HCMV infected cells (11). Understanding whether any of these sites are important for the regulatory mechanisms described here is a focus of future studies.

In summary, these data have provided a strong basis for the understanding of the regulation of UL84 expression in the HCMV infection. Although the mechanism of regulation remains to be fully characterized, we have identified many of the important players in governing proper expression of UL84. Clearly, the IE2 proteins are governing the expression of UL84 protein in a novel fashion that has yet to be described for this family of proteins. We have eliminated the possibilities that this regulation is at the level of transcription of the UL84 mRNA, transport of the mRNA to the cytoplasm, or loading of the mRNA onto polysomes. The lack of accumulation of UL84 protein also does not appear to be due to proteasome or calpain-dependent degradation. It is possible that

translation of the mRNA to termination codon is impaired or that proteolytic degradation involves one of the many other cellular proteases, and studies are in progress to address these questions.

It is interesting that UL84 is the only viral protein that IE2 86 has been shown to interact with, and yet very little UL84 is actually needed to facilitate the production of functional virus. Elucidating the mechanisms governing the interactions between IE2 86, IE2 60, IE2 40 and UL84 should provide insight into how these proteins are regulated throughout the infection and contribution of each of the protein complexes to efficient viral replication.

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The text of Chapter 3, in full, is a reprint of the material as it appears in *Journal of Virology*, 82:11383-11397, 2008. Sanders, R.L., C.J. Del Rosario, E.A.White, and D. H. Spector. Internal Deletions of IE2 86 and Loss of the Late IE2 60 and IE2 40 Proteins Encoded by Human Cytomegalovirus Affect the Levels of UL84 Protein but not the Amount of UL84 mRNA or the Loading and Distribution of the mRNA on Polysomes. The dissertation author was the primary investigator and author of this paper.

CHAPTER 4

IE2 86 and IE2 40 Differentially Regulate UL84 Protein Expression Post-transcriptionally outside the Human Cytomegalovirus Infection Process

ABSTRACT

It has previously been demonstrated during human cytomegalovirus infection that both IE2 86 and IE2 40 are important for the expression of an early-late protein, UL84. Here, we show that expression of the UL84 protein is enhanced upon co-transfection with both IE2 86 and IE2 40, although IE2 40 appears to play a more important role in this regard. The UL84 levels are tightly linked to the amount of IE2 40 present, but this does not appear to be true for IE2 86. RNA remains constant for all corresponding proteins, indicating post-transcriptional regulation of UL84. The first 105 amino acids of UL84 are necessary and sufficient for this phenotype, and this region is also responsible for an interaction with IE2 86 and IE2 40. Treatment with proteasome inhibitors show that UL84 exhibits some proteasome dependent degradation, and UL84 is not protected against this degradation when co-expressed with IE2 86 or IE2 40. UL84 also exhibits an inhibitory effect on IE2 86 and IE2 40 protein levels in these co-transfection assays. Further, we show that the amino acid sequence is important for IE2 40 enhancement of UL84. These results indicate that IE2 86, IE2 40 and UL84 serve to regulate protein expression in a post-transcriptional fashion, and that these proteins are tightly linked in regard to their protein expression patterns outside the context of the infection.

INTRODUCTION

Human cytomegalovirus (HCMV), a β-herpesvirus, is the leading viral cause of birth defects and poses a severe threat to immunocompromised individuals. It has a 240 kbp genome that encodes for at least 150 different open reading frames, only a small portion of which have been well characterized. Gene expression occurs in a temporally controlled fashion and is divided into three major classes: Immediate early (IE), early, and late genes (for a review, see 63). The IE genes serve to shut down host cell defenses and activate expression of early viral genes, while early and late genes serve primarily in viral replication and structure and assembly of the virus, respectively (130, 132). Two major IE (MIE) gene products, IE1 72 and IE2 86, are encoded by the UL122-123 coding region and are important for transactivating viral early promoters, serve in viral promoter repression, and regulate the expression of many host cellular genes in order to allow proper progression of the viral infection (130, 132). Both proteins arise from the same transcript that is alternatively spliced to encode exons 1-4 for IE1 72 and exons 1-3, and 5 for IE2 86. Many important domains have been identified for both IE1 72 and IE2 86, and these proteins have been shown to have some overlapping function, as well as many divergent roles.

Many functional roles have been ascribed to specific domains throughout the entire coding region of IE2 86. DNA binding resulting in transcriptional auto-repression has been shown to involve amino acids (aa) 290-579, while the regions from aa 1-98 and 170-579 appear to be required for some transcriptional activation functions (39, 109, 122, 151, 177-178, 188, 195, 236). In addition, IE2 86 has been shown to interact with many

viral and cellular factors *in vitro*, but many of these have not been confirmed in the context of the infection (for a review, see 63). At late times in the infection, two other proteins arise from exon 5 of IE2 86 using downstream promoters, termed IE2 60 and IE2 40. The IE2 60 (60-kDA) protein is predicted to begin translation at methionine 170 of IE2 86, while the IE2 40 (40-kDA) protein is predicted to begin at methionine 242. The numbering system here reflects the amino acid numbering scheme in the Towne strain of HCMV. Putative TATAA regions for IE2 60 occur in the intron between exons 4 and 5, and just up-stream of the IE2 60 translation initiation site for IE2 40. These proteins have previously been shown to have a role in transactivation of late genes, as well as repression of the MIE promoter (95, 172, 223). Our lab has extensively studied the roles of these two proteins in regard to the later stages of the infection process. Loss of the IE2 60 and IE2 40 proteins was shown to alter the expression of both IE1 72 and IE2 86. We have also identified a role for the IE2 60 and IE2 40 proteins in regard to the expression of two early-late viral proteins, UL83 (pp65) and UL84, and have found that loss of these smaller IE2 proteins results in a reduction of viral production greater than 10 fold (223).

The regulation of UL84 by the IE2 family of proteins has been of great interest in understanding the roles of these proteins during infection. UL84 has previously been shown to interact with IE2 86 (43, 167, 172, 191). It is present at very low levels in early times in the infection, and then accumulates after the onset of viral DNA replication (84). UL84 has also been shown to have a role in *ori-Lyt*-dependent replication and down-regulation of IE2 86 dependent transactivation functions during transient assays (68), and is able to interact with the RNA stem-loop sequence within the RNA/DNA hybrid region

of *ori-Lyt* (42). We have previously shown that UL84 interacts with IE2 86 throughout the entire infection process, and that IE2 60 and IE2 40 can individually interact with UL84 (172).

Using IE2 mutant virus studies, we determined that loss of the IE2 60 and IE2 40 proteins resulted in a significant loss of UL84 expression, and this loss was shown to be post-transcriptional (172, 223). Furthermore, a mutant virus containing a deletion of aa 136-290 of IE2 86 (termed IE2 ΔSX), which also does not express IE2 60 or IE2 40 showed similar results (172). In these studies, it was determined that IE2 40 played a more important role in governing UL84 expression at the later stages of the infection process, given that loss of IE2 40 alone resulted in a significant loss of UL84 (223). Further characterization of the mechanism governing UL84 expression revealed that UL84 RNA could be exported to the cytoplasm and loaded on to the polyribosomes appropriately in IE2 ΔSX mutant infected cells. Analysis using proteasome inhibitors revealed that this loss of UL84 protein expression was proteasome independent, and the stability of the expressed protein was found to be similar to that expressed during the wild-type (wt) HCMV infection (172).

In this report, we have further defined the mechanisms governing UL84 and IE2 expression. The levels of the UL84 protein, but not RNA are slightly upregulated when co-expressed with IE2 86, and much more significantly with IE2 40. We show that the effect of the IE2 proteins on UL84 protein expression does not require other factors that are specific for the infection. The amount of UL84 protein that is expressed is directly dependent on the amount of IE2 40 present, although this is not true for IE2 86.

Additionally, expression of UL84 inhibits the expression of IE2 86 and IE2 40 in these assays. The N-terminal region of UL84 (aa 1-105) is necessary and sufficient for the up-regulation of UL84 protein expression induced by the presence of the IE2 proteins. We find that in contrast to the infection process, UL84 stability is partially governed by the proteasome, but this is independent of the upregulation governed by the IE2 proteins, and the first 105 aa of UL84 do not play a role. Further, a protein-protein interaction is likely necessary for the interactions and phenotypes shown, given that the amino acid sequence of the N-terminal domain of UL84 is important for upregulation governed by IE2 86 and IE2 40. Overall, we have clearly identified important and distinct roles for the IE2 family of proteins and have further characterized the mechanisms by which UL84 expression is governed.

MATERIALS AND METHODS

Cell Culture and 293FT Transfections

293FT cells were purchased and cultured according to manufacturer's instructions (Invitrogen). Media was supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 50 mg/ml geneticin (G418), 1 mM sodium pyruvate, and 10mM non-essential aa (all supplements from Invitrogen). Cells were incubated at 37°C in 7% CO_2. 24 hours (h) before transfection, cells were seeded in a 12 well plate at a density of approximately 80% confluency in media containing no G418. Immediately prior to transfection, 293FT cells were switched into OptiMEM (800 µl/well, Invitrogen). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. 1.6 µg DNA/well (0.8 µg of each plasmid) was transfected per well. Appropriate plasmid DNAs were pre-mixed with OptiMEM (Invitrogen), and then Lipofectamine diluted in OptiMEM was added. Samples were kept at room temperature for 20 minutes, and then the entire reaction was added dropwise to the pre-seeded 293FT cells that had been switched into 800 µl/well of OptiMEM for transfection. The Lipofectamine/DNA mix (200 µl) was allowed to incubate with the cells for 24 h, and then media was removed and replaced with DMEM with no drugs. 24 h later (48 h p.t.), cells were rinsed in phosphate buffered saline (PBS), and then harvested and snap frozen in liquid nitrogen and then placed at -80°C until analysis. For the IE2 40 DNA titration experiment, the same protocol as above was used, except that the DNA was titrated down for each sample, including dilutions of 0.8 µg, 0.4 µg, 0.2 µg, and 0.05 µg. UL84 DNA

concentrations were kept constant, and control plasmid was added to ensure the same amount of total DNA was added to each well.

Plasmid DNA construction

All primers and probes used in these studies can be found in Table 1. The original UL84 plasmid, pTARGET-UL84HA was obtained from Dr. G. Pari (University of Nevada, Reno). The UL84 coding region was removed from this vector using EcoRI sites that flanked either side of the gene, including the HA tag at the C-terminus, and then ligated into the vector pCDNA3 (Invitrogen) with the same sites. To make deletions and amino acid changes, Quikchange Mutagenesis (Stratagene) was performed to create the following mutants in UL84: Δ1-68 aa, Δ1-105 aa, Δ1-135 aa, and Δ1-200 aa. The UL84 frameshift mutant was made by creating 4 different sequential amino acid changes that first shifted the open reading frame to the second possible open reading frame, followed by a change to the third open reading frame. Next, a newly created in-frame stop codon was changed to an alanine to allow continued read-through of the current open reading frame. Following this Quikchange, the open reading frame was shifted back into the original open reading frame to allow read-through of the rest of the UL84 protein. This mutant now encodes a new amino acid sequence for the first 176 aa using a very similar RNA sequence as for wt UL84.

Quikchange mutagenesis was also used to create the IE2 40 deletion mutants. Mutants created include: Δ242-290, Δ243-360, Δ361-420, Δ421-542, and Δ543-579. These mutants were analyzed for expression and appropriate migration on an SDS-PAGE gel in order to confirm that each mutant had been created as expected.

Quantitative real-time PCR and real-time RT-PCR

For the quantitative real-time PCR and real-time RT-PCR analyses, an aliquot of the same cells as used for Western blot analysis was assayed for DNA and RNA quantities, respectively. Following transfection and freezing of the cells, the DNA was prepared using the Invitrogen Miniprep kit or the Norgen RNA/DNA/Protein kit according to manufacturer's instructions. Concentrations were determined by UV spectrophotometry. Quantitative real-time PCR analyses were performedusing the TaqMan Universal PCR Master Mix (Applied Biosystems). In this case, primers and probes were directed against the IE2 proteins, UL84, UL44 and the GAPDH promoter.

For the real-time RT-PCR analyses, RNA was prepared from the same cells as used for Western blot analyses 48 h post-transfection (p.t.) using either the Norgen RNA/DNA/Protein Purification Kit, or the Ambion PARIS kit according to manufacturer's instructions. RNA was DNased using the Ambion Turbo DNA-free kit according to manufacturer's instructions and then quantified using UV spectrophotometry. Samples were diluted down to 12.5 ng/µl and analyzed using the same machine as used for DNA quantification (Applied biosystems, ABI Prism 7000). The real-time RT-PCRs were carried out using the TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) using primers and probes against UL84 (internal region and N-terminal region), UL44, IE2, and G6PD.

Western Blot Analyses

Transfected cells were used following snap freezing in liquid nitrogen and storage at -80°C. Cells were lysed in reducing sample buffer, or protein was prepared using the

Norgen RNA/DNA/Protein Purification Kit according to manufacturer's instructions. Nitrocellulose membranes were blocked in 5% milk in tris-buffered saline containing .05% Tween-20 (TBS-T) and probed with the following antibodies: IE2 MAb 8140 (Chemicon), UL84 MAb (Virusys), HA F-7 MAb (Santa Cruz Biotechnology), UL44 Mab (Virusys) and Actin Mab (Sigma-Aldrich). Horseradish peroxidase-coupled anti-mouse IgG antibody was obtained from Calbiochem. Following secondary antibody incubation and washes, proteins were detected with SuperSignal chemiluminescent substrate (Pierce Biotechnology) according to manufacturer's instructions.

Immunoprecipitations

Transfected 293FTs were lysed in a modified radioimmunoprecipitation buffer containing 10 mM Tris-Hcl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM Dithiothreitol (DTT), 150 mM NaCl, 1% Triton X-100 plus a protease inhibitor cocktail (Sigma-Aldrich) for 30 minutes on ice. Lysed cells were spun down to remove cellular debris for 10 minutes at 4°C, at 10,000 X G and the lysate was allowed to incubate and rotate with Protein G-PLUS agarose beads (Santa Cruz biotechnology) coupled with the IE2 antibody that recognizes IE2 86, IE2 ΔSX, and IE2 40, or an antibody that recognizes an HA tag (clone F-7, Santa-Cruz Biotechnology) overnight at 4°C. The following day, agarose beads were washed twice with the same buffer that was used for lysis, and then resuspended in the same reducing sample buffer used for Western Blot, and heated to 95°C. Then, the supernatant was collected containing the immunoprecipitated proteins. An aliquot of the sample before immunoprecipitation was taken and represents the "Pre" fraction. This fraction was assessed to determine the

amount of protein present before the immunoprecipitation and represents 10% of the immunoprecipitation. All immunoprecipitations were analyzed by Western blot analyses to determine that the appropriate complexes had been pulled down, and to assess which proteins were capable of interaction.

For the *in vitro* immunoprecipitations, IE2 40 proteins (WT and mutant) were synthesized in separate reactions using Promega's Quickcoupled TNT *In Vitro* Transcription/Translation kit according to manufacturers instructions. Briefly, 1 µg of template was used per 100 µl reaction, and allowed to incubate at 37° C for 1 hour. 5 µl of each synthesized protein was used per immunoprecipitation reaction. For the "Pre" lanes, 1 µl of the TNT reaction was used. The "Post" lanes represent 0.5 µl of the TNT reaction, and represent the fraction of protein left following immunoprecipitation. The "IP" lane represents one fourth of the immunoprecipitated fraction.

Proteasome Inhibitor Treatment

293FT cells were cultured as described above and seeded as for transfection. 36 h p.t., cells were treated with 10µM MG132 (Calbiochem) for 12 h, and then harvested at 48 h p.t. Cells were processed for Western Blot analysis as described above. Two other proteasome inhibitors were tested, salinosporamide (Sal A) and lactacystin, in order to confirm the results with MG132. Sal A was used at 100nM and was a gift from Dr. Bradley Moore (Scripps Institute of Oceanography, University of California, San Diego), while lactacystin was used at 10µM and was obtained from Calbiochem. DMSO was used as a vehicle control.

RESULTS

UL84 protein expression is enhanced when co-expressed with IE2 86 or IE2 40.

Studies with IE2 mutant viruses have previously revealed that the expression of UL84 is tightly linked to the expression of IE2 86, IE2 60, and most predominantly with IE2 40 (223, 172). Enhancement of UL84 expression during the infection was shown to occur at the post-transcriptional level, and is independent of the efficiency of RNA loaded onto polyribosomes (172). These data directed our studies to ascertain whether this type of regulation could occur outside of the infection process, and to further delineate the roles of the IE2 proteins in regard to UL84 expression.

To assess whether the IE2 proteins were capable of up-regulating the expression of UL84 in the absence of any other viral proteins or modifications, 293FT cells were transfected with plasmids containing the IE2 86, IE2 40, and UL84 DNAs. These plasmids were either singly transfected, or co-transfected, and then 48 h post transfection (p.t.), cells were harvested and assayed for DNA, RNA, and protein expression. In the case of the singly transfected samples, the quantity of transfected DNA was normalized to the co-transfected samples by adding in empty plasmid DNA so that each of the samples received the same amount of DNA. All expression analyses were also confirmed in COS-7, U373, and HEK-293 cells to assure that expression patterns were not cell type specific. In all cases, each cell type gave similar expression patterns as seen in the 293FT cells, however only the experiments in 293FT cells are shown. When protein expression was analyzed by Western blot, significant differences in each of the proteins were observed when co-transfected (Figure 4.1A). The levels of UL84 protein increased

slightly when co-expressed with IE2 86, and were significantly enhanced when co-expressed with IE2 40. These results are consistent with previous data showing that loss of IE2 40 during the infection results in significantly reduced levels of UL84 protein (223). These experiments clearly demonstrate that UL84 expression is somewhat influenced by the presence of IE2 86, but that the expression of IE2 40 is more important in regulating UL84 protein expression.

The IE2 proteins were also assayed for protein expression in these studies. Each migrated at the appropriate size, although the IE2 40 protein accumulated to slightly higher levels than IE2 86. Interestingly, the levels of each of these proteins were found to be significantly reduced when co-expressed with UL84 (Figure 4.1A). Despite the fact that the UL84 protein levels were different, all IE2 proteins were reduced to a similar degree, indicating that the amount of UL84 present does not influence this phenotype.

The amount of transfected DNA and RNA present in each transfected sample was analyzed by either quantitative real-time PCR or quantitative real-time RT-PCR, respectively. In each case, whether singly transfected or co-transfected, the amount of IE2 86 and IE2 40 DNA was found to be comparable across samples (Figure 4.1B). This was true for the multiple assays that were performed during these studies. Similarly, UL84 DNA in these samples remained constant, whether transfected with IE2 86, IE2 40 or control plasmid. When the levels of the transfected RNA were assessed, each of these samples were found to have relatively equivalent amounts of IE2 86 and IE2 40 RNA, although the levels of the IE2 40 RNA were slightly higher that IE2 86 RNA levels.

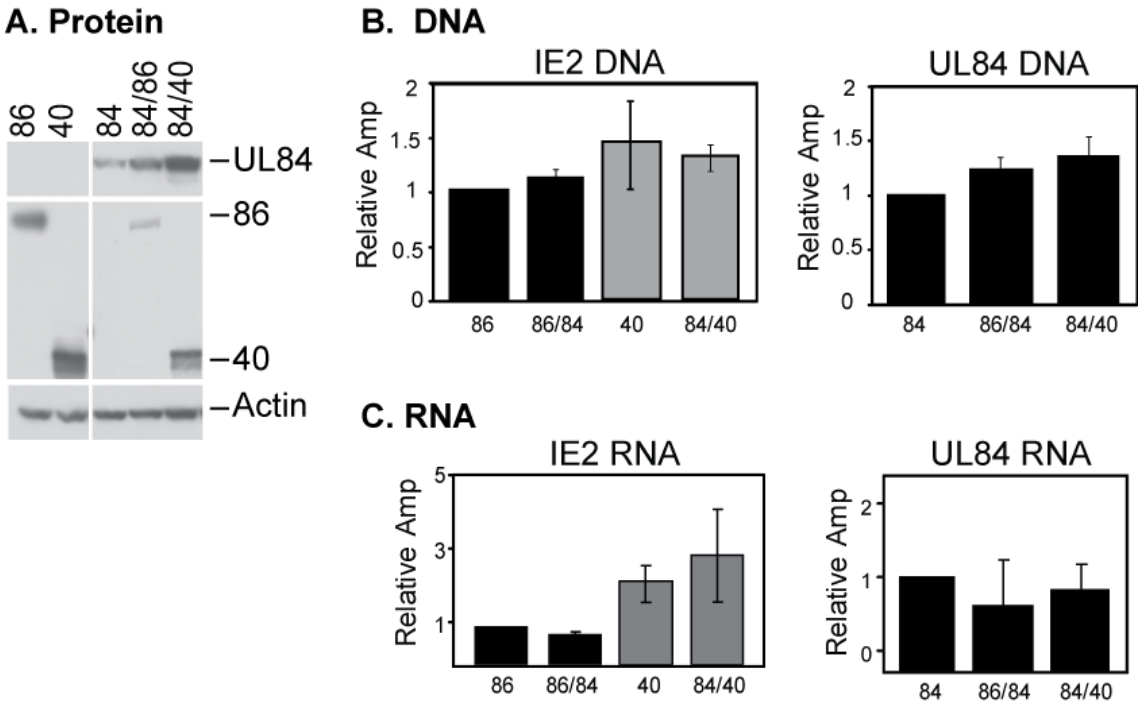


Figure 4.1. UL84 and IE2 Protein, RNA, and DNA expression in transfected 293FT cells. (A) 293FT cells were transfected with either the IE2 86 (86), IE2 40 (40), or UL84 (84) plasmids alone or in combination. 48 h later, UL84, IE2 86, and IE2 40 protein was assayed by Western blot by loading equal amounts of protein per lane. Actin serves as a loading control. (B) Transfected cells with the same constructs as in (A) were assayed for DNA expression by quantitative real-time PCR. IE2 DNAs were assayed using a primer and probe directed against the C-terminus of IE2 86, thus recognizing all forms of the IE2 constructs. UL84 DNA expression was also assayed alone or in combination with the IE2 constructs using primers and probe that recognize the N-terminus of UL84. Each sample was duplicated in each experiment, and the graphs shown are an average of 3 experiments. DNA for each sample was normalized to the GAPDH promoter as a control for the amount of input DNA in each reaction. (C) RNA expression for each of the samples was measured by quantitative real-time RT-PCR. Each of the samples were measured using the same primer and probes as in (B), and are normalized to a housekeeping gene G6PD. Graphs represent at least 2 experiments.

Similar to what was observed in the infection process, despite the increase in protein levels for UL84, RNA levels remained relatively unaffected when co-expressed with any of the IE2 plasmids (Figure 4.1C).

To determine whether cellular processes were necessary to facilitate this regulation, *in vitro* transcription-translation reactions were conducted in which UL84 and IE2 40 or IE2 86 were transcribed and translated in the same reaction, or where IE2 40 or IE2 86 protein was synthesized first, and then added to the UL84 *in vitro* transcription-translation reaction. In either case, no enhancement of UL84 protein expression was observed, indicating that some cellular modifications or processes are necessary for the regulation seen during transfection and infection experiments (data not shown).

UL84 protein expression is stoichiometrically dependent on the amount of IE2 40 present.

To determine how the regulation of UL84 protein expression might be related to the amount of IE2 40 protein present in the cells, the amount of transfected IE2 40 plasmid was titrated down, while UL84 plasmid concentrations remained constant. Starting with 0.8 µg of plasmid, DNA was decreased to 0.4, 0.2, and 0.05 µg of IE2 40 DNA, while 0.8 µg of UL84 DNA was used for all samples. Analysis of both the DNA and RNA expression by quantitative real-time PCR and quantitative real-time RT-PCR revealed appropriate titration of the IE2 40 plasmid (Figure 4.2A and B). The amount of IE2 40 RNA expressed was proportional to the amount of input DNA (Figure 4.2B). In all cases, however, the amount of UL84 DNA and RNA remained relatively unaffected by the presence of the varying levels of IE2 40 (Figure 4.2A and B).

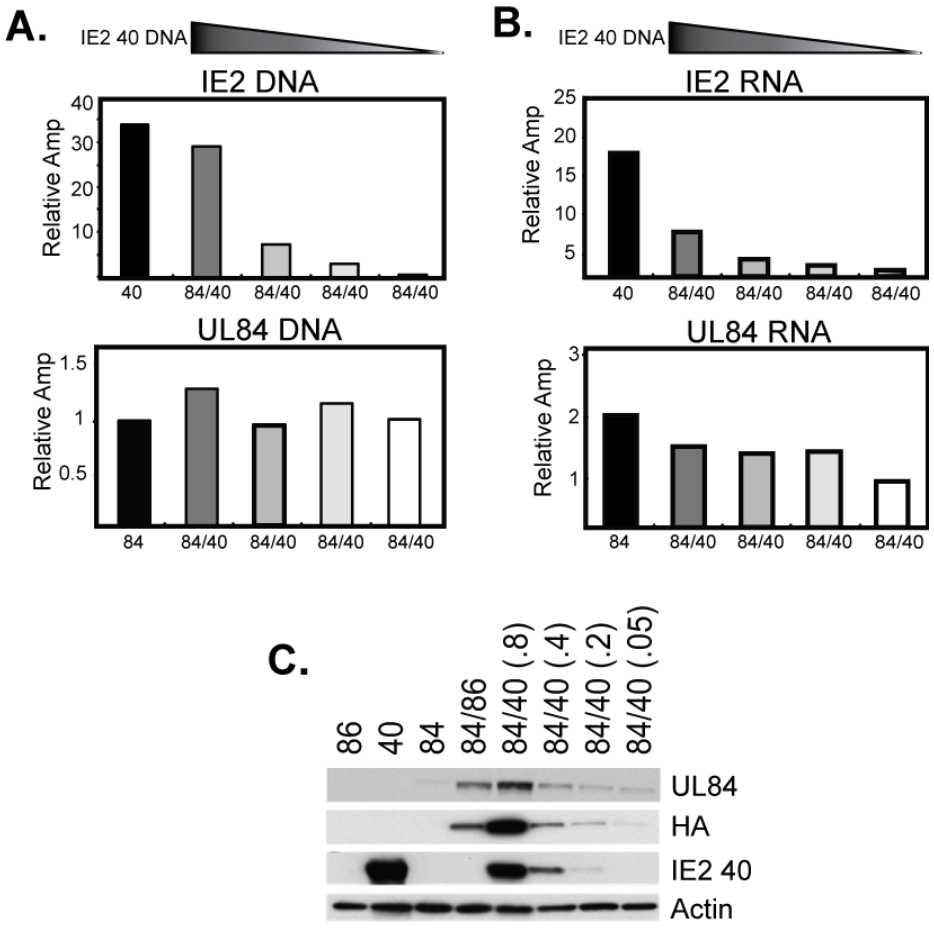


Figure 4.2. Titration of IE2 40 DNA affects UL84 protein expression. (A) Transfected IE2 40 (40) DNA was titrated down, while transfected UL84 (84) DNA remained constant, and analyzed 48 h later. Titrations included 0.8, 0.4, 0.2, and 0.05 µg of DNA. The amount of transfected DNA was analyzed by quanitative real-time PCR and normalized to the GAPDH Promoter as a loading control. Both IE2 and UL84 DNAs were assessed. (B) IE2 40 and UL84 RNAs from the same samples as in (A) were measured by quanitative real-time RT-PCR and normalized to a cellular housekeeping gene, G6PD. (C) UL84 and IE2 40 protein expression was analyzed from the same samples as in (A) and (B) by Western Blot analyses. UL84 expression was also measured with an antibody that recognizes the C-terminal tag, HA to confirm UL84 antibody results. Actin serves as a loading control.

Protein expression of UL84 was directly correlated to how much IE2 40 protein was present. Samples that contained the most IE2 40 protein also expressed the most UL84 protein, and the levels of the UL84 protein was coordinately reduced as the levels of IE2 40 declined (Figure 4.2C). Similar experiments were conducted with IE2 86 titrations, however these data indicated that UL84 expression is not directly linked to the amount of IE2 86 present in the sample. In this case, UL84 expression remained slightly increased compared to the sample with UL84 transfected alone, even when the amount of IE2 86 was decreased over 20 fold (data not shown). This indicates that IE2 86 is likely regulating UL84 protein expression in a different fashion that IE2 40, and that the levels of UL84 protein expression is much more tightly linked to the expression of IE2 40 protein than IE2 86 protein.

Deletions within UL84 affect protein expression and enhancement from the IE2 proteins.

To determine which domains of UL84 were important for the enhanced protein levels, deletions throughout UL84 were created. Specific domains were chosen based on previous literature suggesting that the first 105 aa of UL84 were important for an interaction with IE2 86 (68). Based on these data, we constructed UL84 mutants that lacked either the first 68 (Δ68), 105 (Δ105), 135 (Δ135), or 200 (Δ200) aa (Figure 4.3A). Each of the mutations were confirmed by sequencing, and tested for expression in 293FT cells to determine that each protein was expressed and that the size of each protein was as expected (data not shown and Figure 4.3B). The deletions of aa 1-135 and 1-200 resulted

in similar results as seen with the deletion of the first 105 aa, and are therefore not included in subsequent studies.

Expression of each of the mutants was analyzed by Western Blot analyses. Interestingly, when the UL84 mutants were transfected alone, we observed an increase in the amount of protein expressed as the N-terminal domain was deleted (Figure 4.3B). Deletion of the first 68 aa resulted in a slight enhancement of protein expression, while a further deletion of the first 105 aa results in a significant increase in protein expression levels. However, these levels of protein were still lower than those observed when IE2 40 was present in the sample with the wt UL84 protein (Figure 4.3B). Deletion of the first 68 aa did not result in a loss of enhancement when co-expressed with IE2 86 or IE2 40, however deletion of the first 105 aa resulted in no significant enhancement above the levels expressed when singly transfected. Interestingly, the mutant with a deletion of the first 105 aa was unable to inhibit IE2 86 or IE2 40 protein expression, indicating that this domain is also important for this phenotype. Deletion of the first 135 or 200 aa resulted in similar results as seen with the deletion of the first 105 aa (data not shown).

Interaction of UL84 with IE2 86 and IE2 40 is dependent on the first 105 aa of UL84.

Given that UL84 and IE2 86 and IE2 40 have previously been shown to interact (43, 172, 191), and since deletion analysis of UL84 revealed differences in expression of UL84 protein levels when co-expressed with IE2 86 or IE2 40 (shown above), the interaction between the UL84 mutants and the IE2 proteins was assessed. 293FT cells were singly transfected or co-transfected with the indicated

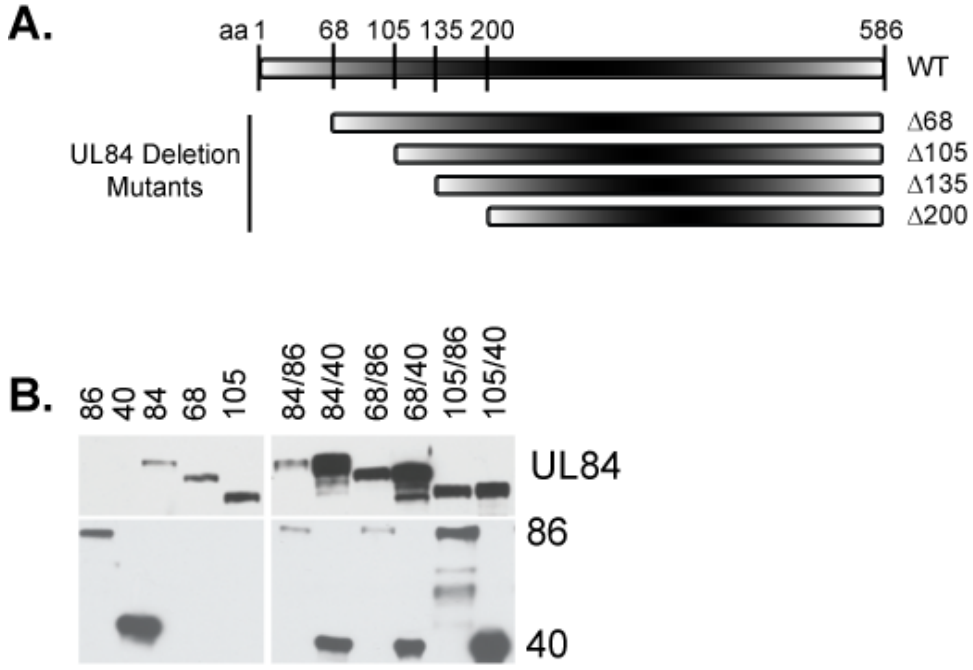


Figure 4.3. The N-terminal 105 aa of UL84 are important for the enhancement of protein expression governed by the IE2 proteins. (A) Deletions throughout the N-terminus of UL84 were created PCR mutagenesis. Deletions of the first 68 (Δ68), 105 (Δ105), 135 (Δ135), and 200 (Δ200) aa were created. Schematic is not to scale. (B) Protein expression of UL84, IE2 86 and IE2 40 was measured using Western blot analyses. Actin serves as a loading control.

plasmids, and then the UL84 mutants were assayed for interaction with the IE2 proteins 48 h later. Cells were harvested, snap frozen in liquid nitrogen, and then lysed and subjected to immunoprecipitation using an antibody that recognizes IE2 86 and IE2 40. Following immmunoprecipitation, Western blot analyses were conducted for each of the transfected proteins. Relative expression of the UL84 protein before immunoprecipitation can be observed in the lanes labeled "Pre" (Figure 4.4). Similar to previous results, the amount of UL84 protein increased as the N-terminal domain was removed. The deletion of aa 1-68 of UL84 resulted in a slight reduction in interaction between IE2 86 and IE2 40. However, the efficiency of binding was still significant. The deletion of aa 1-105 of UL84 resulted in complete loss of interaction with both IE2 86 and IE2 40. (Figure 4.4, IP). These data indicate that the first 105 aa of UL84 are involved in the interaction with both IE2 proteins.

UL84 protein is sensitive to proteasomal degradation, independent of the presence of the IE2 proteins.

To assess whether the lack of UL84 expression when transfected alone in 293FT cells was due to proteasomal degradation, transfected cells were treated with the proteasome inhibitors Lactacystin, MG132, and Sal A. Expression patterns described earlier (Figures 4.3 and 4.4) were maintained in all experiments. All proteasome inhibitors showed the same results, therefore only the results treated with MG132 are shown here. Treatment with MG132 for 12 h prior to the collection of the transfected cells resulted in significantly increased protein expression when UL84 was transfected alone. However, the UL84 levels in the presence of the proteasome inhibitor were still

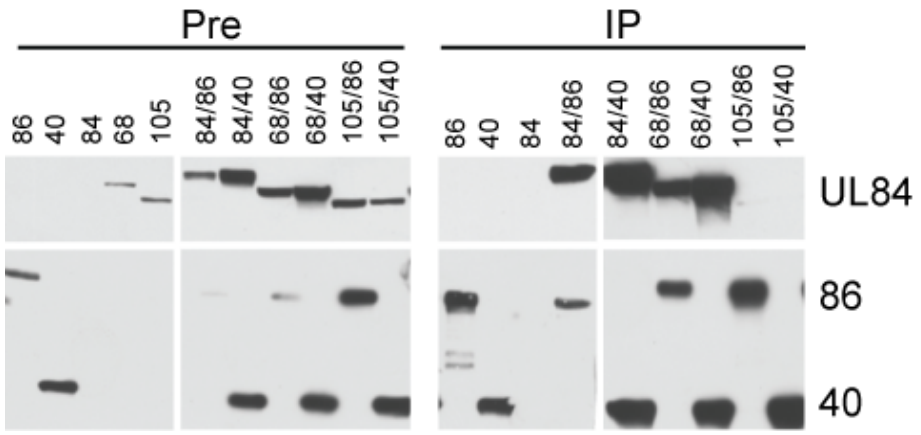


Figure 4.4. The first 105 aa of UL84 are important for an interaction with IE2 86 and IE2 40. wt UL84 and each of the N terminal mutants, Δ68 (68) and Δ105 (105) were assayed for an interaction with both IE2 86 and IE2 40 using immunoprecipitation assays as described in the materials and methods. Expression of each of the proteins was analyzed before immunoprecipitation (Pre) in order to assess the relative expression levels. Following immunoprecipitation, the complexes were eluted from the agarose beads, and are represented in the immunoprecipitation (IP) lanes. UL84 (wt and mutants), IE2 86 and IE2 40 expression was assessed.

significantly lower than the UL84 levels when coexpressed with IE2 40 in the absence of the inhibitor. This was also true for the deletion mutants, Δ68 and Δ105. Interestingly, the protein expression of UL84 also increased when treated with MG132 and co-expressed with either of the IE2 proteins, although the relative increase was slightly less. These data indicate that the IE2 proteins provide some protection from degradation, but they are not sufficient to prevent this degradation entirely. IE2 86 and IE2 40 were not sensitive to the proteasome inhibitor treatment during this period of time, and remained stably expressed throughout the course of the drug treatment.

The first 105 aa of UL84 are sufficient for enhanced expression from the IE2 proteins.

Given that co-expression of the IE2 proteins yielded increased UL84 protein production, and that the first 105 aa of UL84 were shown to be the predominant region of interaction, we next tested whether these aa were sufficient for the enhancement of expression when co-expressed with IE2 86 and IE2 40. To test this, the first 105 aa were cloned onto the N-terminus of another HCMV viral protein, UL44 (Figure 4.6A). This protein was termed UL44-84 Hyb (Hyb). As before, transfections were conducted in 293FT cells and analyzed for protein expression. As a control, IE2 40 was co-transfected with UL44 to determine if IE2 40 could activate UL44 in the same way as UL84. Co-expression of IE2 40 with UL44 had no effect on UL44 protein expression (data not shown, and see below).

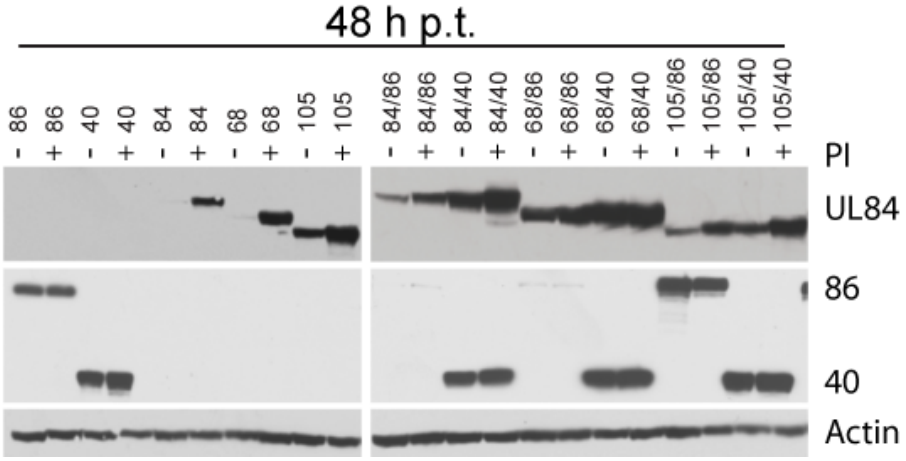


Figure 4.5. Proteasome inhibitor treatment affects the level of UL84 expression, but co-expression with the IE2 proteins does not protect UL84 from proteasomal degradation. 36 h p.t., cells were incubated with a proteasome inhibitor (MG132, 10μM, +) or mock-treated with DMSO (-), and then assayed for protein expression 12 h later. UL84 expression was assayed by Western blot with or without the presence of IE2 86 or IE2 40. Actin serves as a loading control.

However, when IE2 40 or IE2 86 was co-transfected with the UL44-84 Hyb plasmid, significant increases in the hybrid protein expression were observed compared to the singly transfected plasmid (Figure 4.6A). This protein can be detected with either an antibody recognizing the first 105 aa of UL84, or an antibody recognizing UL44. Expression of the hybrid protein did not inhibit expression of IE2 86 or IE2 40, indicating that the first 105 aa of UL84 are not sufficient for the reduction in IE2 protein expression.

To test whether the Hyb protein was also sensitive to proteasomal degradation as observed for the full-length UL84 protein, this mutant was tested for sensitivity to treatment with the proteasomal inhibitors MG132 and Lactacystin (only MG132 is shown). Following treatment with either inhibitor for 12 h before harvest (36-48 h. p.t.), cells were harvested and assessed for protein content by Western Blot analysis. As a control, wt UL84 was assayed to ensure that the proteasome treatment was effective (Figure 4.6C). UL44 was assayed for proteasome sensitivity with and without IE2 86 and IE2 40. No UL44 protein enhancement was seen when the proteasome inhibitor was present. Furthermore, the expression of IE2 86 or IE2 40 did not enhance the expression of UL44. Again, expression of IE2 86 and IE2 40 remained unaffected in all samples. When the Hyb protein was assayed, enhancement from IE2 86 and IE2 40 was observed as before, and no proteasome sensitivity was observed. This indicates that the first 105 aa of UL84 alone are not sufficient for the proteasomal degradation of UL84 observed during these transfection studies, but are sufficient for the enhancement of protein expression when co-expressed with IE2 86 and IE2 40.

The RNA for each of the above samples was analyzed for multiple experiments using quantitative real-time RT-PCR (Figure 4.6D). The IE2 86 and IE2 40 RNAs showed no difference in expression when co-transfected with the UL44 or UL44-84 Hyb plasmid. Similarly, the amount of Hyb and UL44 RNA was analyzed. In each case, no differences in the levels of RNA were observed when either the Hyb or UL44 was cotransfected with IE2 86 or IE2 40, further confirming the previous results that the enhancement provided by the IE2 proteins is post-transcriptional.

Given that the amount of Hyb protein was significantly less than the amount of UL44 protein being expressed during these transfections, the amount of Hyb RNA was compared to that of the UL84 and UL44 RNAs (Figure 4.7). Interestingly, we observed a six to eight fold decrease in the amount of RNA produced during this transfection when compared to UL44. To understand how this compared to the amount of UL84 RNA, new primers and probes were designed that recognized the domain common to both wt UL84 and the Hyb RNA. Using quantitative real-time RT-PCR, the amount of RNA was compared and found to be comparable to wt UL84.

UL84 amino acid sequence is important for the enhancement governed by IE2 40.

To determine whether the actual amino acid coding sequence was necessary for the enhancement governed by IE2 40, we constructed a UL84 mutant that maintained the same RNA sequence (except for the minimal number of base changes required to create the mutant) but encoded for a different amino acid sequence for the first 176 aa (see schematic, Figure 4.8A). This created a mutant that would delineate between the presence of the RNA being important for IE2 40 regulated enhancement, versus the

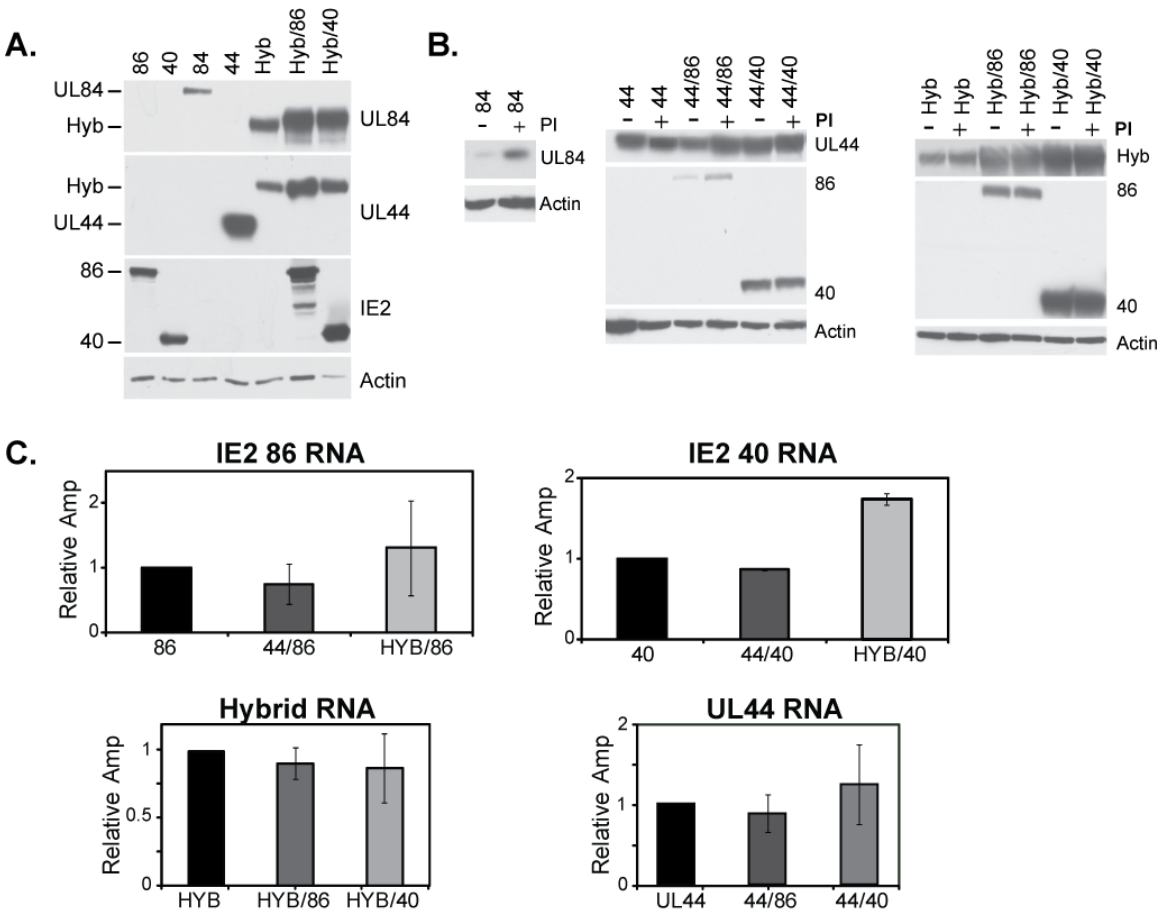


Figure 4.6. The first 105 aa of UL84 are sufficient for enhancement governed by the IE2 proteins, but not for proteasomal degradation. (A) The first 105 aa were added onto another viral protein, UL44 (Hyb). Co-expression of this protein with IE2 86 and IE2 40 was assessed, as was the expression of UL44. The expression of the Hyb protein was assayed using an antibody directed against UL84 and UL44. (B) Proteasome treatment was conducted as in Figure 5, except that the UL44 and Hyb proteins were analyzed in combination with IE2 86 (86) and IE2 40 (40). Cells were either treated with the proteasome inhibitor (+) or mock-treated with DMSO (-). UL84 protein expression was assayed to ensure that the proteasome treatment was effective. Actin serves as a loading control in both (A) and (B). (C) RNA was analyzed for IE2 86 (86), IE2 ΔSX (SX), IE2 40 (40), UL44 (44), and Hyb samples. All samples were normalized to G6PD.

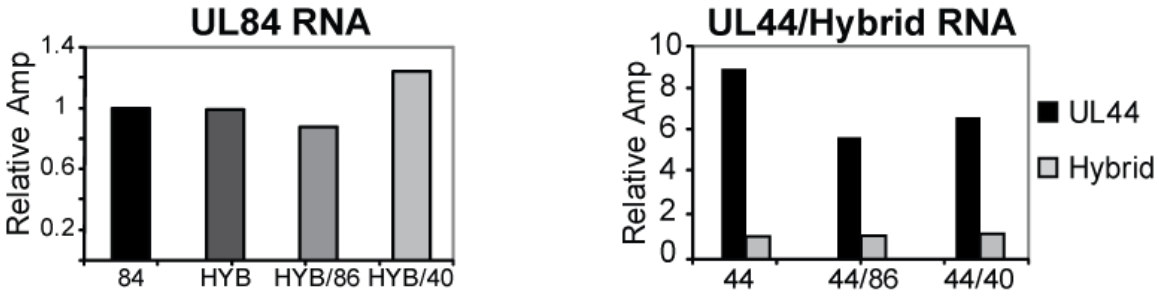


Figure 4.7. Hyb RNA is expressed to the same level as UL84, but much less than UL44. RNA was prepared from the same samples as in Figure 6A and analyzed by quantitative real-time RT-PCR. Primers and probes to the N-terminus of UL84 were used to compare the amount of RNA in the Hyb samples to UL84 (UL84 RNA). Primers and probes to UL44 were used to compare the expression levels of the Hyb RNA to UL44 (UL44/Hybrid RNA).

appropriate amino acid sequence. This new mutant is referred to as UL84-Frameshift (FS) for the remainder of these studies. 293FT cells were transfected with UL84 (wt), IE2 40, IE2 86, and the FS plasmids as before. 48 h p.t., cells were harvested and analyzed for the quantity of DNA, RNA, and protein. DNA was quantitated by quantitative real-time PCR, and showed that equal amounts of the UL84 plasmid and FS plasmid had been transfected in all samples (data not shown). Further, the amount of IE2 40 and IE2 86 DNA was comparable for each of the samples containing this DNA (data not shown). The amount of RNA was also quantitated using quantitative real-time RT-PCR, and this revealed that, similar to wt UL84, the FS mutant was not affected transcriptionally in the presence of IE2 86 or IE2 40. Further, the amount of IE2 86 and IE2 40 RNA remained constant when co-transfected with UL84 or FS DNA (Figure 4.8B).

Next, the protein content for each sample was assessed (Figure 4.8C). UL84 and FS protein was measured using an antibody that recognized a region downstream of aa 176, thus recognizing both proteins. The amount of IE2 86 and IE2 40 protein present was measured with an antibody that recognizes the C-terminal region of IE2 86, and thus recognizes both proteins. As before, UL84 protein expression was enhanced slightly when co-transfected with IE2 86, although this effect again was minimal compared to the significant enhancement governed by IE2 40. However, when the FS mutant was co-transfected with IE2 40, no enhancement of the protein was detected. These data indicate that the low levels of UL84 protein when expressed alone are not solely due to the first

176 aa of UL84. However, this protein sequence is important for IE2 40 enhancement of UL84.

The amount of IE2 86 and IE2 40 protein was also assessed. As before, IE2 86 and IE2 40 protein expression was repressed when co-expressed with UL84 (Figure 4.8C). However, expression of IE2 86 and IE2 40 when co-transfected with FS did not show any inhibition, further confirming that the N-terminal protein sequence is necessary for this event to occur.

To determine if the interaction with IE2 86 and IE2 40 with UL84 was occurring in the FS mutant, immunoprecipitation reactions were conducted as before (Figure 4.8D). Using agarose beads coupled with the IE2 antibody that recognizes both IE2 86 and IE2 40, immunoprecipitations were conducted, either in combination with UL84 or with the FS mutant. As before, UL84 interacted well with IE2 86 and IE2 40. However, the FS mutant was unable to interact with either IE2 86 or IE2 40, indicating that the N-terminal amino acid sequence is important for the interaction between UL84 and IE2 86 and IE2 40. This confirms results with the UL84Δ105 mutant, and indicates that the RNA is not sufficient to facilitate this interaction.

Deletions spanning the length of IE2 40 affect binding of and enhancement from UL84.

To assess whether a particular domain of IE2 40 is involved in the interaction with UL84, deletions were created spanning the length of the IE2 40 protein. The full-length IE2 40 is encoded by amino acids 242-579 of IE2 86. The regions that were deleted in this analysis include amino acids 242-290, 242-360, 361-420, 421-542, and

543-579. These deletions span the entire length of IE2 40 (for schematic, see Figure 4.9A). Each clone retained the HA tag at the C-terminus, allowing detection by Western Blot as well as interaction studies using an anti-HA antibody.

The expression of each of these mutants was tested by transfection in 293FT cells. All proteins expressed well, and ranged in size between ~30-40 kDa. WT IE2 40 expressed to the highest degree, while expression of IE2 40Δ242-290 expressed slightly less. Expression of IE2 40 Δ242-360 and IE2 40 Δ421-542 expressed the least of any of the clones, while IE2 40 Δ361-420 expressed similar to IE2 40 Δ242-290 (data not shown). When assessed by Western Blot analysis in combination with UL84, many of the mutants were unable to enhance UL84 expression to the same degree as WT IE2 40. In particular, IE2 40 Δ361-420 and IE2 40 Δ421-542 enhanced expression the least, while IE2 40 Δ242-290, IE2 40 Δ242-360 and IE2 40 Δ542-579 were intermediate in this regard. When assessed for binding to UL84, all of the IE2 40 mutants displayed a weakened or abrogated ability to interact with the exception of IE2 40 Δ242-290 (data not shown).

Given that UL84 expression is so tightly linked to the amount of IE2 40 present, and that each of the IE2 40 mutants expressed to different levels, we wanted to understand whether the amount IE2 40 being produced was responsible for the differences in activation of UL84, or if the loss of binding to UL84 was important for this phenotype. Since expression levels were variable in 293FT cells, we utilized an *in vitro* system to continue to assess the ability of each of the IE2 40 mutants to bind to UL84.

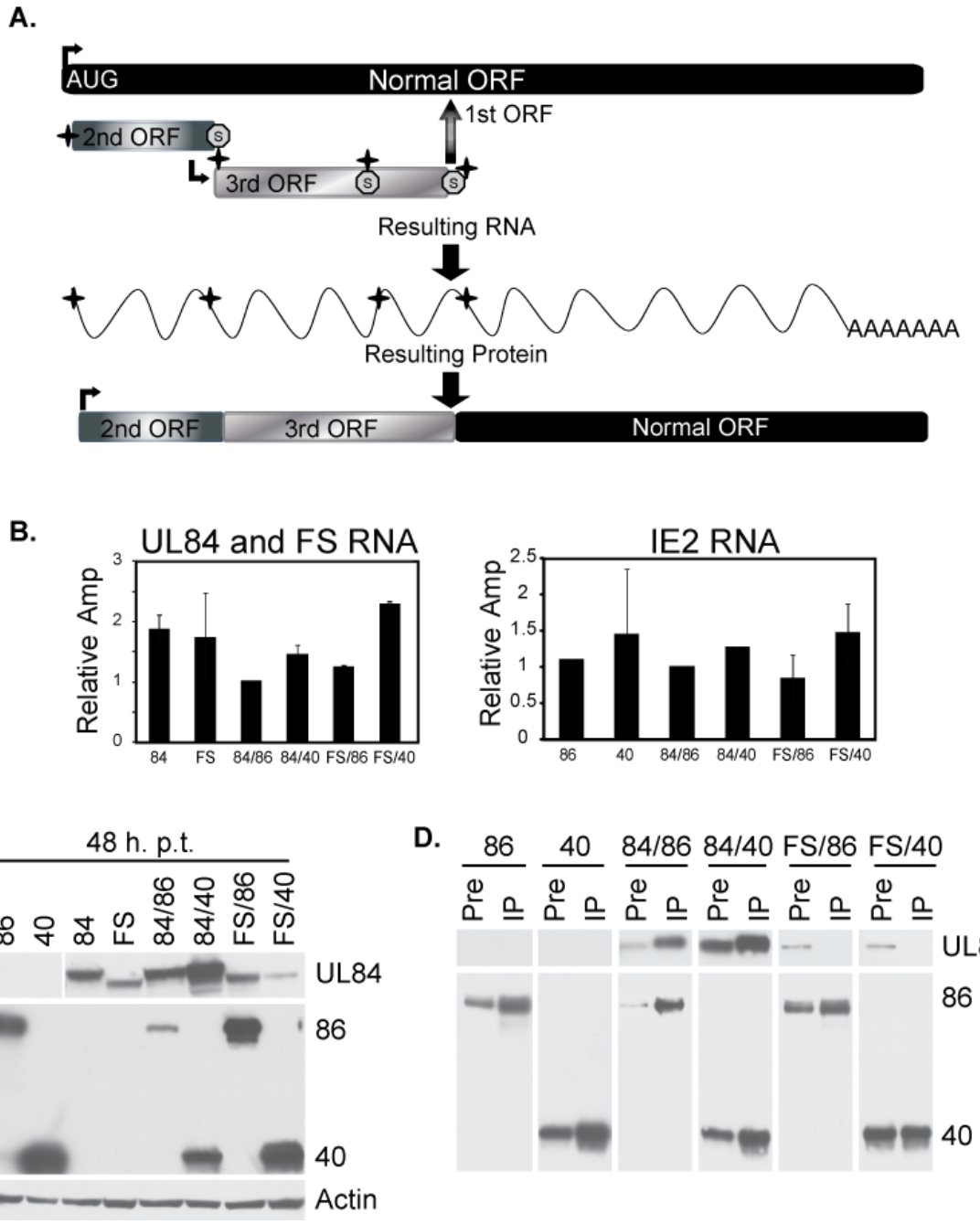
Given that UL84 expression in not enhanced *in vitro*, this made for a simpler system to understand the ability of the IE2 40 mutants to bind to UL84.

To test this, the IE2 40 WT and mutant clones were synthesized in an *in vitro* transcription-translation reaction, as was UL84. Expression of the *in vitro* transcribed and translated protein was tested by Western blot analyses and determined to be comparable for each clone (Figure 4.9B). For the immunoprecipitation reactions, equal amounts of IE2 40 (WT or mutant) and UL84 were used. Anti-HA coupled protein G-PLUS beads were used as before, and immunoprecipitations were performed for 4 hours at 4°C, and then washed 3 times with normal immunoprecipitation buffer. Following washes, the immunoprecipitated proteins were eluted from the agarose beads using reducing sample buffer at 95°C for 5 minutes (see Materials and Methods). The pre corresponds to 1 µl of the *in vitro* transcription/translation reaction, and represents 25% of the amount of protein loaded in the IP lanes. The IP contains all proteins that are associated in the immunoprecipitation reaction. The Post lane represents 12.5% of the IP lane, and corresponds to 50% of the amount of protein present in the "Pre" lane. The pre and post samples represent what is present before and after the immunoprecipitation, respectively, and allows assessment of the efficiency of the immunoprecipitation. Control lanes include IE2 40 alone, and UL84 alone, to test for specificity of the antibody and the IP, respectively.

Each of the IE2 40 mutants gave similar results as seen in the transfected samples (Figure 4.9B). As expected, no UL84 was present in the IE2 40 (40) or UL84 (84) IP lanes. The combination of IE2 40 and UL84 was sufficient for immunoprecipitation, as

expected. Similar to the results seen in the transfected samples, the IE2 40Δ242-290 mutant was able to bind UL84 (Δ242-290). All other mutant forms of IE2 40 were unable to bind UL84 in these experiments. These data corroborated the transfection results, and show that all regions downstream of aa 290 contribute to an interaction with UL84.

Figure 4.8. UL84 amino acid sequence is important for the regulation governed by IE2 86 and IE2 40, and for the interaction of UL84 with the IE2 proteins. (A) Schematic representation of the mutations (star shape) made in the RNA to shift the amino acid sequence into the second and third open reading frames (ORF). The original AUG is depicted and is the site of the first mutation to change the amino acid sequence into the second ORF. A second mutation of the RNA was made at the site of a stop codon (S) and results in a shift to the third ORF. A third mutation changes a stop codon to allow read-through, while the fourth mutation switches the amino acid sequence back to the original ORF at the otherwise encoded stop codon. The resulting RNA has four single nucleotide mutations, while the resulting protein encodes for a new ORF for the first 175 aa, and retains the original ORF for the length of the protein. (B) The RNA for each of the samples was measured by quantitative real-time RT-PCR. Analyses were conducted as in previous experiments, and each of the relative RNA values are shown. (C) The frameshift mutant (FS) was tested by transfection in combination with IE2 86 and IE2 40. 48 h p.t., cells were harvested and assessed for protein expression by Western blot analysis. UL84, FS, IE2 86, and IE2 40 expression was tested. Actin serves as a loading control. (D) Interactions between each of the proteins assessed in (B and C) were analyzed using immunoprecipitation assays. The amount of protein present before the immunoprecipitation is shown (Pre), followed by the amount of protein that was pulled down in the immunoprecipitation (IP). UL84, FS, IE2 86, and IE2 40 expression and interaction was assessed. The FS mutant is recognized by a C-terminal UL84 antibody.


A.
AUG
Normal ORF
1st ORF
2nd ORF
3rd ORF
Resulting RNA
AAAAAAA
Resulting Protein
2nd ORF
3rd ORF
Normal ORF
B.
UL84 and FS RNA
Relative Amp
84
FS
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84/40
FS/86
FS/40
IE2 RNA
Relative Amp
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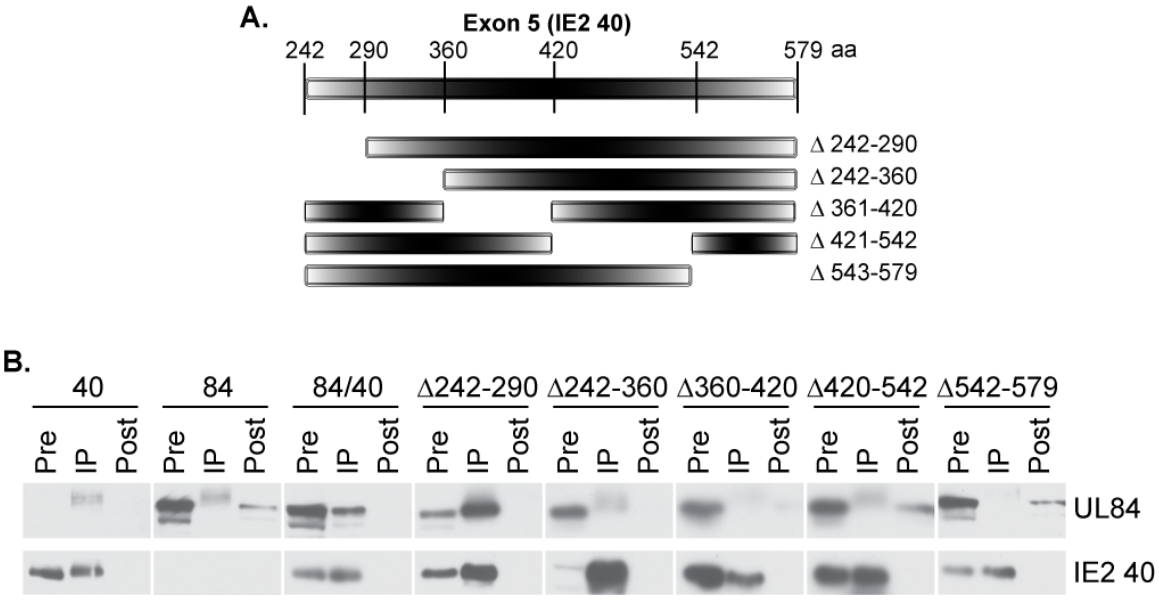

Figure 4.9. Domains throughout IE2 40 are important for an interaction with UL84. (A) Schematic representation of deletions created throughout the coding region of IE2 40. IE2 40 translation begins at aa 242 of IE2 86. Deletions created include Δ242-290, Δ242-360, Δ361-420, Δ421-542, Δ543-579. (B) Immunoprecipitation analysis of *in vitro* translated proteins including all IE2 mutants with WT UL84. Immunoprecipitation lanes (40) and (84) are negative control lanes and include only IE2 40 protein or UL84 protein, respectively. The 84/40 lane serves as a positive control for binding, and all deletion mutants shown were tested in combination with UL84 to assay the ability of the mutant to bind UL84. Pre represents the fraction present before immunoprecipitation, while the IP lane represents proteins that were immunoprecipitated. Post lanes represent the fraction of protein left after immunoprecipitation, and contain only half the amount of protein as present in the Pre fraction. IE2 40 and UL84 were assayed by Western Blot analysis to analyze interactions.

DISCUSSION

Many functions have been ascribed to the MIE proteins during the HCMV infection process. It is known that IE1 72 and IE2 86 serve in shutting down host defenses immediately upon infection. For instance, IE1 72 and IE2 86 have been shown to be important for dysregulating the cell cycle and interfering with apoptosis (31, 117, 136, 226-227, 245). IE2 86 has also been shown to be important for regulating many cellular and viral transcripts throughout the later stages of the infection (For reviews see (63, 132). Much of the regulation governed by IE2 86 is thought to be controlled by protein-protein interactions, given the vast number of proteins that IE2 86 has been shown to interact with (26-27, 32, 39, 41, 64, 77-78, 90, 92, 96-97, 108, 118-119, 176, 178, 188, 191-192, 219, 237). Although IE1 72 and IE2 86 have been extensively studied, the functions of the two early-late proteins, IE2 60 and IE2 40, remain less well understood. Our lab and others have sought to understand the contribution of these proteins to the infection process. In previous studies, our lab has shown that IE2 60 and IE2 40 are important for the late stages of the viral infection, impacting several viral early-late proteins. Interestingly, the regulation of one particular early-late protein, UL84, was shown to be post-transcriptional (171-172, 223).

UL84 has been shown to be important for *ori-lyt*-dependent DNA replication, and is involved in downregulating the ability of IE2 86 to activate some early promoters in transient assays (68). UL84 is the only other viral protein that IE2 86 has been shown to interact with to date. Our previous studies showed that IE2 60 and IE2 40 also have the ability to interact with UL84 individually, independent of IE2 86. Loss of these proteins

in the infection resulted in a significant loss of UL84 protein, but not RNA, expression. In these studies, IE2 40 was found to be more important for regulating UL84 protein expression. Given the known role of IE2 86 as a transcriptional activator, it is important to understand the other potential roles these proteins may be playing in regard to regulating protein expression throughout the infection process.

The current studies have shown that the post-transcriptional regulation of UL84 regulated by IE2 40 can occur outside of the viral infection and in the absence other viral proteins. Similar to the infection process, IE2 86 is able to enhance the expression of UL84 to some degree, while expression of UL84 protein is highly upregulated when co-transfected with IE2 40. This regulation is tightly linked to the amount of IE2 40 present, given that a decrease in the amount of the IE2 40 protein available results in a concomitant loss of UL84 protein expression. These data indicate that the expression of UL84 in both the context of the infection and outside of the infection is tightly linked to the amount of IE2 40 protein present, and this may be one way that HCMV facilitates temporal regulation of protein expression. Given that aberrant expression of many HCMV proteins has been shown to be detrimental to the infection, this method of regulation may be advantageous to the virus in that the ability for fine tuned regulation of expression is achievable. Further, IE2 40 may facilitate the use of many cellular translational processes, which would decrease the burden of the virus to encode many genes to regulate the viral infection in this manner. Given the vast number of proteins IE2 86 has been shown to interact with within the same region encoding IE2 40, we

believe that IE2 40 may play a major role in the regulation thought to be attributed to IE2 86.

These studies led us to continue to identify the domains that are important for the observed regulation. We have found that the first 105 aa of UL84 are important for the interaction with IE2 86 and IE2 40. Further, loss of these aa resulted in loss of the protein enhancement from IE2 86 and IE2 40. These data suggested that a protein-protein interaction is important for this phenotype to occur. In order to address whether the RNA was sufficient to facilitate this interaction, or if the appropriate amino acid sequence is necessary, we created a mutant that maintained the RNA sequence for UL84 except for 4 base changes, but encoded for a different amino acid sequence throughout the N-terminus of UL84. This mutant was not enhanced by the presence of IE2 40, and was unable to interact with IE2 86 or IE2 40. These data further confirmed the hypothesis that the first 105 aa are important for this regulation, and that UL84 RNA sequence is not sufficient for this phenotype. We also showed that the first 105 aa are sufficient for the enhancement from IE2 40 and IE2 86, as these aa attached to the N-terminal region of another HCMV viral protein recapitulated this phenotype.

The first 105 aa of UL84 are likely highly unstructured according to simple bioinformatics analysis (unpublished results, D.H. Spector and R.L. Sanders). It is possible that IE2 40 can act to stabilize UL84 protein expression by aiding in the generation of the correct structure of UL84. In this manner, IE2 40 could potentially act as a chaperone protein, regulating protein folding and expression. Given that the majority of UL84 is in complex with the IE2 proteins (172), it is likely that some type of

regulation such as this is necessary for proper expression of UL84. It is also possible that IE2 40 serves to either remove a translational repressor, or acts as a translational enhancer. The possibility also exists that there are cellular mediators involved in this tightly regulated phenomenon, and that the virus is utilizing one or more of the potential available cellular regulators.

UL84 was also shown to be sensitive to proteasomal degradation in these assays. These data differed from that in the infection (172). There are many possible explanations for this phenotype. It is thought that the virus manipulates the proteasome to be favorable for viral replication (unpublished results, D.H. Spector and K. Tran), and the possibility exists that the proteasome is changed during infection such that many of the viral proteins are not sent to the proteasome for degradation. It is thought that UL84 contains domains that would allow ubiquitination, as well as proteasome dependent degradation (66), but the mechanism by which the proteasome regulates UL84 protein expression levels requires further investigation. It was clear in these studies that the expression of IE2 86 and IE2 40 was not sufficient for protecting UL84 from proteasomal degradation, and that the IE2 proteins provide enhanced expression of UL84 protein levels, above that which is produced when the cells are treated with a proteasome inhibitor.

The domains of IE2 40 that are involved in an interaction with and regulation of UL84 appear to be diverse and complex. Interestingly, deletion of domains throughout IE2 40 resulted in loss of interaction with UL84. Transfection studies suggest that loss of an interaction between IE2 40 and UL84 negates the enhancement facilitated by IE2 40.

Further, domains downstream of aa 290 appear to be involved in this regulation. Given that deletion of any domain downstream of aa 290 resulted in loss of binding in the *in vitro* studies, we hypothesize that multiple domains may contribute to the interaction with UL84. There are many potential hypotheses to explain the loss of interaction between these two proteins. The dimerization domains of IE2 86 and IE2 40 lie in a region common to both proteins, and thus it is possible that dimerization and/or multimerization of these proteins is important for the regulations observed in these studies. Further, the structure of IE2 40 may be affected by the deletions constructed. This would indicate that the interaction with UL84 is dependent on the appropriate structure of IE2 40. Also, it is possible that another cellular protein helps to facilitate the interaction between IE2 40 and UL84, and that this interaction is disrupted. The interaction between IE2 40 and UL84 is highly dependent on the appropriate domains and structure being present.

Many other viruses utilize mechanisms of post-transcriptional regulation to mediate proper expression of both viral and cellular proteins throughout the infection processes (7, 20, 133, 173, 175, 215-216). These mechanisms likely serve as additional control of viral and cellular expression patterns throughout the infection process, making the environment most favorable for productive viral infection. Undoubtedly, there is much to be learned regarding the regulation governed by these proteins, and it is important to identify the mechanisms by which these regulations lead to productive infection.

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The text of Chapter 4, in full, has been submitted for publication of the material as it may appear in *Journal of Virology*, 2009, Sanders, R.L. and D.H. Spector. The dissertation author was the primary investigator and author of this paper.

CHAPTER 5

DISCUSSION

This dissertation describes the analysis of the IE2 family of proteins and elucidation of their roles throughout the infection process. IE2 86 has long been studied as a major player during the infection process and has been shown to have many diverse functions at different stages of the infection. IE2 86 is believed to be a transcriptional activator of both cellular and viral genes, and has been shown to interact with many cellular proteins, likely governing their activities during the infection. Studying mutations in IE2 86 in the context of the infection process has been very difficult, given that it is essential to the infection, and that many regions have been found to be necessary for the proper function of IE2 86. One focus of this dissertation has been to develop a system that allows mutations in essential regions of IE2 86 to be analyzed during the infection. This strategy has involved the development of an inducible expression system that allows for expression of IE2 86 at the onset of the infection. Further, we were able to elucidate roles of the IE2 60 and IE2 40 proteins using this system as well.

Providing appropriate temporal expression of both IE2 86 (immediate-early) and the IE2 60 and IE2 40 proteins (early-late) was an important aspect to consider while creating this system, given that it has been shown that aberrant expression of IE2 86 is deleterious to the infection (53). Further, since IE2 60 and IE2 40 do not arise until later in the infection, and therefore only have roles at the later stages of the infection, we felt it was necessary to provide these during the appropriate times post infection. Our system

utilizes a strategy that allows inducible and selective translation of these proteins following expression of Cre and FLP recombinases. We have constructed viruses that express these recombinases, driven by either the HCMV immediate-early promoter (Cre), or the viral 1.2 kb promoter (FLP), which is only expressed at early-late times post infection. In combination with these viruses, we have constructed cell lines using a lenti-viral based transduction system that allows expression of IE2 86, IE2 60, or IE2 40 following a recombination event that removes a selectable marker upstream of the coding regions for the IE2 proteins. This selectable marker is surrounded in either loxP sites or Frt sites, and upon infection with the virus expressing Cre/FLP, the recombination event commences at the appropriate time post infection. This system has proven to be very effective, and is the first to date to allow complementation of a previously non-viable IE2 mutant virus.

Using this system, we have further identified the contribution of IE2 86, IE2 60, and IE2 40 to the infection process. In regard to a debilitated IE2 mutant virus, IE2 86ΔSX, which also does not express IE2 60 or IE2 40, we were able to elucidate roles of all three IE2 proteins (Chapter 2). This virus normally grows to reduced titers and exhibits very slow cell to cell spread when compared to WT. The expression of WT IE2 86 was able to complement the cell to cell spread, but not the titer produced from this viral infection of human fibroblasts. The expression of both IE2 86 and IE2 40 together had the most beneficial effect on viral progression, aiding in both the titer and spread of the viral infection. The expression of IE2 60 or IE2 40 alone did not aid the virus to any degree, indicating that both IE2 86 and IE2 60/40 must act in concert to achieve optimal

viral conditions. Further, in regard to expression of select viral early-late proteins, the combination of IE2 86 and IE2 40 expressed from the cell lines had the most beneficial effect. Expression of IE2 86 alone was able to complement the expression of UL83 and UL99, while the expression of UL84 protein was increased most significantly when IE2 86 and IE2 40 were expressed in combination. These data corroborated previous analyses that indicated the loss of IE2 40 during the infection process resulted in a reduction in UL84 protein levels (223) and revealed new insight into the regulation governed by the IE2 proteins.

The complementation system developed for this dissertation project was also able to complement a previously non-viable virus, termed IE2 86ΔExon5, which is missing the entire coding region of exon 5 of IE2 86 (Chapter 2). The expression of IE2 86 alone was able to complement this virus to some degree, and the expression of both IE2 86 and IE2 40 was most advantageous to the viral infection. Although this virus still grew slower than WT when grown on the double cell line, this was the first demonstrated example of complementation of previously null virus. We believe that the tight regulation of expression of IE2 86, as well as the levels of protein expressed from these cells allowed this feat to be possible. Many other systems have failed throughout the years (unpublished results, Deborah H. Spector), and this has been a major limitation in studying IE2 mutant viruses. This system can be widely applied to many other HCMV proteins, as well as other proteins which may be toxic when constitutively expressed. Our experience with other inducible expression systems that are regulated at the transcriptional level is that it is difficult to completely eliminate expression of the second

gene in the provided vector. In contrast, translation in eukaryotic cells of the second ORF on a multicistronic RNA, in the absence of an internal ribosome entry site is very uncommon, and control at this level is much more tightly regulated.

Another focus of the studies conducted for this dissertation involved the regulation of the viral early-late protein, UL84. We have found that the IE2 proteins govern expression of this protein in a very unique fashion, and thus have focused a significant portion of the research on understanding what regulations take place, and how these regulations might affect the viral infection. UL84 is the only viral protein found to interact with IE2 86 (167, 191). We have also found that IE2 60 and IE2 40 individually have the ability to interact with UL84 (Chapter 3). We have used IE2 mutant viruses to examine the interactions and regulation of the IE2 proteins and UL84. The mutant virus IE2 86ΔSX (aa 136-290 of IE2 86 removed) exhibits severely reduced levels of UL84 protein, but not RNA, expression. This loss of UL84 was found to be proteasome and calpain independent, and the stability of the protein is comparable to that in the WT infection. We also analyzed the export of the RNA to the cytoplasm, as well as the loading of the RNA onto polyribosomes, and found no difference when compared to the WT infection. Other studies revealed that IE2 40 plays a significant role in regulating UL84 expression (Chapters 2, 3 and 4, and 223), and is likely the primary regulator of UL84 protein expression at late times post infection. These studies also identified that the C-terminal portion of IE2 86 (shared with IE2 60 and IE2 40) is important for an interaction with UL84, indicating that an interaction may be necessary for this regulation to occur.

We have also determined that IE2 86 and IE2 40 can regulate UL84 protein expression in co-transfection assays outside of the viral infection, indicating that no other viral proteins are required for this regulation (Chapter 4). This phenotype does not occur *in vitro* however, indicating that some cellular processes are necessary for the appropriate regulation to occur. Mutational analysis of UL84 identified that the region that is responsible for an interaction with both IE2 86 and IE2 40 lies in the first 105 amino acids of UL84, and that loss of this region abrogates the ability of IE2 86 and IE2 40 to affect the levels of the UL84 protein. Further, the first 105 amino acids are sufficient for enhancement from the IE2 proteins, given that these amino acids added onto another viral protein mimic the effect seen with WT UL84. These studies also show that this region is not sufficient for proteasomal degradation, and that the appropriate amino acid (not just RNA) sequence is necessary for the IE2 proteins to affect the levels of UL84 protein.

These data suggest a number of interesting possibilities regarding the regulation of UL84 during the infection. It has yet to be fully elucidated how the IE2 proteins are allowing enhanced UL84 protein expression, although many key components of this regulation have been identified. Given that this regulation can occur outside of the infection, but requires some cellular components, the possibility exists that one or more cellular proteins is aiding in this phenomenon. IE2 86 has been shown to interact with many cellular proteins during the infection, as has UL84, and thus it is possible that a common or distinct binding partner is necessary for proper UL84 expression. The virus has been shown to modulate many aspects of the cellular environment, making this hypothesis plausible. The cell has many translational systems in place that could

potentially be utilized by the virus in this regard, and thus it would be interesting to assess whether these known cellular translational mechanisms are aiding in proper viral protein expression.

Another interesting hypothesis is that UL84 must associate with the IE2 proteins following translation, to prevent degradation by a pathway yet to be identified. During these studies, we assayed many different degradation pathways, and never elucidated a contributing pathway (unpublished results, R.L. Sanders and D.H. Spector). We believe that IE2 40 is more tightly linked to UL84 protein expression than either IE2 86 or IE2 60, and hypothesize that UL84 protein expression is very tightly governed by IE2 40 through an immediate interaction following synthesis of the protein. It is possible that IE2 40 (and to some extent IE2 86 and IE2 60) is able to protect the UL84 protein from rapid turn-over.

These data also indicate that IE2 40 may be acting as a chaperone-like protein, governing folding, and thus protein expression of UL84. Given that the amount of UL84 protein expressed is so tightly linked to the amount of IE2 40 protein present in the cell, it remains possible that this potential chaperoning like activity allows tight control of the expression ofUL84. During the infection, the expression of IE2 40, IE2 60, and UL84 arise concomitantly, and this may be a way that the virus allows proper temporal expression of these proteins. It is clear that the domains of IE2 40 involved in regulating UL84 expression are complex, and likely involve multiple domains. It is possible that the dimerization and multimerization of IE2 86 and IE2 40 are important for the observed regulations. Further, these domains may be important for interacting with other cellular

proteins that are involved with this process. Other potential mechanisms exist, and clearly there are many important questions to be addressed in order to further understand this important aspect of regulation during the HCMV infection process.

Governing appropriate temporal expression is a major hurdle in producing a productive viral infection, and the mechanisms that I have identified provide one more insight into how this complex virus regulates gene and protein expression. There have been other examples of translational regulation in herpesviruses (7, 20, 133, 173, 175, 215-216), although this field remains poorly understood. Given that so many cellular expression patterns are regulated in a post-transcriptional fashion, it is probable that viruses have developed similar, tightly controlled systems to regulate protein expression.

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