ATTENUATION OF THE HOST INNATE CYTOKINE RESPONSE BY THE HUMAN CYTOMEGALOVIRUS IMMEDIATE-EARLY 2 PROTEIN IE86

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by

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ATTENUATION OF THE HOST INNATE CYTOKINE RESPONSE BY THE HUMAN CYTOMEGALOVIRUS IMMEDIATE-EARLY 2 PROTEIN IE86

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Human cytomegalovirus infects a majority of the human population and is a significant cause of life-long morbidity and mortality in neonates and patients with an impaired immune system. Human cytomegalovirus infection has a profound effect on host cell, and expression of new viral proteins interferes with the ability of the host response to effectively limit virus persistence and the initation of a latent infection. This aim of this dissertation was to identify how human cytomegalovirus attenuates the host innate immune response early during infection. Specifically, I have

employed genetic and biochemical approaches to identify the HCMV immediate-early 2 protein, IE86, as an interferon beta antagonist. IE86 expression also blocks the expression of a number of proinflammatory chemokines, including RANTES, MIG and IL-8 during human cytomegalovirus infection. I have further demonstrated that IE86 mediates the attenuation of cytokine and chemokine expression by targeting the nuclear factor kappa B pathway early during infection. Using gel shift analysis I have demonstrated that IE86 blocks nuclear factor kappa B DNA binding to target promoters, including the interferon beta promoter. Since IE86 is one of the first viral proteins to be expressed during human cytomegalovirus infection, it can rapidly block cytokine and chemokine expression thereby suppressing the antiviral response and limiting the recruitment of effecter cells. The attenuation of the innate immune response by IE86 likely enhances virus replication and contributes to persistence within the host. This work addresses a number of unanswered questions about human cytomegalovirus's interactions with the host, and has identified a previously unrecognized mechanism employed by human cytomegalovirus to evade the host immune response. A better understanding of IE86's ability to attenuate cytokine expression may be key to designing novel antiviral therapy or development of an effective vaccine to prevent human cytomegalovirus infection and disease.

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AD ..... adenovirus
AP ..... activator protein
BAC ..... bacterial artificial chromosome
CARD ..... caspase activation and recruiting domain
CBP ..... CREB binding protein
CHX ..... cycloheximide
CID ..... cytomegalic inclusion disease
CIF ..... cytomegalovirus induced factor
CREB ..... cAMP response element binding protein
CRS ..... cis repressor sequence
DS ..... double stranded
E ..... early
EBV ..... Epstein-Barr virus
EGFR ..... epithelial growth factor receptor
GAPDH ..... glyceraldehyde-3-phosphate dehydrogenase
GAS ..... interferon-γ activated sequence
GFP ..... green fluorescent protein
GPCMV ..... guinea pig cytomegalovirus
HA ..... hemagglutin
HAART ..... highly active antiretroviral therapy
HAT ..... histone acetyltransferase
HCMV ..... human cytomegalovirus
HCV ..... hepatitis C virus
HDAC ..... histone deacetylase
HFF ..... human foreskin fibroblast
HSV ..... Herpes simplex virus
IE ..... immediate-early
IFN ..... interferon
IFNAR ..... type I interferon receptor
IκB ..... inhibitor of NFκB
IKK ..... inhibitor kappa kinase
IL ..... interleukin
IPS ..... interferon-beta promoter stimulator
IRL ..... internal repetitive sequence long
IRF ..... interferon regulatory factor
IRS ..... internal repetitive sequence short
ISG ..... interferon stimulated gene
ISRE ..... interferon stimulated response element
IU ..... international units

KSHV .....Kaposi's sarcoma-associated herpesvirus
L .....late
LCMV .....lymphochoriomenigitits virus
LPS ..... lipopolysaccharide
LT ..... lymphotoxin
LTR .....long terminal repeat
MCMV .....murine cytomegalovirus
MCP ..... monocyte chemotactic protein
MDA ..... melanoma differentiation-associated gene
MHC .....major histocombatability complex
MIEP .....major immediate-early promoter
MIC .....MHC class I-related chain
MIG .....monokine-induced by interferon-γ
MIP ..... macrophage inflammatory protein
MT .....mutant
NFκB .....nuclear factor kappa B
NIH .....National Institutes of Health
NK ..... natural killer
OAS ..... oligoadenylate synthetase
ORF .....open reading frame
PAMP .....pathogen associated molecular pattern
P/CAF ..... p300/CBP associated factor
PKA .....protein kinase A
PKR .....protein kinase R
PP .....phosphoprotein
PRD .....positive regulatory domain
RANTES . regulated upon activation, normal T cell expressed and secreted
RHCMV .....rhesus cytomegalovirus
RHD ..... rel homology domain
RIG ..... retinoic acid-inducible gene
SenV .....Sendai virus
SS ..... single stranded
STAT ..... signal transducer and activator of transcription
TAD .....transactivation domain
TAP .....transporter associated with antigen processing
TBK .....tank-binding kinase
TBP ..... TATA binding protein
TF ..... transcription factor
TIR toll/IL-1R
TK .....thymidine kinase
TLR ..... toll-like receptor

TNFα ..... tumor necrosis factor alpha
TRAIL .....TNF related apoptosis inducing ligand
TRIF ..... TIR domain-containing adaptor-inducing IFN-β
TRL ..... terminal repetitive sequence long
TRS ..... terminal repetitive sequence short
UL ..... unique long
ULBP .....UL16 binding protein
US ..... unique short
UV ..... ultraviolet-irradiated
VSV .....vesicular stomatitis virus
VZV ..... Varicella-zoster virus
WT ..... wild-type

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

HUMAN CYTOMEGALOVIRUS

Taxonomy

Human cytomegalovirus (typically referred to as HCMV), or *human herpesvirus 5* (HHV-5), is the largest member of the *Herpesviridae* family, which includes a number of clinically relevant human viruses such as Herpes simplex virus 1 and 2 (HSV-1 and 2), Epstein-Barr virus (EBV), Varicella-zoster virus (VZV) and Kaposi's sarcoma-associated herpesvirus (KSHV). HCMV is the prototypic member of the *Betaherpesvirinae* subfamily (197). Members of this subfamily are related by species and cell-type specificity, relatively long replication cycles, and similar genome content and organization (197). Cells containing inclusion bodies, what is now considered a hallmark of HCMV infection, were first shown by Ribbert in 1881 (167). However it was not until 1921 when it was first suggested by Goodpasture and Talbert that 'cytomegalia', or cell enlargement, may be caused by a virus. HCMV strains were isolated in the late 1950's (167) and Kluge *et al.* proposed the name 'cytomegalovirus' in 1960 after isolating virus from the urine of infants with CMV disease (112).

Epidemiology and clinical features

HCMV infection is globally distributed with between 30% and 70% of the population infected by adulthood, but can exceed 90% seroprevalence in underdeveloped countries (70). HCMV is widespread due to its capacity for both vertical and horizontal infection. Typical of all herpesviruses, HCMV can establish both an acute, primary infection as

well as establishing a latent infection in cells of the myeloid lineage (132). To satisfy the conditions of a latent infection, the virus must be able to infect cells without any cytopathic effect, nor infectious virion production, the viral genome must be maintained by the host, and importantly the virus must prevent detection or elimination by the host immune response. The strong selective pressure of successful virus infection and persistence within the immunocompetent host has allowed the development of highly effective immune evasion strategies.

Transmission can occur during the acute infection or following reactivation from a latent state. Chronic infection, or persistent productive infection, with HCMV differs from acute infection by exhibiting low levels of virus replication, limited organ involvement and involves the host immune system. HCMV transmission requires direct contact with body fluids from individuals actively excreting virus. Perinatal and breast milk-associated transmission are considered the primary means for HCMV persistence in the population (11). Transfer to uninfected individuals can also occur through contact with body secretions including urine, saliva, tears, semen and cervical secretions since infectious virus can be present in these secretions from months to years. Consequently, non-congenital HCMV is most often sexually transmitted or obtained by children in daycare settings (70).

HCMV infection in an immunocompetent host

Acute HCMV infection is associated with a wide range of symptoms and disease, though high virus replication with multiple organ involvement is a common theme. Symptomatic disease in an immunologically healthy host is a rare event, but most often results in mononucleosis with symptoms ranging from fever and fatigue to hepatitis, splenopathy and

adenopathy (70). Interestingly, it has been reported that prolonged virus replication and viremia can be associated with asymptomatic infection, suggesting that immunity to HCMV infection can prevent organ disease, though is not sufficient to limit virus replication (152). Infection of a normal host, as with acute infection of immune-deficient individuals, results in persistant viremia and virus excretion and the virus can undergo latency in cells of the myeloid lineage (132). To satisfy the conditions of a latent infection, the virus must be able to infect cells without any cytopathic effect, nor infectious virion production, the viral genome must be maintained by the host, and importantly the virus must prevent detection or elimination by the host immune response. Presumably, this strong selective pressure of successful virus infection and persistence within the immunocompetent host has allowed the development of highly effective immune evasion strategies. Reactivation from the latent state is effectively suppressed by the long-term immunity established following primary infection of a competent host (71). However, delayed or absent adaptive immune responses, as with neonates and immunosuppressed patients, can fail to either clear the virus during the primary infection or fail to suppress reactivation from latency resulting in the onset of disease symptoms (213).

Congenital HCMV infection

HCMV is the only herpesvirus that can be readily transmitted transplacentally from mother to fetus. Though HCMV can be transmitted transplacentally, there is a much greater risk of postnatal transfer through breastfeeding (greater than 30% transmission of HMCV to infant following three months breastfeeding with a seropositive mother) (7). Congenital infections occur in approximately 1% of all live births, and only about 5-

10% of these newborns are symptomatic at birth (152). These congenitally infected newborns with symptoms at birth suffer from cytomegalic inclusion disease (CID), which is characterized by a range of symptoms including microcephaly and hepatosplenomegaly among others, and is associated with a poor prognosis (83). Non-central nervous system-associated disease including hepatitis, pneumonitis and myocarditis may be life-threatening. Approximately 20% of these infants die during infancy and the remaining suffer from a range of chronic complications. Brain damage may occur alone or in combination with other symptoms and can range from blindness and deafness to seizures and mental retardation. Importantly, sequelae develop in a large proportion of the infected infants. Fifteen percent of the infants symptomatically infected at birth will develop defects in hearing or impaired intellectual development. A third of all cases in the United States of sensory neural hearing loss in children can be attributed to congenital HCMV infection, and approximately 8000 newborns suffer health problems as a result of congenital infection each year (91).

Transplantation and HCMV infection

Immunocompromised patients are high risk candidates for HCMV and can develop severe disease following either primary infection or reactivation. HCMV is considered a leading cause of graft rejection and death following solid-organ allograft transplantation, and has been referred to as the ‘troll of transplantation’ (15). The immunosuppression therapy associated with transplantation facilitates high level virus replication and end organ disease. Rates of disease in kidney (50%) and liver (61%) transplant patients are greatest following primary infection of a pretransplant seronegative host with seropositive donor, though

reactivation in the seropositive host exceeds 10% with a seronegative donor and 20% with a seropositive donor (11).

AIDS and HCMV infection

HCMV infection is one of the leading opportunistic infections in AIDS patients. Before highly active antiretroviral therapy (HAART), it was estimated that about 25% of adult AIDS patients would develop disease due to HCMV infection (102). Reduction of HIV load by HAART results in recovery of CD4+ T cells and a decrease in CMV replication and disease (148). Underdeveloped countries continue to be burdened by CMV disease in AIDS patients due to a lack of treatment options for HIV infection. Symptoms in AIDS patients range from the more common retinitis to gastrointestinal disease and encephalitis. Infection with HCMV has also been correlated with the clinical progression of HIV infection into AIDS (70, 148). Numerous studies have indicated that HCMV may enhance HIV-1 replication or facilitate reactivation from latently infected cells. In support of this, the vast majority of adults and children infected with HIV are also seropositive for HCMV (152).

Prevention and treatment

Control of HCMV is complicated by the high proportion of asymptomatic individuals with active infections in the population, combined with prolonged virus shedding from months to years in most body fluids. Currently approved therapy for active HCMV infection in immunocompromised patients relies upon three approved antiviral chemotherapeutic drugs: ganciclovir, foscarnet and cidofovir (175). These drugs effectively prevent severe CMV disease in AIDS patients, though treatment is complicated by severe side effects including renal and

hematologic toxicity in some patients (152). Treatment is not necessary in a patient with an intact immune system as infections resolve naturally.

All of the approved anti-CMV drugs selectively target viral DNA replication. Ganciclovir, which is a derivative of the anti-herpes drug acyclovir, is a nucleoside analogue of 2'-deoxyguanosine. The drug is administered as a prodrug, and must first be phosphorylated by the viral-coded UL97 kinase, which is far more efficient in phosphorylating ganciclovir than the cellular kinases. This phosphorylated form of ganciclovir is further phosphorylated by cellular kinases into the triphosphate, active form. The active drug is incorporated in place of dGTP and potently inhibits the viral DNA synthesis by termination of DNA elongation. This complex mechanism of drug activation ensures selective toxicity to infected cells, as the prodrug cannot be phosphorylated in uninfected cells. However, viruses insensitive to ganciclovir are common with mutations most often mapping to the viral UL97 gene.

Foscarnet can be effective in treating ganciclovir-resistant virus infections but is used as a last resort due to the increase in severity of negative side effects. It can be successfully employed in treating mutant strains since it does not require metabolism or phosphorylation by the viral enzyme to become activated.

Passive immunization, as well as anti-herpes drugs, are often administered prophylactically prior to organ transplantation. Transfer of HCMV immune globulin to seronegative recipients of organs from seropositive donors, has been used with varying success to prevent the severity of disease. However, this passive immunization does not prevent HCMV infection (152).

Currently, there is no real method for prevention of congenital infection. In the future, this type of infection will likely only be controlled

with an effective vaccine, especially in populations, where infection during child-bearing years is common, particularly the United States (152). A study in the early 1990's placed the cost to US health care system from congenital HCMV disease to be $1.86 billion annually, or greater than $300,000 per infected child (66). As the problem with symptomatic congenital HCMV infection is largely from primary infection during gestation or within the first few years of life, prevention of this early infection would prevent a lifetime of sickness or disability caused by this virus (11). As such, the Institute of Medicine recently ranked a successful vaccine for HCMV disease prevention at the highest priority, based upon both alleviation of high economic burden and the total years of life without morbidity which can be improved (11).

There are number of ongoing clinical trials with either National Institutes of Health (NIH) or industry funding support for an HCMV vaccine. These include both attenuated viruses and subunit vaccines though the efficacy in preventing HCMV infection and disease has not yet been determined (11). The use of an attenuated virus for vaccination has been long in development. The Towne strain was created though extensive passaging in human fibroblast cells (156). This live attenuated vaccine has been tested in clinical trials with only partial efficacy in transplant recipients (157). New studies are evaluating the use of the vaccine in children and women of child-bearing age (11).

The efficacy of a subunit vaccine using the HCMV glycoprotein gB has been demonstrated in the guinea pig model, where vaccination inhibited CMV intrauterine transmission and decreased pup mortality (176). A gB subunit vaccine has been studied by phase 1 trials (69, 153). This vaccine is safe and immunogenic in humans and is still being evaluated for use (83).

Continued investigation into the molecular biology of HCMV and its interaction with the host will provide more insight into the requirements for an effective HCMV vaccine or other potential targets for anti-HCMV chemotherapy. Work presented in this dissertation identifies a novel immune evasion strategy adopted by HCMV and should provide a stepping stone for novel HCMV vaccine or rational drug development and will be discussed in more detail in a subsequent section.

1.2: MOLECULAR VIROLOGY OF HCMV

Most of the current knowledge about HCMV has been obtained through experimentation with virus infection in cell culture. The laboratory strain of HCMV (AD169) most utilized for HCMV research was propagated *in vitro* to attenuate virulence for use as a live attenuated vaccine (64). After complete sequencing of this strain it was found to contain several deletions including a 15 kilobase pair region corresponding to 19 open reading frames (ORFs) which are present in clinical isolates (46). Many clinical isolates (FIX, Toledo, Towne, TR, and PH) have been sequenced and are available for *in vitro* experimentation. There is very little polymorphism between HCMV strains, with an estimated sequence identity between 90-95% (97, 161).

HCMV replicates within a number of cell types within an infected host including fibroblasts, epithelial, macrophage, smooth muscle and endothelial cell types (182). Productive infection *in vitro* however is limited to primary fibroblasts, endothelial cells and certain differentiated myeloid cells, and a subset of astrocytoma cell lines (98, 147). This limits *in vitro* research with the virus, as primary fibroblasts can only be passaged a limited amount prior to senescence. Recently, primary fibroblasts have been selected with the catalytic subunit of telomerase to effectively create

life-extended fibroblasts (34). These cells support HCMV replication as efficiently as primary fibroblasts and can be used to create effective complementing cells for the propagation of HCMV deletion mutant viruses. Importantly, all tested cellular responses to HCMV infection in these life-extended fibroblasts are indistinguishable from the parental fibroblast cells (34) and data not shown).

CMVs have been identified for many mammalian species ranging from humans and primates to rodents. Numerous essential genes are conserved between the species-specific viruses; however each virus also contains unique genes. The use of animal models of infection (mouse, rhesus, guinea pig) has provided much insight into the function of conserved gene products and pathogenic strategies, however the diversity of gene products and differences in replication requirements make it difficult to draw conclusions about the relevance of the phenotype observed with the animal model to the behavior of HCMV *in vivo*.

Virion structure

Genome

The HCMV infectious virion is composed of the characteristic herpesvirus structure and is represented schematically in Figure 1-1A. At the core of the virus particle, an icosahedral capsid surrounds the linear, double stranded DNA genome. Largest of the herpesviruses at about 240,000 base pairs, this genome codes for at least 150 viral proteins, many of unknown function (210). Mutagenesis studies of the AD169 laboratory strain of HCMV have demonstrated that only about 41 ORFs are essential for virus replication (210). Thus the majority of the genome encodes proteins that are involved in pathogenesis of the virus.

The genome is organized in typical herpesvirus fashion. Two unique regions, unique long (UL) and unique short (US), are divided by inverted repeat regions: internal repeat long and short (IRL, IRS) and terminal repeat long and short (TRL,TRS) (Figure 1-1B). ORFs are located in both unique and repeat regions.

Capsid

The HCMV icosahedral capsid is composed of at least seven proteins. The major capsid protein, pUL86, constitutes most of the total mass of the capsid. Two minor capsid proteins encoded by the UL85 and UL46 ORFs combine with pUL86 to form the capsid pentamers and hexamers. Three additional distinct proteins encoded on the UL80, UL80a and UL80.5 genes are also associated with the capsid and function during capsid assembly and packaging (133).

Tegument

The capsid is surrounded by a proteinacious tegument layer, which contains at least 25 packaged virally-encoded proteins (76). The tegument proteins are heavily phosphorylated (75) and are delivered into the host cell with the nucleocapsid. The two tegument proteins pp65 and pp150 (products of the UL83 and UL32 ORFs respectively) are the most abundantly synthesized viral proteins (75, 191). Most of the tegument proteins are not functionally characterized, though some of the characterized proteins serve important functions during the initiation of HCMV infection.

The tegument protein pp71, the product of the UL82 ORF, is a critical regulator of the major immediate-early promoter (MIEP) involved in “kick-starting” a productive infection (36) and has recently been shown to

activate IE gene expression through inhibition of the transcriptional repressor protein hDaxx (41, 42, 94, 160). Deletion of UL82 dramatically inhibits virus replication (36, 87).

Interestingly, the content of the HCMV tegument is not limited to viral proteins. It has been demonstrated that a number of viral transcripts (35) and host cell proteins including actin, heat shock protein 70, and β_2-microglobulin (14, 198).

Envelope

The virus nucleocapsid and tegument is contained within a host derived lipid envelope, which is studded with both virally-encoded glycoproteins and some host-derived proteins including annexin II (207) and CD13 (77). The HCMV genome encodes up to 60 putative glycoproteins. The viral envelope has been shown to contain 8 major glycoproteins (37), with the UL55 gene product glycoprotein B (gB) being the major constituent (37, 75). gB is the most conserved herpesviral glycoprotein and has a number of ascribed functions including cell binding and entry which is mediated in part through binding to heparin sulfate (55), intercellular spread (27), and targeting of progeny virus during egress (55).

Virus Entry

HCMV infection is initiated by a loose, tethering interaction between the surface glycoprotein gB and heparin-sulfate (55). A cellular receptor for HCMV has not yet been identified, though a number of potential receptors and co-receptors including epithelial growth factor receptor (EGFR) (201) and CD13 (185) have been proposed, though their expression does not correlate with all of the cells which can be infected with HCMV. Despite HCMV's limited host range, most cells can support

the initial events of HCMV infection including binding and entry, however IE gene expression and DNA replication does not ensue. The fact that HCMV can bind and enter most cells supports the conclusion that HCMV may utilize multiple cellular receptors and has also hampered the identification of the necessary surface receptor. Regardless of the cellular receptor, after stable attachment HCMV fuses with the host cell membrane in a pH-independent manner (54).

Viral gene expression

Unlike other herpesviruses, infection with HCMV does not result in a global shutdown of host cell transcription (133). Indeed, HCMV infection has a profound effect on host cell signaling and gene expression. Infection of human primary fibroblasts with the lab-adapted AD169 strain of HCMV has been shown by differential display (217) and microarray experiments (39, 181, 216) to regulate the transcription of hundreds of cellular genes involved in not only the cellular response to infection, but also apoptosis and cell cycle regulation. Coincident with this increase in cellular transcription, HCMV gene expression is highly efficient and organized temporally during a productive infection. Viral genes are categorized as immediate-early (IE), early (E), or late (L) based upon expression kinetics (Fig. 1-2).

The IE genes are expressed within the first few hours postinfection of permissive cells. IE genes rely mainly upon host factors for their expression and by definition are transcribed in the absence of newly synthesized viral or cellular proteins.

Immediate-early transcription is controlled by the immediate-early promoter/enhancers, which contains binding sites for numerous mammalian transcription factors including NFκB, Sp1, CREB/ATF, p53

and AP1 (133), as well as two binding sites analogous to the interferon gamma activated sequence (GAS) for binding to type II interferon induced-transcription factors. Interestingly, IE gene expression was enhanced by treatment IFN-γ, and this effect was abrogated by mutation of the GAS elements within the major immediate-early promoter (MIEP) (145). However, the requirement for host transcriptional activation or interferon signaling in IE gene expression has not been clearly established. The specific role of the individual factors in IE gene transcription is a matter of much debate. The MIEP controls the expression of the two IE genes IE1 and IE2, which are the first and most abundantly expressed viral genes during the early hours following HCMV infection. The strong IE promoter/enhancer yields two major differentially spliced transcripts which encode IE1/IE72 and IE2/IE86. These proteins share 85 amino acids in the amino terminus, but are differentially spliced into UL123/IE1 or UL122/IE2 (188). IE72 and IE86 are crucial for HCMV infection, and as such an IE72 deletion mutant virus exhibits a severe growth defect, and an IE86 deletion mutant virus has not been propagated to date (82). In addition, there are a number of other immediate-early genes (TRS1/IRS1, UL36/37, and US3) that are not expressed from the MIEP that play important roles during the initial stage of infection. The IE gene products are primarily responsible for the transactivation of the E and L viral gene promoters. IE proteins also function to modulate the host cell environment through cell cycle modulation, inhibition of apoptosis, and transcriptional activation of cellular genes (45). The identification of a novel function of IE86 in attenuating the host cytokine response will be the focus of the research in this dissertation and will be discussed more extensively later. The collective effect of all IE gene expression is to initiate a productive infection and optimize the host cell environment for virus replication.

The E gene products are involved in the DNA replication process and encode the DNA replication machinery including the DNA polymerase (UL54) and the DNA processivity factor (UL44). Also the E genes are involved in optimizing the cellular environment for viral DNA synthesis. The UL112-UL113-encoded regulatory proteins are involved in organization of replication compartments, as well as regulating expression of core replication proteins (133). During a productive HCMV infection, host cell DNA replication is shutdown, most likely due to piracy of the essential host cell factors and machinery for viral DNA replication.

The viral gene expression program culminates in the expression of the viral late genes, which mostly encode the structural components of the HCMV virion. In general, tegument, capsid and glycoproteins are encoded by late genes and their expression is dependent on viral DNA synthesis and coincides with virus assembly, packaging and egress through the cellular endocytic pathway.

Immediate-early 2 protein (IE86)

IE86 is a 579 amino acid nuclear phosphoprotein. A growing list of regulatory functions has been attributed to the IE2 gene product IE86. The two separable primary roles of IE2 during a productive infection are 1) to control the transition from the IE to E and L gene expression and 2) the down-regulation of the MIEP during the transition to the late phase of infection to inhibit IE1/IE2 gene transcription (133). As a DNA binding viral protein transactivator, IE86 potently activates both viral and cellular promoters. The mechanism of this transactivation has yet to be elucidated, but is most likely effected through numerous functional interactions of IE86 with cellular transcription factors and accessory proteins. This transactivation for the most part is sequence independent,

similar to the well-studied adenoviral transactivator E1A, though a report has shown that IE86 upregulates a subset of genes in a site-specific manner (32). IE86-mediated autoregulation however is site-specific, requiring a 14-15 base pair DNA element, CG-N_{10}-CG, the cis repressor sequence (CRS). Two CRS regions are located between the TATA box and transcriptional start site of the MIEP. IE86 binds to the CRS in an homo-dimeric form and this represses transcription by sterically blocking RNA polymerase II recruitment to the preinitiation complex, without blocking recruitment of other required basal transcription factors (117).

The importance of IE86 for virus replication is underlined by the inability to generate an IE86 knock out mutant virus. Unfortunately, this has hampered studies on the function of IE86. A number of IE86 mutant viruses can be propagated *in vitro*, but complementation of a full deletion mutant, or point mutants in the essential carboxy terminal region has been unsuccessful to date. Stable expression of IE86 appears to be toxic to cells, and non-functional mutants are often selected for in these cell lines. Thus, most experiments examining the function of IE86 during HCMV infection rely upon transient expression using plasmid transfection or virus-mediated gene delivery.

Expression of IE86 in human fibroblasts has been shown to have a number of physiologic effects. The most probable reason for the toxicity of IE86 expression is due to its numerous cell cycle effects. Expression of IE86 has been shown to block progression at the G1/S border in various cell lines and primary fibroblasts. Additionally it has been reported that IE86 induces the cyclin E promoter (32) and associtated kinase activity (186), which mimicks the arrest observed in cells infected with HCMV (12).

IE86 is post-translationally modified by phosphorylation and sumoylation. These secondary modifications appear to be essential not

only for the function of IE86, but also for controlling the switch from autoregulation to trans-activation. Structure-function analysis of IE86 using deletion and point mutations has begun to reveal domains that are required for interactions with other viral and cellular proteins and also for some of the specific functions of IE86 including transactivation, autorepression, cell cycle regulation, and inhibition of apoptosis. IE86 transactivation is attributed to acidic transactivation domains (TAD) located in the amino terminal 85 aa and a portion of the carboxy terminus between 544 and 579 aa (12). The DNA binding, dimerization and autorepression domains overlap in the carboxy terminus between amino acids 290 and 579. Data supports the conclusion that autorepression requires IE86 dimerization and direct DNA binding, whereas transactivation does not require these functions. A zinc finger domain is also present in the carboxy terminus. Point mutations in this region abrogate DNA binding, though full deletion of the sequence does not. This suggests that small conformational changes induced by point mutations can potently affect the function of IE86 (12). Interaction domains with a number of host transcription factors (including pRb, p53, c-Jun, Egr-1) and accessory factors (including TBP, CREB, TFIIB/D, CBP and P/CAF) have only loosely been defined to large regions within the N- and C-termini of IE86 (45).

IE86 expression immediately following virus penetration is one of the key events required to initiate a productive infection. The cellular environment is shaped by host genes transcriptionally activated by IE86 expression, including cell-cycle regulatory genes. Cyclin E, which is directly activated by IE86 binding to its promoter, promotes cell cycle progression to early S phase and helps prepare the cell for viral DNA replication (32). IE86 also functions to prevent one of the classical host

responses to virus infection, programmed cell death or apoptosis, mediated in part through functional interactions of IE86 with the tumor suppressor proteins p53 (45, 218). The multi-functional nature of this viral transactivator protein suggests that additional effects on the host cell have not been described for IE86. This work identifies a novel function of IE86 during HCMV infection: attenuation of the host innate cytokine response through specific inhibition of NFκB DNA binding.

THE INNATE RESPONSE TO INFECTION

Coincident with virus infection and initiation of a viral gene expression program which ultimately will result in cell take-over and death, the host cell is also coordinating a rapid response intended to limit virus replication and prevent spread to other uninfected cells (Fig. 1-3).

Virus detection

Virus infection is detected by a variety of host cell receptors which result in the activation of multiple, redundant pathways that converge at the level of Type I interferon transcriptional induction (72). Two distinct classes of virus sensors are present in the host cell, extracellular/membrane bound or cytoplasmic receptors. These sensors initiate signaling events which result in transcription factor activation.

Extracellular and endosomal pathways

Sensor receptors bound to the cellular surface play an important role in detection of both virus and viral products in the extracellular environment. Toll-like receptors (TLR) consist of a family of membrane receptors with extracellular domains designed to detect distinct pathogen-associated molecular patterns (PAMPs) including double-stranded RNA,

which is a common product of virus replication and is detected by TLR3 (6). Other TLRs recognize single-stranded RNA (TLR7/8) and DNA (TLR9). TLRs are not limited to virus detection and distict receptors recognize bacterial lipopolysaccharide (LPS), peptidoglycan and a variety of other non-viral PAMPs. The main viral TLRs (TLR3/7/8/9) are located within endosomal compartments, not on the cell surface, and contact with PAMPs occurs during endosomal-mediated internalization of virus or virus products (72). The gene expression profile induced by TLRs varies depending upon the signaling adapters associated with the cytoplasmic domains. Most TLRs are dependent upon the MyD88 adapter protein, and can initiate an inflammatory response through activation of the NFκB and AP1 pathways (110). TLRs associated with the toll/IL-1R (TIR) domain-containing adaptor-inducing IFN-β (TRIF) adaptor molecule, which includes TLR3 and TLR4, can also function to activate IFN-β, aTLR2, which classically recognizes bacterial peptidoglycan (110), has recently been shown to be necessary for HCMV-induced inflammatory gene expression (53).

s the transcription factor IRF-3 is activated in addition to NFκB and AP1 (110). These transcription factors, as described below, participate in the IFN-β enhanceosome. TLR2, which classically recognizes bacterial peptidoglycan (110), has recently been shown to be necessary for HCMV-induced inflammatory gene expression (53).

Cytoplasmic pathway

As all TLRs which recognize viral PAMPs are not localized in endosomes and not on cell membrane, a mechanism must exist for detection of endosome-independent virus entry. The cytoplasmic proteins retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-

associated gene 5 (Mda5) contain a helicase domain that can directly bind to dsRNA and a caspase-recruiting domain (CARD)-like domain that can mediate the activation of downstream IRF-3 and NFκB through the adapter molecule IPS-1. IPS-1, which also contains a CARD domain, interacts with RIG-1/Mda5 and activates the kinases required for IRF-3, NFκB and AP1 activation (110).

Recent reports have also demonstrated the existence of cytosolic dsDNA sensors, though the identification of the proteins remains undetermined (100, 190). This class of sensor may be more important during infection with a virus with a dsDNA genome like HCMV.

Virus-activated transcription factors

Interferon regulatory factors (IRFs)

IRFs are a family of transcription factors that have been implicated in the transcriptional regulation of antiviral and stress responses, cytokine signaling and cell cycle control. There are nine identified IRFs and members consist of an N-terminal DNA binding domain and a C-terminal transactivation domain. The two inducible IRFs, IRF-1 and IRF-7 can both participate in the activation of innate immune genes, although they are not necessary for virus-induced IFN-β expression. Unlike the other IRFs, IRF-3 is constitutively expressed in uninfected cells in an inactive, hypophosphorylated state. Mouse knockout studies have clearly shown IRF-3 to be required for the induction of IFN-β in response to virus infection *in vivo* and in cell culture (173). Mice with a targeted deletion of IRF-1 do not exhibit any IFN-β defect in response to virus infection (125). Characteristic of all IRFs, activation is mediated by phosphorylation on specific serine/threonine residues within the carboxy terminal region of the proteins. C-terminal phosphorylation of IRF-3 is actually specific to virus

infection and has been shown to be mediated by toll like receptor 3 (TLR3), RIG-I and Mda5. Aside from virus infection, bacterial LPS can signal IRF-3 activation through TLR4 and a number of stimuli have been shown to result in N-terminal IRF-3 phosphorylation including stress and DNA damaging agents. Virus induced phosphorylation in the case of IRF-3 has recently been shown to be mediated by the virus-activated, canonical IKK members TBK1 and IKKε (65, 93). TBK1 has further been shown to be the critical IRF-3 kinase during virus infection in fibroblasts and during TLR signaling. The C-terminus of IRF-3 structurally obscures a nuclear import signal. Phosphorylation on key serine residues results in a conformation change which reveals the nuclear import signal, and facilitates IRF-3 homodimerization and nuclear translocation (121). This active form of IRF-3 can participate in a complex with CBP/p300 and bind specifically to many promoters involved in the innate host immune response including IFN-β, regulated upon activation normal T cell expressed and secreted (RANTES), and ISG15. IRF-3 dependent genes contain specific cis elements within their promoter, which have similarity to the interferon stimulated response element (ISRE) of interferon stimulated genes. Mutational analysis of IRF-3 has revealed that constitutively activated IRF-3, by a phosphomimetic mutation on a key serine residue, results in the expression of a subset of interferon stimulated genes including ISG15, ISG54, ISG56, ISG60, and GBP1. These IRF-3 target genes are induced by interferon treatment, though can be induced by IRF-3 in an interferon-independent manner (80). Transcription of these target genes does not require new protein synthesis, and thus are quickly transcribed following virus detection and can function prior to or concurrent with Type I interferon production. Some genes that require IRF-3 for their activation, including IFN-β and RANTES, are not directly

induced by constitutive activation of IRF-3. These genes require additional factors, in particular NFκB, to cooperatively induce the transcriptional expression.

Viruses capable of preventing the activation and subsequent nuclear translocation of IRF-3 can severely attenuate the host interferon response. A number of viral proteins have been shown to disrupt these activation pathways and block formation of the downstream IFN-β enhanceosome. The Ebola virus VP35 protein and the hepatitis C virus NS3/4A protease interfere with the phosphorylation of IRF-3 and therefore blocks its ability to translocate to the nucleus and activate transcription (18, 68). The rotavirus NSP1 protein and the human papillomavirus E6 protein directly bind to IRF-3 and inhibit its ability to translocate to the nucleus (79, 168). Other viral proteins have been shown to interact with CBP/p300 and alter its interaction with IRF-3 (105).

Nuclear factor kappa B (NFκB)

The nuclear factor kappa B (NFκB) transcription factor family is central to immune and inflammatory responses, as well as controls cell growth and survival (49). The NFκB binding element can be found in more than 150 cellular gene promoters (149). Those encoding cytokines, cytokine receptors, adhesion molecules and growth regulators are positively regulated by this family of transcription factors and include genes encoding IL-6, IL-8, RANTES, TNFα, IFN-β, c-Jun and c-Myc. The genes are classified as immediate or delayed, based upon expression kinetics following NFκB activation. The NFκB response element in the promoter of ‘immediate type’ target genes is accessable to NFκB binding, whereas chromatin modification is required for transcription factor access to the response element in ‘delayed type’ target genes (166). Thus

transcription of ‘delayed type’ target genes additionally requires the recruitment of NFκB with histone acetyltransferases.

NFκB is activated by a plethora of inducing agents including tumor necrosis factor alpha (TNFα), LPS and TLR signaling and is considered a hallmark of viral infections (149). NFκB target genes range from innate cytokines to genes involved in normal cell processes. In light of its ability to promote the expression of proteins involved in both the innate and adaptive immune responses, NFκB may coordinate aspects of these immune functions required for resistance to infection. As such, activation of NFκB during virus infection has been viewed as a host protective response (149). Strong support for this view comes from experiments using mice lacking various NFκB factors. These mice are more susceptible to infection with various viruses including influenza and lymphocytic choriomenigititis virus (LCMV) (194).

The NFκB family consists of five genes, which give rise to seven proteins: Rel (cRel), RelA (p65), RelB, NFκB1 (p105/p50) and NFκB2 (p100/p52). All members share a rel homology domain (RHD) which enables both homodimeric and heterdimeric pairing between members as well as DNA binding. Only RelA, c-Rel and RelB contain transactivation domains within their C-terminal region. p50 and p52, which are processed by ubiquitin-dependent processing of the carboxy termini of the larger p105 and p100 precursor proteins respectively, only contain a DNA binding domain (172). NFκB target gene specificity is gained by the formation of homo- and heterodimeric complexes between the members, though the classical heterodimer complex is composed of p50/p65. This complex participates in the IFN-β enhanceosome (Fig. 1-4).

Normally retained in the cytoplasm bound to the inhibitor complex IκB, NFκB is activated by the phosphorylation and degradation of the IκB

inhibitor, which unmasks the nuclear localization signal of NFκB and facilitates rapid nuclear translocation (3). IκB is phosphorylated on the α subunit on serines 32/36 by the inhibitor kappa kinase (IKK) complex. This complex consists of three members, IKKα and IKKβ are both kinase subunits and a structural, non-catalytic IKKγ (NEMO) subunit. Phosphorylation of IκBα results in polyubiquitination and degradation in a proteosome dependent manner. NFκB members then freely translocate to the nucleus where they can activate target gene expression through binding to DNA κB elements, which are composed of a remarkably loose consensus sequence. 5'-GGGRNNYYCC-3' (R,purine; N,any; Y,pyrimidine) (74). Mutation of IκBα on serines 32/36 results in a non-phosphorylatable dominant inhibitor of NFκB. Therefore, NFκB transcriptional activation does not require *de novo* protein synthesis and occurs rapidly after exposure to inducing stimuli or virus infection.

Inhibitor degradation and nuclear translocation of NFκB is sufficient for activation, though maximal activity requires post-translational modifications (49). This second level of transcriptional regulation occurs in the nucleus following translocation (172). The p65 subunit is specifically phosphorylated by a number of kinases in both the RHD and the transactivation domain. Protein kinase A (PKA) phosphorylates serine 276 in the RHD which promotes an interaction with CREB Binding Protein (CBP)/p300, modulates NFκB DNA binding and oligomerization, and enhances NFκB-dependent transcription (215). Further enhancement of transcriptional activity is achieved by phosphorylation in the transactivation domain on serine 536 by IKKs. These phosphorylation events are additionally required for acetylation of lysine 310 by CBP (50).

Despite many viral and cellular NFκB target genes, not all genes are expressed when NFκB is induced (149), as is the case for ISRE-containing interferon stimulated genes. Since more than one transcription factor or promoter modification (i.e. local acetylation) is usually required to induce effective transcription, individual genes are activated selectively under specific circumstances. Moreover, depending on the receptor or the transduction molecules required, different cell types react differently to a given stimulus, conferring specificity on the transcriptional response to NFκB activation (149). Interestingly, though NFκB binding sequence specificity is important in determining which dimer is recruited to a given promoter, rather than determining binding affinity, the sequence does determine which cofactors will form a productive interaction with the NFκB dimer (119).

Many virus infections result in the activation of NFκB, though it is unclear whether virus replication is enhanced in a cellular environment with activated NFκB. Regardless of NFκB stimulation, certain viruses have evolved strategies to evade NFκB-dependent immune responses and there are many examples of viral NFκB antagonists. Poxviruses encode a number of proteins that interfere with the activation of NFκB. Vaccinia virus encodes a viral variant of the TLR adapter protein MyD88. The viral protein A52R can act as a dominant-negative form of MyD88, to inhibit MyD88-dependent TLR signaling (29). In addition, other poxviruses, as well as the human immunodeficiency virus Vpu protein, can prevent NFκB activation by blocking IκBα degradation (172). African swine fever virus (ASFV) expresses a non-phosphorylatable homolog of IκB, A238L, which can bind NFκB dimers and prevent translocation (158). Other viral gene products including EBV's ZEBRA and adenovirus E1A can prevent IKK kinase activity or the phosphorylation of IκBα (149).

AP1

AP1 is the final virus-activated transcription factor which binds to the IFN-β promoter (Fig. 1-4) and plays an essential role in transcriptional induction (2). AP1 is a heterdimeric complex of the transcription factors c-Jun and ATF-2 (51). Similar to NFκB, AP1 is induced in response to multivariate stimuli aside from virus infection through phosphorylation by mitogen activated protein kinases (MAPK), including JNK (47).

Interferon beta (IFN-β)

Isaacs and Lindenmann first identified interferon in 1957 by showing that supernatant derived from inactivated influenza virus-infected chick membranes protected uninfected membranes from infection with live influenza virus infection (99). This supernatant contained the soluble proteins now referred to as interferons, which are potent activators of numerous biological effects ranging from developmental and antiproliferative activities to antiviral and immune-modulatory capabilities (19). In regard to antiviral activity, interferons have been shown to be effective against a broad range of RNA and DNA viruses (23). Interferons (IFNs) are classified as Type I or Type II. Type I IFNs mostly refer to IFN-α and IFN-β, though a number of similar, though distinct species have been described as Type I including IFN-ω, IFN-δ, and IFN-τ. Type II IFN only refers to IFN-γ. IFN-α is composed of a family of structurally similar proteins which are encoded on 14 separate genes on chromosome 9, and due to post-transcriptional modifications there are an estimated 22 subtypes. IFN-β however is derived from only one gene on chromosome 9, with only a single identified species and is approximately 30% identical to the IFN-α species. Both IFN-α and IFN-β are glycosylated, small molecular weight (~20 kDa) and bind to the same IFN receptor (19).

Despite the multiple and redundant mechanisms of virus detection, all pathways result in the production of IFN-β by activating the transcription factors necessary for the formation of an enhanceosome (203). This convergence of signaling pathways to activate the interferon response exemplifies the importance of rapid induction regardless of the viral stimuli. Thus IFN-β is the first and most important innate defense against virus infection. As such, IFN-β transcription is a tightly regulated process to prevent expression of the cytokine in the absence of virus infection. Induction involves the activation of a number of signal transduction cascades and the recruitment of transcription factors including NFκB, interferon regulatory factors-1/3/7 (IRF-1, IRF-3 and IRF-7), and ATF-2/c-jun (AP1) to their respective DNA binding elements (positive regulatory domains, PRD) within the interferon beta promoter to form an enhanceosome that facilitates preinitiation complex formation (2, 128, 140, 209). The IFN-β promoter contains several positive and negative acting cis-elements (173). The positive regulatory elements make up the virus-inducible enhancer, and consists of binding sites for the p65/p50 NFκB dimers (PRDII), the ATF-2/c-Jun complex AP1 (PRDIV), and IRF family members bind to the PRDI and PRDIII regions (Fig. 1-4) (173). The elements cooperatively bind their respective transcriptional activators to direct IFN-β induction in response to virus infection (173). The subsequent recruitment of RNA polymerase II complexed with CBP is then required for rapid activation of IFN-β gene expression (209).

Mouse knockout and inhibitor studies have shown that some, though not all of the factors that can participate in enhanceosome formation are required for IFN-β production. Mice deficient in IRF-3 are more susceptible to viral infections, and are devoid of IFN-β expression (173). IFN-β enhanceosome formation is also dependent upon the activity

of chromatin modifiers including CBP and P/CAF (140), which are recruited to the promoter in a complex with RNA polymerase II. Additional control of enhanceosome formation is through the architectural component HMGI(Y). The acetylation status of HMGI(Y), controlled by CBP and P/CAF, acts as a transcriptional switch to control enhanceosome stability (139). All steps leading up to IFN-β transcription, as well as all the factors required for enhanceosome formation and transcription itself, may act as potential targets for viral IFN antagonism.

Interferon signaling

Subsequent to IFN-β transcription, the cytokine is translated and secreted from the infected cells. Despite usage of the same receptor, the diverse IFN species have a wide range of cellular effects and potency, which likely comes from the manner and affinity of the interaction with the receptor. Evidence suggests that this receptor complex contains multiple IFN binding sites (96). The Type I IFN receptor complex (IFNAR) is composed of two subunits and is present in low numbers (100-5000 molecules/cell) on the cell surface (19). IFN-β can act in both an autocrine and paracrine fashion to both amplify the antiviral response in infected cells and signal to uninfected cells. IFN binds to the IFNα/β receptor (IFNAR), which is expressed on all cell types (19). Receptor binding results in cross-phosphorylation of the Jak1 and Tyk2 tyrosine kinases, which are constitutively bound to the cytoplasmic domain of the heterdimeric IFNAR (189). The activated kinases recruit and phosphorylate the signal transducers and activators of transcription 1 and 2 (STAT1/2). Activated STATs homo- and heterdimerize and associate with IRF-9 to form the interferon stimulated gene factor 3 (ISGF3). This complex translocates to the nucleus and activates hundreds of genes

containing the consensus interferon stimulated response element (ISRE) within their promoters. These ISGs, which include (2'-5')-oligoadenylate sythetase (OAS), protein kinase R (PKR), ISG56, ISG15, TLRs and IRF-7 are the effectors of interferon signaling and are responsible for establishing the 'antiviral state', which not only makes neighboring cells refractory to virus infection, but also acts to limit virus replication in infected cells, as well as inducing an apoptotic cascade to eliminate any virus infected cells.

The function of most of these interferon stimulated genes (ISGs) is currently unknown, but proteins involved in translational control, apoptosis, virus sensing and transcriptional activators have been shown to be induced by the interferon signaling pathway. The better understood ISGs, OAS and PKR, are activated only in the presence of the common viral signature, dsRNA. These enzymes interfere with host cell protein synthesis by independent mechanisms. OAS uniquely polymerizes adenosine in a 2'-5' manner to produce oligomers that activate the latent RNase L, which cleaves mRNA. PKR is a dsRNA-binding serine/threonine kinase inhibits protein synthesis though phosphorylation and inactivation of the eukaryotic translation initiation factor eIF2α (23). ISG15 is a ubiquitin-like protein that can be conjugated to a number of signaling molecules including PLC-γ1, Jak1 and ERK. The effect of this 'ISGylation' has not been elucidated, but has been linked to regulation of JAK-STAT signaling (122). IRF-7, which can participate in the IFN-β enhanceosome similar to IRF-3 through binding to the PRDIII, is only expressed in response to IFN signaling and acts to amplify the IFN-β transcriptional response.

Chemokines

Chemokines (chemoattractant cytokines) are essential to the host response to viral infection, which makes them common viral targets. The innate response is important in directing and enhancing the adaptive response, to ensure high numbers of memory T cells specific to HCMV antigens (11). Cells produce chemokines (either induced directly upon infection or by interferon stimulation) which act to link the host innate immune response to the cell-mediated adaptive immune response. These small, secretory proteins, which include RANTES, MIG, and IL-8, aid in viral clearance by attracting leukocytes including macrophages, natural killer (NK) cells and T-cells to the site of infection, by enhancing the cytotoxic activity of NK and T cells, and by blocking entry of viruses that use chemokine receptors for entry into the host cell (24, 52, 166). Leukocyte recruitment however can act independently of interferon, as numerous chemokines are transcriptionally induced by virus-activated transcription factors, including NFκB and IRF-3 (127). A virus-mediated block to chemokine induction may sever the link between the innate and adaptive immune responses and prevent virus elimination by activated T and NK cells. However, as viruses have evolved, they have developed mechanisms to block these antiviral responses induced by chemokines, thereby allowing for viral persistence within the infected host. A number of viruses encode proteins that function to block the expression of interferon and chemokines (17, 135, 195), in addition to expressing chemokine analogues (113, 155, 180), chemokine binding proteins (5, 151, 200, 204), and virus-encoded chemokine receptors (4, 114, 164, 169, 192).

HCMV modulation of the host immune responses

With a genome of about 230 kb, HCMV encodes for proteins which antagonize nearly every component of the host response in order to enhance pathogenesis and immune evasion. A number of studies have identified HCMV gene products involved in attenuating both the innate and adaptive arms of the host response. The ability of HCMV to counter innate responses likely enhances replication and spread from the infected cell. The ability of HCMV to replicate efficiently in immunosuppressed individuals underscores the role of host-mediated control of virus replication, in particular, the ability of a healthy host to suppress virus replication and reactivation and identifies a delicate balance between immune responses and viral antagonism. However, the host response does not prevent HCMV from establishing a persistent infection or undergoing latency, so the virus must have evolved strategies to counter the innate host response and evade clearing by the cell-mediated adaptive immune responses.

Initial infection with HCMV results in a transient state of immunosuppression, which is potentially mediated by infection of dendritic cells, which are central to immune surveillance and antigen presentation during virus infection (9). Important during the innate response to infection, dendritic cells potently induce NK cells through production of cytokines and chemokines including IFN-β (10).

Cell-mediated immunity is necessary for control of HCMV infection. CD8+ T cells that recognize HCMV antigens are essential for clearing HCMV, though not sufficient to prevent virus persistence or latency. In addition, A well-studied immune evasion strategy adopted by HCMV is the inhibition of major histocombatability complex (MHC) class I antigen presentation on cell surfaces to avoid detection and cell-mediated lysis by

cytotoxic T lymphocytes (CTLs) (123). Numerous genes including US2, US3, US6 and US11 encode for proteins that function to decrease the expression of MHCI proteins on the cell surface. US3 retains the MHCI proteins in the endoplasmic reticulum. US6 binds to the TAP complex on the ER luminal side to prevent peptide loading. And finally, US2 and US11 function to export the heavy chain to the cytosol, allowing for ubiquitin-dependent degradation via proteosome. Another protein encoded by the UL83 gene, pp65, phosphorylates the immediate-early proteins to prevent degradative processing by the proteosome.

Since the virus is decreasing the MHC class I surface expression, it must have a means to prevent lysis by NK cells, which are mostly activated by lack of MHC class I expression. For this purpose, HCMV makes its own MHC class I homologue (UL18) to act as a decoy for the NK cells. UL40 inhibits NK cells by increasing the expression of HLA-E. The UL16 gene encodes a protein which binds to UL16 binding proteins and MIC-b (205). This interaction retains the proteins in the cytosol. Since these proteins normally function by activating NK cells through interactions on the cell surface, UL16 is effective in limiting the activated NK cell response. Additionally, the surface expression of another NK cell activating receptor, MICA, is decreased during HCMV infection, which results in decreased killing by NK cells. Interestingly, a commonly occurring MICA allele variant lacks the cytoplasmic tail, but retains the ability to activate NK cells. This allele MICA*008 is refractory to HCMV downregulation and may be indicative of the host's attempt to overcome HCMV's evasion tactics (219). *In vivo*, low NK cell cytotoxicity has been linked to severe disseminating HCMV disease and increased mortality in bone marrow recipients (23). Thus, despite numerous attempts to disarm

the host response, the replication of HCMV continues to be controlled by the cellular response.

HCMV uses redundant mechanisms to suppress the host cytokine response and has been shown to target chemokine function by the production of functional chemokine mimics (UL143) and G-protein coupled chemokine receptors which have been shown to recognize RANTES, MCP-1 and Fractalkine (US28, UL21.5) (78, 200). HCMV also encodes a number of chemokine receptor homologues. These G protein coupled receptors (US27, US28, UL33, UL78) may act as chemokines-sinks, which prevents the bound cytokines (RANTES, MCP-1, and Fractalkine) from signaling to the immune cells. In addition, HCMV expresses an IL-10 homolog, which shifts cellular response by decreasing T cell activation.

Despite extensive knowledge about HCMV's interference with activation and detection by immune effecter cells, little is known about the signaling events which result in their activation; i.e. the effect of HCMV infection on cytokine production. HCMV infection has been previously associated with altered or defective interferon signaling (131). Recently, the immediate-early 1 protein IE72 was shown to attenuate signaling through the IFNAR by association with STAT1 and STAT2 and preventing their binding to target promoters. We and others have shown that expression of IE72 prior to IFNα/β treatment resulted in reduced expression of the interferon stimulated gene ISG54 (154). HCMV also expresses dsRNA binding proteins, TRS1/IRS1, which potentially could act upstream of IFN-β transcriptional induction to prevent virus-induced transcription factor activation (86).

SUMMARY

Infection with human cytomegalovirus is characterized by both productive infection and life-long latency regardless of a competent host immune response to virus infection. This requires evasion or attenuation of multiple host antiviral strategies from detection to virus elimination. The innate cytokine response, exemplified by IFN-β, is essential for control of virus infection and viruses that can interfere with this response are better adapted to persist within the infected host. This dissertation is based upon the hypothesis that HCMV can attenuate the cytokine response at the level of transcriptional induction during the initial stage of infection. The studies presented here outline our effort to identify an HCMV cytokine antagonist and elucidate a viral defense against the host cytokine response.

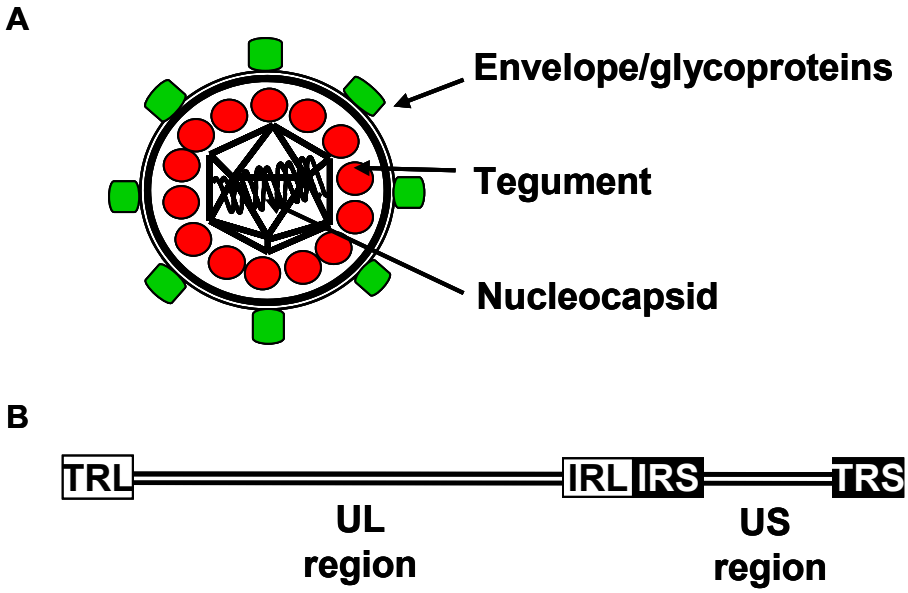

Figure 1-1. HCMV virion structure. (A) Schematic representation of the HCMV infectious virus particle. **(B)** The HCMV double stranded DNA genome. Two unique regions, unique long (UL) and unique short (US), are divided by inverted repeat regions: internal repeat long and short (IRL and IRS) and terminal repeat long and short (TRL and TRS).

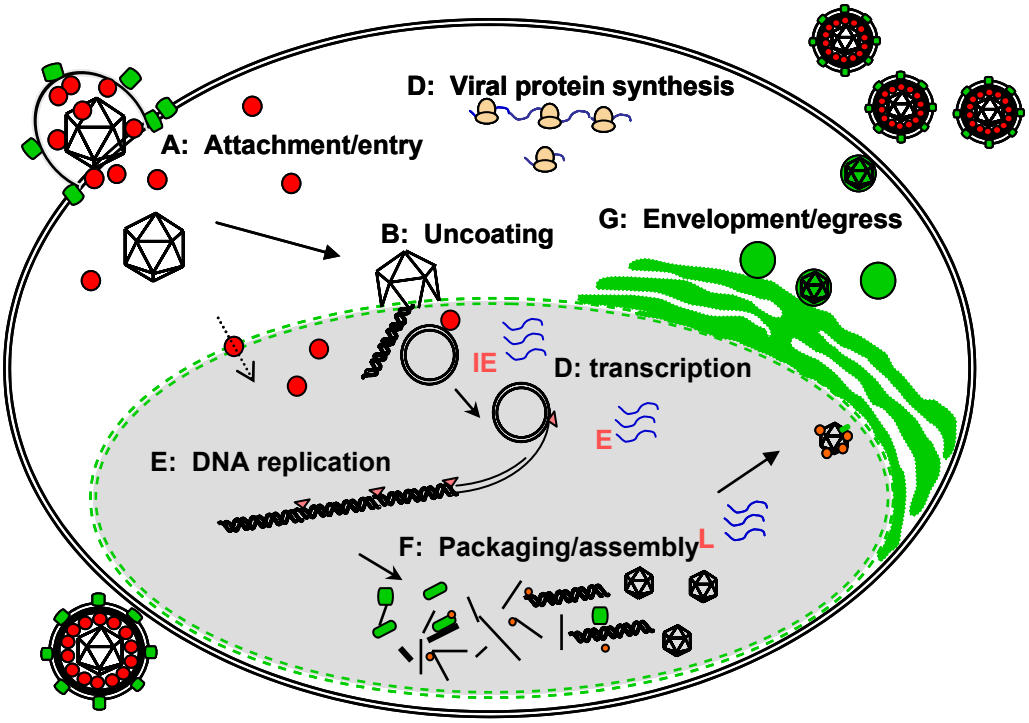

Figure 1-2. HCMV replication cycle. Schematic representation of some of the important events during HCMV infection. The HCMV replication cycle spans over a hundred hours postinfection for maximal virus production. **(A)** The cycle is initiated by virus binding and entry of the host cell. The nucleocapsid and tegument are released into the cytoplasm. **(B)** The nucleocapsid is shuttled to the nuclear envelope where it disassembles and injects the dsDNA genome into the nucleus through a nuclear pore. **(C)** During a productive infection, there are three major phases of viral gene expression. The IE genes are transcribed within the first 4-6 hours of infection. All of the viral messages are transported to the cytosol for translation **(D)**. The IE proteins return to the nucleus and transactivate the E gene promoters. The Early genes are synthesized prior to DNA replication. Included in the E class are the viral proteins required to activate the cell to a metabolic state conducive for viral DNA replication as well as proteins involved in the replication process itself. **(E)** Viral DNA synthesis begins 18-24hpi and is followed by expression of the Late genes, which are the structural components of the virion. **(F-G)** Once the late gene products are synthesized in abundance, there is virus packaging and finally egress via the endocytic pathway at about 96hpi.

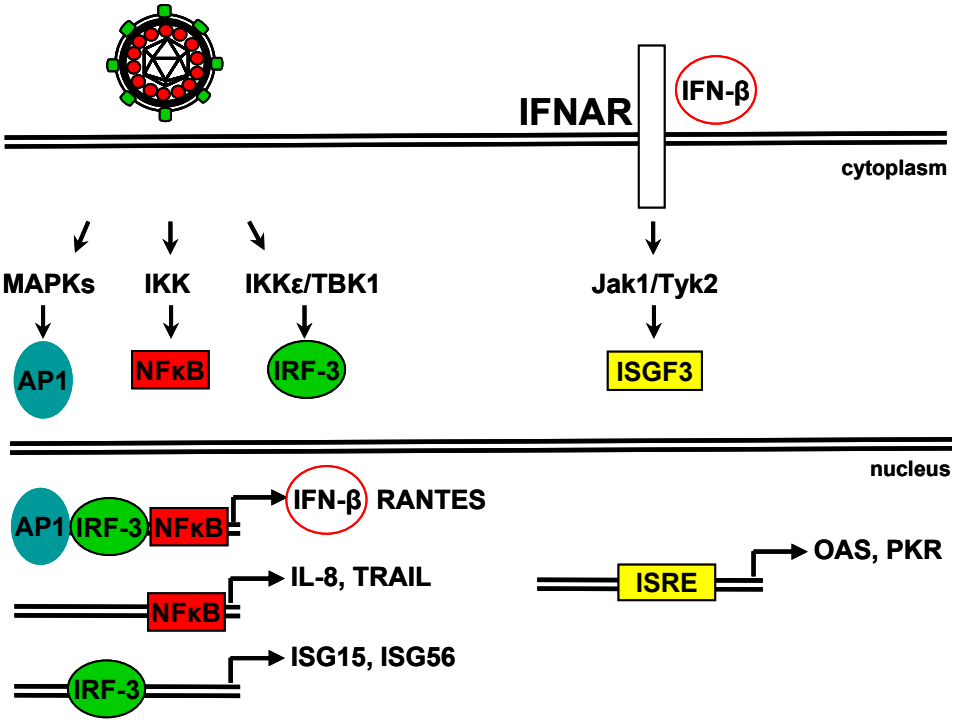

Figure 1-3. Innate host response to virus infection. Schematic representation of the innate immune response to virus infection. Virus infected cells respond to infection by inducing numerous transcriptional programs that ultimately lead to the expression of cellular genes that limit viral replication and spread. This response is characterized by the induction of cytokines and proinflammatory chemokines. Central to this response, multiple transcription factors coordinate the induction of IFN-β, which functions in an autocrine and paracrine fashion to induce a plethora of interferon stimulated genes to establish an "antiviral state" with the infected cell and the surrounding tissue.

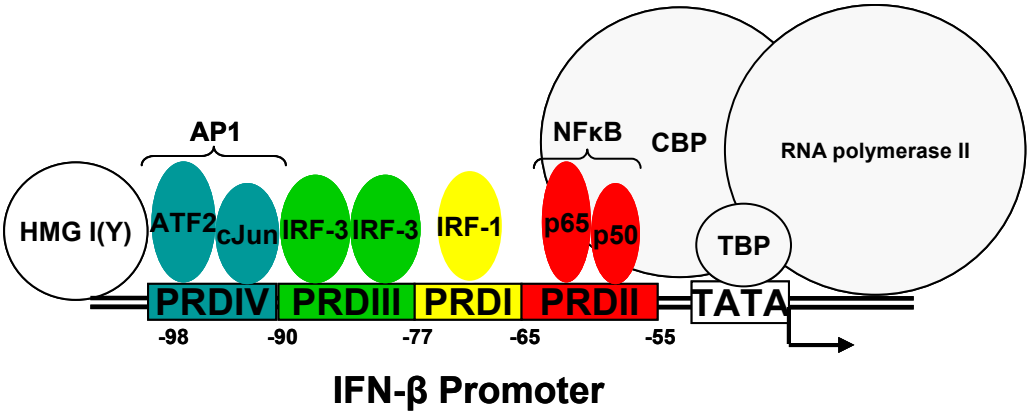

Figure 1-4. IFN-β enhanceosome. Schematic representation of the IFN-β promoter and enhanceosome. Transcription factors ATF2/cJun (AP1), IRF-3, IRF-1, and p65/p50 (NFκB) are shown bound to their respective positive regulatory domains (PRDs). Transcriptional induction further requires CBP/p300 and the HMGI(Y) architectural protein.

CHAPTER 2
MATERIALS AND METHODS

Cell culture and virus infections

Telomerase 12 human foreskin fibroblast (HFF) cells (34) , 293 and Phoenix A (provided by Gary Nolan) cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (Gemini), 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37^{o}C. HCMV stocks were purified by ultracentrifugation in an SW40 rotor for one hour at 20,000 rpm. Purified virus was resuspended in serum free media and used for infection, as previously described (39). For HCMV infection, cells were infected at an indicate multiplicity of infection with either purified wild-type HCMV (strain AD169), purified UV-irradiated (360 mJ/cm^2 in a Stratalinker) HCMV, or purified recombinant HCMV. Sendai virus (Cantell strain; Charles River labs) infections were performed using 100 Hemagglutin (HA) units/ml as previously described (195). Cells were treated with IFN-β (500 IU/ml) (11410-1; PBL), TNFα (50ng/ml) (sc-4564; Santa Cruz), or cycloheximide (100 μg/ml) (c-7698; Sigma) in serum-free DMEM.

Antibodies

The following antibodies were obtained from commercial sources: α-pp65 (1205-S; Rumbaugh-Goodwin Institute); α-pp28 (1207, Rumbaugh-Goodwin Institute),α-tubulin (TU-02; Santa Cruz); α-IE1/2 (MAb810; Chemicon); α-p50 (sc-7178; Santa Cruz); α-IκBαP (sc-8404; Santa Cruz); α-IκBα (sc-203; Santa Cruz); α-IRF-3 (sc-9082; Santa

Cruz); and α-GFP (sc-8334; Santa Cruz); α-adenovirus hexon (MAB8043; Chemicon). pp71 antibodies were a generous gift from T. Shenk and have been previously described (108).

Oligonucleotides

Primers for shuttle vector construction

83RF F, 5'-GGATCCGATATCATTTCGGGACAACGGCG-3';
83RF R, 5'-AGATCTACACTCGCGGTCCACATCCC-3';
83LF F, 5'-AGATCTCCACGCAGCGGCCCTTGATG-3';
83LF R, 5'-GGATCCCATGCATCGCCTCGACGCCC;
83Stop F, GATATCCtagactaGTCtagTTTCGGGGCACGTGCTGAAAGC;
83Stop R, 5'-GCAGCAAGTCGATATCGAAAAAGAAGAGC-3';
IRF3ΔN F 5'-AAGCTTATGGGAACCCCAAAGCCACGG-3';
IRF3ΔN R 5'-TCTAGATCAGCTCTCCCCAGGGCCCTG-3'.

Real Time PCR primers

IFNβ F, 5'-CAGCAATTTTCAGTGTCAGAAGCT-3';
IFNβ R, 5'-TCATCCTGTCCTTGAGGCAGTAT-3';
GAPDH F, 5'-CTGGGCTACACTGAGCACCAG-3';
GAPDH R, 5'-CCAGCGTCAAAGGTGGAG-3';
RANTES F, 5'-TGCTGCTTTGCCTACATTGC-3';
RANTES R, 5'-TTGCCACTGGTGTAGAAATACTCCTT-3';
MCP2 F, 5'-AGCAGAGAGGTTGAGAACAACCCA-3';
MCP2 R, 5'-AGCGCTGCAGAAACCTTCATCTTG-3';
MIP1A F, 5'-TGTCCTGTCTCTCCTCA-3';
MIP1A R, 5'-CATTGGTGCTGAGAGCG-3';
MIG F, 5'-TTGAATCAGCCTACAGGCCTCACA-3';
MIG R, 5'- TGCACTGGAGAGAAGAAAGGCACT-3';
IL8 F, 5'- AGAAACCACCGGAAGGAACCATCT-3';
IL8 R, 5'-AGAGCTGCAGAAATCAGGAAGGCT-3'.

EMSA probes

PRDII: 5'-GGGAAATTCCGGGAAATTCC-3'
mPRDII: 5'- GGCAAATTGCGGCAAATTGC-3'

Cloning and generation of recombinant viruses

HCMV BAC shuttle vectors

To construct the pGS284ΔUL83 shuttle vector, UL83 flanking regions corresponding to nucleotides 118,881-119388 and 121,000-121,500 of the HCMV AD169 strain genome were amplified by PCR using the primers 83RF F and 83RF R for the right flanking region and 83LF F and 83LF R for the left flanking region of UL83. These PCR products were then cloned into the pGEMT-Easy vector (Clontech) to yield pUL83Flanks. pUCK4 (199) was digested with *BamHI* to excise the kanamycin resistance cassette which was then cloned into *BamHI* digested pUL83Flanks to yield pΔUL83Kan. pΔUL83Kan was digested with *BglII* (which drops out the UL83 flanks and kanamycin cassette) and was cloned into *BglII* digested pGS284 (184) to yield pGS284ΔUL83. pGS284ΔUL83 contains a kanamycin cassette in place of the UL83 coding region from nucleotides 121,000 to 119,388. To construct the pGS284-UL83Stop shuttle vector, the entire UL83 open reading frame (ORF) with both flanks was first amplified with 83RF R and 83LF F primers and cloned into pGEMT-Easy cloning vector to create pGEMTE-UL83R. The stop codon mutations were created by using PCR product generated with the 83Stop F and 83Stop R primers, mutating the UL83 sequence to create stop codons in all three open reading frames starting at nucleotide 119,388. This PCR product was TA cloned into pGEMT-Easy to create pGEMTE-UL83StopRV. pGEMTE-UL83Stop was created by replacing the *EcoRV* fragment of pGEMTE-UL83R with a the EcoRV fragment from pGEMTE-UL83StopRV. Additionally, this mutation created a novel *SpeI* site to facilitate screening. pGEMTE-UL83Stop was then digested with *BbsI* to remove the region containing the stop codons and cloned into

pGS284ΔUL83 that was digested with *BbsI* to create pGS284-UL83Stop. All constructs used in these studies were sequence verified.

HCMV BAC mutagenesis and allelic exchange

All viral mutants were generated using previously reported allelic exchange protocols (184, 211). Briefly, the pADCREGFP (42) bacterial artificial chromosome (BAC), the pADCREGFPΔUL83 and pADCREGFP-UL83Stop BACs were generated by standard allelic exchange procedures described previously. The shuttle vector pGS284ΔUL83 was used for recombination with the pADCREGFP BAC to generate the ΔUL83 virus. The shuttle vector pGS284-UL83Stop was used for recombination with the pADCREΔUL83 BAC to generate the UL83Stop virus. Following allelic exchange, all mutant BACs were screened by restriction enzyme digest, Southern blot analysis, and direct sequencing to confirm proper recombination and incorporation of the desired mutations. Recombinant viruses were generated as described previously (184, 211). Briefly, BAC DNA was transfected (~10 μg) into 5 x 10^6 human foreskin fibroblasts via electroporation (950μF, 260V). Cells were seeded into dishes and infectious virus harvested when 100% cytopathic effect was observed. Wild-type and UL83 recombinant viruses generated from BAC DNA were propagated as described previously (36). Infectious titers for all viruses were determined at the same time by plaque assay as described (36).

Recombinant adenoviruses

pAdIE2 was constructed by cloning the IE2 cDNA from pCGNIE2 (218) into pADTrack (88) via *KpnI* to create pADTrack-IE2. Adenovirus expressing the IκBα super repressor (IκBαSR) was generated by removing the IκBαSR cDNA from the pRep4-IκBαSR plasmid (provided by

Aubrey Thompson) via a KpnI/HindIII double digestion and cloning the cDNA fragment into the pADTrack (88) vector that was also digested with KpnI and HindIII. The resulting plasmid was termed pADTrack-IκBαSR.

Adenovirus was generated according to the AdEasy protocol (88). The generation of replication defective adenoviruses expressing IE86, pp65 and GFP have previously been described (195). Adenoviruses were propagated and titered on 293 cells as previously described (195). Importantly, expression from all adenoviruses was confirmed by Western blot analysis. Adpp65 and AdGFP have previously been described (109). Adenovirus transduction has been previously described (142) and was enhanced by adding 1μl/ml Lipofectamine (Invitrogen) to the virus inoculum.

Recombinant retroviruses

pLXSN-IRF3ΔN was generated by using primers IRF3ΔN F and IRF3ΔN R to PCR amplify the IRF3ΔN open reading frame using pCMVBL-IRF3ΔN (121) as template. The PCR product was TA cloned into the pGEMT-Easy vector (Promega) and was subsequently sequenced. The IRF3ΔN cDNA was then removed by EcoRI digestion and cloned into the EcoRI digested pLXSN vector (Clontech) to create pLXSN-IRF3ΔN.

Retrovirus stocks were prepared as described previously (111). Briefly, 20μg of the pLXSN or pLXSN-IRF3ΔN plasmids were transfected into Phoenix A cells using Lipofectamine reagent (Invitrogen). 48 hours after transfection, supernatant containing retrovirus was collected and cell debris removed via centrifugation (3,000 x g for 10 min). Polybrene (4μg/ml) was added to the retrovirus containing inoculum during infections.

Following transduction, IRF3ΔN expression was confirmed by Western blot analysis.

Plaque Assay

HFF or 293 cells were seeded into 6 well culture dishes and were infected with serial dilutions of HCMV once the cells were confluent. Following a 2 hour adsorption period, the virus inoculum was removed and replaced with complete DMEM. 2-3 days postinfection the wells were overlayed with 0.75% low melting point agarose in DMEM supplemented with 20% fetal bovine serum. Infections were monitored for plaque formation or GFP expression and fixed overnight in 10% formalin 15-20 days (HCMV) or 5-7 days (adenovirus) postinfection. Agarose plugs were removed and the cells were stained with 0.05% methylene blue to facilitate plaque counting.

Real Time PCR

RNA was DNAse treated (DNA free, Ambion) and 2μg was reverse transcribed using Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol. cDNA was then used as template for real time PCR. All reactions were performed in duplicate using SYBR green dye (ABgene) and standard conditions on a BioRad iCycler.

Northern blot analysis

RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol and quantitated on a Nanodrop spectrophotometer. Northern blot analysis was performed as previously described (196). Briefly, total RNA (6-10μg) was separated by

electrophoresis on a 1% formaldehyde gel and transferred to Nytran Supercharge membranes using a Turboblotter (Schleicher & Schuell) according to the manufacturer's instructions. Membranes were crosslinked using a Stratalinker and probed overnight with ^{32}P-labeled probes generated by random priming in ULTRAhyb (Ambion) hybridization buffer at 48°C. Membranes were then washed twice in low Stringency wash buffer (0.1X SSC, 0.1% SDS) and twice in high stringency wash buffer (2X SSC, 0.1% SDS) at 45°C and exposed to film for autoradiography.

Western blot analysis

Western blots were conducted as previously described (33). Briefly, cells were washed in phosphate buffered saline (PBS) and harvested with a cell scraper, collected by centrifugation, and lysed in RIPA buffer (50mM Tris-HCL, 1% NP-40, 0.25% Na-deoxycholate) with proteinase inhibitor cocktail (Roche). Cellular debris was removed by centrifugation and the supernatant fluids reserved. The protein concentration was determined by Bradford assay (30). Equal amounts (40μg) of protein were resolved by electrophoresis in the presence of sodium dodecyl sulfate (SDS) on 8.5-10% polyacrylamide gels (SDS-PAGE). Proteins were transferred to nitrocellulose membrane (Optitran; Schleicher & Schuell) and probed with primary and secondary antibodies. Immunoreactive proteins were detected by the ECL chemiluminescent system (Amersham).

Dimerization assay

Dimerization assays were conducted as previously described (101). Briefly, cells were washed in PBS and harvested with a cell scraper, collected by centrifugation, and lysed in RIPA buffer without Na-deoxycholate (50mM Tris-HCL, 1% NP-40). Cellular debris was removed by centrifugation and the supernatant fluids reserved. The protein concentration was determined by Bradford assay (30). Equal amounts (10μg) of protein were resolved in the absence of SDS by electrophoresis in 10% polyacrylamide gels. Proteins were transferred to nitrocellulose membrane (Optitran; Schleicher & Schuell) and probed with primary and secondary antibodies. Immunoreactive proteins were detected by the ECL chemiluminescent system (Amersham).

Immunofluorescence assay

Cells were seeded onto sterilized coverslips in 6 well culture dishes and infected the following day with virus. Cells were washed twice with PBS and subsequently fixed with 4% paraformaldehyde for 20 min. Cells were permeabilized with PBST (PBS, 0.1% Triton X-100, 0.05% Tween 20) for 25 min at room temperature, and incubated with blocking solution (PBST, 0.5% BSA, 1% goat serum) for an additional 30 min. Cells were then incubated with primary antibody for 1h at room temperature, washed three times in PBST and incubated with secondary antibodies conjugated to Alexa-488 or Alexa-546 for 1h. Slides were washed in ddH_2O and nuclei stained with Hoechst (0.5μg/ml) for 5 min. Coverslips were sealed on slides and cells visualized using a Zeiss Atto Arc HBO 110W Upright Microscope.

Enzyme linked immunosorbent assay (ELISA)

ELISAs were performed according to manufacturer's protocol in 96 well dishes. Supernatent from infected cells was assayed directly for the presence of IFN-β protein. Colorimetric readings were measured in a microplate reader at A595 and compared to and standard curve prepared using supplied IFN-β. Data represent the average of two independent experiments.

Electrophoretic Mobility Shift Assay (EMSA)

NFκB specific EMSAs were performed as previously described (8, 212). Briefly, nuclear extracts were prepared by lysing cells in cytosolic isolation buffer (10 mM HEPES pH 7.6, 60 mM KCL, 1 mM EDTA, 0.1% NP-40, 1 mM dithiothreitol, proteinase inhibitor cocktail) and sedimenting nuclei by centrifugation (3,000 x g for 10 min). Nuclei were then washed in lysis buffer lacking NP40, and subsequently lysed in nuclear lysis buffer (20 mM Tris-HCl pH 8, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, proteinase inhibitor cocktail) and quantitated by Bradford assay. Nuclear extracts (10μg) were then incubated for 10 minutes in 19 ul of extract buffer (10 mM Tris-HCl pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 7.5 mM $MgCl_2$, 1μg poly(dI-dC)). Unlabeled specific competitor (PRDII) or non-specific competitor (mPRDII) double standed oligonucleotides were added during this incubation step when indicated. Double stranded oligonucleotides containing two NFκB binding sites from the positive regulatory domain II (PRDII) region of the IFN-β promoter (8) were end-labeled using [γ-^{32}P] ATP plus T4 polynucleotide kinase and added to the reaction mixture (250,000 cpm). Binding mixtures were incubated at room temperature for 30 min. Samples were separated

on a pre-run 6% polyacrylamide gel (60:1 polyacrylamide:bis). Gels were then dried and exposed to film for autoradiography.

CHAPTER 3
IE86 ATTENUATES VIRUS-INDUCED IFN-β PRODUCTION

INTRODUCTION

HCMV infection has a profound effect on the host cell transcriptome. Microarray studies have demonstrated that HCMV infection regulates a number of genes involved in the host antiviral response (39, 181). Interestingly, the expression of a number of these genes was enhanced significantly when viral gene expression was inhibited, suggesting viral proteins may actively block the expression of these genes. One of the genes regulated in this fashion was IFN-β. Infection with transcriptionally inactive UV-irradiated virus or infection with wild-type virus in the presence of cycloheximide (CHX) resulted in a dramatic increase in IFN-β expression, when compared to wild-type HCMV infection (39). These results suggested that *de novo* viral gene expression is required to block the induction of IFN-β and that expression of this viral gene product(s) may attenuate the host antiviral response during HCMV infection. However, the newly synthesized HCMV gene product responsible for blocking the induction of IFN-β has not been identified. In this study we examined the role of HCMV gene expression on the inhibition of IFN-β and sought to identify an HCMV IFN-β antagonist.

RESULTS

IFN-β inhibits HCMV replication

To assess the biological significance of suppressing the expression of IFN-β during HCMV infection, we examined what effect IFN-β has on

HCMV replication. Human foreskin fibroblasts (HFF) were pre-treated with 500 IU/ml IFN-β (PBL) for 24 hours, treated with IFN-β after HCMV infection or pre and post-treated with IFN-β during HCMV infection. Cells were infected with wild-type HCMV (strain AD169) a multiplicity of 0.1 plaque forming units (pfu)/cell. Ten days postinfection, cultures were harvested and infectious virus was quantified by plaque assay. As shown in Figure 3-1, pre-infection (horizontal hatched bar), or post-infection (cross hatched bar) treatment of HFF cells with IFN-β inhibited HCMV virus production by greater than 99% when compared to untreated (black bar) control cells. Virus production was inhibited by greater than 99.9% if cells were both pre-treated and treated during HCMV infection (open bar) with IFN-β. These results demonstrate that IFN-β can efficiently block HCMV replication in HFF cells and suggests the ability to block the production of IFN-β would be advantageous for viral replication.

HCMV gene expression attenuates IFN-β production

We first examined the kinetics of IFN-β expression following infection with wild-type HCMV and UV-inactivated HCMV to confirm that HCMV infection transcriptionally regulates IFN-β expression. HFF cells were infected with WT-HCMV, UV-irradiated HCMV or WT-HCMV in the presence of CHX and assayed for IFN-β RNA expression 6 hours postinfection. Figure 3-2A demonstrates the robust IFN-β induction following infection with UV-inactivated HCMV or infection with wild-type virus in the presence of CHX (lanes 3 and 5). Little or no IFN-β transcript is detected in mock, wild-type HCMV infected cells or cycloheximide-treated cells (lanes 1, 2 and 4). We also probed for the immediate-early 1 transcript to demonstrate that our UV-irradiation protocol effectively blocks viral transcription, and GAPDH is included as a loading control (Fig. 3-2A).

The IFN-β response to UV-irradiated HCMV was further confirmed by using the TR clinical strain of HCMV. As shown in Figure 3-2B, infection of HFF cells with the wild-type TR strain results in reduced IFN-β transcript levels when compared to UV-irradiated-TR virus infection, confirming that our results are not specific to the AD169 laboratory strain of HCMV. We next determined the kinetics of IFN-β induction following infection with UV-inactivated virus. Cells were infected with either purified wild-type virus or UV-inactivated virus at a multiplicity of 5 pfu/cell. RNA was harvested at various times postinfection and used for Northern blot analysis. As shown in Figure 3-2C, IFN-β RNA was barely detectable at any time after wild-type HCMV infection. However, IFN-β transcript levels were induced by 4 hours and reached maximal levels by 12 hours postinfection with UV-inactivated virus. Blots were also probed for expression of the HCMV immediate-early 2 transcript to demonstrate our UV-irradiation protocol effectively blocks viral transcription (Fig. 3-2C). These results demonstrate that IFN-β RNA levels are induced following infection with UV-inactivated virus. To determine if IFN-β was secreted during infection with wild-type virus or UV-inactivated virus, we performed a quantitative IFN-β specific ELISA on the supernatants from infected cells. Figure 3-2B shows that IFN-β protein is synthesized and secreted from the infected cells, and that there is a significant increase in the amount of IFN-β produced in response to infection with UV-inactivated virus when compared to wild-type infection. Taken together, these results demonstrate that a newly synthesized gene product, which is expressed within the first 4 hours post HCMV infection, is required to efficiently block the induction of IFN-β. With these criteria, we next sought to identify the HCMV IFN-β antagonist.

HCMV mutant viruses block IFN-β expression

Our first approach to identify the HCMV IFN-β antagonist utilized HCMV deletion mutants in immediate early genes (IE1) or genes previously shown to be involved in attenuating the host antiviral response to HCMV infection (UL1-20, US2-11, UL21.5). Collectively, this panel of deletion mutant viruses represents about 20% of the HCMV genome. To determine whether HCMV mutant viruses were still able to block expression of IFN-β, cells were infected with either purified wild-type HCMV, UV-irradiated HCMV, ΔUL1-11, ΔUL11-20, ΔUS2-11, ΔIE1, or ΔUL21.5 virus. RNA was isolated 6 hours postinfection and assayed for IFN-β by Northern blot analysis. As shown in Figure 3-3, infection with UV-irradiated HCMV resulted in a robust induction of IFN-β expression. However, infection with WT-HCMV and the panel of HCMV mutant viruses did not significantly induce the expression of IFN-β, suggesting the HCMV IFN-β antagonist was not deleted from any of these mutant viruses or multiple, redundant genes are involved in the attenuation of IFN-β expression.

IE86 protein expression blocks IFN-β production

As our approach to identify an IFN-β antagonist using deletion mutants did not yield the viral gene, we next wanted to test other genes for which deletion mutants were not available. We used a replication defective adenovirus expression system to express candidate viral genes prior to infection with UV-irradiated HCMV, and then assayed for IFN-β expression 6 hours postinfection (see schematic Figure 3-4A). We predicted that if the viral gene expressed using the adenovirus can function as an IFN-β antagonist, then infection of transduced cells with UV-HCMV will not result in IFN-β expression. To test this prediction, we

transduced cells with a panel of adenoviruses expressing the HCMV immediate early proteins IE72 and IE86, two tegument proteins pp65 and pp71, and the control green fluorescent protein (GFP) at a multiplicity of 3 pfu/cell in the presence of 1 µl Lipofectamine. 24 hours post-transduction, cells were then infected with UV-inactivated HCMV and assayed by Northern blot for IFN-β expression 6 hours postinfection. As shown in Figure 3-4B, wild-type HCMV infection or transduction with adenovirus alone did not induce IFN-β expression (lanes 2 and 3), but infection with UV-inactivated HCMV alone resulted in a robust induction of IFN-β RNA levels (lane 4). Expression of IE72, pp71, pp65 or GFP prior to infection with UV-inactivated virus had little effect on IFN-β RNA induction (compare lane 4 with lanes 5, 7, 8, and 9). However, expression of IE86 prior to infection with UV-inactivated virus efficiently blocked the induction of IFN-β (compare lanes 4 and 6). Western blots are included in Figure 3-4B to demonstrate protein expression of the various genes at the time of infection with UV-HCMV. The level of secreted IFN-β from transduced cells infected with UV-inactivated HCMV was also evaluated. As shown in Figure 3-4C, expression of IE86 prior to infection with UV-inactivated HCMV dramatically reduced the secretion of IFN-β. There was no inhibition of IFN-β secretion in cell expressing IE72, pp65, pp71 or GFP prior to infection with UV-inactivated HCMV when compared to UV-HCMV alone. We also determined if IE86 could block the induction of IFN-β RNA expression following infection with wild-type HCMV in the presence of CHX. HFF cells were transduced with replication-defective adenoviruses that express either IE86 or GFP 24 h prior to infection with wild-type HCMV in the presence or absence of CHX. As shown in Figure 3-4D, infection with HCMV, or the addition of CHX alone to uninfected cells had no effect on IFN-β induction (lanes 2 and 4). However, cells infected with

wild-type HCMV in the presence of CHX resulted in robust IFN-β RNA expression (lane 3). Expression of IE86 prior to infection with wild-type HCMV in the presence of CHX efficiently attenuated the induction of IFN-β (lane 6), whereas prior expression of GFP had no effect on the induction of IFN-β (lane 5). These results demonstrate that the HCMV IE86 protein can attenuate HCMV-induced IFN-β RNA and protein secretion, so we next needed to confirm that IE86 expression during a wild-type virus infection is necessary to attenuate IFN-β expression.

An HCMV IE86 mutant virus fails to block IFN-β expression

Ectopic expression of IE86 attenuates HCMV-induced IFN-β expression. So we next sought to confirm that IE86 expression was necessary to block IFN-β expression during HCMV infection. To determine whether IE86 inhibits IFN-β expression during a wild-type infection, we utilized an IE2 mutant virus, termed IE2ΔSX, which has amino acids 136-290 deleted from exon 5 of IE2, and is fused at its carboxy-terminal to the green fluorescent protein (GFP). IE2ΔSX is viable but expresses IE86 at dramatically reduced levels and with delayed kinetics when compared to IE86 expression during wild-type or a revertant virus infection (171). Cells were infected with purified UV-irradiated HCMV, IE2ΔSX virus, or IE2-Rev virus (a revertant virus of IE2ΔSX). RNA was isolated at various times after infection and assayed for IFN-β expression by Northern blot analysis. As shown in Figure 3-5, infection with both UV-irradiated HCMV and the IE2ΔSX virus resulted in a robust induction of IFN-β expression. However, infection with the IE2-revertant virus did not induce the expression of either gene. Interestingly, the level of induction observed following infection with the IE2ΔSX virus closely paralleled that observed following infection with UV-inactivated virus.

These results further support the claim that IE86 can function as an IFN-β antagonist in the context of a productive HCMV infection. We next wanted to assay whether IE86 can attenuate IFN-β when induced by non-HCMV viral stimuli.

IE86 inhibits Sendai virus-induced IFN-β production

Cells have evolved multiple strategies to detect virus and induce IFN-β expression. To address whether IE86's ability to block induction of IFN-β expression was specific to HCMV or if it could also block induction of IFN-β by other viruses, an experiment was performed using wild-type Sendai virus, which is a potent inducer of IFN-β expression through the RIG-I pathway (203). HFF cells were infected for 6 hours with purified HCMV at a multiplicity of 5 pfu/cell. The cells were then washed with PBS and super-infected with 100 hemagglutinin units/ml of Sendai virus. RNA and media were harvested 16 hours after Sendai virus infection and Northern blot and ELISA analysis was performed for IFN-β expression. Sendai virus infection alone dramatically induced IFN-β RNA accumulation (Fig. 3-6A, lane 3) and IFN-β secretion (Fig. 3-6C). However, pre-infection with wild-type HCMV blocked the induction of IFN-β transcript (Fig. 3-6A, lane 4) and protein secretion (Fig. 3-6C) that is induced during wild-type Sendai virus infection. To confirm that HFF cells infected with HCMV were still susceptible to Sendai virus infection, blots were also probed for the Sendai virus N-transcript. Both mock-infected and HCMV infected cells that were infected with Sendai virus efficiently expressed the Sendai virus N transcript (Fig. 3-6A-B). These results suggest that the HCMV gene product that is blocking the interferon response during HCMV infection is also capable of inhibiting the response induced by Sendai virus. To determine if IE86 could block the induction of IFN-β during

Sendai virus infection, HFF cells were transduced with adenoviruses that express either IE86 or GFP. 24 hours after transduction, the cells were infected with Sendai virus. RNA and supernatants were harvested 6 hours post Sendai virus infection and assayed for IFN-β. As shown in Figure 3-6B, expression of GFP prior to Sendai virus infection had no effect on the induction of IFN-β following Sendai virus infection. However, prior expression of IE86 efficiently blocked accumulation of IFN-β transcript and the secretion of IFN-β induced by Sendai virus infection (Fig. 3-6B and Fig. 3-6D, compare lanes 3 and 4). Additionally, IE86 was able to block the induction of IFN-β following infection with vesicular stomatitis virus (data not shown). Blots were also probed for the Sendai virus-specific N-transcript to eliminate the possibility that adenovirus transduction prevents infection by Sendai virus. Collectively, these results confirm that IE86 can block virus-induced IFN-β production.

DISCUSSION

Our results demonstrate that HCMV gene expression is required to efficiently block the production of IFN-β following HCMV infection and that the HCMV IE2 gene product IE86 is responsible for this inhibition. Using two independent methods, ectopically expressed IE86 and an IE86 mutant virus, we show that IE86 expression is necessary to attenuate IFN-β expression during HCMV infection. We also demonstrate that IE86 can block the induction of IFN-β following Sendai virus infection, demonstrating this function of IE86 is not limited to a productive HCMV infection.

Induction of IFN-β transcription is a tightly regulated process that involves the activation of a number of signal transduction cascades and the recruitment of transcription factors including NFκB, interferon

regulatory factor-3 (IRF-3), and ATF-2/c-jun to the interferon beta promoter to form an enhanceosome that facilitates rapid preinitiation complex formation (2, 128, 140, 209). IFN-β enhanceosome formatioṇ is also dependent upon the activity of chromatin modifiers including CBP and P/CAF (140), which are recruited to the promoter in a complex with RNA polymerase II.

The mechanism by which IE86 blocks IFN-β expression has yet to be defined. IE86 has previously been shown to interact with other components of the IFN-β enhanceosome including CBP and P/CAF (40, 179), suggesting that IE86's interaction with these proteins may play a role in blocking IFN-β enhanceosome formation and activation.

The ability of HCMV to inhibit IFN-β expression, like other viruses, has evolved a specific mechanism to circumvent a major arm of the host response to virus infection. Despite new observations that other IE gene products target aspects of the innate response; this is the first identification of an IFN-β antagonist expressed during HCMV infection. The immediate-early 1 protein IE72 attenuates IFN signaling by preventing STAT binding to ISG promoters (154). Additionally, the TRS1/IRS1 gene products have recently been identified as dsRNA binding proteins, which potentially function to interfere with the activation of the transcription factors necessary for IFN-β (86). Thus, within the first few hours postinfection, HCMV expresses at least three immediate early proteins with nonredundant functions in antagonizing the innate interferon response: TRS1/IRS1, IE86, and IE72, which target virus detection, IFN-β transcriptional induction, and IFN signaling through the IFNAR respectively.

The effect of IE86 on the transcriptional induction of other innate cytokines will require further examination, as their transcription is regulated similar to IFN-β.

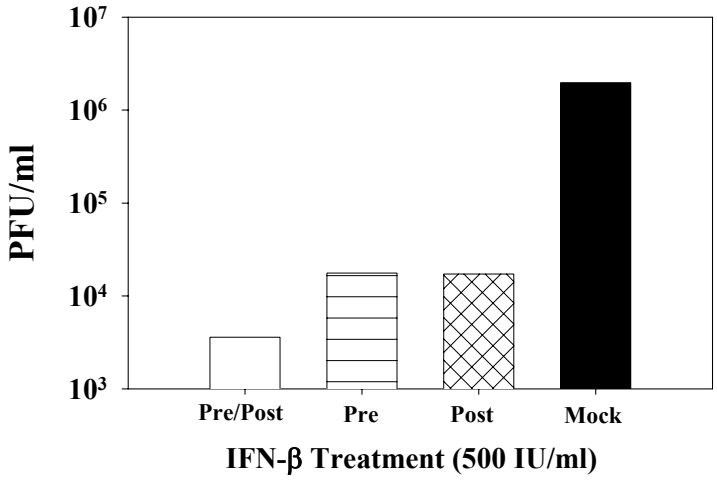

Figure 3-1. IFN-β inhibits HCMV replication. HFF cells were mock treated or treated with 500 IU/ml IFN-β. Cells were then infected with HCMV at a multiplicity of 0.1 pfu/cell. After one hour incubation, the inoculum was replaced with fresh medium either with or without 500 IU/ml IFN-β. Virus was harvested 10 days postinfection and quantified by plaque assay on HFF cells. Conditions: mock treatment (black bar); Pre-treatment alone (horizontal bar); Post-treatment alone (cross-hatched bar); and continuous treatment (open bar). Data represents the average of two independent experiments.

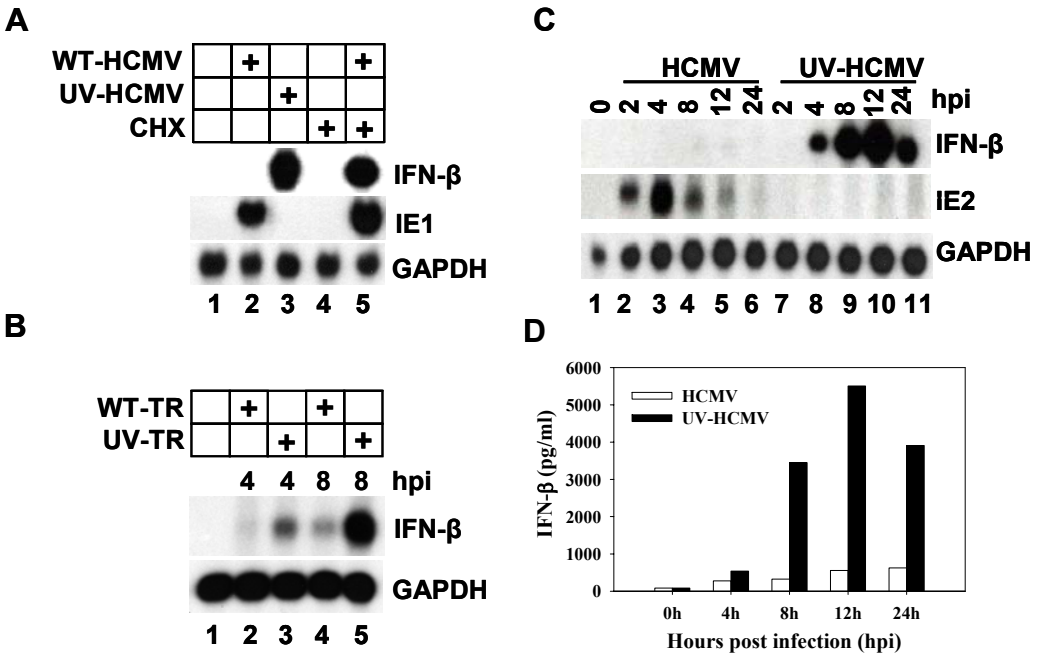

Figure 3-2. **HCMV gene expression attenuates IFN-β expression. (A)** HFF cells were either mock-infected, infected with HCMV, UV-inactivated HCMV, or wild-type HCMV in the presence of 100 μg/ml cycloheximide (CHX), or treated with cycloheximide alone. RNA was isolated 6 hours post treatment and analyzed for IFN-β, IE1, and GAPDH transcript by Northern blot. **(B)** HFF cells were either mock-infected, infected with a clinical HCMV strain (TR) or UV-inactivated HCMV (UV-TR). RNA was isolated 4 and 8 hours postinfection and analyzed for IFN-β and GAPDH by Northern blot. **(C)** HFF cells were infected at a multiplicity of 5 pfu/cell with either HCMV or UV-inactivated HCMV. RNA was harvested at various times postinfection and assayed for IFN-β, IE2, and GAPDH by northern blot. **(D)** Supernatants from cells infected with either wild-type HCMV (open bars) or UV-inactivated HCMV (black bars) were harvested and assayed for IFN-β secretion by ELISA. Data represents the average of two independent experiments.

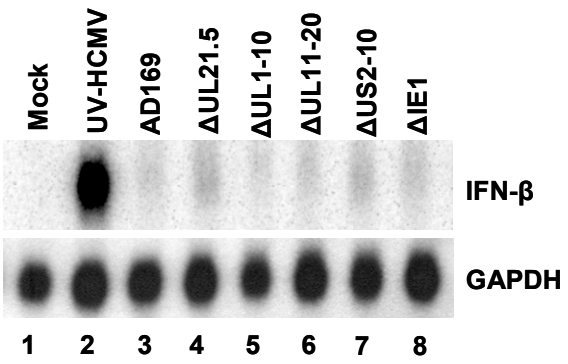

Figure 3-3. HCMV mutant viruses block IFN-β expression. HFF cells were mock-infected (M) or infected with UV-irradiated HCMV, wild-type strain AD169, ΔIE1, ΔUL1-11, ΔUL11-20, ΔUS2-10, or ΔUL21.5 at a multiplicity of 5 pfu/cell. RNA was isolated 6 hours postinfection and was assayed for IFN-β and GAPDH expression by Northern blot analysis.

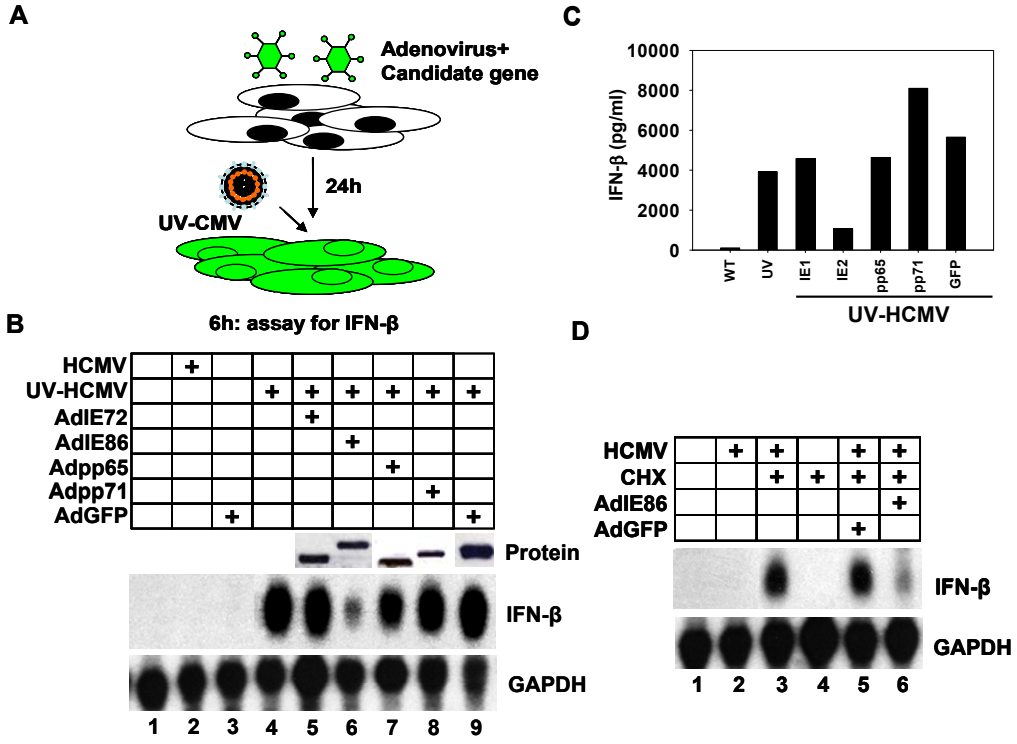

Figure 3-4. IE86 blocks HCMV-induced IFN-β expression. (A) Schematic overview of the IFN-β antagonist assay. **(B)** HFF cells were transduced with replication-defective adenoviruses expressing IE1, IE2, pp65, pp71 or GFP for 24 hours. Transduced cells were then infected with UV-HCMV at a multiplicity of 5 pfu/cell. RNA was harvested 6 hours postinfection with UV-HCMV and analyzed for IFN-β and GAPDH transcript. Expression of IE72, IE86, pp71, pp65, and GFP protein expressed from the adenoviruses is also shown. **(C)** Supernatants from infected samples as described in (B) were assayed for IFN-β secretion by ELISA. Data represents the average of two independent experiments. **(D)** HFF cells were transduced with replication-defective adenoviruses expressing either IE2 or GFP for 24 hours. Transduced cells were then infected with HCMV in the presence of 100 µg/ml cycloheximide (CHX) or treated with CHX alone. RNA was harvested 6 hours postinfection and assayed for IFN-β and GAPDH by Northern blot.

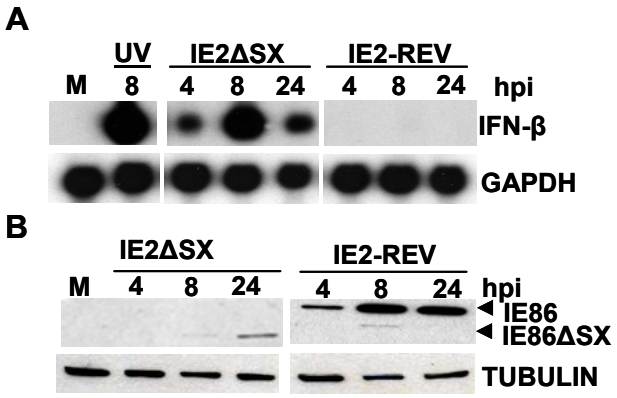

Figure 3-5. An HCMV IE86 mutant virus fails to block IFN-β expression. **(A)** HFF cells were mock-infected (M) or infected with IE2ΔSX, IE2ΔSX-REV, or UV-irradiated HCMV at a multiplicity of 5 pfu/cell. RNA was isolated 4, 8 and 24 hours postinfection and was assayed for IFN-β and GAPDH expression by Northern blot analysis. **(B)** HFF cells were mock-infected or infected with IE2ΔSX or IE2ΔSX-REV at a multiplicity of 5 pfu/cell. Protein was isolated 4, 8 and 24 hours postinfection and was assayed for IE86 and tubulin expression by Western blot.

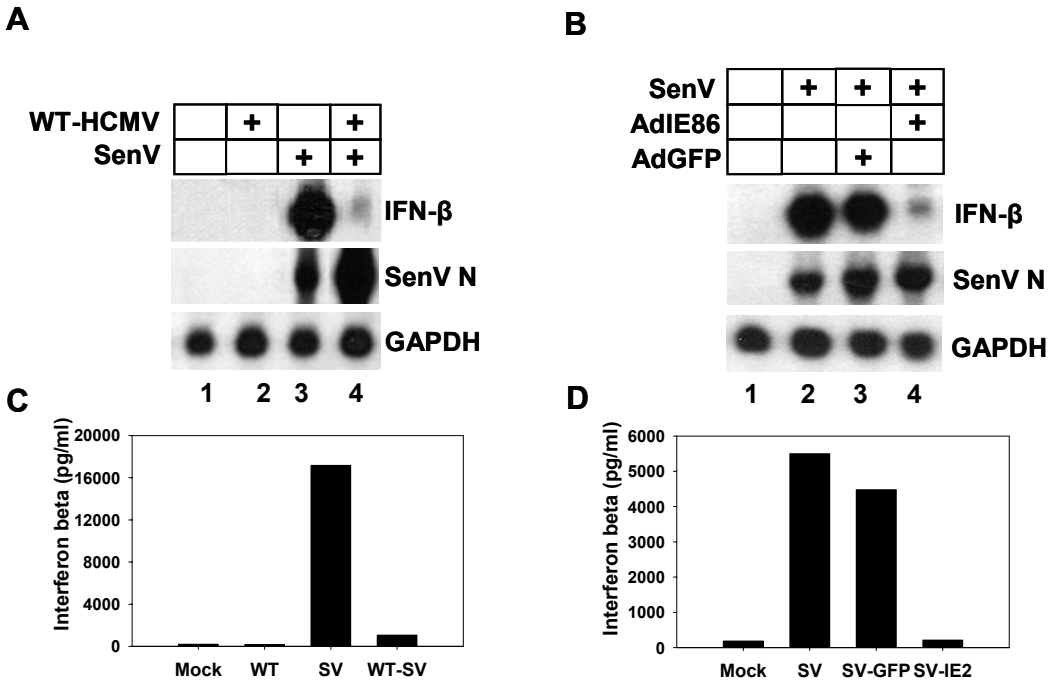

Figure 3-6. IE86 expression blocks Sendai virus-induced IFN-β expression. **(A)** HFF cells were either mock-infected or infected with HCMV at a multiplicity of 5 pfu/cell. Six hours postinfection the cells were super-infected with Sendai virus (100 HAU/ml). RNA was isolated 16 hours after Sendai virus infection and assayed for IFN-β, GAPDH, and Sendai virus N transcript by northern blot. **(B)** Supernatants from infected samples described in panel (A) were assayed for IFN-β secretion by ELISA. Data represent the average of two independent experiments. **(C)** HFF cells were either mock-transduced or transduced with replication-defective adenoviruses for 24 hours that express either IE86 or GFP. Transduced cells were then infected with Sendai virus. RNA was isolated 6 hours post Sendai virus infection and assayed for IFN-β, GAPDH, and Sendai virus N transcript by northern blot. **(D)** Supernatants from infected samples described in panel (C) were assayed for secretion of IFN-β by ELISA. Data represent the average of two independent experiments.

CHAPTER 4
ROLES OF IE86 AND pp65 IN ATTENUATION OF HOST RESPONSE

INTRODUCTION

Independent of IFN-β expression, HCMV infection regulates the expression of a number of proinflammatory cytokines/chemokines including: RANTES (1, 22, 38, 39, 81, 216), monokine-induced by interferon-γ (MIG) (1, 38, 39), monocyte chemotactic protein-1, and 2 (MCP-1 and 2) (38, 92), macrophage inflammatory protein-1 alpha (MIP-1α) (1, 39), and interleukin-8 (IL-8) (39, 53, 56, 141, 164). Interestingly, the expression of these cytokines was significantly enhanced when HCMV gene expression was inhibited, suggesting that one or more newly synthesized HCMV-encoded protein(s) may actively block the expression of these genes during infection (22, 39, 73, 92, 217).

In addition to our results that IE86 blocks IFN-β production during HCMV infection, two independent reports using microarray analysis and viral deletion mutants showed that the HCMV UL83-encoded protein pp65 could partially inhibit the induction of IFN-β and a number of chemokines following HCMV infection (1, 38). There is significant overlap in the signaling pathways and virus-activated transcription factors that regulate the expression of IFN-β and the various chemokines regulated by HCMV. The goal of this study was to determine the relative contributions played by IE86 and pp65 in the inhibition of IFN-β expression and to determine if IE86 can block the expression of proinflammatory chemokines following viral infection.

RESULTS

HCMV gene expression attenuates chemokine expression

Previous reports have demonstrated that infection with transcriptionally inactive UV-irradiated HCMV or infection with wild-type HCMV in the presence of cycloheximide results in a dramatic induction of cytokine and chemokine expression, when compared to wild-type infection (22, 39, 73, 92, 217). These results suggest that a newly synthesized HCMV gene product can inhibit the induction of these genes during infection. To confirm the previous results we assayed for the expression of key cytokines and chemokines by Northern blot. HFF cells were infected for 8 h at a multiplicity of 5 plaque forming units (pfu) per cell with either purified wild-type HCMV or purified UV-inactivated HCMV. As shown in Figure 4-1, infection with UV-inactivated HCMV results in a robust induction of IFN-β, RANTES, MIG, and MCP-2 expression when compared to wild-type infection. Blots were also probed for the immediate-early 1 (IE1) transcript to confirm that our UV-irradiation protocol efficiently blocks viral gene expression. Thus HCMV gene expression regulates the expression of proinflammatory chemokines. Given that pp65 has been linked to cytokine and chemokine regulation during HCMV infection, we next sought to elucidate the individual contributions of both IE86 and pp65 regarding the inhibition of IFN-β and chemokine expression during HCMV infection.

Delayed IE86 expression correlates with cytokine induction

To compare the roles of IE86 and pp65 in attenuating IFN-β and chemokine induction, we used two HCMV mutant viruses. The first was an IE2 mutant virus, termed IE2ΔSX, which has amino acids 136-290 deleted from exon 5 of IE2, and is fused at its carboxy-terminal to the

green fluorescent protein (GFP). IE2ΔSX is viable but expresses IE86 at dramatically reduced levels and with delayed kinetics when compared to IE86 expression during wild-type or a revertant virus infection (171). The second mutant was a UL83 deletion mutant virus termed ΔUL83. The ΔUL83 mutant virus has the UL83 ORF corresponding to nucleotides 119,388-121,000 of the AD169 genome replaced with a kanamycin cassette. The ΔUL83 virus was constructed to be identical to the previously described RVAd65 UL83 deletion mutant (177). Cells were infected with purified UV-irradiated HCMV, IE2ΔSX virus, IE2-Rev virus (a revertant virus of IE2ΔSX) or ΔUL83 virus. RNA was isolated at various times after infection and assayed for IFN-β and RANTES expression by Northern blot analysis. As shown in Figure 4-2A, infection with both UV-irradiated HCMV and the IE2ΔSX virus resulted in a robust induction of IFN-β and RANTES expression. However, infection with the IE2-revertant virus did not induce the expression of either gene. Interestingly, the level of induction observed following infection with the IE2ΔSX virus closely paralleled that observed following infection with UV-inactivated virus. As previously described (1, 38), infection with the ΔUL83 virus also resulted in an increase in both IFN-β and RANTES expression (Fig. 4-2B). However, this increase in expression is delayed and significantly reduced when compared to the response following infection with either the IE2ΔSX virus or UV-inactivated HCMV. This suggests that there is a greater cytokine response following infection with a virus that does not express IE86, at early times during infection, when compared to a virus that does not express pp65.

The expression of pp65 could be affected during infection with the IE2ΔSX virus or deletion of pp65 could somehow alter the expression of IE86 during infection. To address these possibilities, we examined the

protein levels of IE86 and pp65 following infection with the IE2ΔSX, ΔUL83 or wild-type virus. As previously reported, infection with the IE2ΔSX virus resulted in a dramatic reduction and delay in IE86 expression when compared to wild-type HCMV (171). The truncated form of IE86 was not detected until 24 hours after infection, whereas IE86 was abundantly expressed by 4 hours following wild-type infection. There was also a slight decrease in the expression of pp65 at 4 hours postinfection with the IE2ΔSX virus when compared to the wild-type virus (Fig. 4-3A). These results are consistent with previous reports demonstrating the IE2ΔSX virus expresses lower levels of pp65 (171). However, pp65 was clearly present within the infected cell by 4 hours postinfection with the IE2ΔSX virus. The levels of two other tegument proteins pp71 and pp28 were approximately the same following infection with either the IE2ΔSX or wild-type virus (Fig. 4-3A). As expected, the ΔUL83 virus did not express pp65. Surprisingly, there was a delay in the expression of IE86 following infection with the ΔUL83 virus when compared to the wild-type virus (Fig. 4-3B). IE86 was abundantly expressed by 4 hours postinfection following wild-type infection, whereas IE86 was not expressed until 8 hours postinfection with the ΔUL83 virus. We obtained similar results when a second UL83 deletion mutant virus termed RVAd65 (177) was used (data not shown). Interestingly, there was also a significant delay in the expression of pp71 following infection with the ΔUL83 virus (Fig. 4-3B). Taken together, these results identify a connection between delayed IE86 expression and increased accumulation of IFN-β and RANTES transcripts.

IE86 blocks virus-induced IFN-β and RANTES expression

Since there was a decrease in the expression of pp65 following infection with the IE2ΔSX virus, we could not rule out the possibility that

decreased expression of pp65 was also contributing to the cytokine induction observed during infection with the IE2ΔSX virus. Therefore, we wanted to determine whether expression of IE86 or pp65 alone was capable of blocking virus-induced cytokine expression. To accomplish this, we used replication-deficient adenoviruses that express IE86, pp65 or GFP. Cells were transduced with adenovirus that express either IE86, pp65, or GFP at an MOI of 3 pfu/cell. 24 hours after transduction the cells were infected with either UV-HCMV (Fig. 4-4A and B) or Sendai virus (Fig. 4-4C and D). RNA was isolated 8 hours postinfection, reverse transcribed, and real time PCR was performed to quantitate the abundance of IFN-β and RANTES transcripts. As shown in Figures 4-4A-D, infection with either UV-irradiated HCMV or Sendai virus alone resulted in a dramatic accumulation of IFN-β and RANTES message. However, expression of IE86 prior to infection with UV-HCMV or Sendai virus greatly reduced or abolished the induction of IFN-β or RANTES (Fig. 4-4A-D). Prior expression of either pp65 or GFP had no effect on the induction of IFN-β or RANTES following either type of infection. A Western blot is also shown (Fig. 4-4E) to confirm the expression of IE86, pp65 and GFP at the time of infection. These results confirm the role of IE86 as an IFN-β antagonist and also demonstrate its ability to block the induction of RANTES following virus infection. These results additionally demonstrate that ectopic expression of pp65 does not block the induction of IFN-β or RANTES during virus infection. They further suggest the increased cytokine response observed in Figure 4-2B following infection with the ΔUL83 virus is not the result of abolishing pp65 expression, but is likely due to the delayed expression of IE86. We next wanted to determine why infection with the ΔUL83 virus elicits an IFN-β and chemokine response.

IE protein expression restored with ΔUL83 infection of pp71 cell line

The decrease in pp71 and IE86 protein expression observed following infection with ΔUL83 suggests that deletion of pp65 affects multiple viral proteins. The tegument protein pp71 is a critical regulator of immediate early gene expression, so we first wanted to determine whether the delay in pp71 expression was leading to a decrease in IE86. To test this, we infected HFF cells stably expressing pp71 (42) with WT-HCMV, ΔUL83 virus and ΔUL82 virus and assayed for IE proteins by Western blot 4 and 8 hours postinfection. Shown in Figure 4-5, IE gene products IE72 and IE86 are expressed with wild-type kinetics following infection of pp71 expressing cells with both ΔUL82 and ΔUL83 mutant viruses. These results suggest that deletion of the UL83 open reading frame (ORF) affects pp71 expression.

Generation of a UL83 stop codon mutant virus

The delay in pp71 protein expression following infection with the ΔUL83 virus (Fig. 4-3B) suggests that deletion of the UL83 ORF from the viral genome in some way alters expression of the downstream UL82 ORF, which encodes for pp71 (see ORF schematic Fig. 4-6A). In order to determine whether loss of pp65 expression or deletion of the UL83 gene sequence from the genome is responsible for the delay in pp71 expression and increase in cytokine induction observed following infection with the ΔUL83 virus, we constructed a second UL83 viral mutant. This mutant, termed UL83Stop, contains point mutations that introduce stop codons in all three reading frames 35 base pairs downstream of the UL83 start codon. Therefore, like the ΔUL83 virus, the UL83Stop virus will not be capable of expressing pp65. However, unlike the ΔUL83 virus, the UL83Stop virus does not delete any genomic sequences and still contains

the entire UL83 ORF (Fig. 4-6A), and therefore should not interfere with the expression of pp71. The UL83Stop virus was constructed by standard allelic exchange protocols described previously (184, 211).

To confirm proper recombination and insertion of the stop codons within the UL83 ORF, restriction enzyme analysis of wild-type, ΔUL83 and UL83Stop BAC DNA was performed. As indicated by the asterisk in Figure 4-6B, wild-type and UL83Stop DNA digested with *XhoI* generates the predicted 9.5 kb fragment that is absent from ΔUL83 digested DNA due to a novel *XhoI* site in the kanamycin cassette. The stop codon mutations within UL83Stop also creates a novel *SpeI* restriction enzyme site in the UL83 ORF (Fig. 4-6A). To confirm deletion of UL83 from ΔUL83 and distinguish UL83Stop DNA from wild-type DNA, PCR was used to amplify across the recombination junction to produce a specific fragment using purified wild-type, ΔUL83 or UL83Stop BAC DNA as template. As shown in Figure 4-6C, there is a 1.5 kb PCR product generated by using wild-type and UL83Stop DNA as template. No amplification product was observed using ΔUL83 BAC DNA. Digestion of the 1.5 kb UL83Stop PCR fragment with *SpeI* produced a 1.0 kb fragment and a 0.5 kb fragment, indicating the stop codon mutations were properly located within the viral genome. The fragment amplified from WT DNA was not digested with *SpeI*.

Lastly, to confirm the UL83Stop is unable to express pp65, cells were infected with wild-type, ΔUL83 or UL83Stop virus, cell lysates were prepared 72 hours postinfection and subjected to Western blot analysis. As expected, both the ΔUL83 and UL83Stop viruses do not express pp65, whereas pp65 is abundantly expressed following infection with the wild-type virus (Fig. 4-6D).

Replication of ΔUL83 is delayed compared to wild-type and UL83Stop viruses

To compare the ΔUL83 and UL83Stop viruses we first performed a multi-step growth curve analysis. HFF cells were infected at an MOI of 0.1 with WT-HCMV, ΔUL83, and UL83Stop. Virus was harvested at 4, 8, 15 and 20 days postinfection and quantified by plaque assay. Figure 4-7A shows an early delay in virus replication with the ΔUL83, when compared to both WT-HCMV and UL83Stop.

We then examined the expression of IE86 and pp71 following infection with either wild-type or UL83Stop virus. HFF cells were infected with either virus and cell lysates were prepared at various times postinfection. As shown in Figure 4-7B, the expression of IE86 and pp71 following UL83Stop virus infection occurred with the same kinetics and to the same levels that are observed following wild-type infection. These results suggest that the delay in IE86 and pp71 expression observed with the ΔUL83 virus (Fig. 4-3B) is the result of deleting the UL83 ORF from the genome and were not due to abolishing pp65 expression.

pp65 is not responsible for blocking IFN-β and RANTES expression

We next compared the cytokine response following infection with the UL83Stop and ΔUL83 mutants. Fibroblasts were infected with wild-type, UV-HCMV, ΔUL83, or UL83Stop virus. Cells were harvested and RNA extracted 8 hours postinfection and assayed for IFN-β expression by Northern blot analysis. As shown in Figure 4-8A, infection with UV-HCMV resulted in a strong induction of IFN-β transcript. Infection with ΔUL83 also resulted in IFN-β transcript accumulation, although it was not nearly as robust as that observed for UV-HCMV. Interestingly, infection with either wild-type or UL83Stop virus completely blocked the induction of

IFN-β (Fig. 4-8A). Similar results were obtained for RANTES expression (data not shown). Northern blotting was used to determine if prior infection with the UL83Stop virus could block Sendai virus-induced cytokine expression. Cells were infected with wild-type, ΔUL83, or UL83Stop virus for 8 hours and then infected with Sendai virus. RNA was isolated 8 hours post Sendai virus infection and assayed for IFN-β and RANTES expression. As shown in Figure 4-8B, infection with Sendai virus alone results in a dramatic induction of both IFN-β and RANTES expression. However, when cells were infected with either wild-type or the UL83Stop virus prior to Sendai virus infection, the induction of both IFN-β and RANTES was almost completely blocked, demonstrating that an HCMV gene other than pp65 which is expressed within the first 8 hours of infection was responsible for this inhibition. Prior infection with the ΔUL83 virus also resulted in an attenuated cytokine response. However, this inhibition was not as dramatic as that observed for the wild-type virus or UL83Stop virus. Since the UL83Stop and ΔUL83 viruses do not express pp65, the difference in their ability to inhibit the cytokine response following viral infection is likely due to the different mutation strategies adopted to prevent pp65 expression.

UL82 mutant virus fails to block cytokine induction

Infection with the ΔUL83 virus results in a cytokine and chemokine response which we show is likely due to a decrease in pp71. Thus infection with the pp71 deletion mutant should also result in a cytokine and chemokine response. To test this, HFF cells were infected with WT-HCMV, UV-HCMV, ΔUL83 and ΔUL82 viruses at a multiplicity of 5 pfu/cell. Cells were harvested 4, 8, and 12 hours postinfection and assayed for IFN-β by Northern blot. Figure 4-9 shows that similar to the

ΔUL83 mutant virus, infection with the ΔUL82 virus results in an increase in IFN-β message when compared to WT-HCMV infection.

HCMV IE86 blocks induction of proinflammatory chemokines

We also examined the ability of IE86 to block the induction of a number of other chemokines that were previously reported to be blocked by pp65 following infection with the ΔUL83 virus. Cells were transduced with adenovirus that express IE86, pp65, or GFP and then infected with UV-HCMV for 8 h. RNA was isolated and assayed by real time PCR for expression of IFN-β, RANTES, MCP-2, MIG, MIP-1α and IL-8. As shown in Table 4-1, infection with UV-HCMV resulted in a robust induction of all cytokines and chemokines tested when compared to wild-type infection. All chemokines were induced to levels similar to that observed for UV-HCMV in cells expressing either pp65 or GFP, demonstrating that these gene products are not capable of blocking chemokine induction during HCMV infection. However, cells expressing IE86 effectively blocked chemokine induction following UV-HCMV infection. ISG56, which is induced by both WT and UV-HCMV (39, 181), was included to show that the effect of IE86 is specific and not all antiviral genes are regulated by IE86. These results demonstrate that IE86 not only functions as an IFN-β and RANTES antagonist but also functions to inhibit the expression of numerous chemokines during HCMV infection.

DISCUSSION

The host innate immune response is the primary barrier to virus infection at the cellular level. Inflammatory chemokines are a key component of this innate response. Secreted from both infected and uninfected cells, chemokines facilitate the recruitment and activation of the

effector cells responsible for viral clearance. A virus-mediated block to chemokine induction would likely sever the link between the innate and adaptive immune responses and prevent virus elimination by activated T and NK cells. Therefore, a general suppression of chemokine function would likely enhance viral replication and/or pathogenesis (78), In fact, HCMV uses redundant mechanisms to attenuate the host response and has been shown to target inflammatory chemokine function by the expression of chemokine mimics (113, 155), chemokine binding proteins (164, 200) and G-protein coupled chemokine receptors (134, 192). This report identifies yet another mechanism of chemokine inhibition by HCMV, in which the immediate-early 2 protein IE86 blocks expression of proinflammatory cytokines at the level of mRNA abundance.

Previous reports have demonstrated that HCMV gene expression can attenuate the host proinflammatory cytokine response and this attenuation is dependent on a newly synthesized viral protein expressed early during infection (22, 39, 73, 92, 216, 217). We have shown that the HCMV immediate-early 2 gene product IE86 can efficiently block the induction of IFN-β following viral infection (195). Additionally, two labs have independently reported microarray studies that demonstrate an increase in the cellular antiviral cytokine response during infection with a UL83 deletion mutant, suggesting the tegument protein pp65 is involved in attenuating cytokine expression (1, 38). Therefore, we wanted to investigate the respective roles that IE86 and pp65 play during the immediate early events following virus infection, and determine if either protein is responsible for suppressing the host cytokine response.

To determine the relative roles of IE86 and pp65, we used two HCMV viral mutants. The first mutant, termed IE2ΔSX (171), is an IE2 mutant that expresses a truncated IE86 protein that is expressed with

severely delayed kinetics. The second mutant, termed ΔUL83, is a UL83 deletion mutant which does not express pp65. Following infection with the IE2ΔSX virus we observed a dramatic induction in both IFN-β and RANTES expression when compared to wild-type infection. The level of induction observed following infection with the either the IE2ΔSX virus or UV-irradiated HCMV were quite similar (Fig. 4-2A). Infection with the ΔUL83 virus also resulted in an induction of IFN-β and RANTES expression, confirming previous microarray results. However, when compared to the IFN-β and RANTES levels observed following infection with the IE2ΔSX virus or UV-inactivated HCMV, the induction observed following infection with the ΔUL83 virus was severely attenuated; suggesting pp65 could only be partially responsible for blocking the response. Western blot analysis of viral proteins expressed at early times after infection revealed that IE86 expression is not only delayed in the IE2ΔSX virus as reported (171), but is also impaired following infection with the ΔUL83 virus. Together, these results support the conclusion that impaired IE86 expression correlates with increased cytokine expression.

Experiments with the IE2ΔSX and ΔUL83 mutants suggest that IE86 can more efficiently attenuate cytokine expression when compared to pp65. To test this, we used replication-defective adenoviruses that express IE86 or pp65 to determine if prior expression of either protein could block the expression of cytokines induced following infection with UV-HCMV or Sendai virus. Prior expression of IE86 efficiently blocked IFN-β and RANTES expression following infection with either UV-HCMV or Sendai virus. The results demonstrate that IE86 not only functions as an IFN-β antagonist but also can function as a RANTES antagonist. Interestingly, prior expression of pp65 had no effect on the induction of IFN-β and RANTES expression observed following UV-HCMV or Sendai

virus infection. These results suggest that the delay in IE86 expression observed following infection with the ΔUL83 virus is likely responsible for the increased cytokine response, and not a lack of pp65 expression.

We also observed a delay and a decrease in the expression of pp71, a key regulator of IE86 expression (36), following infection with the ΔUL83 virus. This raised the possibility that deletion of the UL83 ORF from the viral genome may have also affected expression of the downstream UL82 ORF which encodes for pp71. In this scenario, the reduced expression of pp71 observed following ΔUL83 infection would be responsible for the observed delay in IE86 expression, which in turn would lead to the modest IFN-β and RANTES induction observed in Figure 4-2B. To test this possibility, and determine if pp65 expression was involved in blocking cytokine induction, we constructed an additional UL83 null mutant. This mutant, termed UL83Stop, contains stop codon mutations in all three reading frames 35 bp down stream of the UL83 AUG codon. The UL83 sequence containing these stop codon mutations was used to repair the deleted sequence within the ΔUL83 genome. Importantly, the UL83Stop mutant does not have any UL83 or other genomic sequence deleted, but is still unable to express pp65. When cells were assayed for IE86 and pp71 expression following infection with the UL83Stop virus, we observed no difference in the levels or kinetics at which these proteins were expressed when compared to wild-type infection. Most importantly, infection with the UL83Stop virus now blocked the expression of IFN-β and RANTES to the same levels observed following wild-type infection (Fig. 4-8A). In addition, prior infection with the UL83Stop mutant also blocked the induction of IFN-β and RANTES observed during Sendai virus infection (Fig. 4-8B). These results demonstrate that the induction of IFN-β and RANTES observed following infection with the ΔUL83 virus is not

the result of blocking pp65 expression, but more likely is the result of inhibiting the expression of pp71, which in turn regulates IE86 expression. In support of this hypothesis we have demonstrated that infection of pp71-expressing fibroblasts with the ΔUL83 virus results in wild-type kinetics and expression of IE86 (Fig. 4-5). In addition, infection with a UL82 deletion virus (42), resulted in an IFN-β and chemokine response similar to that observed following infection with the ΔUL83 virus (Fig. 4-9). In fact, since the phenotype of the UL82 deletion mutant (*i.e.* reduced expression of IE genes) is multiplicity-dependent, we observed an even greater cytokine response following infection with the ΔUL82 virus at a low multiplicity, when compared to the ΔUL83 virus (data not shown). Thus, using a UL83 stop codon mutant and a pp65-expressing adenovirus, we have demonstrated that pp65 is not involved in blocking the expression of IFN-β, RANTES, MIG, MCP-2, IL-8, or MIP1-α during HCMV infection. However, this does not mean that other genes identified by microarray analysis following infection with the ΔUL83 virus are not controlled by pp65. It also suggests that in addition to the chemokines identified here, IE86 may also regulate a number of other cellular genes which were previously identified as being regulated following ΔUL83 infection by gene array analysis.

Our results demonstrate that HCMV gene expression is required to efficiently block chemokine induction following HCMV infection and that the immediate-early 2 gene product IE86, in the absence of other HCMV gene products, is capable of blocking this induction during both HCMV and Sendai virus infection. Importantly, this is the first report to demonstrate the ability of IE86 to function as a proinflammatory cytokine antagonist. However, as reported by others some chemokines are modestly induced during a wild-type virus infection, albeit at a much lower

level when compared to inactivated virus infection (1, 22, 38, 39, 48, 56, 73, 130, 141, 216). Therefore, it is important to note that the low level of chemokine expression observed following wild-type infection may play an important role in the viral life cycle.

Similar to the induction of IFN-β transcription (203), inflammatory cytokines transcribed in response to virus infection require the activation of numerous transcription factors which include IRF-3, NFκB and AP1 (127, 136). An IE86-mediated block to cytokine induction may be facilitated though inhibition of transcription factor activation or binding to the various cytokine promoters.

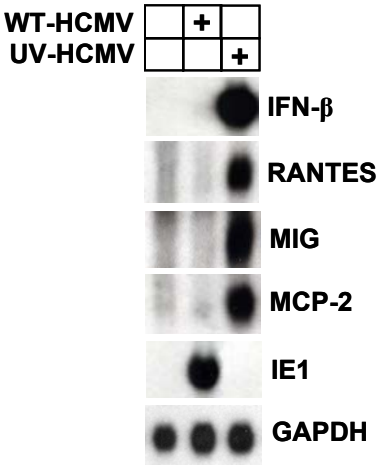

Figure 4-1. HCMV gene expression attenuates chemokine expression. HFF cells were mock-infected, infected with wild-type HCMV (WT) or infected with UV-inactivated HCMV (UV-HCMV) at a multiplicity of 5 pfu/cell. RNA was isolated 8 hours postinfection and analyzed for IFN-β, RANTES, MIG, MCP-2, IE1, and GAPDH transcript accumulation by Northern blot analysis.

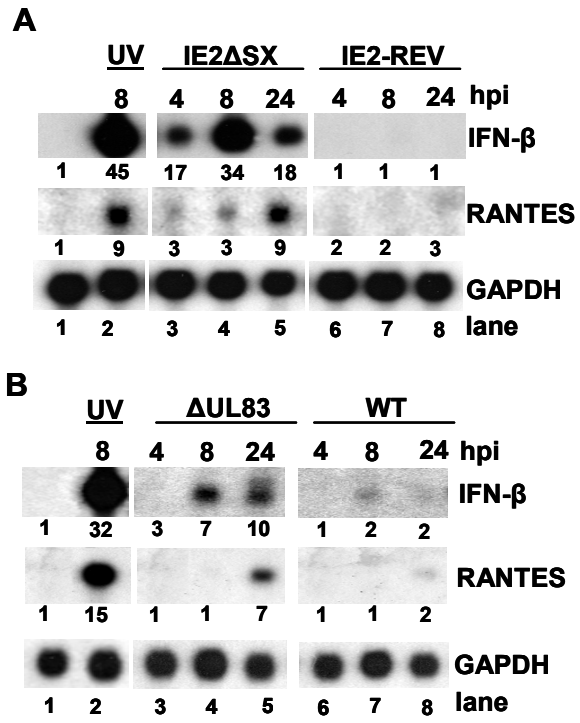

Figure 4-2. pp65 mutant virus blocks cytokine expression more efficiently than an IE86 mutant virus. HFF cells were mock-infected (M) or infected with IE2ΔSX, IE2ΔSX-REV **(A)**, ΔUL83 or wild-type **(B)** virus at a multiplicity of 5 pfu/cell. RNA was isolated 4, 8 and 24 hours postinfection and was assayed for IFN-β, RANTES and GAPDH expression by Northern blot analysis. IFN-β and RANTES expression was quantified by phosphorimage analysis and standardized to GAPDH expression. Numbers below each blot represent the fold increase over mock-infected cells.

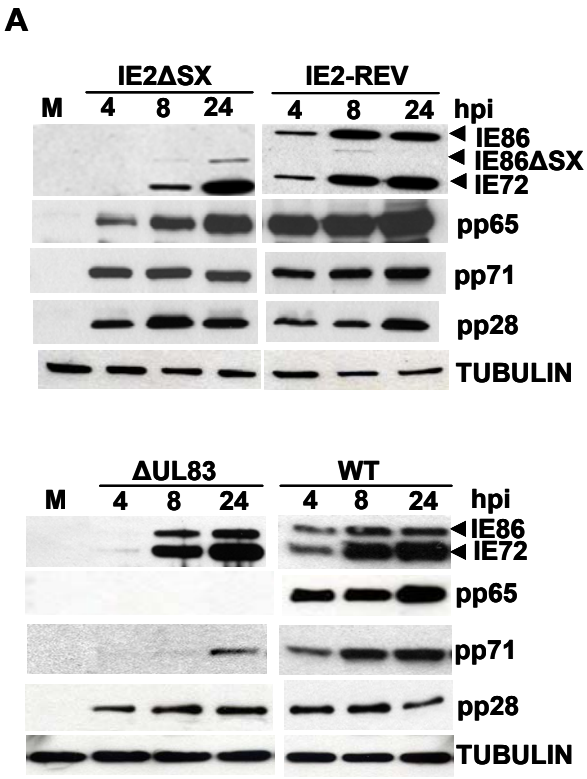

Figure 4-3. ΔUL83 virus fails to express IE proteins with wild-type kinetics. HFF cells were mock-infected (M), infected with IE2ΔSX or IE2ΔSX-REV virus **(A)**, or infected with ΔUL83 or wild-type virus **(B)** at a multiplicity of 5 pfu/cell. Cell lysates were harvested 4, 8 and 24 hours postinfection and assayed for IE86, pp65, pp71, pp28, and tubulin expression by Western blot analysis.

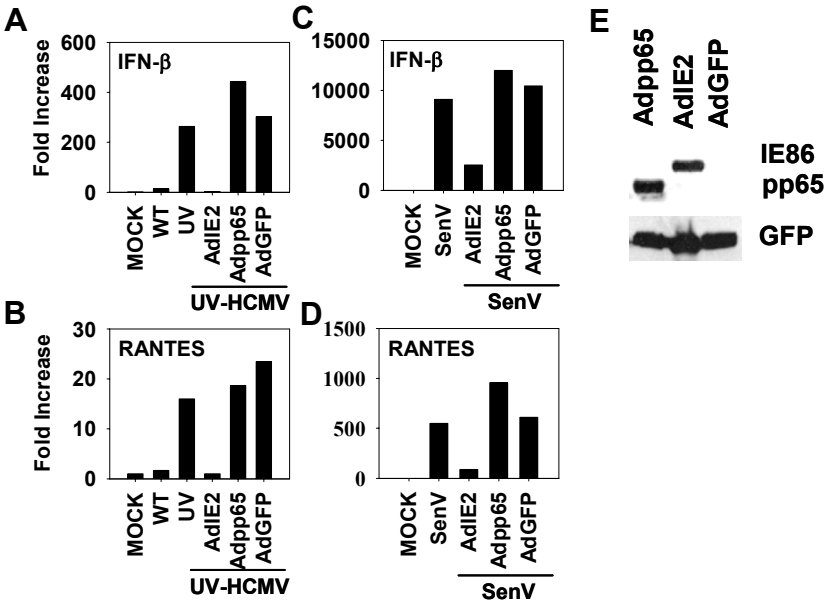

Figure 4-4. IE86 blocks virus-induced RANTES expression. HFF cells were transduced with replication-defective adenoviruses expressing IE86, pp65 or GFP. 24 h later the transduced cells were infected with UV-HCMV **(A & B)** or with 100 HA units/ml of Sendai virus **(C & D)**. RNA was isolated 8 hours postinfection and analyzed for IFN-β (A & C) and RANTES (B & D) expression by Real Time PCR. Transcript levels were standardized to GAPDH levels and represent the average of two independent experiments. Data represent fold increase over mock-infected cells. **(E)** HFF cell lysates were harvested 24 hours after transduction with the given adenovirus and assayed for IE86, pp65, and GFP expression by Western blot analysis.

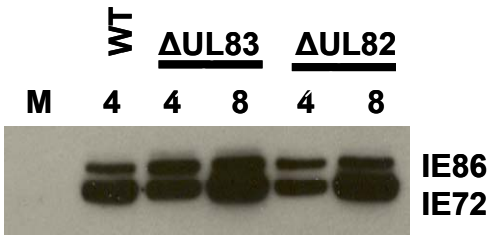

Figure 4-5. IE expression restored with ΔUL83 infection of pp71 cell line. HFF cells were mock-infected (M), infected with ΔUL83, ΔUL82 or wild-type virus at a multiplicity of 5 pfu/cell. Cell lysates were harvested 4, 8 hours postinfection and assayed for IE72 and IE86 expression by Western blot analysis.

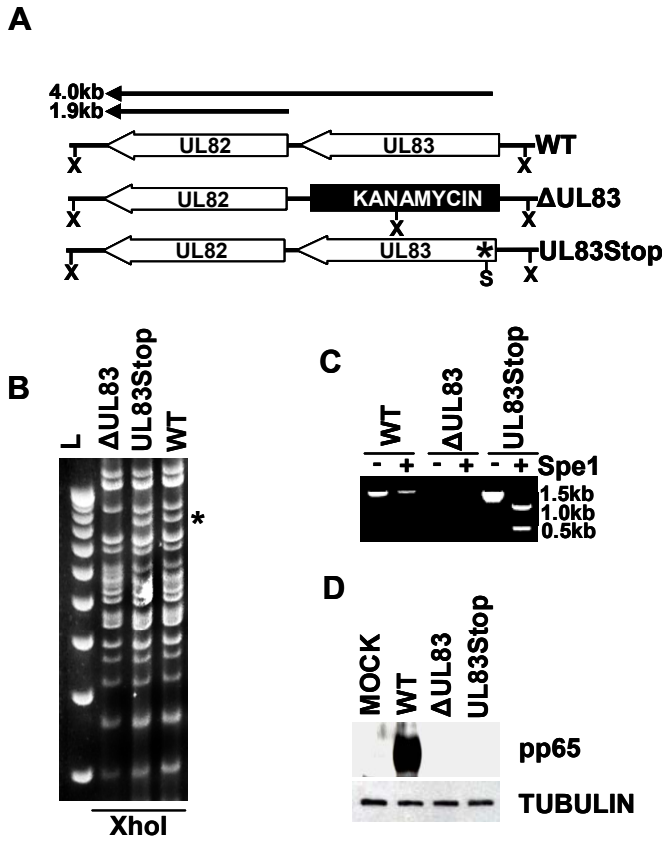

Figure 4-6. Characterization of a UL83 stop codon mutant virus. (A) Schematic representation of the UL83 genomic region depicting the UL83 and UL82 ORFs, the 4.0kb UL82 and UL83 co-terminal transcript, the *XhoI* (X) and *SpeI* (S) restriction sites, and the organization of the ΔUL83 and UL83Stop mutant viruses The asterisk (*) represents the stop codon mutation within the UL83Stop virus. **(B)** Wild-type, ΔUL83, or UL83Stop BAC DNA was digested with *XhoI* and the fragments separated by agarose gel electrophoresis. The asterisk (*) indicates the diagnostic fragment which is absent in the ΔUL83 digest and present in the WT and UL83Stop digests. **(C)** WT, ΔUL83, or UL83Stop BAC DNA was used as template to amplify a specific genomic region spanning the 5' end of UL83 ORF and the adjoining flanking sequence. The PCR product was then digested with *SpeI* to confirm that the stop codon was properly inserted within the UL83 ORF. **(D)** HFF cells were either mock-infected, or infected with wild-type (WT), ΔUL83, or UL83Stop virus. Cell lysates were prepared 72 hours postinfection and assayed for pp65 expression by Western blot analysis. Tubulin expression was included as an internal loading control.

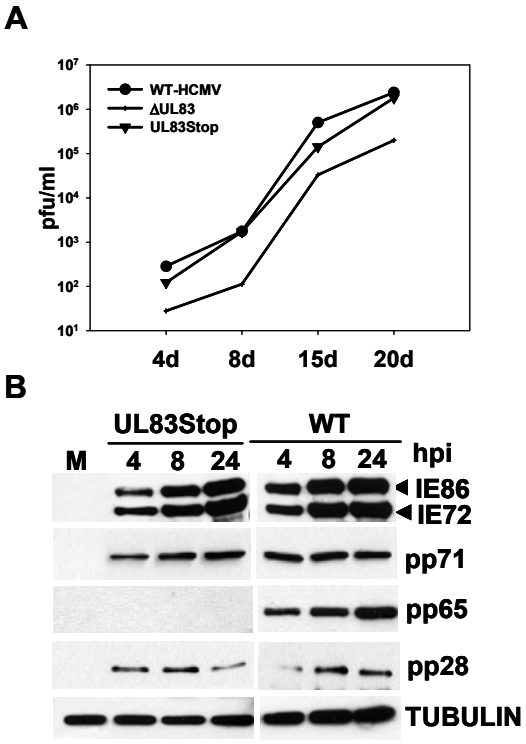

Figure 4-7. UL83Stop virus expresses IE and pp71 proteins with wild-type kinetics. (A) Growth curve analysis comparing ΔUL83 and UL83Stop to wild-type virus infection. **(B)** HFF cells were either mock-infected, infected with wild-type HCMV or the UL83Stop virus at a multiplicity of 5 pfu/cell. Cell lysates were prepared at the indicated times postinfection and assayed for IE86, pp65, pp71, pp28 and tubulin expression by Western blot analysis.

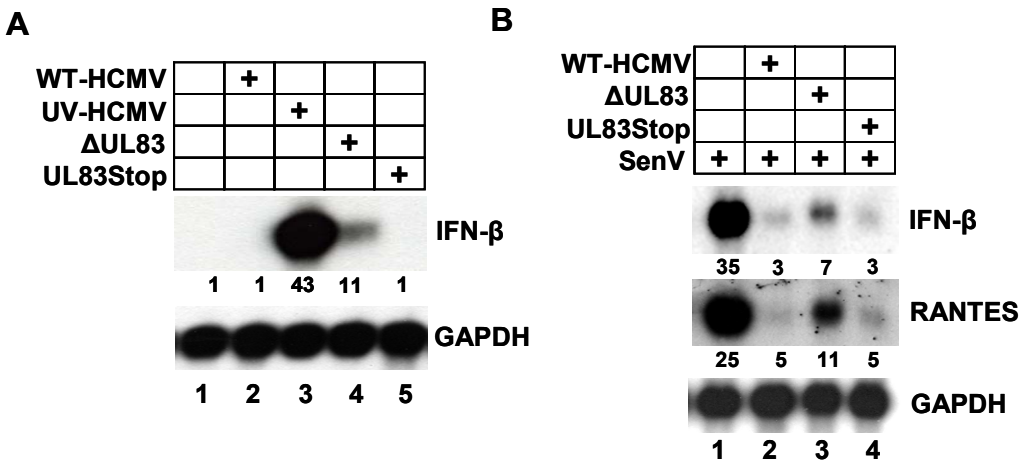

Figure 4-8. The UL83Stop virus attenuates cytokine induction. (A) HFF cells were mock-infected or infected with wild-type, ΔUL83 or UL83Stop virus at a multiplicity of 5 pfu/cell. RNA was isolated 8 hours postinfection and assayed for IFN-β and GAPDH by Northern blot analysis. **(B)** HFF cells were either mock-infected or infected with wild-type, ΔUL83 or UL83Stop virus at a multiplicity of 5 pfu/cell. 8 hours postinfection the cells were super-infected with Sendai virus (100 HAU/ml). RNA was isolated 8 hours after Sendai virus infection and assayed for IFN-β, RANTES and GAPDH by Northern blot analysis. The expression of IFN-β and RANTES was quantified by phosphorimage analysis and standardized to GAPDH expression. Numbers below each blot represent the fold increase over mock-infected cells.

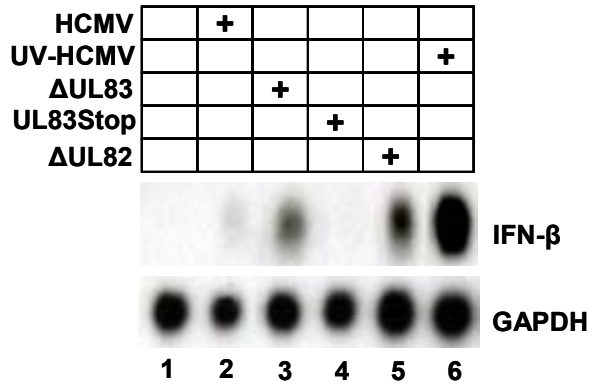

FIGURE 4-9. ΔUL82 virus fails to block cytokine expression. HFF cells were mock-infected (M) or infected with WT-HCMV, UV-HCMV, ΔUL83, UL83Stop or ΔUL82 virus at a multiplicity of 5 pfu/cell. RNA was isolated 6 hours postinfection and was assayed for IFN-β and GAPDH expression by Northern blot analysis.

TABLE 4-1. IE86 blocks virus-induced cytokine and chemokine expression[a]

				UV-HCMV		
GENE	MOCK	HCMV	UV-HCMV	AdIE86	Adpp65	AdGFP
IFN-β	1.0	13.8	263.0	2.4	442.6	302.0
RANTES	1.0	1.7	16.0	1.0	18.6	23.4
MCP-2	1.0	5.9	43.7	10.2	71.0	50.2
MIG	1.0	10.2	57.7	10.9	43.7	42.2
MIP-1α	1.0	1.1	6.5	1.4	11.3	8.0
IL-8	1.0	0.2	3.5	0.9	3.7	6.5
ISG56	1.0	1783.0	2195.0	1448.2	1260.7	2048.0

[a] Data is presented as fold increase over mock infection. Experiments were performed in duplicate and standardized to GAPDH.

CHAPTER 5
IE86 ATTENUATES NFκB DNA BINDING

INTRODUCTION

Induction of IFN-β transcription involves critical signal transduction cascades which result in the recruitment and binding of cellular transcription factors to form an enhanceosome on the IFN-β promoter (203). Previous work has demonstrated that the cellular transcription factors NFκB and IRF-3 are required for enhanceosome formation and IFN-β transcription (2). Inhibition of either the NFκB or IRF-3 pathway abrogates IFN-β transcription. A number of viruses have evolved mechanisms to inhibit IFN-β expression by targeting the IRF-3 or NFκB pathways which allows for viral persistence within the infected host. For example, the Ebola virus VP35, rotavirus NSP1 and human papillomavirus E6 proteins block specific steps required for the activation of IRF-3 (18, 79, 168). In addition, the NS3/4A protease cleaves the RIG-I signaling adapter molecule IPS-1, which prevents the activation of both NFκB and IRF-3 during hepatitis C virus infection (31, 67, 124, 129).

The studies presented in Chapters 3 and 4 showed that the immediate-early 2 gene product IE86 can efficiently block expression of IFN-β and a number of proinflammatory chemokines (195, 196). However the mechanism by which IE86 blocks the induction of IFN-β and these chemokines has not been elucidated. Given that activation of both NFκB and IRF-3 are required for IFN-β transcription, and that IRF-3 and NFκB are regulated during HCMV infection (59, 60), it is reasonable to suspect that IE86 may target one or both of these pathways to block IFN-β expression. In this report we examine the effect of IE86 on the IRF-3 and

NFκB pathways during HCMV infection and sought to address the mechanism by which IE86 blocks IFN-β expression.

RESULTS

Inhibition of IRF-3 or NFκB blocks IFN-β induction

We and others have previously reported that IFN-β expression is attenuated during wild-type HCMV (WT-HCMV) infection, when compared to infection with transcriptionally inactive UV-irradiated HCMV (UV-HCMV) (Fig. 5-1A, compare lanes 2 and 3) (39, 181, 195). We have also demonstrated that the HCMV immediate early 2 gene product IE86 can efficiently block the induction of IFN-β following infection with UV-HCMV (Fig. 5-1A) (195). However, the mechanism by which IE86 blocks IFN-β expression has not been elucidated.

The IRF-3 and NFκB transcription factors are required for IFN-β transcription following infection with a number of viruses (2, 8, 59, 93, 146, 174, 203, 209). We therefore hypothesized that IE86 may target the IRF-3 and/or NFκB pathway to inhibit IFN-β expression. However, we first needed to confirm that both IRF-3 and NFκB are required for IFN-β expression following infection with UV-HCMV. To test this, human foreskin fibroblasts (HFFs) were transduced with replication defective viruses expressing either a non-phosphorylatable form of the IκBα repressor, IκBαSR, (which blocks NFκB activation) (8) or a dominant negative IRF-3 protein, IRF3ΔN, (which blocks IRF-3 activation) (121). Transduced cells were then mock-infected or infected with WT-HCMV or UV-inactivated HCMV. RNA was isolated 8 hours postinfection and assayed for IFN-β expression by Northern blot. As shown in Fig. 5-1B, expression of IκBαSR prior to infection with UV-HCMV efficiently inhibited the induction of IFN-β expression (compare lanes 3 & 4). IFN-β

expression was also inhibited when IRF-3ΔN was expressed prior to UV-HCMV infection (Fig. 5-1C, compare lanes 3 & 4). However, infection with a control virus did not block the induction of IFN-β following UV-HCMV infection. Together, these initial experiments establish that induction of IFN-β during UV-HCMV infection requires IRF-3 and NFκB, and thus IE86 may target one or both of these pathways to block IFN-β expression.

IRF-3 activation and target gene expression are not attenuated by IE86

We next determined if IRF-3 is activated during HCMV infection and whether IE86 is capable of blocking IRF-3 activation. IRF-3 is constitutively expressed and retained in the cytoplasm of uninfected cells. The C-terminus of IRF-3 structurally obscures a nuclear import signal. Phosphorylation on key serine residues results in a conformation change which reveals the nuclear import signal, and facilitates IRF-3 homodimerization and nuclear translocation (121).

To assess IRF-3 activation during HCMV infection, we examined IRF-3 phosphorylation, IRF-3's ability to homodimerize, IRF-3's ability to translocate to the nucleus, and IRF-3's ability to activate gene expression. To monitor the phosphorylation state of IRF-3, HFF cells were either mock-infected or infected with WT-HCMV, UV-HCMV or Sendai virus. Cell lysates were harvested 6 hours postinfection and assayed for IRF-3 expression by Western blot. As shown in Figure 5-2A, a slower migrating form of IRF-3, which is consistent with hyperphosphorylation (121) is observed, following infection with WT-HCMV, UV-HCMV and Sendai virus, suggesting that IE86 does not block IRF-3 phosphorylation. To investigate this more directly, we assayed for IRF-3 homodimerization, which requires IRF-3 phosphorylation (121). HFF cells were transduced

with replication defective adenoviruses that express either IE86 or GFP and then mock-infected or infected with WT-HCMV or UV-HCMV. As shown in Figure 5-2B, only monomeric IRF-3 was present in mock-infected or GFP transduced cells (lanes 1 and 2). However, IRF-3 was present as a dimer in cells that were infected with WT-HCMV or UV-HCMV (lanes 3 and 4). Importantly, expression of IE86 prior to UV-HCMV infection did not block IRF-3 dimerization (compare lanes 4 and 5).

We next examined if HCMV infection and/or IE86 expression could block IRF-3 nuclear translocation. HFF cells were mock-infected or infected with WT-HCMV or UV-HCMV and fixed for immunofluorescent staining 3 hours postinfection. As shown in Figure 5-2C, IRF-3 is localized in the cytoplasm of mock-infected cells. However, upon infection with either WT-HCMV or UV-HCMV, IRF-3 translocates to the nucleus. Additionally, prior expression of IE86 had no effect on IRF-3's ability to translocate to the nucleus following UV-HCMV infection (Fig. 5-2D). Lastly, we determined whether IE86 could attenuate the expression of IRF-3 dependent genes. To test this, HFF cells were transduced with replication defective adenoviruses that express either IE86 or GFP. Transduced cells were then mock-infected, infected with WT-HCMV or UV-HCMV and assayed for expression of the IRF-3 dependent gene interferon stimulated gene 15 (ISG15) (59, 80, 121). As shown in Figure 5-2E, we observed a dramatic increase in ISG15 expression following both WT-HCMV and UV-HCMV infection. Importantly, prior expression of IE86 was unable to block the induction of ISG15 following UV-HCMV infection. However, prior expression of IRF-3ΔN efficiently blocked the expression of ISG15 (data not shown) (59). Similar results were obtained for the IRF-3 dependent genes ISG54, ISG60 and GBP1 (data not shown). Collectively, these results demonstrate that IE86 does not block

IRF-3 phosphorylation, homodimerization, nuclear translocation, or gene expression.

IE86 does not inhibit NFκB activation

We next assayed the ability of IE86 to prevent NFκB activation during HCMV infection. Normally retained in the cytoplasm bound to the inhibitor complex IκB, NFκB is activated by the phosphorylation and degradation of the IκB inhibitor, which unmasks the nuclear localization signal of NFκB and facilitates rapid nuclear translocation (3). To assay the activation of NFκB, we first assayed for the phosphorylation and degradation of the inhibitor IκBα following treatment with TNFα, a potent inducer of NFκB activation in the presence and absence of IE86. HFF cells transduced with IE86 or GFP were treated with 50 ng/ml TNFα for 30 minutes and cell lysates were prepared for Western blot analysis. As shown in Figure 5-3A, treatment with TNFα resulted in the phosphorylation and degradation of IκBα (lane 4). In addition, prior expression of IE86 did not prevent TNFα-induced IκBα phosphorylation or degradation (compare lanes 4 and 5).

We next determined if HCMV infection or IE86 expression could block the nuclear translocation of NFκB by examining the subcellular localization of the p50 subunit of NFκB. As shown in Figure 5-3B, NFκB is predominantly localized in the cytoplasm of mock-infected cells. However, upon infection with WT-HCMV or UV-HCMV, NFκB (p50) is rapidly translocated to the nucleus. Again, prior expression of IE86 was unable to block the nuclear translocation of p50 following infection with UV-HCMV (Fig. 5-3C) or TNFα treatment (data not shown). Similar results were obtained when we examined the nuclear translocation of the p65 subunit of NFκB (data not shown).

IE86 expression blocks NFκB DNA binding

Since NFκB is activated and translocated to the nucleus in the presence of IE86, we determined if HCMV infection or IE86 expression could attenuate NFκB binding to the IFN-β promoter. Therefore, electrophoretic mobility shift assays (EMSA) were performed using the NFκB binding site within the IFN-β promoter as probe (8). Cells were mock-infected or infected with WT-HCMV or UV-HCMV. Nuclear extracts were prepared, incubated with labeled probe, and assayed for NFκB binding. As shown in Figure 5-4A, NFκB binding was observed following both WT-HCMV and UV-HCMV infection. However, NFκB binding was significantly enhanced following infection with UV-HCMV when compared to WT-HCMV infection (compare lanes 2 and 3). To confirm the binding observed following infection with UV-HCMV was specific for the NFκB binding site within the IFN-β promoter we performed a competition assay with unlabeled probe. As shown in Figure 5-4B, the addition of unlabeled probe (PRDII) to extracts from UV-HCMV infected cells efficiently blocked NFκB binding in a dose dependent manner. However, NFκB binding was not inhibited when unlabeled probe that contains a two basepair mismatch within the NFκB binding site (mPRDII) was added to the reaction. These data suggest that HCMV gene expression and potentially IE86 may attenuate NFκB binding to its target sequence within the IFN-β promoter.

To test whether IE86 blocks NFκB DNA binding during HCMV infection, we utilized an IE2 mutant virus termed IE2ΔSX in which IE86 expression is both attenuated and severely delayed when compared to wild-type infection (171, 196). Cells were infected with WT-HCMV, UV-HCMV, IE2ΔSX or a pp65 mutant virus termed UL83Stop (196). Nuclear extracts were prepared 6 hours postinfection and assayed for NFκB binding. As shown in Figure 5-5A, infection with the IE2ΔSX virus results

in a dramatic increase in NFκB DNA binding when compared to WT-HCMV infection (compare lanes 2 and 3). In addition, NFκB DNA binding following infection with the IE2ΔSX virus was similar to that observed following infection with UV-HCMV (compare lanes 3 and 5), whereas NFκB binding following infection with the UL83Stop virus was only slightly above the levels observed in mock-infected cells. A Western blot is included in Figure 5-5A to show the expression of IE86, pp71 and pp65 following infection with these viruses. The Western blot for pp71 was included to confirm that cells were infected with approximately an equal number of virus particles.

We next determined if IE86 expression in the absence of virus infection could block UV-HCMV induced NFκB DNA binding. Cells were transduced with adenovirus expressing IE86, pp65, IκBαSR, or GFP. Transduced cells were infected with UV-HCMV and nuclear extracts prepared for EMSA 6 hours postinfection. As shown in Figure 5-5B, prior expression of IE86 or IκBαSR efficiently inhibited NFκB DNA binding following UV-HCMV infection (compare lanes 4 and 7 to lane 3). However, prior expression of pp65 or GFP had no effect on NFκB DNA binding activity.

Finally, we assayed for IE86's ability to attenuate NFκB dependent gene expression. As shown in Figure 6, expression of the NFκB dependent genes tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (13) and interleukin-6 (IL-6) (120) were induced following infection with UV-HCMV. However, expression of IE86 prior to infection with UV-HCMV effectively inhibited the expression of TRAIL and IL-6, whereas expression of GFP had no effect on their expression (Fig. 5-6).

IE86 attenuates TNFα signaling

We next determined if IE86 was capable of blocking NFκB dependent DNA binding and/or gene expression following exposure to stimuli other than HCMV infection. To test this, cells were transduced with adenoviruses that express IE86 or GFP and then infected with Sendai virus or treated with TNFα and assayed for NFκB DNA binding activity. Both Sendai virus and TNFα are potent inducers of NFκB (8). As shown in Figure 5-7A, infection with Sendai virus results in a robust induction of NFκB DNA binding to the IFN-β promoter (compare lanes 1 and 2). Interestingly, prior expression of IE86 inhibited NFκB DNA binding induced by Sendai virus infection (compare lanes 2 and 3). In addition, expression of IE86 inhibited the NFκB DNA binding activity observed following TNFα treatment (Fig. 5-7B, compare lanes 2 and 3).

Lastly, we examined the effect of IE86 expression on TNFα-induced gene expression. Cells were transduced with adenovirus expressing IE86, GFP or IκBαSR, and then treated with TNFα. RNA was isolated 6 h post treatment and assayed by Northern blot for expression of the TNFα-induced, NFκB dependent genes interleukin-8 (IL-8) (116), interleukin-6 (IL-6) (120) and RANTES (137). As shown in Figure 5-7C, treatment of cells with TNFα induced the expression of IL-8 and RANTES (compare lane 1 and 2). Expression of IL-8, IL-6 and RANTES following TNFα treatment is also dependent on NFκB signaling since their expression is effectively blocked in the presence of the IκBαSR (compare lanes 2 and 5). Interestingly, expression of IE86 prior to TNFα treatment effectively blocked the expression of IL-8, IL-6 and RANTES (Fig. 5-7C, compare lanes 2 and 3). Taken together, our results demonstrate that IE86 effectively inhibits both virus-induced and TNFα-induced NFκB DNA binding and gene expression.

DISCUSSION

Previous reports have demonstrated that HCMV can attenuate the expression of IFN-β and proinflammatory chemokines and this attenuation is dependent on a newly synthesized viral protein expressed early during infection (39, 181). We recently demonstrated that the HCMV immediate-early 2 gene product IE86 can efficiently block the induction of IFN-β and a number of chemokines following HCMV infection (195, 196). However, the mechanism by which IE86 inhibits IFN-β and chemokine expression has remained elusive. Therefore, we set out to investigate how IE86 attenuates IFN-β and chemokine production during HCMV infection.

The IRF-3 and NFκB pathways are required for IFN-β induction following certain viral infections (2). In addition, a number of viruses express proteins that specifically target the IRF-3 and/or NFκB pathway in order to inhibit the expression of IFN-β (18, 31, 62, 79, 129, 168, 187, 202). Therefore, we examined if either pathway is required for the induction of IFN-β observed following infection with UV-HCMV. Using dominant negative repressors that block activation of IRF-3 or NFκB we were able to demonstrate that inhibition of either pathway will block IFN-β induction following infection with UV-inactivated HCMV (Fig. 5-1B-C). Therefore, IE86 may target either the IRF-3 or the NFκB pathway to block IFN-β expression.

Others have demonstrated that IRF-3 is activated at early times after HCMV infection (26, 38, 58, 59, 81, 144, 159, 208). Our results confirm these previous observations and demonstrate that IRF-3 is phosphorylated, homodimerizes, translocates to the nucleus, and activates target gene expression following both wild-type and UV-HCMV infection (Fig. 5-2A-C and E), suggesting that IE86 does not inhibit IRF-3 activation. Using a replication defective adenovirus that expresses IE86

we were able to directly examine if IE86 is capable of blocking IRF-3 activation. As shown in Figure 5-2, expression of IE86 prior to infection with UV-HCMV did not inhibit IRF-3 homodimerization, nuclear translocation, or target gene expression. These results are in agreement with a recent report by DeFillipis *et al.* which used siRNA directed against IRF-3 to demonstrate that a subset of genes induced during WT-HCMV infection, including IFN-β and ISG15, require IRF-3 activation (59). Together, these results demonstrate that IRF-3 is activated and that IE86 does not target the IRF-3 pathway during HCMV infection.

Analysis of the early events in NFκB activation revealed that IE86 does not prevent the phosphorylation or degradation of the α subunit of the IκB inhibitor (Fig. 5-3A), nor does IE86 prevent the nuclear translocation of the NFκB subunits p50 or p65 (Fig. 5-3B-C and data not shown). However, using electrophoretic mobility shift assays, we demonstrate that IE86 can attenuate NFκB DNA binding activity. First, infection with wild-type HCMV results in an increase in NFκB DNA binding to the IFN-β promoter when compared to mock-infected cells (Fig. 5-4A, compare lanes 1-2), confirming previous studies that demonstrate HCMV infection results in both NFκB activation and DNA binding activity (53, 60, 63, 212). However, NFκB DNA binding was significantly enhanced following infection with UV-HCMV (Fig. 5-4A), suggesting that viral gene expression and more specifically IE86 may be involved in attenuating NFκB DNA binding activity. We used two independent methods to determine that IE86 is capable of attenuating NFκB DNA binding. First, we utilized the IE2ΔSX virus, which has amino acids 136-290 deleted from exon 5 of IE2 (171). The IE2ΔSX virus is viable but expresses IE86 at dramatically reduced levels and with delayed kinetics when compared to IE86 expression during wild-type or revertant virus infection (171). We

have previously demonstrated that infection with the IE2ΔSX virus results in an increase in IFN-β and RANTES transcript accumulation similar to that observed following UV-HCMV infection (196). Therefore, if IE86 is involved in blocking NFκB DNA binding we would predict that infection with the IE2ΔSX virus would result in an increase in NFκB DNA binding when compared to wild-type infection. As demonstrated in Fig. 5-5A, NFκB DNA binding was dramatically enhanced following infection with the IE2ΔSX virus when compared to wild-type infection (compare lane 2 and 5). In addition, NFκB DNA binding following infection with the IE2ΔSX virus was comparable to that observed following infection with UV-HCMV (compare lane 3 and 5). Infection with a control mutant virus, UL83Stop, had no effect on NFκB DNA binding activity and looks identical to wild-type HCMV infection (compare lanes 2 and 4). We also utilized an adenovirus expression system to demonstrate that expression of IE86 prior to infection with UV-HCMV inhibits NFκB DNA binding to the IFN-β promoter, whereas expression of the HCMV tegument protein pp65 or GFP had no effect on NFκB DNA binding (Fig. 5-5B). We supported these results by demonstrating the IE86 expression efficiently blocked the expression NFκB dependent genes (Fig. 5-6). Furthermore, we report that IE86 can attenuate TNFα signaling by blocking NFκB DNA binding (Fig. 5-7B).

Our results show that IE86 can function as an NFκB antagonist and suppress both virus and TNFα-induced NFκB DNA binding activity and subsequent NFκB dependent gene expression. This suppression of cytokine and chemokine expression during HCMV infection likely provides for a cellular environment that is conducive to viral replication and persistence. This report identifies a novel strategy employed by HCMV to attenuate the host antiviral cytokine and chemokine response early in

infection by suppressing NFκB DNA binding. This augments a growing list of mechanisms by which HCMV inhibits cytokine and/or chemokine function. These mechanisms include the expression of chemokine mimics (155), the expression of chemokine binding proteins (200) and the expression of G-protein coupled chemokine receptors (4, 164, 192). Additionally, a recent report suggests that HCMV may express additional proteins involved in blocking inflammatory chemokine expression. Jarvis *et al.* demonstrated that HCMV expresses a late protein during infection that is capable of attenuating both TNFα and IL-1β induced chemokine expression (103). Interestingly, the mechanism by which this late viral protein inhibits chemokine expression involves blocking the activation of NFκB by preventing IκBα phosphorylation. Therefore, it is likely that HCMV may express at least two different proteins (IE86 and a yet unidentified late protein) that target NFκB activation at different steps and function at different stages during HCMV replication to block IFN-β and chemokine expression.

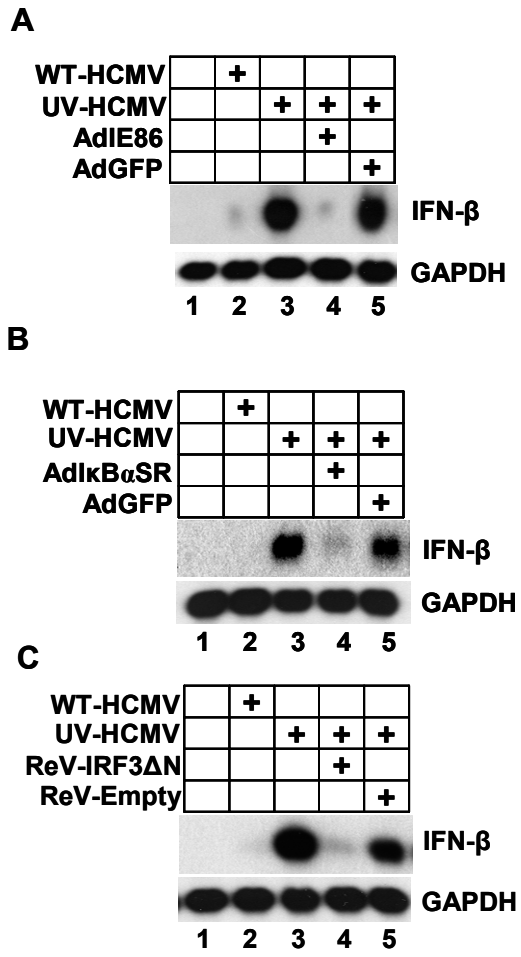

Figure 5-1. IRF-3 and NFκB are both required for HCMV-induced IFN-β expression. HFF cells were transduced with adenoviruses expressing GFP, IE86 **(A)**, and IκBαSR **(B)** or retroviruses expressing IRF-3ΔN and empty vector **(C)**. Transduced cells were then mock-infected or infected with WT-HCMV or UV-inactivated HCMV. RNA was isolated 8 hours postinfection and analyzed by Northern blot for IFN-β and GAPDH transcripts.

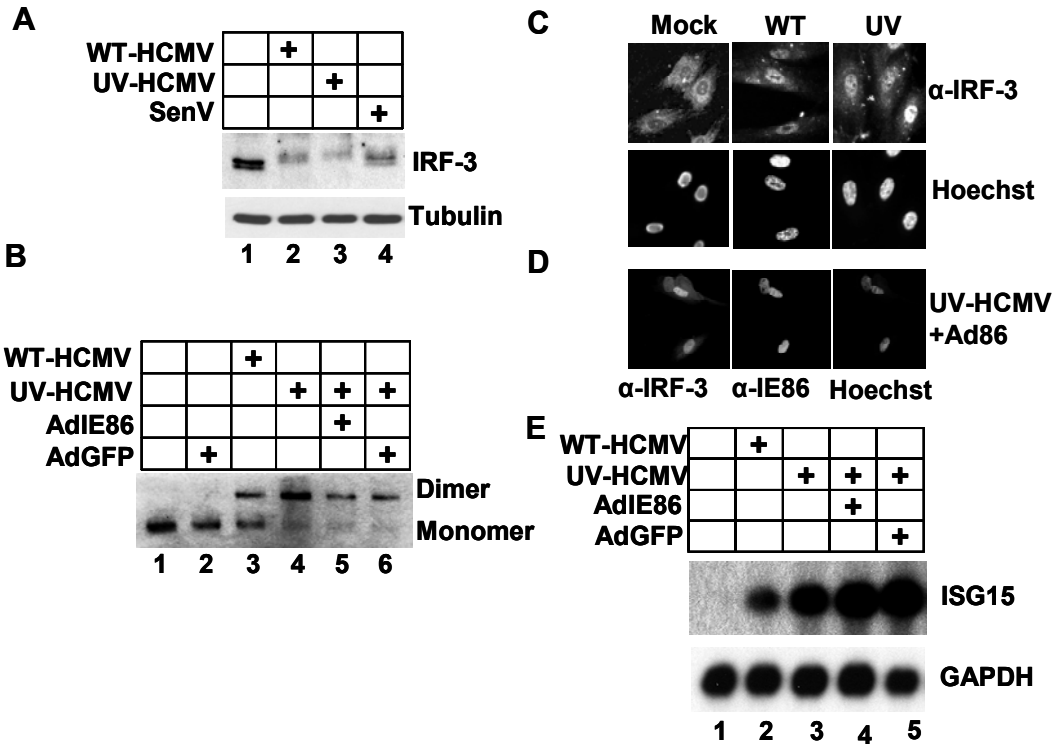

Figure 5-2. IE86 does not prevent IRF-3 activation or target gene expression. **(A)** HFF cells were mock-infected or infected with WT-HCMV, UV-HCMV or Sendai virus. Cell lysates were prepared 6 hours postinfection and assayed for IRF-3 and tubulin expression by Western blot analysis. **(B)** HFF cells were transduced with adenoviruses expressing IE86 or GFP. 24 hours post-transduction, cells were mock-infected, or infected with wild-type or UV-HCMV at a multiplicity of 5pfu/cell. Cell extracts were prepared 6 hours postinfection and assayed for IRF-3 dimerization by native gel electrophoresis and Western blot analysis using an IRF-3 antibody. HFF cells **(C)** or HFF cells transduced with adenovirus expressing IE86 **(D)** were seeded onto coverslips and either mock-infected, infected with WT-HCMV, or infected with UV-HCMV. Cells were fixed 3 hours postinfection and assayed for IRF-3 and IE86 localization by immunofluorescence assay. Nuclei were stained with Hoechst. **(E)** HFF cells were transduced with adenoviruses expressing IE86 or GFP for 24 hours. Cells were then infected with WT-HCMV or UV-HCMV at a multiplicity of 5 pfu/cell. RNA was isolated 8 hours postinfection and assayed for ISG15 and GAPDH expression by Northern blot.

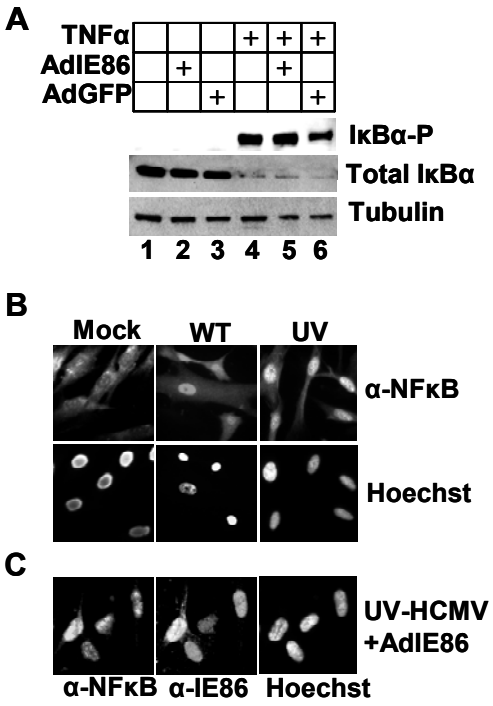

Figure 5-3. IE86 does not prevent NFκB activation. (A) HFF cells were mock transduced or transduced with adenoviruses expressing IE86 or GFP and treated with 50 ng/ml TNFα. Cell lysates were prepared 30 min post treatment and assayed for phosphorylated-IκBα, total IκBα and tubulin by Western blot. HFF cells **(B)** or HFF cells transduced with adenovirus expressing IE86 **(C)** were seeded onto coverslips and either mock-infected, infected with WT-HCMV or UV-HCMV. Cells were fixed 3 hours postinfection and assayed for NFκB (p50) and IE86 localization by immunofluorescence assay. Nuclei were stained with Hoechst.

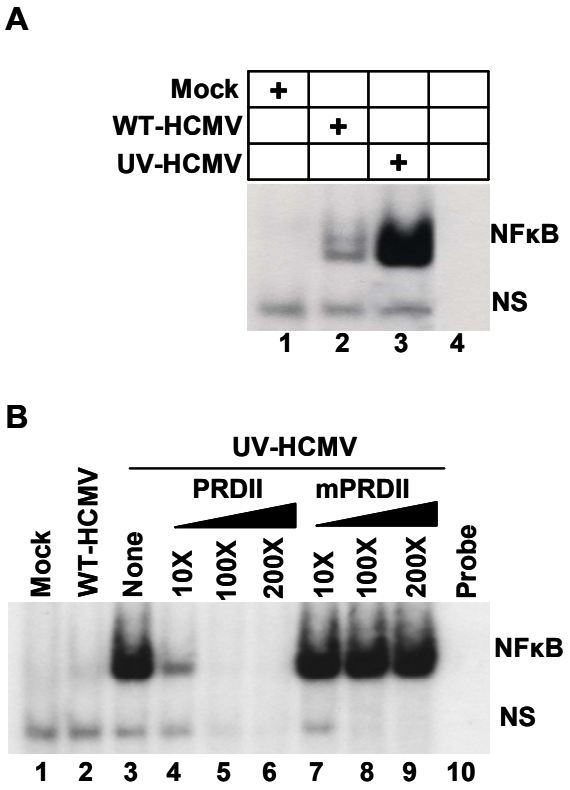

Figure 5-4. NFκB DNA binding is attenuated by HCMV gene expression. **(A)** HFF cells were mock-infected or infected with WT-HCMV or UV-HCMV at a multiplicity of 5 pfu/cell. Nuclear extracts were prepared 6 hours postinfection and assayed by EMSA for NFκB binding to the PRDII region of the IFN-β promoter. NS indicates a non-specific shift. **(B)** HFF cells were infected with WT-HCMV or UV-HCMV. 6 hours postinfection nuclear lysates were isolated and assayed for NFκB binding. A competition analysis was performed on UV-HCMV extracts by adding unlabeled specific competitor oligonucleotide probe (PRDII) or a mutated probe sequence (mPRDII) in increasing concentrations to the binding mixture to confirm specificity of the NFκB DNA binding.

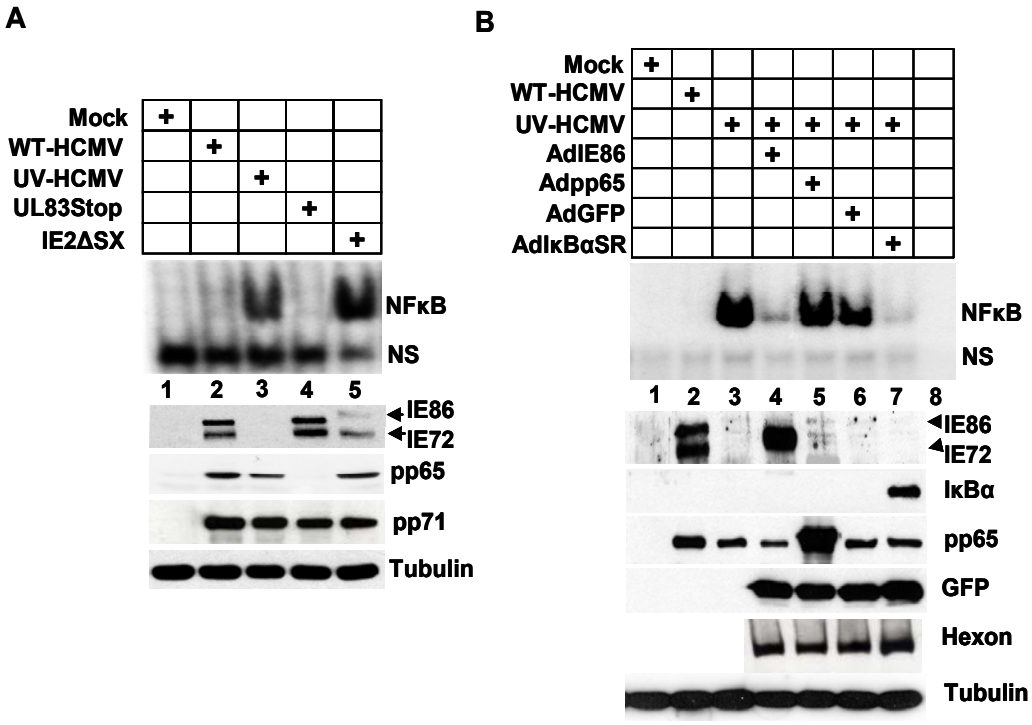

Figure 5-5. IE86 attenuates HCMV-induced NFκB DNA binding activity. (A) HFF cells were infected with HCMV, UV-HCMV, UL83Stop virus or IE2ΔSX virus at a multiplicity of 5pfu/cell. Nuclear extracts were prepared 6 hours postinfection and assayed for NFκB binding by EMSA. A Western blot is also included to confirm expression of the various viral proteins. **(B)** HFF cells were transduced with adenoviruses expressing IE86, pp65, GFP or IκBαSR, and then infected at a multiplicity of 5 pfu/cell with UV-HCMV. Nuclear lysates were prepared 6 hours postinfection and assayed for NFκB binding by EMSA. A Western blot is included to confirm expression of IE86, pp65, IκBα, GFP, adenovirus hexon and tubulin.

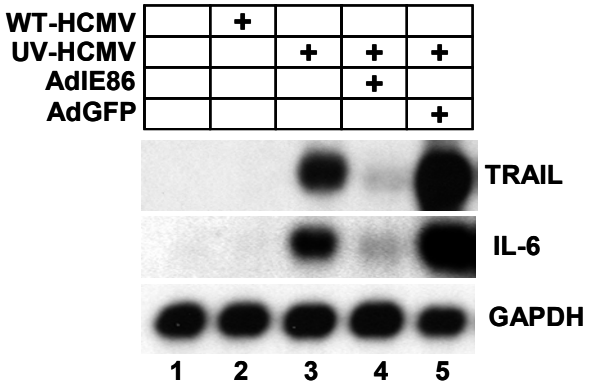

Figure 5-6. IE86 attenuates NFκB target gene expression. Cells were transduced with adenovirus expressing either IE86 or GFP and then infected at a multiplicity of 5pfu/cell with UV-HCMV. RNA was isolated 8 hours postinfection and assayed for TRAIL, IL-6 and GAPDH expression by Northern blot.

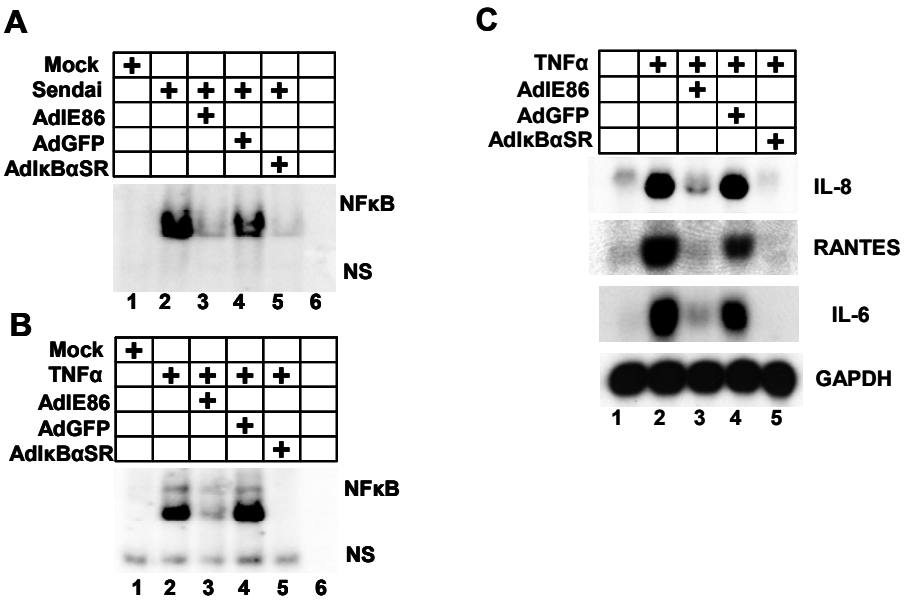

Figure 5-7. IE86 attenuates TNFα signaling. HFF cells were transduced with adenovirus expressing IE86, GFP or IκBαSR and then infected with Sendai virus at 100 HAU/ml **(A)** or treated with 50 ng/ml TNFα **(B)**. Nuclear extracts were prepared 6 hours postinfection or treatment and assayed for NFκB binding by EMSA. NS indicates a non-specific shift. **(C)** Cells were transduced with adenovirus expressing IE86, GFP or IκBαSR for 24 h. Transduced cells were then treated with TNFα. RNA was isolated 6 h post treatment and assayed for IL-8 and RANTES expression by Northern blot.

CHAPTER 6
DISCUSSION AND FUTURE DIRECTIONS

Identification of an HCMV IFN-β antagonist

The goal of this dissertation was to identify the viral gene product capable of preventing HCMV-induced IFN-β expression, and to determine the mechanism by which IFN-β expression is blocked. The research outlined here addresses a number of unanswered questions about HCMV's interactions with the host, and identifies a previously unrecognized mechanism employed by HCMV to evade the host immune response through attenuation of NFκB-dependent arm of the innate immune response. A number of published reports have hypothesized that HCMV codes for a gene that can attenuate IFN induction, though a candidate viral gene had not been proposed (21, 26, 39, 163, 217). These hypotheses were based upon observations that inactivation of HCMV by multiple mechanisms or infection in the presence of inflammatory cytokines results in a robust IFN-β response following virus infection. Under these conditions, the host cell can clearly detect HCMV infection, though without virus inactivation the IFN-β response to virus infection is greatly attenuated.

Chapter 3 outlines the genetic and biochemical approaches utilized to identify the immediate-early 2 gene product IE86 as an IFN-β antagonist. Since IE86 is one of the first viral proteins to be expressed during HCMV infection, it can rapidly block cytokine and chemokine expression thereby dampening the antiviral response and limiting the recruitment of effecter cells. The attenuation of the innate immune response by IE86 likely enhances HCMV replication and contributes to

HCMV persistence within the host. The biological relevance of blocking IFN-β is exemplified by the severe inhibition in HCMV replication observed following treatment with IFN-β. Experiments described in Fig. 3-1 and elsewhere (143) demonstrate that treatment with IFN-β effectively inhibited HCMV replication in cell culture greater than 99%. Previous data has shown that IE1/IE72 can dampen IFN signaling by preventing STAT binding to target promoters (154). Clearly, the presence of IE1/IE72 is not sufficient to protect HCMV from the antiviral effects of IFN treatment, exemplifying the importance of attenuation in IFN expression.

In addition to data confirming exogenous IFN treatment inhibits HCMV infection, previous data shows that infection with HCMV in the presence of the inflammatory cytokines TNF-like lymphotoxin alpha (LTα) or interleukin 1 beta (IL-1β) inhibited HCMV replication in an IFN-β dependent manner (21, 163). Benedict *et al* showed that HCMV and LTα molecules cooperate to induce IFN-β (21). The group hypothesized that HCMV has developed a specific strategy to suppress induction of IFN-β and tumor necrosis factor signaling prevents this block. Similar to what we observe following wild-type HCMV infection, they showed that infection with wild-type virus alone does not result in IFN-β transcription, though treatment of the cells with LTα during HCMV infection resulted in IFN-β transcription by 4 hours postinfection (21). Interestingly, IE2 transcript levels were decreased with the LTα treatment at early times postinfection (21) supporting our hypothesis that an early delay in IE86 expression results in an increased cytokine response to virus infection, as we observed following infection with the ΔUL83 and IE2ΔSX and ΔUL82 mutant viruses (Figs. 4-2A-B, 4-9). This suggests that LTα treatment decreases immediate early gene expression coincident with potent activation of NFκB, and combined with virus-induced activation of IRF-3,

resulted in an antiviral response. Our data presented in Fig. 5-7B-C suggests however that HCMV IE86 will also function to prevent this phenomenon, as TNFα signaling through NFκB is blocked in the presence of IE86. This is perhaps more relevant, as TNFα-related cytokines, such as IE86-regulated, TNFα-related TRAIL (Fig. 5-6), not only induce NFκB activation (178), but also require NFκB for their transcriptional induction as well (13).

IFN-β therapy for the treatment of HCMV infection

Without viral intervention, mediated by immediate expression of IE86, a maximal cellular IFN-β response to HCMV infection could effectively prevent virus replication and persistence. IFN-α/β is currently used as antiviral therapy for hepatis C virus, Kaposi's sarcoma-associated herpesvirus and viral myocarditis, as well as a number of non-viral diseases including multiple sclerosis and hairy cell leukemia (28). Our data which identifies and confirms that IE86 attenuates the IFN-β response may justify the use of IFN-β for anti-HCMV therapy or prophylactic treatment for patients at risk for HCMV infection. Logic dictates if HCMV blocks this early IFN response in order to enhance replication and persistence, then addition of exogenous IFN-β or prevention of this antagonistic function of IE86, would facilitate an innate response more capable of overcoming the burden of HCMV infection.

A previous study examined the efficacy of IFN administration during murine cytomegalovirus infection (MCMV). They found that daily low dose (10 international units(IU)/g) oral administration of IFN-α/β significantly reduced virus replication in the liver and spleen of BALB/c mice after challenge with MCMV *in vivo* (28). Low dose IFN therapy appears to be more effective in treating virus infection and is not associated with the flu-

like side effects that are common with conventional IFN treatment using high cytokine doses (maximum allowable dose of 5 million international units IFN injected intramuscularly, intravenously, or subcutaneously). An added benefit of oral administration is that the gastrointestinal tract has many IFNAR dense tissues which effectively respond to ingested IFNs, and as the cytokine does not enter the circulation, neutralizing antibodies are not generated, as has been described for greater than 40% of the patients following high dose intramuscular or intravenous injections (28).

Experiments in cell culture have shown that treatment with IFN-α/β in conjunction with the anti-herpes chemotherapeutic agents ganciclovir and acyclovir to treat HCMV infections synergistically inhibits virus replication (165, 183). This combination therapy may facilitate lower effective doses, with reduced toxicity, during treatment of HCMV disease or for prophylactic use prior to organ transplantation.

Effect of pp65 deletion on pp71 expression and HCMV replication

The goal of Chapter 4 was to identify the relative roles of pp65 and IE86 during infection and to determine which viral protein was necessary to attenuate cytokine and chemokine induction. Western blot analysiss presented here supports the view that UL82 (pp71) gene is affected by deletion of the UL83 open reading frame (ORF). Encoded by the UL82 gene, the tegument protein pp71 has been shown to be essential for HCMV infection at low multiplicities. In fact, a deletion mutant for UL82 exhibits a significant delay in IE86 gene expression during a low multiplicity infection and must be propagated on complementing cells (36). UL83 is transcribed on a 4 kb bicistronic message with UL82. An additional 1.9 kb transcript, co-terminal with the larger transcript, is separately transcribed from a TATA box located between the two ORFs

and contains only the UL82 open reading frame (Fig. 4-6A). The literature at this point is not clear which transcript is important for pp71 expression (57, 90). As there is only 180 base pair separation between the genes, deletion of UL83 may delete essential promoter regulatory elements for pp71 transcribed on the 1.9 kb message. Alternatively, substitution of the UL83 gene with a resistance cassette may prevent the transcription or subsequent pp71 translation from the 4 kb transcript. This is supported by a study from Dal Monte *et al*. using stably expressed UL83 antisense RNA (57). They found that pp71 transcription and protein synthesis were severely reduced following infection of wild-type virus into pp65-antisense expressing cells versus a control cell line, suggesting that targeting the UL83 gene can affect pp71 expression and impair virus replication (57). It is also unclear whether this phenotype of the ΔUL83 virus is the result of a decrease in newly synthesized pp71, or if the pp71 is not packaged to wild-type levels in the tegument of ΔUL83 virions.

Regardless of the complicating effect of UL83 deletion on pp71 expression, construction of a stop codon mutant virus, UL83Stop, effectively showed that pp65 was not required to attenuate the host cytokine response (Fig. 4-8), as well as pp65 expression is not required for proper pp71 expression. This virus can now be used to identify true pp65-regulated genes, or to establish the role of pp65 during HCMV infection. These experiments also highlight the importance of proper virus characterization.

Mechanism of immune detection of HCMV by the host cell

HCMV infection results in the activation of both the NFκB and IRF-3 pathways. Despite antagonism of the NFκB arm of this innate response, the mechanism by which these pathways are activated in response to

HCMV is currently unclear. As discussed in the introduction, IRF-3 and NFκB can be activated by the dsRNA sensor, RIG-I. Sendai virus has been shown to induce IFN-β in a RIG-I-dependent fashion (193), though current data suggests that the host cell does not require RIG-I to respond to HCMV infection (150). Regardless of the activation of IFN-β through the RIG-I pathway, IE86 expression attenuates Sendai virus-induced expression, despite the fact that HCMV does not signal through RIG-I (Fig. 3-6).

Further data suggests that the activation of NFκB, and not IRF-3, requires TLR2 signaling during HCMV infection. TLR2, unlike the other virus-specific toll-like receptors is found on the cell surface and not located within an endosome. TLR2 recognizes peptidoglycan in response to bacterial infection (110), but has recently been shown to be necessary for HCMV-induced inflammatory gene expression (53). The HCMV glycoproteins gB and gH appear to interact directly with TLR2, and this interaction is necessary for NFκB activation (25). Cells which do not express TLR2, or express a dominant negative form of TLR2, do not respond to HCMV infection by activating NFκB (25). In contrast to these results, treatment with purified gB protein will also result in the expression of a number of IRF-3 target genes (181). This complicates the situation as TLR2 signaling not only does not result in IRF-3 activation (110), but the TLRs necessary for IRF-3 activation are expressed on the cells that do not respond to HCMV infection (25). This may suggest a unique association of TLR2 with other surface receptors or signaling molecules which expand the signaling capability of TLR2 to include both the activation of NFκB and IRF-3.

The pathways used by the host cell for RNA virus detection (RIG-I/Mda5, TLRs) appear to be non-redundant and evolved to specifically

detect virus-associated motifs. It is not surprising that new research is revealing a cellular mechanism to detect viral dsDNA. These unidentified sensors are capable of detecting non-self DNA in the cytoplasm and respond with the expression of IFN-β as a consequence of both NFκB and IRF-3 (100, 190). Interestingly, transfection of HCMV genomic dsDNA results in the activation of these pathways, providing strong evidence of a unique mechanism of herpesvirus detection (100). This may however not account for immune activation during a normal HCMV infection, as genomic DNA is delivered directly to the nucleus and would be potentially protected by the capsid from cytosolic DNA sensors.

Identification of the signaling molecules necessary for innate response to HCMV infection would likely provide further insight into other mechanisms by which HCMV evades the host response. Though HCMV does not prevent the activation of these pathways, there may be other viral strategies to dampen the response or redirect the signals to ensure the cellular environment is better suited for HCMV replication.

Transcription factor activation during HCMV infection

The goal of Chapter 5 was to determine whether IE86 expression interferes with either of the main transcription factor signaling pathways necessary for IFN-β induction, IRF-3 or NFκB. The current literature contains conflicting results in regard to HCMV-induced transcription factor activation. Data can be found for both IRF-3 and NFκB to support either a positive or negative impact of HCMV on the transcription factor activation and dependent gene expression.

IRF-3

The activation status of IRF-3 has been a matter of much debate recently, though many independent studies have demonstrated that IRF-3 is activated at early times after HCMV infection (26, 38, 58, 59, 81, 144, 159, 208), reports have also identified the tegument protein pp65 as a potential IRF-3 antagonist. IRF-3 was first shown by EMSA to be contained within an HCMV-induced transcription factor complex, termed the cytomegalovirus-induced factor (CIF). Identification of nuclear IRF-3 during HCMV infection coincided with the identification of a number of IRF-3 target genes, including ISG15 and ISG54, which are induced by both wild-type HCMV infection and by treatment of cells with purified gB, in an interferon independent fashion (39, 59, 144, 181, 196). These results suggest that regardless of any effect of HCMV on IRF-3, the pathways upstream of IRF-3 are activated by HCMV virion binding to the host cell membrane and the end result or IRF-3 signaling, IRF-3 target gene expression, is not prevented by virus infection. More detailed biochemical analyses, in addition to the results presented in Fig. 5-2, have recently confirmed the phosphorylation, dimerization and nuclear translocation of IRF-3 during HCMV infection, regardless of viral gene expression (38, 58, 59). In direct contrast to these results, the report by Abate et al. proposed that the tegument protein pp65 functions to attenuate the innate response though interference with IRF-3 nuclear translocation. These conclusions are based upon experiments using the pp65 deletion mutant virus (ΔUL83), which has an additional defect in IE gene expression early in infection. Though we observe a similar innate response to this pp65 deletion virus, infection with a pp65 stop codon mutant (UL83Stop) virus does not result in an increase in IFN-β or RANTES expression. Both viruses lack pp65, but the UL83Stop virus

expresses IE86 with wild-type kinetics. In addition, prior expression of pp65 did not prevent virus-induced IFN-β or chemokine expression. Our results argue that pp65 expression has no inhibitory effect on the innate response, and thus the true function of pp65 during virus infection remains elusive. If in fact pp65 attenuates IRF-3 signaling, IRF-3 and the subsequent target gene expression would not be induced by HCMV infection, regardless of virus-inactivation. UV-irradiation or infection in the presence of cycloheximide effectively prevents viral gene expression, though does not prevent the delivery of the tegument and pp65 into the host cell. IFN-β would therefore not be induced during infection with HCMV regardless of viral gene expression, which is not the phenotype that is observed following infection with UV-HCMV or infection with wild-type virus in the presence of cycloximide (Fig. 3-2). Interestingly, rhesus CMV (RhCMV) infection potently blocks the innate response including IRF-3 dependent genes, and this attenuation does appear to be due to a tegument component (58).

Since HCMV induces IRF-3, it's reasonable to hypothesize that IRF-3 activation does not negatively impact HCMV replication. Indeed, results from our lab suggest that expression of the dominant negative IRF-3ΔN does not inhibit HCMV infection (data not shown). It is however possible that IRF-3 expression enhances HCMV replication, has a stimulatory effect on viral gene expression, or expression of the IRF-3 target genes may provide for a cellular environment better suited for HCMV replication.

NFκB

NFκB continues to be a highly debated transcription factor in the HCMV field. From the initial steps in activation to the regulation of NFκB

dependent genes during virus infection, data can be found to support an inhibitory or stimulatory role of NFκB during the HCMV replication cycle. This dissertation supports the view that NFκB is not required for a productive HCMV infection, as the necessity for NFκB during virus infection would be complicated by IE86-mediated functional suppression. The current data however does not rule out promoter-specific inhibition of NFκB, where IE86 may prevent NFκB association with the IFN-β promoter, but not with other, non-antiviral NFκB target gene promoters.

The major immediate early promoter (MIEP) contains four distinct binding sites for NFκB, and gel shift assays have confirmed that the factors can indeed site-specifically bind to the promoter (118, 126, 162, 170). As this promoter/enhancer is considered one of the strongest viral or cellular regulatory elements, it has long been dogma that high level immediate early gene expression is dependent upon these NFκB binding to its cognate elements within the MIEP to activate transcription (44, 60, 133). Infection with wild-type HCMV has been shown to result in NFκB activation and DNA binding. These results support the view that HCMV IE gene expression is facilitated or enhanced by activation of host transcription factors. Evidence that NFκB is necessary of HCMV gene expression comes from experiments using chemical inhibitors of NFκB including MG132, aspirin, and indomethacin (60, 61). Infection in the presence of these inhibitors effectively inhibited IE gene expression and blocked virus replication. Though these inhibitors will inhibit NFκB, additional effects of these drugs have also been reported, as they are not specific inhibitors of the NFκB pathway, and complicate any conclusions drawn from these experiments. It is important to note that in the presence of activated NFκB, target gene expression would not be regulated, as is observed for a wild-type HCMV infection (39).

Several lines of evidence support the view that NFκB is not required for IE gene transcription. Experiments performed in our lab and others (20, 63), demonstrate that prior expression of a dominant negative inhibitor of the NFκB pathway (IκBαSR) does not prevent accumulation of IE72/IE86 within the first four hours postinfection. In Figure 6-1, HFF were transduced with adenoviruses expressing GFP or the IκBαSR. The transduced cells were then infected with wild-type HCMV at a high multiplicity. Protein was isolated 6 and 24 hpi. As shown, expression of IE72 and IE86 is not affected by inhibition of NFκB. Further support comes from mutational analysis of the NFκB binding sites within the MIEP of both the human and murine cytomegalovirus (MCMV) genomes revealed that deletion of NFκB cognate elements did not negatively impact virus replication *in vitro* or in the mouse model, nor did infection of fibroblasts lacking the p65/p50 subunits of NFκB (20, 84). In addition, treatment of cells with TNFα, LTα, IL-1β or overexpression of the NFκB activating kinase IKK prevented HCMV replication by inducing IFN-β in an NFκB dependent manner (21, 63, 163). Our data support the hypothesis that NFκB is not required for IE transcription, as our experiments specifically target the NFκB pathway.

This report identifies the first HCMV-encoded NFκB antagonist. A previous report by Browne and Shenk observed a similar phenotype following infection with the ΔUL83 mutant virus that was reported by Abate et al, in that cytokines and chemokines were induced following infection with the mutant virus. Interestingly, they found that the ΔUL83 virus induced NFκB DNA binding relative to wild-type virus. However, they did not demonstrate that ectopically expressed pp65 could block NFκB DNA binding, or induction of IFN-β. These results support our data that the delay in IE86 expression observed with the ΔUL83 virus results in an

increase in NFκB and expression of its target genes. We showed here that infection with the UL83Stop virus blocked NFκB DNA binding as efficiently as wild-type virus, and importantly ectopic expression of pp65 did not prevent HCMV-induced NFκB DNA binding or target gene expression (Fig. 5-5 and Table 4-1).

NFκB appears to play a non-essential role during a lytic infection with HCMV, though this does not rule out a role for the transcription factor during HCMV latency or persistant infections. A recent study found a differential requirement for NFκB during infection with gammaherpesvirus-68. Similar to the results obtained for HCMV infection, they found that lytic replication, both in cell culture and in the mouse model, is not prevented by expresson of the NFκB super repressor, IκBαSR. They did however observe a 90% reduction in the number of latently infected lymphocytes. This suggests that NFκB may be important for the establishment of gammaherpesvirus latency (115). Similarly, NFκB p50 promoted latency of the human immunodeficiency virus long terminal repeat (LTR) through changes in chromatin acetylation which prevented RNA polymerase II recruitment and transcriptional induction. Local histone deacetylation was mediated by interaction of p50 and histone deacetylase-1 (HDAC1) with the LTR (206). This appears to be a basal regulatory mechanism for NFκB target genes, where HDACs are recruited to the promoters by p50 to repress transcriptional expression in the absence of NFκB activation stimuli. Importantly, p50 homodimers can be detected in the nucleus, albeit at very low levels, in the absence of stimuli, but lack the transactivation domain necessary to activate transcription (214).

Potential mechanisms of NFκB antagonism

NFκB is sensitive to viral attack at many stages of activation. Data presented in Fig. 5-3 suggests that many of the common viral targets of the NFκB pathway (IKK activity, IκB phosphorylation and degradation, inhibition of nuclear translocation, and downregulation or selective degradation of the transcription factors) are not affected by IE86 expression. Thus, all steps leading up to transcription factor accumulation in the nucleus are detectable in the presence of ectopically expressed IE86. These results suggest that IE86 may function to directly inhibit NFκB subunits from recognizing or binding to the cognate element within target promoters, or modifies the factors to decrease their DNA binding capacity. This may be a similar function to the adenovirus E1A protein, which can efficiently block select NFκB target genes, including inducible nitric oxide synthase, by preventing NFκB activation at the level of IκBα phosphorylation or binding to target promoters (43).

The mechanism by which IE86 prevents NFκB DNA binding is presently unclear. As IE86 interacts with many cellular transcription and accessory factors, a logical first step for identification of this mechanism antagonism would be to determine whether IE86 directly interacts with NFκB. To date there are no reports of any direct interactions between IE86 and members of the NFκB family members. We have been unable to detect an interaction between IE86 and the NFκB subunits p50 or p65 during HCMV infection by immunoprecipitation assay (data not shown). This however does not rule out a low affinity, or low level, interaction that is not detectable in that assay. Overexpression of the viral and cellular proteins may be required to identify an interaction. There is significant overlap in the interacting partners of the NFκB family members with proteins that can interact with IE86, including TBP, CBP, CREB, TFIIB,

p53 and Jun (45). Therefore, IE86 binding to the NFκB subunits or a necessary interacting partner may attenuate sequence-specific DNA binding. This specific method of NFκB antagonism has been reported for the EBV IE protein BZLF1. BZLF1 directly interacts with NFκB p65 and has been shown to induce the nuclear accumulation of p65 (85). Similar to IE86, expression of BZLF1 prevents NFκB DNA binding and target gene expression without preventing the upstream steps in NFκB activation (138). Thus, if a direct interaction of IE86 with an NFκB family is responsible for this attenuation, then overexpression of the NFκB protein would titrate out the IE86 and reduce the inhibitory effect of IE86. In addition, overexpression of the NFκB factors could potentially inhibit the classical functions of IE86 during HCMV infection, as has been described for BZLF1 (138).

Alternatively, IE86 expression may result in a modification of NFκB members, in which case a physical interaction may not be required. As discussed in the introduction, inhibitor degradation and nuclear translocation of NFκB is sufficient for activation, as is observed in the presence of IE86, however maximal activity requires post-translational modifications of the RelA/p65 subunit (49). p65 is specifically phosphorylated by a number of kinases in both the amino-terminal DNA binding domain and the carboxy-terminal transactivation domain. Protein kinase A phosphorylates serine 276 in the DNA binding domain (RHD) which promotes an interaction with CREB Binding Protein (CBP)/p300, modulates NFκB DNA binding and oligomerization, and enhances NFκB-dependent transcription (215). Further enhancement of transcriptional activity is achieved by phosphorylation in the transactivation domain on serine 536 by IKKs. These phosphorylation events are additionally required for acetylation of lysine 310 by CBP/p300 (50). An IE86-

mediated block to p65 phosphorylation or acetylation may account for the attenuated NFκB DNA binding. Support for this hypothesis comes from a previous report examining the effect of IE86 expression on the function of the tumor suppressor p53. This study demonstrated that the interaction of IE86 with CBP and p300 inhibited their acetyltransferase activity on p53 and the histones of p53-dependent promoters. This resulted in a dose-dependent decrease in the gene expression of the p53 responsive gene p21 *in vivo*, as measured by chromatin immunoprecipitations (95). Like p65, acetylation of p53 by CBP/p300 is required for maximal transcriptional activation of target gene expression. Thus, an interaction of IE86 with CBP/p300 may prevent a post-translational modification of p65 in order to prevent NFκB DNA binding and target gene expression. Inhibition of CBP/p300 local histone or transcription factor acetylation may be a mechanism utilized by IE86 to prevent target gene expression from a number of signaling pathways. Further studies will be required to determine whether additional pathways that require acetylation are suppressed by IE86.

Identification of essential domains of IE86 required for blocking NFκB and cytokine expression

It is difficult to identify specific functions of IE86 that are required during HCMV infection in the absence of an IE2 deletion mutant virus. Extensive mutation analyses of IE86 have begun to identify specific domains within IE86 that are required for the two primary functions of IE86 transactivation and autorepression, as well as some post-translational modifications including sumoylation (12). The IE2 mutant virus used in these studies, IE2ΔSX, contains two specific defects in IE86. IE86 is expressed with delayed kinetics, and the IE86 that is expressed contains

an internal deletion of amino acids 136-290. This large deletion contains sequences that have been identified as interaction regions for a number of cellular transcription factors and accessory proteins including TATA-binding protein (TBP) (45, 107). To differentiate between these two defects in IE86 expression, further studies must be conducted using adenovirus-expressed IE86ΔSX protein. If this mutant protein can still function as an IFN-β antagonist, then the delayed kinetics of IE86 during IE2ΔSX virus infection is responsible for the dramatic induction of NFκB and the downstream cytokine expression observed in Fig. 4.2. Furthermore, if this mutant protein does not prevent virus-induced cytokine induction, a more careful analysis of the region can be conducted to potentially identify small deletions or point mutations which abrogate IE86's ability to attenuate cytokine induction.

As described in the introduction, IE86 has two principle roles of during a productive infection which can be classified as transactivation of viral promoters to progess from the IE to the E and L stage of infection, and to autoregulate or repress transcription from the MIEP. As a promiscuous viral transactivator, IE2 potently activates both viral and cellular promoters. The mechanism of this transactivation has yet to be elucidated, but is most likely effected through numerous functional interactions of IE86 with cellular transcription factors and accessory proteins. This transactivation for the most part is sequence independent, whereas IE86 mediated autoregulation is site-specific, requiring a 14-15 base pair DNA element, CG-N_{10}-CG, the cis repressor sequence (CRS). Two CRS regions are located between the TATA box and transcriptional start site of the IE1/IE2 genes. IE86 binds to the CRS in an homo-dimeric form and this autorepression appears to result from a steric block of RNA

polymerase II recruitment to the preinitiation complex, without blocking recruitment of other required basal transcription factors (117).

Structure-function analysis of IE2 may identify the mechanism by which IE86 prevents cytokine transcriptional activation. Initial experiments were conducted to determine whether IE86 DNA binding, which is essential for autorepression of the MIEP, is required to block IFN-β induction. Point mutations in the zinc finger (IE86mZn) and the dimerization region (IE86mDI) effectively prevent IE86 DNA binding to the CRS (106, 107). Adenoviruses were constructed which express IE86mZn and IE86mDI and were used to transduce HFF cells prior to UV-HCMV infection. As shown in Fig. 6-2, expression of these mutant IE86 proteins blocked IFN-β expression as efficiently as the wild-type IE86 protein. This suggests that IE86 DNA binding is not required to block cytokine expression. Further support for this comes from analysis of the IFN-β promoter sequence, which does not contain any sequence analogous to the CRS. Additional adenoviruses may now be constructed which express mutant IE86 proteins defective either transactivation, or which no longer interact with cellular transcription and accessory factors, including CBP, TBP and p53.

Though there is not an IE86 deletion mutant virus available, there are currently a number of small deletion or point mutant viruses available, including a temperature sensitive HCMV IE2 mutant virus (89). However, this mutant will not definitively demonstrate what role IE86 plays in blocking IFN-β during HCMV infection, as it is not a true deletion mutant. But rather will be useful in identifying the mechanism by which IE86 regulates expression of IFN-β. The TS510 virus expresses IE86 protein at both the permissive and non-permissive temperatures. The IE86 protein expressed at the non-permissive temperature is only defective in its ability

to transactivate target promoters. If another function of the protein is responsible for blocking IFN-β expression, such as a protein/protein interaction as hypothesized here, then the IE86 mutant protein will potentially block IFN-β expression at both the permissive and non-permissive temperatures. This virus will be instrumental in allowing us to identify the mechanism by which IE86 blocks IFN-β expression, allowing us to differentiate between IE86's transactivation function and some other function of the protein.

Propagation of an HCMV IE86 mutant virus for vaccine purposes

As discussed in the introduction, there is a growing need for preventative vaccine against HCMV infection. Current vaccine candidates include the live attenuated Towne strain and a subunit vaccine against the major glycoprotein gB (176), though their efficacy in preventing HCMV infection has not been determined. Due to the strict species specificity of cytomegaloviruses, vaccines for HCMV cannot be studied in an animal model, and therefore animal cytomegaloviruses must be studied (176). Though murine and guinea pig CMVs, which are the best studied animal models of CMV infection, contain functional homologues of IE86, further work will be necessary to identify whether a similar attenuation of NFκB occurs during infection with MCMV or GPCMV, and to determine the IE gene that is necessary for this attenuation.

The effect of enhanced IFN-β on vaccine efficacy is supported by data in the murine model that showed DNA vaccination with the gB gene with concurrent IFN-β immunotherapy was more protective against MCMV challenge than DNA vaccination alone (16). Given the present data identifying the role of IE86 in antagonizing the innate response to HCMV infection, an IE86 mutant virus may be an attractive vaccine candidate.

This mutant virus, constructed using point mutations identified by structure-function analysis to be essential for IE86-mediated NFκB antagonism, would be unable to prevent the initial host IFN-β and chemokine response. Following inoculation with this virus for vaccination, the host would mount an innate cytokine response. This response would not only inhibit or delay virus replication through IFN-β signaling and the activation of an antiviral state, but would also effectively result in enhanced recruitment and activation of cytotoxic T cells and NK cells through production of proinflammatory chemokines (See model in Fig. 6-3). IE86 mediated inhibition of cytokine and chemokine expression during the innate response to virus infection may sever the link to the adaptive, cellular response. This innate response shapes the adaptive immune response, as these cytokines and chemokines are critical for the activation and recruitment of effecter cells. Inhibition of their expression by IE86 may result in a weaker adaptive response, in that less CTL and NK cells are recruited and activated at the site of infection. This would facilitate higher level HCMV replication and potentially result in HCMV persistence and latency. Therefore, infection with a mutant HCMV virus defective in IE86 expression may allow for a more robust host innate response, which would more effectively result cell-mediated clearance of HCMV infected cells.

Importantly, since the primary function of IE86 is to transactivate downstream viral gene expression, this delay in virus replication and functional IE86 expression may result in reduced expression of other HCMV immunomodulatory genes involved in both the downregulation of MHC class I molecules and blocking NK cell activating receptor expression. Thus the IE86 mutant virus would not only be ineffective in preventing the activation and recruitment of CTL and NK cells, but also the

HCMV-infected cells would be more easily detectable by these activated cells due to impaired viral immunomodulatory gene expression. This would allow for more efficient virus clearance by both CTLs and NK cells which are recruited to the site of infection that would not normally detect HCMV infected cells as a result of HCMV immune evasion. These innate responses could potentially prevent HCMV from establishing a latent or persistant infections. So in effect, inhibition of this cytokine attenuation by IE86 could prevent downstream evasion strategies employed by the virus and effectively disarm HCMV.

Closing remarks

An emerging theme is beginning to be revealed for herpesvirus interactions with the host. Recent reports for herpes simplex virus type 1, varicella-zoster virus, Epstein-Barr virus and human cytomegalovirus support the view that herpesvirus infection globally suppresses inflammatory signaling by attenuation of NFκB activation (103, 104). This antagonistic function, now attributed to IE86 for HCMV infection, may be conserved between herpesviruses and may account for their success in establishing persistant and latent infections. Elucidating the molecular mechanism by which IE86 attenuates the NFκB dependent cytokine and chemokine response may be a stepping stone for development of novel antiviral drugs which work by blocking HCMV's ability to attenuate the host response. Lastly, HCMV mutant viruses defective in IE86 expression may be better vaccine candidates for treatment and prevent of human cytomegalovirus infection and disease.

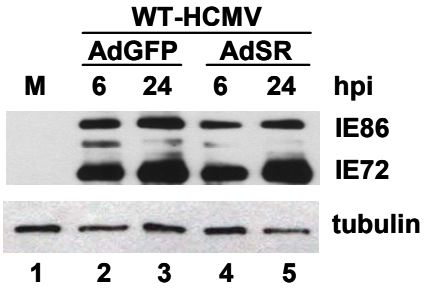

Figure 6-1. IE gene expression does not require NFκB activation. HFF cells were transduced for 24 hours with adenovirus expressing GFP or IκBαSR and then infected with WT-HCMV at a multiplicity of 5 pfu/cell. Cell extracts were prepared 6 and 24 hpi and assayed for IE and tubulin expression by Western blot.

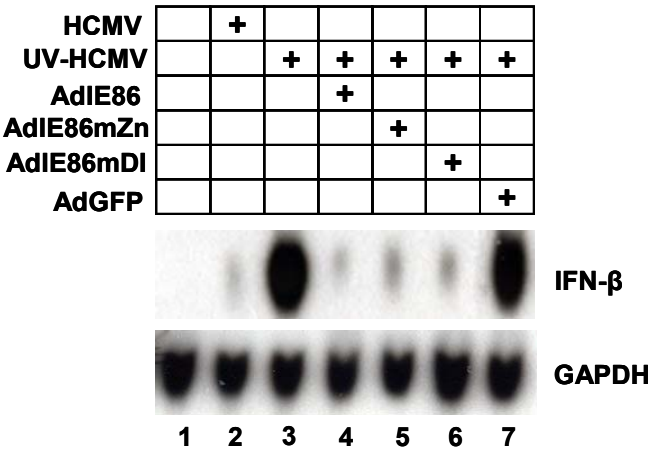

Figure 6-2. IE86 DNA binding is not required to block IFN-β expression. HFF cells were transduced for 24 hours with adenovirus expressing IE86, IE86mZn, IE86mDI, and GFP and then mock-infected or infected with WT-HCMV or UV-HCMV at a multiplicity of 5 pfu/cell. RNA was isolated 6 hpi and assayed for IFN-β and GAPDH expression by Northern blot.

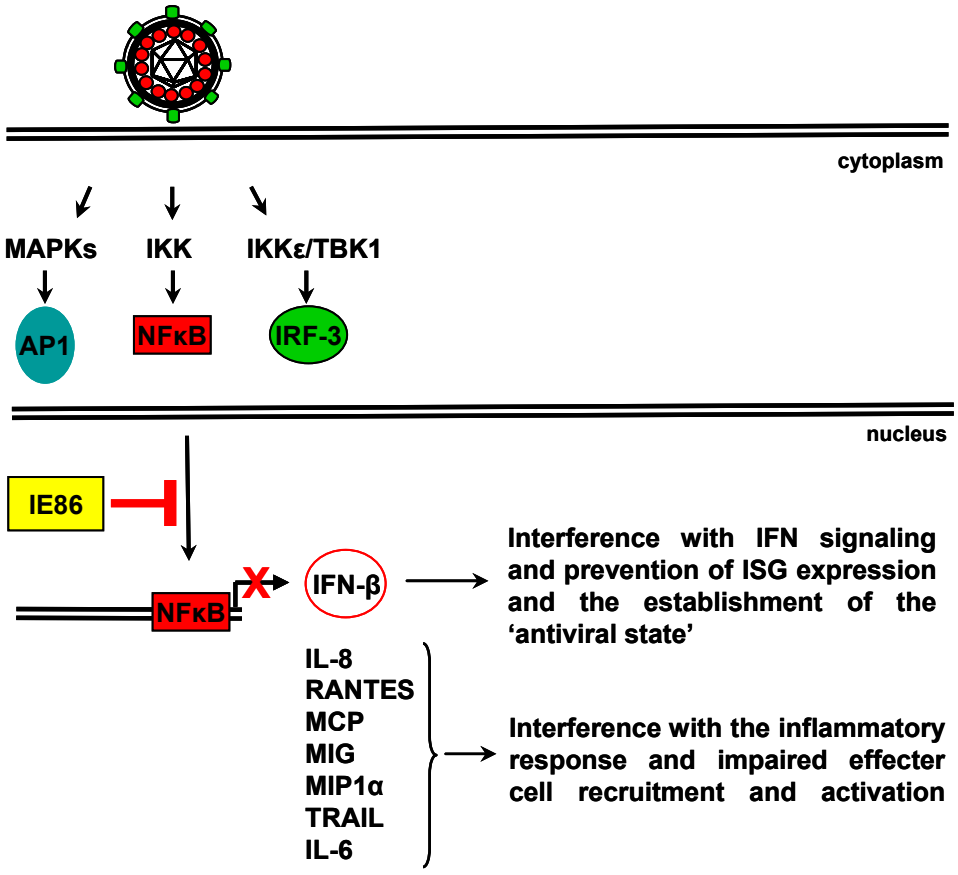

Figure 6-3. Schematic representation of the mechanism by which IE86 attenuates the host innate cytokine response. IE86 expression during a wild-type HCMV infection can target NFκB after the initial activation and translocation. Inhibition in NFκB DNA binding efficiently attenuates the expression of NFκB target genes, which includes a large assemblage of innate cytokines and chemokines.

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