The Role of Human Cytomegalovirus Immediate Early Proteins in Cell Growth Control: A Dissertation

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THE ROLE OF HUMAN CYTOMEGALOVIRUS IMMEDIATE EARLY PROTEINS IN CELL GROWTH CONTROL

A Dissertation Presented
By
Jonathan Patrick Castillo

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of:

Doctor of Philosophy

October 30, 2002

Program in Immunology and Virology
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THE ROLE OF HUMAN CYTOMEGALOVIRUS IMMEDIATE EARLY PROTEINS IN CELL GROWTH CONTROL

A Dissertation Presented

By

Jonathan Patrick Castillo

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ACKNOWLEDGMENTS

This dissertation is dedicated to my late uncle, Nelson de la Paz, M.D. Dr. de la Paz passed away in November of 1995 from complications associated with cancer. During my childhood, my uncle would always remind me to do my best both in school and in everything I did. Looking back at how my uncle lived his life, I recall that he was very dedicated to his family, his friends and to his profession. But what I remember most about my uncle was that he was a kind and gentle man who loved to enjoy life. Although my uncle neither persuaded nor urged me to pursue my doctorate, I believe that in some way, he had a definite influence in my decision to follow the path that I have chosen. He has and always will be a role model for the type of person that I aspire to be.

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ABSTRACT

The proper maintenance of the pathways governing cell growth is critical to ensure cell survival and DNA fidelity. Much of our understanding of how the cell cycle is regulated comes from studies examining the relationship between DNA viruses and the mechanisms of cell proliferation control. There are numerous examples demonstrating that viruses can alter the host cell environment to their advantage. In particular, the small DNA tumor viruses, which include adenovirus, simian-virus 40 (SV-40), and human papillomavirus (HPV), can modulate the host cell cycle to facilitate viral DNA replication. Due to the fact that these viruses infect quiescent, non-cycling cells and lack the necessary enzymes and resources to replicate their DNA (e.g. DNA polymerase), the small DNA tumor viruses must activate the host cell replication machinery in order to expedite viral DNA replication. The capacity of these viruses to perturb normal cell proliferation control is dependent upon their oncogene products, which target p53 and members of the Retinoblastoma (RB) family of proteins and inactivate their respective functions. By targeting these key cell cycle regulatory proteins, the small DNA tumor viruses induce the infected host cells to enter S-phase and activate the components involved with host cell DNA synthesis thereby generating an environment that is conducive to viral DNA replication.

In contrast, the larger, nuclear-replicating DNA viruses such as those from the family Herpesviridae, do not share the same stringent requirement as the small DNA viruses to induce the infected host cell to enter S-phase. The herpesviruses encode
many of the components to stimulate nucleotide biosynthesis and the necessary factors to facilitate virus DNA replication including a viral DNA polymerase and other accessory factors. Additionally, many herpesviruses encode gene products that arrest the host cell cycle, in most instances, prior to the G\textsubscript{i}/S transition point. Inducing cells to growth arrest appears to be a prerequisite for the replication of most herpesviruses.

However, in addition to encoding factors that inhibit the cell cycle, many herpesviruses encode proteins that can promote cell cycle progression in a manner similar to the small DNA tumor virus oncoproteins. By targeting members of the RB family and p53 protein, the herpesvirus proteins induce S-phase and activate S-phase associated factors that play a role in DNA replication. In this manner, the herpesviruses may promote an environment that is favorable for DNA replication.

Consistent with the other herpesviruses, human cytomegalovirus (HCMV) induces human fibroblasts to growth arrest. However, in other cell types, virus infection causes cells to enter S-phase. In addition, HCMV replication requires several cellular factors that are present only during S-phase. Furthermore, HCMV induces the activation of S-phase-associated events as well as the increased expression of numerous S-phase genes following infection.

HCMV encodes two immediate early (IE) gene products, IE1-72 and IE2-86, which can interact with members of the RB family of proteins. Additionally, the IE2-86 protein can bind to and inhibit p53 protein function. Given the functional resemblance between the HCMV IE proteins and the oncoproteins of the small DNA
tumor viruses, we hypothesized that expression of the HCMV IE proteins could modulate cell cycle control.

Specifically, we determined that expression of either IE1-72 or IE2-86 can induce quiescent cells to enter S-phase and delay cell cycle exit following serum withdrawal. Moreover, IE2-86 mediates this effect in the presence or absence of p53, whereas IE1-72 fails to do so in p53-expressing cells. Furthermore, both IE1-72 and IE2-86 induce p53 protein accumulation that is nuclear localized.

Because IE1-72 fails to promote S-phase entry in cells expressing p53 and induces p53 protein levels, the mechanism by which IE1-72 alters p53 levels was examined. IE1-72 elevates p53 protein levels by inducing both p19^ARF protein and an ATM-dependent phosphorylation of p53 at Ser^15. IE1-72 also promotes p53 nuclear accumulation by abrogating p53 nuclear shuttling. As consequence of this IE1-72-mediated increase in p53 levels, p21 protein is induced leading to a p21-dependent growth arrest in cells expressing IE1-72.

These findings demonstrate that the HCMV IE proteins can alter cell proliferation control and provide further support to the notion that HCMV, through the expression of its IE proteins, induces S-phase and factors associated with S-phase while blocking cell DNA synthesis, to possibly generate an environment that is suitable for viral DNA replication.
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CHAPTER I
Introduction

A. The Family *Herpesviridae*

The herpesviruses are large DNA viruses that are distributed in nature. The herpesvirus virion is comprised of four concentric layers: (1) an inner core containing the viral DNA in the form of a torus, surrounded by (2) an icosahedral capsid, an amorphous structure referred to as (3) the tegument, and a (4) lipid-protein envelope. The herpesviruses contain linear, double stranded DNA that ranges in length from 120 to 250 kbp (121).

All of the herpesviruses share several properties that are distinct for this family of viruses. Each herpesvirus encodes most of the factors they require to increase the pool of deoxyribonucleotides and to synthesize their DNA. Viral DNA synthesis and capsid assembly occur in the nuclei of herpesvirus-infected cells (159). A productive herpesvirus infection results in the destruction of the infected cell.

Another feature of the herpesviruses is their ability to establish latent infections within their host for an indefinite period of time. The viral genomes likely persist in the form of episomes within the nuclei of the infected host cell. The latent herpesviruses express a certain subset of latency-associated genes and retain the capacity to replicate and cause disease upon activation (121).
The herpesviruses vary in their host-cell specificity, replication kinetics, speed at which they lyse cells, and the cells that they latently infect.

The family *Herpesviridae* is divided into three subfamilies: alphaherpesviruses, betaherpesviruses, and gammaherpesviruses (100). The alphaherpesviruses infect a wide host range, grow rapidly, and establish latency in sensory ganglia (122). This subfamily includes herpes simplex virus (HSV). The betaherpesviruses, on the other hand, have a restricted host range and replicate much slower than the alphaherpesviruses. Examples of the betaherpesviruses include both human and murine cytomegaloviruses and these viruses can establish latency at numerous sites including lymphoreticular tissue, secretory glands, kidneys, and other tissues (159). The gammaherpesviruses target cells of lymphoid origin. Specifically, they infect T- or B-lymphocytes and establish latency in lymphoid tissue (121). Members of this subfamily include Epstein-Barr virus (EBV) and Kaposi's sarcoma associated Herpesvirus (KSHV) (100).

B. Cytomegalovirus

1. Human Cytomegalovirus

Human cytomegalovirus (HCMV) is a ubiquitous, species-specific beta-herpesvirus that, like other herpesviruses, can establish life-long latency in its host following primary infection. The HCMV genome consists of a linear, double-stranded DNA that is 230,000 base pairs in length, which is encased within an
icosahedral capsid and surrounded by tegument and a lipid bilayer envelope containing numerous HCMV-encoded glycoproteins (102) (Figure 1.1). The HCMV genome encodes for over 200 open reading frames (ORFs), and is divided into two covalently linked segments, designated Uₐ (unique long) and Uₛ (unique short), which are flanked by inverted repeat sequences (Figure 1.2).

HCMV, like other betaherpesviruses, is highly species-specific and will therefore only productively infect human cells, preferably those of fibroblast origin. Unlike the other herpesviruses, HCMV has a slow replicative cycle (>24 h) and requires a longer period of time to induce its cytopathic effects (102). HCMV-infected cells typically become enlarged (cytomegalia) and eventually develop nuclear and cytoplasmic inclusions that are characteristic of HCMV infection (113). In vivo, the virus targets a wide variety of cells including epithelial and endothelial cells, fibroblasts, smooth muscle cells, and peripheral blood leukocytes, which include monocytes and granulocytes (133).

2. Epidemiology

Although the virus is endemic within the population, HCMV infection rarely causes symptomatic disease in healthy, immunocompetent individuals but manifests itself as a lifelong persistent infection. The reactivation of HCMV commonly occurs in immunocompromised and immunosuppressed individuals and is the causative agent of a variety of maladies such as HCMV-associated pneumonitis and retinitis, which are the most prevalent HCMV-associated
Figure 1.1. Illustration of HCMV virion and structural organization.

Figure acquired from the following website: http://www.biografix.de/. Reproduced with permission from Dr. Marko Reschke.
Figure 1.2. Diagram illustrating the organization of the HCMV genome.

The HCMV genome consists of linear double stranded DNA that is divided into two covalently linked segments, designated $U_L$, (unique long) and $U_S$, (unique short). The DNA segments are flanked by inverted repeat sequences (depicted as: a, an, a'm, b, b', c', and c).
problems, detected in these patients (113). Additionally, reactivation of latent HCMV or transmission of the virus to organ transplant recipients may result in complications such as disseminated viremia and in some instances, organ dysfunction. HCMV also poses a serious threat to the health of HIV-positive individuals because HCMV may accelerate the development of AIDS as well as contribute to the morbidity associated with increased immunodeficiency (113). Likewise, HCMV infection is problematic for pregnant women and children, especially infants. HCMV is recognized as the most common congenital viral infection and is the leading cause of various neurological abnormalities associated with an infectious agent during early childhood (61). Infants congenitally infected with HCMV are more prone to hearing loss, chorioretinitis, and other disorders involving the perceptual organs (e.g. inner ears and eyes) and the central nervous system.

3. Association with proliferative disease

There is a plethora of evidence that suggests a link between HCMV and a number of proliferative disorders. Although HCMV does not appear to be oncogenic, HCMV exhibits the capacity to transform rodent embryo fibroblasts (6, 15) and in some instances, human cells (48) in vivo. Additionally, the detection of HCMV DNA and antigen in tumor tissues isolated from patient biopsies along with elevated HCMV antibody titers in these patients imply a relationship between HCMV and several cancers including cervical carcinoma, prostate...
cancer, and adenocarcinoma of the colon (37, 130). However, it is unlikely that HCMV directly induces cancer since the low incidence rates observed for each of the cancers linked to the virus do not reflect the ubiquitous nature of HCMV within the population. It has been suggested that HCMV may act as a co-etiologic agent in the development of tumors through a "hit and run" mechanism (90, 131) in which HCMV promotes cellular transformation by causing genetic instability or by preventing cells from undergoing apoptosis. Thus it appears that HCMV may contribute to tumor formation, but its specific role in the transformation process remains undefined.

In contrast to human cancer, a more apparent relationship between HCMV and atherosclerosis exists. Atherosclerosis is characterized by the formation of fibrous fatty lesions along blood vessel walls accompanied by inflammation (123) resulting in the occlusion of blood flow in the affected vessel. Several lines of evidence hint at a contributory role for HCMV in the atherosclerosis process. The results from numerous epidemiological studies show that atherosclerosis patients exhibit high levels of anti-HCMV antibodies and that HCMV-seropositive individuals are more susceptible to developing vascular disease (1, 52, 97, 137). Additionally, HCMV induces several events that may accelerate the atherosclerotic process. HCMV increases the expression of scavenger receptors in smooth muscle cells (SMCs) leading to increased lipid accumulation (138, 172). Also, HCMV infection of endothelial cells induces the expression of several chemokines and adhesion molecules that enhance the migration and adherence
of inflammatory cells to the endothelium (7, 143). Moreover, detection of viral DNA and antigen in fibrous lesions and in cells present in fatty streaks (endothelial cells and SMCs) (98, 112), as well as in the arterial walls of coronary artery disease patients (60, 64), imply that HCMV presence may be a factor in atherosclerosis development.

Recent evidence suggests that HCMV may also play a role in the development of restenosis, a coronary artery disorder that is characterized by the overproliferation, migration, and accumulation of arterial SMCs along the vessel wall following coronary angioplasty (139, 140). In contrast to atherosclerosis, the occlusion resulting from the accumulation of SMCs is often worse than the initial blockage. The detection of viral DNA and protein in SMCs from restenotic lesions suggests an association between HCMV presence and restenosis. Additionally, epidemiological evidence suggests that a correlation exists between HCMV-seropositivity and restenosis occurrence. HCMV-seropositive patients exhibit a higher propensity of developing restenosis following coronary angioplasty as compared to HCMV-seronegative patients (173). Moreover, studies using rat CMV in a rodent carotid artery model demonstrate that the rates of restenosis following angioplasty are higher in rat CMV-infected animals as compared to uninfected ones (174).

Although there is no definitive proof that HCMV causes restenosis, it has been hypothesized that the virus promotes certain events that contribute to restenosis. HCMV infection enhances SMC migration in vitro (175).
may be attributed to the increased expression of various chemokines such as RANTES, monocyte chemoattractant protein-1 (MCP-1), and interleukin-8 (IL-8) by HCMV-infected endothelial cells and SMCs (99, 143, 148). HCMV infection of endothelial cells also induces the expression of surface adhesion molecules that augment the migration and adhesion of SMCs and inflammatory cells to the endothelium (143). In addition, HCMV encodes a chemokine receptor (Us28) that when expressed on the surface of SMCs, promotes their cellular migration in vitro (148). Taken together, these findings imply a strong link between HCMV and the induced migration of SMCs that may contribute to restenosis.

In contrast to our understanding of HCMV and SMC migration, the relationship between HCMV and the overproliferation of SMCs remains vague. Studies examining HCMV effect on cellular proliferation have yielded conflicting results. Early studies on HCMV and the cell cycle suggested that HCMV-infected cells exhibit increased rates of DNA synthesis (5, 51). Notably, HCMV infection induces proliferation in SMCs in vitro that may be attributed in part, to the HCMV-mediated increase in NFκB activity and platelet-derived growth factor (PDGF) receptor in these cells (141, 175). Additionally, HCMV inhibits apoptosis in response to various stimuli (90, 176). In this manner, HCMV may contribute to the development of restenosis by promoting the overproliferation of SMCs in vivo and abrogating their ability to undergo apoptosis. However, contrary to the proliferative effects mediated by the virus, HCMV infection causes human fibroblasts, the model system for HCMV research, to undergo growth arrest (19,
35, 69, 88). Given the divergent effects that HCMV mediates on the cell cycle, it remains unclear if HCMV contributes to the overproliferation of SMCs in vivo and this brings into question the issue of how HCMV alters the cell cycle in general.

C. Viruses and the cell cycle

1. Targeting of the RB proteins by the small DNA tumor viruses

There is a precedent for DNA viruses altering cell cycle control to their advantage. In particular, the small DNA tumor viruses, such as adenovirus, simian virus 40 (SV-40), and human papillomavirus (HPV), can each perturb the replication machinery of the host cell to facilitate the replication of their viral DNA (121). Their ability to overcome the normal regulation of cell proliferation control is dependent upon their oncogene products, which target and inactivate members of the RB family of proteins. The RB protein family consists of three members, p107, p130, and pRb, whose function is to maintain cells in a quiescent state as well as regulate the transition from G0/G1 to S-phase by modulating the activity of the E2F family of transcription factors (56). The E2F proteins play an essential role in regulating the expression of genes required for DNA replication (151). Active hypophosphorylated RB proteins repress both E2F activity and the expression of E2F target genes by binding to E2F proteins at their C-terminal transactivation domain and recruiting transcriptional repressors (56). When the RB proteins become hyperphosphorylated by cyclin/cyclin-
dependent kinase complexes activated during mid-late G₁, the RB proteins no longer bind E2F proteins resulting in the derepression of E2F target genes (109).

Each of the small DNA tumor viruses encodes proteins that can bind to the RB proteins, displace their interaction with E2F, and alleviate the repression of E2F proteins. The E1A protein of adenovirus, large T antigen of SV-40, and the E7 protein of HPV each contain an LxCxE motif that facilitates their binding to the RB "pocket domain" thereby displacing E2F proteins from their interaction with the RB proteins at this region (108). The displacement of the E2F proteins from the RB proteins enables them to transactivate the promoters of their target genes, including genes required for S-phase (32, 81). Because each of the small DNA tumor viruses infect quiescent cells and lack certain components needed to replicate their viral DNA, it is thought that targeting the RB proteins allows for the induction of S-phase genes thereby generating an environment conducive to viral DNA replication.

2. Targeting of p53 protein by the small DNA viruses

In response to aberrant growth signals or cellular stresses, such as viral infection, DNA damage, or hypoxia, the p53 protein becomes activated (118). Mutations within the p53 gene that results in loss of p53 activity are detected in almost half of all human tumors (153). Additionally, many tumors that contain wild-type p53 often contain mutations in the pathways that regulate p53 protein levels and activity. Further evidence that p53 is an important tumor suppressor is
that p53−/− mice rapidly develop tumors when compared to their wild-type littermates (36). The p53 tumor suppressor protein is a sequence-specific DNA binding transcription factor that transactivates the promoters of many p53-responsive target genes including the cyclin-dependent kinase inhibitor, p21, to mediate cellular growth inhibition (41, 57). Besides inducing growth arrest, p53 activation results in apoptosis (154).

In addition to the viral oncoproteins that target the RB family members, each of the small DNA tumor viruses express proteins that complex with and inactivate p53 to prevent an infected cell from undergoing p53-mediated growth arrest or apoptosis. The adenovirus E1B 55kDa protein and the large T antigen of SV-40 bind p53 and inhibit its function (11, 87, 96, 126, 163). In contrast, the HPV E6 protein promotes the degradation of p53 through the ubiquitin-dependent proteolytic system (128). In all of these instances, expression of the viral oncoproteins alters p53 function and/or levels within infected cells to override this host response to virus infection. Thus, the ability to disrupt RB-mediated repression of E2F and abrogate p53 activity facilitates the DNA replication of each of the small DNA tumor viruses.

3. Large DNA viruses

The larger, nuclear replicating DNA viruses such as the herpesviruses, seem to mediate a different effect on the cell cycle as compared to the small DNA tumor viruses. In contrast to the small DNA tumor viruses, the
herpesviruses have evolved a replication process that does not appear to require an S-phase-like environment. All of the herpesviruses encode enzymes and factors that are involved in enhancing the pool of available deoxyribonucleotides within the infected host cell (121). The herpesviruses also encode a viral DNA polymerase as well as accessory proteins that play a role in viral DNA replication. Furthermore, many of the herpesviruses encode proteins that cause cells to growth arrest. Therefore, it appears that inducing cell cycle arrest within the infected host cell is an integral part of the herpesvirus replication strategy.

HCMV has been shown to have varying effects on the cell cycle. Some studies show that HCMV infection stimulates cellular DNA synthesis (5, 142). In contrast to these observations, many studies on HCMV effects on the cell cycle show that HCMV induces a G1, and occasionally a G2/M, growth arrest in infected human fibroblasts (19, 35, 69, 88). Although these more recent studies demonstrate that HCMV induces fibroblasts to undergo what has been described as a "G1 arrest", biochemically these cells exhibit hallmarks of early S-phase entry including pRb hyperphosphorylation, increased E2F transcriptional activity, elevated cyclin E and cyclin A kinase activity, and expression of many S-phase genes such as dihydrofollic reductase (DHFR), DNA polymerase alpha, proliferating cell nuclear antigen (PCNA), topoisomerase II, ORC 1, and the minichromosome maintenance (MCM) proteins 3-7 (19, 35, 70). In addition, HCMV infection of human endothelial cells, rodent smooth muscle cells, or a differentiated monocytic cell line biases these cells towards S-phase (132, 175).
These observations suggest that HCMV mediates differing effects on the cell cycle.

The ability of HCMV to manipulate the cell cycle may be attributed to its ability to modulate the activities of certain cell cycle regulatory proteins. Besides altering the levels of phosphorylated pRb and inducing E2F transcription activation function, HCMV increases p53 levels by decreasing p53 degradation to enhance its stability in infected cells (44, 69). Additionally, HCMV sequesters p53 into distinct foci within the nuclei of infected cells (44). HCMV also promotes the nuclear localization of Cdk2 and stimulates Cdk2 activity in infected cells, which is necessary for HCMV replication (18, 20). This increase in Cdk2 activity is attributed in part to the HCMV-mediated degradation of p21 (27). Furthermore, it has been hypothesized that HCMV modulates the proteins that regulate the cell cycle through its immediate early (IE) proteins.

D. HCMV immediate early proteins

1. General background

Analogous to the other members of the family Herpesviridae, expression of HCMV genes occurs in a temporal order. The first set of viral gene products to be expressed is classified as immediate early, followed by the expression of the early genes, and finally, the late gene products (121). The IE genes do not require de novo protein synthesis for their expression. The most abundantly transcribed HCMV IE gene products originate from ORFs encoded in the major
IE region located within the U₆ segment of the viral genome and that are under the control of the major IE promoter (MIEP) (157). Transcription from the MIEP gives rise to several spliced mRNA species (Figure 1.3) (144). The initial and most abundant transcript originates from the U₆.123 region and gives rise to a spliced 1.95 kb mRNA composed of exons 1 through 4 and encodes a 491 aa (72 kDa) nuclear phosphoprotein referred to as IE1-72 (also known as IE1 or IE72) (146). Transcription through the other IE gene, U₆.122, gives rise to two major transcripts, a 2.25 and a 1.7 kb mRNA, that have the same first three exons as IE1-72 mRNA but contain a novel exon, exon 5, in place of exon 4 as a result of alternative splicing (145). The 2.25 kb mRNA encodes a 579 aa (82-86 kDa) nuclear protein, IE2-86 (also known as IE2 or IE86), and the 1.7 kb mRNA encodes for a 425 aa (55 kDa) protein, IE2-55 (also known as IE55). IE2-55 is identical to IE2-86 except for a 154 aa deletion between residues aa 365 and 519 resulting from a splicing event within exon 5 (77). Because all three of the HCMV IE proteins contain the same first three exons, they all share the same 85 aa in their N-terminal sequence (144) (Figures 1.4 and 1.5). However, the remaining sequences in each of the IE proteins differ and likely account for the divergent activities exhibited by each protein.

2. HCMV IE1-72

IE1-72 mRNA is the initial and most abundant viral transcript expressed during HCMV infection (146). Studies utilizing HCMV IE1-72-deletion mutants
Figure 1.3. HCMV major IE proteins and their location within the genome.
Schematic diagram representing the organization of the HCMV DNA genome
and the relative position of the ORFs encoding the major IE gene products. The
major IE transcripts are expressed from the major IE promoter (MIEP; shown in
box with vertical lines) and are alternatively spliced to yield the major IE proteins.
Arrows represent transcripts and broken lines depict splicing events/sites.
show that IE1-72 is not absolutely required for HCMV infectivity and viral DNA replication (53, 101), but does contribute to the efficient replication of HCMV. IE1-72 primarily functions as a transcriptional co-activator of numerous HCMV gene promoters (53, 67). The contribution of IE1-72 to HCMV replication most likely revolves around its ability to transactivate the expression of early viral genes that facilitate the replication process. Additionally, IE1-72 activates expression from its own promoter, the MIEP, through the induction of NFκB (28, 83).

In addition to viral promoters, IE1-72 activates the expression of several cellular gene promoters. A region located in the C-terminus of IE1-72 is necessary for IE1-72 transcriptional co-activation (ref) (Figure 1.4). Although IE1-72 does not bind to DNA directly, the transient expression of IE1-72 is sufficient to stimulate transcription from a number of TATA-less promoters including the cellular DHFR and DNA polymerase alpha promoters, in addition to certain promoters containing TATA-box elements (59, 94, 156). IE1-72 expression enhances AP-1 activity and is sufficient to induce AP-1-driven transcription in reporter constructs (79). Moreover, IE1-72 contributes to the induction of Rel/NF-κB transcription by interacting with the transcription factor Sp-1 to enhance p65 promoter transactivation (164, 165).
Figure 1.4
Figure 1.4. The HCMV IE1-72 protein and its regulatory domains.

Schematic diagram of HCMV IE1-72 and location of the functional and protein binding domains. Note that many of the functional domains have only been grossly mapped. Mapping of some functional domains are still unclear. Motif abbreviations: Zn, zinc finger; LZ, leucine zipper; LR, leucine-rich; AD, acidic domain.
3. HCMV IE2-86

The importance of the IE2-86 protein to HCMV replication is exemplified by the inability to generate infectious HCMV mutants lacking IE2-86. The results from several alternative approaches to assess the role of IE2-86 in HCMV replication show that IE2-86 is essential for virus production (93). The failure of HCMV to replicate in the absence of IE2-86 is consistent with its role as a potent transactivator of numerous viral early genes including UL112/113 and others that participate in viral replication (67). In addition to activating the expression of HCMV early and late genes, IE2-86 can transactivate other viral promoters such as the HIV-1 LTR (156, 162).

IE2-86 can transactivate the promoters of many cellular genes. Some of this transactivation occurs through a TATA-box-dependent mechanism that involves the interaction of IE2-86 with components of the basal transcription complex including the TATA box-binding protein (TBP), TFIIB, and TFIID (24, 55, 72, 73) (Figure 1.5). Additionally, IE2-86 exhibits TBP associated factor (TAF)-like activity as demonstrated by the rescue of cells containing defective TAF proteins by IE2-86 (91).

Besides functioning as a strong transactivator, IE2-86 contains a domain that allows it to function as a repressor that downregulates expression from the MIEP by binding to the so-called cis-repression sequence (crs) that lies within the MIEP (114) (Figure 1.5). The fact that IE2-86 induces the expression of several
HCMV IE2-86

REGION SHARED WITH IE1-72

TRANSACTIVATION

AUTOREPRESSION

DNA-BINDING

TBP-BINDING

TFII-BINDING

p53-REPRESSION

p53-BINDING

pRb-BINDING
Figure 1.5. The HCMV IE2-86 protein and its regulatory domains.

Schematic diagram of HCMV IE2-86 and location of the functional and protein binding domains. Note that many of the functional domains have only been grossly mapped. Mapping of some functional domains are still unclear (e.g. pRb binding). HLH, helix-loop-helix domain.
viral and cellular genes while auto-regulating its own expression implies multiple roles for IE2-86 in relation to regulating gene expression during the viral infection process.

4. HCMV IE2-55

The IE2-55 gene product is encoded by a transcript that is a splice variant of the IE2-86 gene product and contains a conserved N-terminus as well as a 155aa deletion at aa 365-519 in the C-terminus. The region that is deleted has been shown to be required for many of the functions attributed to IE2-86 including transcriptional activation and DNA binding (29, 92, 115, 129). Consequently, unlike IE2-86, IE2-55 fails to transactivate HCMV early promoters and to repress the MIEP at the crs (80). Additionally, IE2-55 fails to dimerize with IE2-86 (29) and the inability of IE2-55 to dimerize may contribute to its failure to transactivate the promoters of HCMV early genes.

5. HCMV IE proteins and the cell cycle

In addition to their ability to enhance the transcription of viral and cellular gene promoters, the HCMV IE proteins modulate components of the cell cycle machinery. Both IE1-72 and IE2-86 exhibit similar properties to the small DNA tumor virus oncoproteins in that the IE proteins target members of the RB family of proteins. However, unlike the E1A, large T antigen, and E7 proteins, the HCMV IE proteins lack the consensus pocket domain-binding motif, LxCxE, and
therefore, do not bind to the RB proteins at their pocket domains (43, 54, 71). Moreover, in contrast to the viral oncoproteins, which target all three RB family members, the HCMV IE proteins bind to specific RB family members, IE1-72 binds p107 and IE2-86 interacts with pRb (43, 54, 116).

The interaction between IE1-72 and p107 occurs through a domain contained within the first 85 aa of the N-terminus of IE1-72 (Figure 1.4) (71, 116). Binding of IE1-72 to p107 alleviates the p107-mediated repression of E2F-responsive promoters and abrogates p107-mediated growth suppression (116). Additionally, it has been suggested through in vitro kinase assays that IE1-72 can exhibit kinase activity and phosphorylate p107 and p130 to disrupt their interaction with E2F4 (111). A putative kinase domain has been identified on IE1-72 spanning from aa 173 to 197 (Figure 1.4). Besides p107 and p130, IE1-72 phosphorylates the E2F proteins, E2F1, E2F2, and E2F3 in vitro (111) and can interact with E2F1 (94), however the significance of these events to the cell cycle is not known.

Analogous to IE1-72, IE2-86 interacts with an RB family member, pRb (43, 54). Attempts to map the pRb-binding domain have shown that several regions on IE2-86 spanning from aa 85 to 364 can interact with pRb (Figure 1.5) (refs). Binding of IE2-86 to pRb relieves the pRb-mediated repression of E2F responsive promoters (43, 54). However, the precise mechanism by which IE2-86 blocks pRb function is unclear. IE2-86 induces the expression of numerous E2F-target genes including factors associated with the bioenzymatic machinery
necessary for DNA replication. For example, IE2-86 induces an increase in the mRNA levels of c-myc, cyclin E, cdk2, E2F1, ribonucleotide reductase 1 and 2, thymidine synthetase, MCM3, and MCM7 (136). The results from this study imply that IE2-86 disruption of pRb-E2F complexes may free E2Fs to transactivate their target genes.

In addition to pRb, IE2-86 also interacts with p53 and p21 (132, 140). The domain identified on IE2-86 that interacts with p53 is contained within the first 135 aa of the N-terminus (Figure 1.5). IE2-86 binds to the N-terminus of p53 and this interaction inhibits p53 transactivation activity (152). Studies to elucidate IE2-86 effect on the cell cycle showed that IE2-86 blocks cell cycle progression in G1 (19, 35, 69, 88). However, subsequent studies indicated that IE2-86 induces cells to enter S-phase (105). The ability of IE2-86 to promote S-phase entry is attributed in part to IE2-86 transactivation of the cyclin E promoter and induction of E2F activity (17, 136).

Despite the absence of the dimerization, DNA binding, and transactivation domains, the putative pRb binding sites that were identified on IE2-86 (43, 54) are present on IE2-55. It is unclear whether IE2-55 mimics IE2-86 by binding to pRb and consequently exhibiting similar effects on the cell cycle as IE2-86. There is an additional line of evidence that suggests that IE2-55 may have the capacity to affect similar aspects of the cell cycle. It has been shown that transient expression of IE2-55 induces NF-κB levels by somehow transactivating the p105/p50 promoter and enhancing the transactivation of the p65 promoter via
Sp-1 (164, 165). In contrast to IE2-86, expression of IE2-55 fails to enhance the DNA binding activity of Sp-1. Therefore, it appears that IE2-55 cooperates with Sp-1 to transactivate the NF-κB promoter though a different mechanism. Furthermore, one might predict that the induction of NF-κB by IE2-55 should increase protection from apoptosis and for that reason contribute to a more favorable environment for virus replication.

Besides targeting RB family members and p53, the HCMV IE proteins, IE1-72 and IE2-86, co-localize to sites in the nucleus referred to as nuclear domain 10 (ND10) at which DNA replication, transcription, pre-mRNA splicing, and ribosome assembly take place (3, 66). Although the precise function of these structures, also referred to as promyelocytic leukemia protein (PML) oncogenic domains (PODs), is not known, there is a correlation between the nuclear distribution of the ND10/PODs and normal cell growth (95). Under normal conditions, the ND10/PODs appear as nuclear punctate bodies that are present through most of the cell cycle except during mitosis. However, in instances where a loss of cell growth control occurs, the nuclear distribution of ND10/PODs is disrupted (95).

Evidence suggests that disruption of the ND10/PODs is necessary for efficient viral DNA transcription and replication. In the case of adenovirus, SV-40, and herpes simplex virus-1 (HSV-1), viral DNA transcription initiates at the ND10/PODs (65). Adenovirus and HSV-1, along with HCMV, encode proteins that co-localize with and disrupt the ND10/PODs. The HCMV IE1-72 protein can
interact with and disrupt the ND10/PODs and can also promote the dispersal of PML protein from these structures (Figure 1.4) (2, 3). In contrast, IE2-86 co-localizes with PML in the ND10/PODs but neither disrupts the ND10/PODs nor induces the nuclear redistribution of PML (3, 4). Although the IE proteins co-localize with ND10/PODs and some of their associated proteins, the consequences of these events on the cell cycle remains unclear.

D. Thesis Aims

The objective of this dissertation was to determine whether the HCMV IE proteins could modulate the host cell cycle, presumably to promote an environment conducive to viral DNA replication. Given the ability of HCMV IE1-72 and IE2-86 to target specific RB family members for inactivation, and in the case of IE2-86, to bind to and inactivate p53, it was hypothesized that these HCMV IE proteins modulate the cell cycle in a manner similar to the small DNA tumor virus oncoproteins. The data presented in this thesis confirm that these IE proteins can alter the cell cycle. Specifically, the experiments in Chapter III demonstrate that expression of these IE proteins can alter the cell cycle by inducing quiescent cells to enter S-phase and by delaying cells from exiting the cell cycle. The inability of IE1-72 to promote S-phase entry as well as delay cell cycle exit in the presence of p53 prompted us to examine IE1-72 effect on p53 protein and activity. The experiments in Chapter IV address the multiple pathways by which IE1-72 induces p53 protein accumulation and the results
described in Chapter V detail the consequences of the IE1-72-mediated induction of p53 in cells. These findings provide further insight into the relationship between the HCMV IE proteins and the cell cycle.
CHAPTER II
Materials and Methods

A. Cells

Cells from a rat embryo fibroblast cell line, REF52, were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO BRL), supplemented with 5% fetal bovine serum (FBS) (HyClone, Inc.), 5% fetal calf serum (FCS) (HyClone, Inc.) and 1% penicillin-streptomycin (GIBCO BRL). Early passage wild-type (WT) as well as genetically matched p53-deficient (p53<sup>−/−</sup>) mouse embryo fibroblasts (MEFs) (passages 2-7), Mdm2<sup>−/−</sup>/p53<sup>−/−</sup> MEFs, and SAOS-2 were generous gifts from Stephen Jones (University of Massachusetts, Medical School Worcester, MA). The p19ARF<sup>−/−</sup> MEFs were a generous gift from Charles Sherr (St. Jude's Medical Center, Memphis, TN). Early passage p21<sup>−/−</sup> and genetically matched WT MEFs were obtained from Tyler Jacks (Massachusetts Institute of Technology, Boston, MA). Human embryonic lung fibroblasts (HELs) were a generous gift from Eng-Sheng Huang (University of North Carolina, Chapel Hill, NC). All of the aforementioned MEFs, SAOS-2 cells, and HEL cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Human dermal fibroblasts from ataxia-telangiectasia patients (GM03395C; AT) as well as age matched, normal dermal fibroblasts (GM00316B; WT) were obtained from the Coriell Institute for Medical Research (Camden, NJ). The AT and WT human fibroblasts were cultured in minimal
essential media supplemented with 15% FBS, essential and non-essential amino acids, vitamins, and 1% penicillin-streptomycin. All cells were incubated in a 37°C incubator supplemented with 5% carbon dioxide (CO₂).

B. Recombinant adenoviruses

A recombinant adenovirus encoding an HCMV (AD169) IE1-72 cDNA under the control of the HCMV promoter/enhancer region from position -299 to +69 (AdIE1-72) and a recombinant adenovirus encoding an HCMV (Towne) IE2-86 cDNA under the control of the HCMV promoter/enhancer region from position -299 to +60 were kindly provided by Gary Hayward (Johns Hopkins University, Baltimore, MD) (3). A recombinant adenovirus encoding an empty vector cDNA was generated (81) and was used as a negative control in our experiments. A recombinant adenovirus containing a β-galactosidase (β-gal) expression cassette under the control of the HCMV enhancer (Adβgal) (82) was also used as a negative control in our experiments. An E2F-1 cDNA-encoding recombinant adenovirus (AdE2F1) was used a positive control in our cell cycle analyses (81). Recombinant adenoviruses encoding wild-type p53 cDNA (Adp53) (82) and a mutant form of p53 containing mutations at all of its phosphorylation sites (Adp53N/C) (8, 120) were used in our heterokaryons assays. Viruses were grown and titered in a human embryonic kidney cell line (293 cells) and subsequently purified on cesium-chloride gradients (110). Virus titers representing the number of infectious particles per unit of volume were
determined by immunohistochemical staining of the adenovirus hexon with an anti-adenovirus antibody (Biodesign International).

C. Virus infections

Different multiplicities of infection (MOIs) representing the number of viral particles used to infect a specific cell population were used for the different recombinant adenoviruses with optimal doses empirically determined. Prior to infection, cells were washed with PBS and with serum-free DMEM. The recombinant adenoviruses were diluted in serum-free DMEM supplemented with penicillin and streptomycin and then added to the cells. Infections were incubated at 37°C in 5% CO₂ for 1 h with plates being rocked every 15 min. After 1 h, the viral inoculum was removed and replaced with DMEM containing the appropriate serum concentration and the cells were cultured at 37°C.

D. Cell cycle analysis

a. Bromodeoxyuridine (BrdU) incorporation

Cells were plated at sub-confluent densities onto 6-well plates (Falcon) and cultured under normal conditions prior to infection with the recombinant adenoviruses. For the cell cycle exit analysis, cells were subjected to serum starvation by culturing in media containing low concentrations of serum post-infection (p.i). For the S-phase induction experiments, cells were rendered quiescent by serum starvation prior to infection. The conditions (serum
concentration and culture time) employed to induce quiescence were empirically
determined for each cell type examined. After infection, cells were cultured
under the appropriate serum starvation conditions. At 12 h prior to harvesting,
cells were labeled with bromodeoxyuridine (BrdU) by adding BrdU (10μM final
concentration) to the culture medium. At different times p.i., the culture medium
was removed and the cells were washed twice with PBS. Cells were then fixed
in ethanol (90% final concentration) for 5 minutes at room temperature. Fixed
cells were washed twice with PBS then incubated in hydrochloric acid (HCl) (2N
final concentration) for 30 minutes at room temperature. Afterwards, sodium
tetraborate (0.1M final concentration) was added to the cells and were then
subjected to two washes with PBS and one with PBS containing 0.5% Tween-20
(PBS/Tween-20). The cells were subsequently blocked with 1% BSA diluted in
PBS/Tween-20 for 30 minutes at room temperature. Immunohistochemical
staining for BrdU incorporation was done by incubating cells with a mouse anti-
BrdU monoclonal antibody (Boehringer Mannheim) diluted 1:500 in PBS/Tween-
20 containing 1% BSA for 1 h at room temperature in a humidified chamber.
Cells were washed with PBS/Tween-20 and incubated with a biotin-conjugated
goat-anti mouse Ig secondary antibody (Vector Laboratories) diluted 1:250 in
PBS/Tween-20 for 1 hour at room temperature in a humidified chamber.
Afterwards, cells were washed and then incubated with a streptavidin-conjugated
substrate (Vector Laboratories) diluted 1:200 in PBS for 30 minutes at room
temperature. The cells were washed with PBS and then incubated at room
temperature with Vectastain DAB substrate (DAB kit, Vector Laboratories) as recommended by the manufacturer, to visualize stained nuclei. Scoring for BrdU positive cells was done by counting the number of cells that stained positive for BrdU incorporation per cell population. A minimum 300 total cells spread over 10 or more fields was scored for each cell population from each experiment.

b. Propidium iodide (PI) staining

Cells were plated at sub-confluent densities prior to infection with the appropriate recombinant adenoviruses. To process the samples for flow cytometry analysis, the cells were first trypsinized, pelleted, washed with PBS, re-pelleted, and resuspended in 400 μl of PBS. All centrifugations were done at 500 x g for 5 minutes at 4°C. The cells were subsequently fixed in cold ethanol (70% final concentration). Fixed cells were washed twice with PBS and then incubated in HCl (2N final concentration) containing pepsin (0.2 mg/ml) for 30 minutes at room temperature. Afterwards, 0.1M sodium tetraborate was added and the cells were washed with PBS then blocked with PBS containing 1% BSA. Cells were washed three times with PBS and then resuspended in 0.5 ml PBS containing PI and RNase A (0.5 mg/ml). Flow cytometry analysis was performed by the UMMS Flow Cytometry Core Facility, (Worcester, MA), and the data was processed using the FlowJo FACS analysis program (Treestar Inc., CA). S-phase cells were defined as the population of cells having greater than 2N but less than 4N DNA content.
E. Immunohistochemical staining

Semiconfluent cultures of cells were infected with the appropriate recombinant adenoviruses. At different times p.i., cells were washed with PBS and then fixed with formaldehyde diluted in PBS (0.37% final concentration) for 5 minutes at room temperature. The cells were washed with PBS and then incubated in methanol (100% final concentration) for 5 minutes at room temperature. Following several washes with PBS/Tween-20, the cells were incubated with the primary antibody diluted in PBS/Tween-20 containing 1% BSA for 1 hour at room temperature. The cells were washed with PBS/Tween-20 and bound antibody was detected using a Vectastain DAB substrate kit as described by the manufacturer. p53 protein was detected with an anti-p53 monoclonal antibody (Ab-1, Oncogene Research Products) at a 1:500 dilution and p21 protein was detected with an anti-p21 monoclonal antibody (F-5; Santa Cruz Biotechnology) at a 1:250 dilution.

F. Western blot analysis

Cells were infected with the appropriate recombinant adenoviruses and subsequently harvested at various times post infection. Whole cell extracts were prepared by first washing the cells with cold PBS and then lysing them in 100 μl of whole cell extract buffer (50 HEPES (pH 7.9), 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 % Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.3 mM sodium orthovanadate, 2 mM sodium fluoride, 2 μg/ml
apoptinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin) by incubation for 30 min on ice.

To assess p21 protein, cells were washed once with cold PBS then scraped into 1 ml of RIPA buffer (PBS, 0.1% NP-40, 1% SDS, 0.5% sodium deoxycholate, 0.1% sodium vanadate, 0.1% PMSF, and 0.3% aprotinin) and incubated on ice for 1 h. Soluble proteins were isolated by centrifugation at 13,000 rpm in a microcentrifuge, with the resulting supernatant collected and stored at -70°C.

Aliquots of cell extracts were analyzed by SDS-PAGE, and the resolved proteins transferred to nitrocellulose membranes by electroblotting. Membranes were incubated in blocking solution (5% non-fat milk dissolved in tris-buffered sulfate (TBS) containing 0.1% Tween-20 (TBS/Tween-20)) for 1 h at room temperature. Afterwards, membranes were incubated with primary antibody diluted in TBS/Tween-20 containing 5% blocking solution at 4°C overnight on an orbital shaker. HCMV IE1-72 and IE2-86 protein expression was detected with a mouse anti-cytomegalovirus monoclonal antibody (MAB810; Chemicon International, Inc.) at a 1:500 dilution. p53 was detected with an anti-p53 polyclonal antibody (Ab-7; Oncogene Research Products) at a 1:2000 dilution, the phospho-Ser$^{15}$ form of p53 was detected with a polyclonal antibody specific for this modification (#9284; Cell Signaling Technology) at a 1:1000 dilution, p21 was detected with an anti-p21 polyclonal antibody (C-19; Santa Cruz Biotechnology) at a 1:500 dilution, p19$^{ARF}$ was detected with an anti-p19$^{ARF}$ polyclonal antisera (NB 200-106; Novus Biologics) used at (0.5 μg/ml), MDM2 was detected with an anti-MDM2 monoclonal antibody (Ab-2; Oncogene Research Products) at a 1:500
dilution, and actin was detected with an anti-actin polyclonal antibody (I-19; Santa Cruz Biotechnology) at a 1:500 dilution. The membranes were washed with TBS/Tween-20 for three 10 min intervals at room temperature and then incubated with secondary antibody diluted in TBS/Tween-20 containing 5% blocking solution at room temperature for 1 h. Immunoreactive proteins were detected by using a chemiluminescence kit (Amersham) according to the manufacturer’s recommendations.

G. Northern blot analysis
Total cellular mRNA (25 μg/lane) was resolved by electrophoresis under denaturing conditions in formaldehyde-agarose gels, containing 1% agarose, 3-(N-morpholino) propanesulfonic acid, EDTA, and formaldehyde. After separation, the RNA was transferred to nylon membranes by capillary blotting overnight at room temperature. Blots were prehybridized overnight in prehybridization buffer (10% dextran sulfate, SSPE, 1% SDS, 0.5% non-fat milk, yeast total RNA) at 50°C. A p53 cDNA probe (SJ58, a generous gift from Stephen Jones) was digested with KpnI and labeled with 32P. Following prehybridization, the membrane was incubated in 5ml of prehybridization solution containing the labeled probe at 50°C. Membranes were washed twice with 2x SSPE for 20 min at 50°C before exposure to film or a phosphorimager screen.

H. Monitoring p53 nuclear shuttling
Heterokaryons were generated as described (168). Essentially, subconfluent cultures of SAOS-2 cells (3 x 10^5 cells per 60mm plate) were coinfected with Adp53 (MOI = 20) and either AdlE1-72 or AdCon at the indicated MOI. Following infection, cells were cultured in normal media at 37°C for 1.5 h. The SAOS-2 cells were washed twice with PBS, then trypsinized, re-plated with an equal number of MDM2^{+/−}/p53^{+/−} MEFs onto glass coverslips in 35mm plates, and incubated overnight at 37°C. Afterwards, MDM2^{+/−}/p53^{+/−} MEFs and SAOS-2 cells were washed twice with PBS and then incubated in media containing cyclohexamide (50 μg/ml) for 20 min at 37°C. MDM2^{+/−}/p53^{+/−} MEFs and SAOS-2 cells were subsequently washed twice with PBS and then incubated for 2 min in 50% polyethylene glycol (MW 3350, SIGMA) dissolved in DMEM containing penicillin-streptomycin to facilitate cell fusion. The treated MDM2^{+/−}/p53^{+/−} MEFs and SAOS-2 cells were washed twice with PBS and incubated in media containing cyclohexamide for 1 hr at 37°C. MDM2^{+/−}/p53^{+/−} MEFs and SAOS-2 cells were then fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and permeabilized with cold 0.2% Triton X-100 in PBS for 10 min at 4°C.

To identify heterokaryons and determine the location of p53 in the heterokaryons, cells were blocked with 10% FBS in PBS for 15 min at room temperature prior to incubation with primary antibodies. The cells were doubly stained with an antibody specific for human, but not murine, Ku nuclear antigen to distinguish human and murine nuclei, together with an antibody that
recognizes p53. Human Ku-86 was detected using an anti-Ku86 monoclonal antibody (sc-5280; Santa Cruz Biotechnology) diluted at 1:200 and p53 was detected using an anti-p53 monoclonal antibody (Ab-1; Oncogene Research Products) diluted at 1:250. The p53N/C mutant was detected using an anti-p53 monoclonal antibody (1:250 dilution) that recognized an epitope (aa 46-55) that was not affected by the mutation to p53 (Ab-2; Oncogene Research Products). A monoclonal antibody recognizing the two subunits of the human Ku protein (p70/p80) (Ab-2; Oncogene Research Products) was used at a dilution of 1:250 to identify human nuclei. After incubation with the primary antibodies, the cells were washed three times with PBS and then incubated with an isotype-specific fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (Southern Biotechnology, Inc.) diluted 1:250 and an isotype specific rhodamine red-X (RRX)-conjugated monoclonal antibody (Southern Biotechnology, Inc.) diluted 1:200, to detect p53 and human Ku-86, respectively. All of the described antibodies were diluted in 10% FBS/PBS and were incubated in a humidified chamber for 1 hour at room temperature. Cells were washed three times with PBS and once with distilled water. Glass coverslips containing the cells were removed from the 35mm plates and mounted onto glass slides with Vectashield containing 4',6'-diamidino-2-phenylindole (DAPI) (H-1200, Vector Laboratories, Inc.) according to manufacturer’s recommendations. p53 nuclear shuttling was scored by assessing the localization of p53 protein in human – murine heterokaryons using a Zeiss immunofluorescent microscope. Results were
presented as the percentage of heterokaryons in which p53 shuttled from human to murine nuclei.

I. Luciferase assays

HEL fibroblasts were plated at subconfluent densities onto 6-well plates and grown overnight at 37°C. Cells were co-transfected with a plasmid encoding an IE1-72 cDNA (pcDNA3-IE1-72) or a control plasmid (pcDNA3) (2 μg/well), along with a p21-specific reporter construct, WAF-1-Luc (generously provided by Karen Vousden, National Cancer Institute, Frederick, MD) (41) (1μg/well), using Lipofectamine Plus (Invitrogen Inc.). A renilla luciferase-expressing plasmid, pRL-TK (kindly provided by Zdenka Matijasevic, UMMS, Worcester, MA), was also included (0.25μg/well) as an internal control to normalize transfection efficiencies between samples. Transfection reactions were incubated for 5 h in 37°C. Afterwards, transfection media was replaced with DMEM containing 10% FBS and cells were incubated at 37°C for 24 h. The cells were then lysed and luciferase activity was determined using a Dual-Luciferase reporter assay system (Promega). Results are presented as fold induction of p21 promoter activity relative to the control plasmid transfected cells.
CHAPTER III

Effect of HCMV IE1-72 and IE2-86 Expression on the Cell Cycle

A. Expression of HCMV IE1-72 and IE2-86 alters the cell cycle distribution of asynchronously cycling cells

The small DNA tumor viruses, through the ability of their viral oncoproteins to bind to RB family members and induce E2F transactivation functions, can alter the distribution of cells towards S-phase (108). HCMV expresses two IE gene products, IE1-72 and IE2-86, that can interact with RB family members and induce E2F activity (43, 54, 116, 136). However, it is unclear whether the expression of these IE proteins can alter the distribution of cells towards S-phase. To address this issue, asynchronously cycling REF52 cells were infected with recombinant adenoviruses encoding cDNAs for either IE1-72 or IE2-86, and analyzed for alteration in cell cycle distribution over time.

We utilized recombinant adenovirus technology to express the individual HCMV IE proteins in cells. We used two recombinant adenoviruses, AdIE1-72 and AdIE2-86, in our study (3). To confirm that both recombinant adenoviruses expressed each IE product and to determine if their expression could alter growth control, we infected REF52 cells or MEFs with either AdIE1-72 or AdIE2-86. The REF52 cells line is an immortalized cell line that is permissive to infection with recombinant adenoviruses. REF52 cells efficiently undergo growth arrest in response to serum withdrawal and they express wild-type p53 and RB family
members. They were also the cells used to examine the effects of the adenovirus E1A and E1B proteins on cell growth control (87). Extracts from REF52 cells infected with either AdIE1-72 or AdIE2-86 were analyzed for HCMV IE protein expression. As shown in Figure 3.1, the IE1-72-encoding and IE2-86-encoding recombinant adenoviruses expressed the appropriate HCMV IE proteins albeit at levels different from those observed in the HCMV-infected cells. Cells infected with AdIE2-86 expressed lower levels of IE2-86 protein as compared to the HCMV-infected cells. Specifically, a higher concentration of extract (100 ug) was needed to detect IE2-86 protein in the AdIE2-86-infected cells. In contrast, cells infected with AdIE1-72 expressed the IE1-72 protein at levels exceeding those observed in HCMV-infected HELs. To maintain consistency in our analysis of the IE proteins, 100 ug of extract was used from the AdIE1-72-infected cells and this four-fold difference in concentration may account for the disparity in the levels of IE1-72 protein detected in these cells versus the HCMV-infected cells. Therefore, we utilized these recombinant adenoviruses in our analysis.

The cell cycle distributions from IE1-72-expressing and IE2-86-expressing cells were compared with that of the control virus (AdCon) infected cells. With the expression of either IE1-72 or IE2-86, the distribution of randomly cycling REF52 cells was altered relative to that of the control virus-infected cells. IE1-72 expression caused an increase in the percentage of REF52 cells in S-phase at the later times post-infection (Figure 3.2-B; Table 3-1). Cells expressing IE1-72
Figure 3.1. IE1-72 and IE2-86 expression in REF52 cells infected with AdIE1-72 and AdIE2-86.

Western blot analysis of whole cell extracts from REF52 cells infected with AdIE1-72 or AdIE2-86 (MOI = 250) (100 μg/lane) or HEL cells infected with HCMV (Towne) (MOI = 5) (25 μg/lane). Cells were harvested at the different hours post infection (hpi) indicated. HCMV IE proteins were detected with an anti-cytomegalovirus monoclonal antibody specific for a common determinant found on both IE proteins.
Figure 3.2
Figure 3.2. HCMV IE1-72 and IE2-86 expression disrupts cell cycle progression in asynchronously cycling cells.

Randomly cycling REF52 cells were infected with AdIE1-72, AdIE2-86, or AdCon (MOI = 500). Following infection, cells were fixed at the indicated times post-infection and stained with PI. The levels of PI incorporated by the cells were measured by flow cytometry analysis to assess cellular DNA content. (A) Plots from REF52 cells stained with PI depicting DNA content versus cell number; (B) histograms summarizing flow cytometry analyses performed on REF52 cells which depict the percentage of cells in each phase of the cell cycle are shown in (A). The averaged percentages from two separate experiments are shown.
Table 3.1. Distribution of REF52 cells in S-phase following IE1-72 or IE2-86 expression

<table>
<thead>
<tr>
<th>Recombinant adenovirus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdCon</td>
<td>12.3%</td>
<td>16.3%</td>
<td>17.5%</td>
</tr>
<tr>
<td>AdIE1-72</td>
<td>13.5%</td>
<td>26.4%</td>
<td>35.4%</td>
</tr>
<tr>
<td>AdIE2-86</td>
<td>38.8%</td>
<td>32.0%</td>
<td>44.4%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Recombinant adenoviruses used at a MOI = 500

<sup>b</sup> Times at which cells were harvested and fixed. Results are shown as percent cells in S-phase.
exhibited almost a two-fold increase in the percentage of S-phase cells as compared to the control virus-infected cells at 48 h (26.4% vs. 16.3%, respectively) and 72 h (35.4% vs. 17.5%, respectively) (Figure 3.2-B).

Concomitant with this increase in the percentage of S-phase cells, there was a decrease in the percentage of cells in the G\textsubscript{1} phase of the cell cycle following IE1-72 expression as compared to the control virus-infected cells. At both 48 h and 72 h post-infection, a lower percentage of G\textsubscript{1} cells were observed in the IE1-72-expressing cell population as compared to the control population (48 h: 49.8% vs. 65.6%; 72 h: 51.1% vs. 69.5%, respectively). A similar analysis was performed using asynchronously cycling WT MEFs. Similar to the results observed with the REF52 cells, there was a slight increase in the percentage of S-phase cells (1.5-fold) following IE1-72 expression as compared to the control virus-infected cells (Figure 3.3; Table 3-2).

IE2-86 expression had a more dramatic effect on the cell cycle distribution of REF52 cells than IE1-72. As was observed in the cells expressing IE1-72, the distribution of the cells was altered such that there was an increase in the percentage of cells in S-phase (Table 3-1). By 24 h post-infection, over one-third of the IE2-86-expressing cells appeared to be in S-phase as compared to a smaller percentage of the control virus-infected cells (38.8% vs. 12.3%, respectively). The same trend was observed at the later time points with IE2-86 expression maintaining a two- to three-fold increase in the percentage of S-phase cells as compared to the control virus-infected samples (Table 3-1).
Figure 3.3. HCMV IE1-72 and IE2-86 expression alters the normal cell cycle distribution of $p53^{+/+}$ MEFs.

Randomly cycling $p53^{+/+}$ MEFs were infected with AdIE1-72, AdIE2-86, or AdCon (MOI = 500). Following infection, cells were fixed with at the indicated times post-infection and stained with PI. The levels of PI incorporated by the cells were measured by flow cytometric analysis to assess cellular DNA content. Data presented as the fold-increase in the percentage of S-phase cells relative to control virus-infected cells.
Table 3-2. Distribution of WT MEFs in S-phase following IE1-72 or IE2-86 expression

<table>
<thead>
<tr>
<th>Recombinant adenovirus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdCon</td>
<td>15.6%</td>
<td>10.0%</td>
<td>9.9%</td>
</tr>
<tr>
<td>AdIE1-72</td>
<td>18.2%</td>
<td>16.5%</td>
<td>18.8%</td>
</tr>
<tr>
<td>AdIE2-86</td>
<td>43.6%</td>
<td>40.4%</td>
<td>38.0%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Recombinant adenoviruses used at a MOI = 500

<sup>b</sup> Times at which cells were harvested and fixed. Results are shown as percent cells in S-phase and represent an average of two experiments.
Moreover, IE2-86 expression caused an increase in the percentage of G₂/M-phase cells as compared to the percentage observed for the control samples. By 24 h, almost one-half of the IE2-86-expressing cells (44.1%) were in G₂/M-phase, whereas a smaller proportion of the control virus-infected cells (17.1%) were in G₂/M-phase. Concomitant with the increase in the percentage of S-phase cells, IE2-86 expression caused a three to four-fold decrease in the percentage of cells in G₁ (Figure 3.2).

A similar outcome was observed in WT MEFs. IE2-86 expression mediated an increase in the percentage of S-phase cells (Table 3-2). As shown in Figure 3-3, IE2-86 expression caused a two- to three-fold increase in the percentage of S-phase cells as compared to the control virus-infected cells. Taken together, these results indicate that HCMV IE2-86, and IE1-72 to a lesser extent, can influence the cell cycle distribution of randomly cycling fibroblasts by biasing the distribution towards the S- and G₂/M-phases of the cell cycle.

B. HCMV IE2-86 expression induces quiescent cells to enter S-phase and delays cell cycle exit

Since HCMV IE2-86 expression altered the cell cycle distribution of randomly cycling fibroblasts, we examined whether it could also alter the cell cycle under more stringent measures of growth control. To approach this issue, we examined the ability of IE2-86 to (1) induce quiescent cells to enter S-phase and (2) inhibit cells from exiting the cell cycle. To address whether IE2-86 can
A. S-PHASE INDUCTION

B. CELL CYCLE EXIT

Figure 3.4
Figure 3.4. IE2-86 expression induces quiescent REF52 cells to proliferate and delays cell cycle exit in REF52 cells.

(A) REF52 were rendered quiescent by culturing in the presence of 0.25% serum and then infected with AdIE2-86 (at the MOI indicated) or AdCon (MOI = 500). Cells were maintained in media containing 0.25% serum following infection. (B) Asynchronous cultures of REF52 cells were infected with AdIE2-86 or AdCon (MOI = 500) and then subjected to culture in media containing reduced serum (0.25%) until harvested. Cells were pulsed with 10mM BrdU for 12 h prior to harvesting at the indicated times post-infection. Immunohistochemical staining for BrdU incorporation was performed using an anti-BrdU monoclonal antibody and the number of BrdU-positive cells was scored. The average from two separate experiments are shown in (A) and (B). Error bars represent the standard error of the mean.
induce quiescent cells into S-phase, we expressed IE2-86 in quiescent, serum-depleted cultures of REF52 cells and examined the cells for S-phase induction by determining the number of cells that incorporated the proliferation marker, BrdU. Over 90% of the control virus-infected cells underwent growth arrest following serum withdrawal. Upon addition of serum to the culture medium, over 75% of the cells re-entered the cell cycle. IE2-86 expression in quiescent fibroblasts resulted in almost a 10-fold increase in the population of BrdU positive cells relative to the control virus-infected population (Figure 3.4-A). The ability of IE2-86 to induce S-phase in quiescent cells appeared to be dose dependent, since the strength of the proliferative response correlated with the dose of AdIE2-86.

To determine if IE2-86 expression can delay cells from exiting the cell cycle, we expressed IE2-86 in a population of asynchronously cycling REF52 and monitored for changes in cell cycle exit kinetics following culture in reduced serum. Optimization experiments demonstrated that culturing REF52 cells for 36 h in reduced serum (0.25%) was sufficient to induce the majority of cells to exit the cell cycle as measured by BrdU incorporation (data not shown). REF52 cells were cultured in reduced serum for a minimum of 36 h after infection with the recombinant adenoviruses and pulsed with BrdU for 12 h prior to harvest to assess the impact of IE2-86 on cell cycle exit. By 36 h after serum withdrawal, over 90% of the control virus-infected cells became quiescent (Figure 3.4-B). As expected, cells cultured under normal conditions (10% serum) continued to proliferate.
Cell cycle exit in fibroblasts expressing IE2-86 was delayed as compared to control virus-infected cells. At the highest MOI of AdIE2-86 used, over one-third of the population of cells (34.8%) continued to incorporate BrdU through 42 h following serum withdrawal, whereas less than 10% of control virus-infected cells incorporating BrdU was much lower (8.2%).

To confirm that the cell cycle alterations were due to effects from IE2-86 expression and not a consequence of the recombinant adenovirus approach we employed, we transiently transfected two different plasmids containing IE2-86 cDNAs into REF52 cells prior to serum withdrawal. Using this approach, we obtained BrdU incorporation results that were similar to those obtained with the AdIE2-86-infected cells. Specifically, 40% of the IE2-86-expressing cells stained positive for BrdU incorporation (Table 3-3). These results suggest that IE2-86 expression can delay cell cycle exit for extended periods following serum withdrawal.

Taken together, these findings indicate that IE2-86 modulates cellular proliferation control by inducing growth-arrested cells to enter S-phase and by delaying cells from exiting the cell cycle.

C. Expression of IE2-86 induces proliferation and delays cell cycle exit in WT and p53−/− MEFs

Viral oncoproteins from the small DNA tumor viruses such as adenovirus, SV-40, and HPV have the ability to bind to p53 and inhibit its activity. Several
Table 3-3. Delayed cell cycle exit in cells following IE2-86 expression

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>36h</th>
<th>48h</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHM121&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.5%</td>
<td>45.5%</td>
</tr>
<tr>
<td>pcDNA3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.7%</td>
<td>17.6%</td>
</tr>
<tr>
<td>pcDNA3-IE2-86&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.0%</td>
<td>43.4%</td>
</tr>
<tr>
<td>pcDNA3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.9%</td>
<td>22.5%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plasmids transfected into subconfluent cultures of REF52 cells at 1 μg/well.

<sup>b</sup> Percentage of cells stained positive for IE2-86 and BrdU.

<sup>c</sup> Percentage of cells stained positive for BrdU.
groups have demonstrated that HCMV IE2-86, in addition to binding to pRb, can
interact with p53 and block apoptosis. Additionally, HCMV-infected cells exhibit
elevated p53 protein levels and this may be coincident with the HCMV-mediated
growth arrest observed in these viral infections (69, 104). Because IE2-86 can
bind to p53, we wanted to determine whether targeting of p53 by IE2-86 was
required for its ability to induce S-phase entry as well as delay growth arrest.

To address this issue, we examined the effects of IE2-86 expression in
early-passage MEFs lacking p53 (p53−/−) and their wild-type counterparts (WT).
Although both cell types were derived from the same strain of mice (36), the WT
and p53−/− MEFs required different culturing conditions to yield an optimal level of
growth arrest. The WT MEFs required culturing in 0.25% serum for 48 h to
induce quiescence in these cells, and the p53−/− MEFs required culturing in 0.1%
serum for 60 h to induce arrest (data not shown). The re-addition of serum to the
culture medium stimulated both populations of quiescent MEFs to enter S-phase.

Growth-arrested MEFs were infected with varying doses of AdIE2-86 or
control virus (MOI = 500) and then pulsed with BrdU 12 h prior to harvest to
assess DNA replication levels. Quiescent WT MEFs expressing IE2-86 were
induced to incorporate BrdU as evidenced by the increased percentage of BrdU
positive cells observed relative to control (Figure 3.5-A). By 48 h post-infection,
over 30% of the IE2-86-expressing p53+/− MEFs (MOI = 500) incorporated BrdU
as compared to 10% of the control virus-infected cells.
Figure 3.5

A.

WT MEFs

B.

p53 -/- MEFs
Figure 3.5. IE2-86 expression induces S-phase in quiescent WT and p53−/− MEFs.

(A) Early passage wild-type and (B) p53−/− MEFs were cultured under low serum conditions to induce growth arrest. Cells were then infected with AdIE2-86 (at the MOI indicated) or with AdCon and maintained under low serum conditions after infection. Cells were pulsed with BrdU for 12 h prior to harvesting at the indicated times post-infection. BrdU-positive cells were identified by immunohistochemical staining using an anti-BrdU monoclonal antibody and the number of BrdU-positive cells was scored. Results are representative of two separate experiments for each cell type.
IE2-86 exhibited a similar effect when expressed in quiescent p53−/− MEFs (Figure 3.5-B). While the culture conditions were sufficient to arrest growth for approximately 80% of the p53−/− MEFs, by 48 h post-infection, IE2-86 expression induced almost a four-fold increase in the percentage of BrdU positive p53−/− MEFs (MOI = 500; 84%) as compared to the control virus-infected population (23%). The fold induction in BrdU positive cells was similar in WT and p53−/− MEFs. Therefore, the ability of IE2-86 to induce S-phase from quiescent cells is apparently independent of p53 protein.

We next determined if p53 targeting was required for IE2-86 to delay growth arrest following serum withdrawal. Asynchronously cycling WT and p53−/− MEFs were first infected with AdIE2-86 or AdCon and then cultured in reduced serum to induce growth arrest. Similar to the effects seen in the REF52 cells, IE2-86 expression delayed the ability of WT MEFs to exit the cell cycle following culture in 0.25% serum (Figure 3.6-A). By 48 h post-infection, the percentage of WT MEFs still BrdU positive following IE2-86 expression and serum withdrawal was almost three-fold higher than the percentage observed with the control virus-infected cells (MOI = 500; 39.8% vs. 13.6%, respectively).

Delayed growth arrest by IE2-86 was also observed in cells lacking p53 (Figure 3.6-B). Expression of IE2-86 in the p53−/− MEFs resulted in a three-fold higher percentage of BrdU positive cells relative to the control virus-infected population at 72 h post-infection (46.1% vs. 15.2%, respectively). Taken
Figure 3.6
Figure 3.6. IE2-86 expression delays cell cycle exit in WT and p53\(^{-}\) MEFs. (A) Early passage wild-type and (B) p53\(^{-}\) MEFs were infected with AdIE2-86 (at the MOI indicated) or AdCon (MOI = 500) and then cultured in media containing reduced serum (0.25\%). Cells were pulsed with BrdU for 12 h prior to harvesting at the indicated times post-infection. Cells were immunohistochemically stained for BrdU incorporation using an anti-BrdU monoclonal antibody and the number of BrdU-positive cells was scored. Results are representative of two separate analyses for each cell type.
together, these findings suggest that p53 targeting is not required by IE2-86 to induce quiescent cells to enter S-phase or to delay cell cycle exit.

D. HCMV IE1-72 expression does not induce quiescent cells to enter S-phase and fails to delay cell cycle exit

Since IE1-72 disrupted the normal distribution of randomly cycling cells following the expression of IE1-72 in REF52 cells, we wanted to determine whether IE1-72 could influence cell proliferation control in a manner similar to IE2-86. To address whether IE1-72 could induce growth-arrested cells to enter S-phase, we expressed IE1-72 in serum-starved, quiescent REF52 cells and measured S-phase induction by BrdU incorporation. Expression of IE1-72 had no apparent effect on quiescence since the percentage of BrdU positive cells observed in the AdIE1-72-infected populations was comparable to the percentage observed in the control virus-infected cells (Figure 3.7-A). By 48 h post-infection and at each dose of AdIE1-72 used, less than 10% of the IE1-72-expressing cells were positive for BrdU. This suggests that IE1-72 cannot induce cells to re-enter the cell cycle from quiescence.

To address whether IE1-72 could retard cell cycle exit, we expressed IE1-72 in REF52 cells and subjected them to reduced serum conditions. Unlike IE2-86 expression, IE1-72 expression did not delay cell cycle exit in the REF52 cells (Figure 3.7-B). Analysis at earlier times post-infection and post-serum withdrawal did not show any delay in the kinetics of cell cycle exit following IE1-
A. S-PHASE INDUCTION

B. CELL CYCLE EXIT

Figure 3.7
Figure 3.7. IE1-72 expression fails to induce quiescent REF52 cells to enter S-phase and does not delay cell cycle exit in REF52 cells.

(A) REF52 cells were rendered quiescent by culturing in the presence of 0.25% serum and then infected with AdIE1-72 (at the MOI indicated) or AdCon (MOI = 500). Following infection, cells were maintained in media containing 0.25% serum. Results from one representative experiment are shown. (B) Asynchronous cultures of REF52 cells were infected with AdIE1-72 (at the MOI indicated) or AdCon (MOI = 500) and then subjected to serum withdrawal. Cells were pulsed with BrdU for 12 h prior to harvesting. Immunohistochemical staining for BrdU incorporation was performed using an anti-BrdU monoclonal antibody and the number of BrdU-positive cells was scored. The average from two separate experiments is shown. Error bars represent the standard error of the mean.
72 expression in the REF52 cells. Taken together, these findings suggest that IE1-72 can influence cell cycle progression in asynchronously cycling cell populations. However, it is ineffective in altering the cell cycle under more stringent growth conditions such as inducing quiescent cells to enter S-phase or in delaying cell cycle exit following serum withdrawal.

E. Expression of HCMV IE1-72 induces S-phase entry and delays cell cycle exit in the absence of p53

Although IE1-72 expression caused a modest change in the cell cycle distribution of randomly cycling cells, it failed to induce S-phase entry in quiescent REF52 cells and was unable to delay cell cycle exit following serum withdrawal. This outcome was unexpected given the fact that IE1-72 has been shown to bind to p107 and induce E2F activity (116). Since p53 can induce growth arrest under certain conditions, we asked if the presence of p53 prevents IE1-72 from inducing its proliferative effects.

To address this issue, IE1-72 was first expressed in serum-starved WT and p53−/− MEFs and S-phase induction was monitored by BrdU incorporation. Quiescent WT MEFs failed to re-enter the cell cycle following the expression of IE1-72 as evidenced by the similar percentage of BrdU positive cells relative to the control population (Figure 3.8-A). In contrast, expression of IE1-72 was able to induce the growth-arrested p53−/− MEFs to enter S-phase (Figure 3.8-B). By 48 h post-infection, over a two-fold increase in the percentage of BrdU positive cells
Figure 3.8

A.

WT MEFs

B.

p53−/− MEFs
Figure 3.8. IE1-72 expression induces S-phase only in the absence of p53.

(A) Early passage wild-type and (B) p53-/− MEFs were cultured under low serum conditions to induce quiescence. Cells were then infected with AdIE1-72 (at the MOI indicated) or AdCon (MOI = 500) and maintained under low serum conditions following infection. Cells were pulsed with BrdU for 12 h prior to harvesting at the indicated times post-infection. BrdU-positive cells were identified by immunohistochemical staining using an anti-BrdU monoclonal antibody and the number of BrdU-positive cells was scored. Results are representative of two separate analyses for each cell type.
was observed in the IE1-72-expressing cells as compared to the control virus-infected cells (MOI = 500: 43% vs. 21%, respectively). The ability of IE1-72 to induce cell cycle re-entry was apparent even at the latest time point examined (72 h) where over six-times as many IE1-72-expressing cells (MOI = 500) scored positive for BrdU as compared to the control virus-infected population. The re-addition of serum had a nominal effect on the p53- MEFs, which may be attributed to the stringent conditions required to render these cells quiescent.

To address whether p53 hinders IE1-72 from delaying cell cycle exit, IE1-72 was expressed in WT and p53- MEFs prior to serum withdrawal. IE1-72 expression did not perturb the WT MEFs from exiting the cell cycle following serum withdrawal (Figure 3.9-A). At each of the time points analyzed, the percentage of BrdU positive cells observed in each of the IE1-72-expressing cell populations was less than or equal to the percentage observed in the control virus-infected population. These results are consistent with those obtained by expressing IE1-72 in REF52 cells suggesting that IE1-72 has no effect on delaying cell cycle exit following serum withdrawal in cells containing p53.

IE1-72 had a different effect in the p53- MEFs. Expression of IE1-72 delayed cell cycle exit in the p53- MEFs following culture in 0.1% serum (Figure 3.9-B). After 72 h, over one-third of the IE1-72-expressing cells remained cycling as compared to a smaller percentage of the control virus-infected cells (MOI = 500: 36% vs. 16.5%, respectively). The ability of IE1-72 to delay cell cycle exit was apparent even at the latest time point examined (96 h). Taken together,
Figure 3.9
Figure 3.9. IE1-72 expression delays cell cycle exit in the absence of p53.

(A) Early passage wild-type and (B) p53/- MEFs were infected with AdIE1-72 (at the MOI indicated) or AdCon (MOI = 500) and then serum starved. Cells were pulsed with BrdU for 12 h prior to harvesting at the indicated times post-infection. Cells were immunohistochemically stained for BrdU incorporation using an anti-BrdU monoclonal antibody and the number of BrdU-positive cells was scored. Results are representative of two separate analyses for each cell type.
these findings suggest that p53 can mask the proliferative capacity of IE1-72. In the absence of p53, IE1-72 expression can induce quiescent cells to enter S-phase and can delay cell cycle exit following serum withdrawal.

F. Expression of either HCMV IE1-72 or IE2-86 induces p53 protein accumulation

We have shown that both IE1-72 and IE2-86 can modulate the cell cycle. Expression of the IE2-86 protein has a more prominent effect on proliferation control. It induces S-phase and delays cell cycle exit under all of the growth conditions that we tested. Similarly, we found that IE1-72 has the capacity to exert comparable effects on proliferation control, but only in the absence of p53. Given that expression of the small DNA tumor virus oncoproteins can lead to the accumulation of p53 protein (108), and that under certain circumstances p53 accumulation leads to growth arrest at G1 (153), we determined whether expression of the IE proteins affected p53 protein levels. Although other groups have previously demonstrated that expression of HCMV IE2-86 causes an elevation in the levels in p53 protein in human cells (103, 140), we wanted to verify whether the expression of IE1-72 and IE2-86 induced p53 protein in the rodent fibroblasts used in our analysis.

To address this issue, we infected asynchronously cycling REF52 cells with AdIE1-72, AdIE2-86, or AdCon, and then immunohistochemically stained for p53 protein. Infection with AdCon did not have an effect on p53 levels in the
REF52 cells. In contrast, expression of either IE1-72 or IE2-86 resulted in increased p53 protein accumulation in cells (Figure 3.10). In both instances, the accumulation of p53 protein was localized primarily to the nucleus. This extends the earlier observation demonstrating that the IE2-86 induces p53 protein and causes p53 to become nuclear localized (103, 140). A similar effect was observed in the IE1-72-expressing cells. As a positive control, we infected cells with AdE2F1, a recombinant adenovirus that expresses E2F1, a factor that members of our group have previously shown to induce p53 protein accumulation in rodent fibroblasts (82). As expected, expression of E2F1 caused p53 protein to accumulate in most of the cells.

To further analyze the effect of IE1-72 on p53 protein, we examined p53 levels in IE1-72-expressing WT MEFs by Western blot analysis. As compared to the levels of p53 detected in the control virus-expressing WT MEFs, the cells expressing IE1-72 exhibited almost a two- to three fold increase in the levels of p53 that was apparent as early as 24 h post-infection (Figure 3.11). This outcome is consistent with the result observed in Figure 3.10 and suggests that IE1-72 induces p53 protein accumulation.
Near-confluent cultures of REF52 cells were infected with AdIE1-72, AdIE2-86, or AdCon (MOI 500) and cultured in normal media following infection. Cells were infected with AdE2F1 served as a positive control. At 24 h post-infection, cells were harvested, fixed with formaldehyde, and immunohistochemically stained for p53 using an anti-p53 monoclonal antibody.
Figure 3.11. IE1-72 expression induces p53 protein in WT MEFs.

Western blot analysis was performed on whole cell extracts from WT MEFs infected with either AdIE1-72 or control virus. Cells were harvested at the indicated times post-infection. An extract from Mdm2-/-/p53-/- MEFs was included as a negative control in our analysis. Endogenous p53 protein was detected using a commercially available anti-p53 polyclonal antibody. Actin levels were probed as a loading control.
G. Discussion

We initially set out to characterize the effects of HCMV IE1-72 and IE2-86 on the cell cycle by modeling our analysis of the HCMV IE proteins to that of the studies performed on the human adenovirus E1A and E1B proteins due to the fact that these proteins can perturb the cell cycle by targeting members of the RB family of proteins and p53. The results presented in this chapter suggest that expression of the HCMV IE proteins can modulate the cell cycle, presumably to promote an environment that is conducive for viral replication. Specifically, we found that expression of IE2-86 can drive cells out of quiescence and into S-phase as well as delay cells from exiting the cell cycle into G0. We also observed that IE1-72 could mediate effects similar to those of IE2-86, but only in cells lacking p53. Moreover, we found that expression of IE1-72 or IE2-86 induces p53 protein accumulation in rodent fibroblasts.

Although rodent fibroblasts are not permissive to HCMV replication, we utilized these cells in our analysis because these were the cell types used in the studies of the effects of adenovirus E1A and E1B on the cell cycle (87). The rat fibroblast cell line (REF52) expresses wild-type p53 and RB family members and responds very well to serum withdrawal by undergoing growth arrest. This particular characteristic was vital to our study and enabled us to examine closely the effects of IE1-72 and IE2-86 on the cell cycle under varying levels of growth stringency. Moreover, the use of the REF52 cells in our initial experiments allowed us to establish the conditions necessary for our subsequent studies in
wild-type and p53-deficient mouse fibroblasts. By incorporating fibroblasts from wild-type and p53-null mouse embryos in our study, we could assess the effect of IE1-72 and IE2-86 on the cell cycle in the presence or absence of p53 in cells that are genetically identical without having to worry about complications, such as genetic variability, arising from the use of transformed cell lines that lack or contain mutated forms of p53.

Several reports indicate that HCMV induces certain cells such as human fibroblasts to undergo growth arrest following infection (19, 35, 69, 88). While our findings for IE1-72 and IE2-86 are not consistent with these observations, they are consistent with other reports showing that HCMV infection promotes cell cycle progression in other cell types. Specifically, these studies show that HCMV induces DNA synthesis and promotes events that are characteristic of early S-phase such as pRb hyperphosphorylation, increased cyclin E and Cdk2 protein expression, and activation of cyclin E-cdk2 activity, (20, 69). Thus, these findings suggest that by influencing activities that are associated with S-phase entry, HCMV has the capacity to induce an S-phase-like environment in infected cells. Moreover, HCMV infection induces S-phase in other cell types including endothelial cells, smooth muscle cells, and monocytes (132, 175), it appears that the virus mediates divergent effects on the cell cycle.

The ability of HCMV to differentially affect the cell cycle may be attributed in part to IE1-72 and IE2-86. Because both IE proteins target certain RB family members, one would expect expression of IE1-72 or IE2-86 to influence cell
proliferation in a positive manner. To properly assess this, we employed a strategy where we tested the ability of the IE proteins to influence cellular proliferation under varying levels of growth stringency. Specifically, we examined the effect of IE protein expression on cycling cells (low stringency), cells undergoing growth arrest (medium stringency), and quiescent cells (high stringency). We found that IE2-86 expression alters the distribution of randomly cycling cells towards S-phase and delays cells from exiting the cell cycle. These results suggest that IE2-86 exerts a growth-promoting effect on the cell cycle in all of the growth stringency conditions tested and implies that IE2-86 can modulate the cell cycle to generate an S-phase-like environment in cells.

IE2-86 exerts a similar effect on the cell cycle under high levels of growth stringency. In particular, we found that IE2-86 expression induces quiescent cells to enter S-phase. This observation is consistent with results from other contemporary studies showing that IE2-86 induces entry into early S-phase (105, 160). Given that IE2-86 binding to pRb is sufficient to relieve the pRb-mediated repression of E2F-responsive promoters (43, 54), it is not surprising that IE2-86 expression can drive cells out of G0/G1-phase and into S-phase. Moreover, IE2-86 induces cyclin E expression and can modulate other factors associated with S-phase entry including E2F1, thymidine kinase, and DNA polymerase α (17, 136). Taken together, these findings suggest that IE2-86 can promote a cellular environment that is conducive to proliferation.
We also examined IE1-72 protein to determine if it could influence cell proliferation control. Analogous to IE2-86, there are several lines of evidence that suggest a link between IE1-72 and overcoming cell cycle control. IE1-72 binds to p107 and can overcome the p107-mediated repression of an E2F-responsive promoter (116). Additionally, IE1-72 can phosphorylate the pocket proteins, p107 and p130, as well as several members of the E2F family of transcription factors in vitro (111). Although the significance of these events to the cell cycle is not known, the IE1-72-induced modification of p107 and p130 may influence their ability to repress E2F. Finally, IE1-72 can activate expression from the DHFR and DNA polymerase α promoters (59, 94), both of which are induced during the transition from G1 to S-phase and are factors required for cellular DNA synthesis. Based on these lines of evidence, we assumed that IE1-72 expression could mediate a proliferative phenotype similar to the one observed for IE2-86-expressing cells. Contrary to the observations for the IE2-86-expressing cells, we found that IE1-72 expression does not alter the cell cycle in normal rodent or murine fibroblasts. However, in the absence of p53, IE1-72 expression was sufficient to induce S-phase entry and delay cell cycle exit in MEFs. Thus, these observations indicate that under certain conditions, IE1-72 has the capacity to alter cell cycle control.

The inability of IE1-72 to modulate the cell cycle in the presence of p53 implies a role for p53 in negatively influencing the effect of IE1-72 on the cell cycle. This notion is reinforced by our observations of nuclear p53 accumulation
and increased p53 levels following IE1-72 expression. Given that increases in p53 protein correlate with an increase in p53 protein activity (153), the observed elevation in nuclear p53 protein levels following IE1-72 expression coupled with the lack of a p53-inactivating function ascribed to IE1-72 may account for the inability of IE1-72 to modulate the cell cycle in wild-type fibroblasts. Therefore, it is feasible that the presence of increased p53 protein levels negates the proliferative capacity of IE1-72 through an undefined mechanism.

We also observed nuclear p53 protein accumulation in cells following IE2-86 expression. This observation is consistent with the ability of IE2-86 to stabilize p53 protein levels in cells (44). However, in contrast to IE1-72, IE2-86 can interact with p53 and block its transactivation function (140, 152). In addition to p53, IE2-86 interacts with p21 and abrogates its activity (132). Thus, the capacity of IE2-86 to promote S-phase entry and delay cell cycle exit despite inducing p53 protein in wild-type cells is likely attributed to its ability to inhibit p53 and p21 function.

The results presented in this chapter indicate that the HCMV proteins IE1-72 and IE2-86 are capable of altering cell cycle control. Additional evidence demonstrating the proliferative capacity of the IE proteins stems from a study showing that HCMV infection increases rat SMC proliferation (175). Although non-permissive for HCMV replication, IE1-72 and IE2-86 expression still occurs in the rat SMCs. Taken together, these findings support the idea that HCMV, through the expression of the IE proteins, positively influences cell proliferation.
and provides at least two potential mechanisms by which HCMV can modulate the cell cycle in vivo. In one instance, HCMV infection of quiescent cells may induce these cells to re-enter the cell cycle and promote entry into S-phase. Another possibility is that following HCMV infection, cycling cells continue to progress through the cell cycle due to their inability to growth arrest.
CHAPTER IV

Mechanisms by which HCMV IE1-72 induces p53 protein accumulation

A. HCMV IE1-72 does not affect p53 RNA levels

In the previous chapter, we observed that expression of either IE1-72 or IE2-86 induces p53 protein accumulation in rodent fibroblasts. This finding is consistent with other reports demonstrating that HCMV induces p53 protein (69, 104). The ability of HCMV to alter p53 levels may be attributed to the IE proteins. IE2-86 binds p53 and can transactivate the p53 promoter (103, 140). Unlike IE2-86, IE1-72 cannot bind to p53 and has no effect on the p53 promoter (103). Because IE1-72 expression can alter p53 protein levels in cells, we wanted to elucidate the mechanism(s) by which IE1-72 induces p53 protein accumulation. Given that IE1-72 can function as a transcriptional co-activator of numerous cellular, as well as, viral promoters, we asked if IE1-72 induces p53 protein levels by enhancing its transcription. Specifically, we looked for changes in p53 mRNA levels in REF52 cells expressing IE1-72. We found that IE1-72 expression did not affect p53 mRNA levels in cells (Figure 4.1). This outcome is consistent with the results from transient p53 promoter/reporter assays showing that IE1-72 fails to activate expression from a p53-promoter reporter construct (103). We included mRNA extracts from both mock- and AdE2F1-infected cells as a negative control since E2F1 does not activate expression from the p53 promoter (153). As expected, there was no change in p53 mRNA levels
Figure 4.1. IE1-72 expression does not affect p53 mRNA levels.

Northern blot analysis was performed on mRNA extracts from REF52 cells infected with either AdIE1-72 or a control virus (AdCon) (MOI = 250). Extracts from mock-infected and AdE2F1-infected cells (MOI = 500) were included as controls. mRNA extracts were harvested at 48 and 72 h post-infection. A p53 cDNA probe was used to detect p53 mRNA (expected size = 2.8 kb).
observed between the AdE2F1-infected cells and mock-infected cells. The inability of IE1-72 to affect p53 expression suggests that IE1-72 modulates p53 protein levels through a post-transcriptional mechanism.

B. Expression of HCMV IE1-72 induces p19ARF protein

Because IE1-72 did not affect the expression of p53, we considered the other ways by which IE1-72 could alter p53 protein levels in cells. Normally, p53 has a very short half-life in cells ($t_{1/2} = 20$ min). This is attributed primarily to the MDM2 protein, which promotes the degradation of p53 by the proteosome (45, 46, 58, 63). Functioning upstream of MDM2 is p19ARF, a nucleolar protein that facilitates the stabilization of p53 protein through its ability to bind MDM2 and inhibit its activity (75, 117, 158). Consequently, increases in p19ARF protein enhance the stability of p53 thereby augmenting the levels of p53 protein in cells (117, 170). We examined p19ARF protein levels in REF52 cells expressing IE1-72 via Western blot analysis and found that compared to control virus-infected cells, IE1-72 expression caused almost a three-fold increase in p19ARF protein (Figure 4.2). Therefore, it appears that IE1-72 could modulate p53 protein levels by increasing the levels of p19ARF protein in cells.

C. HCMV IE1-72 expression induces p53 protein levels in the absence of p19ARF

Given that the stabilization of p53 protein can occur through other mechanisms besides the p19ARF/MDM2 pathway, we measured p53 protein
Figure 4.2. IE1-72 expression induces p19ARF protein.

Western blot analysis was performed on whole cell extracts from REF52 cells infected with either AdIE1-72 or a control virus (Adβgal) (MOI = 250). Cells were harvested at the indicated times p.i. Extracts were probed for endogenous p19ARF protein using an anti-p19ARF polyclonal antibody. Actin levels were probed as a loading control.
levels in p19ARF−/− MEFs to determine if p19ARF is necessary for p53 induction following IE1-72 expression. As shown in Figure 4.3, low levels of p53 protein were detected in the uninfected and control virus-infected cell populations demonstrating that infection with the recombinant adenoviruses does not alter p53 levels. Increased levels of p53 protein were observed in the IE1-72-expressing cells in which IE1-72 expression caused up to a two-fold increase in p53 protein levels as compared to the control virus-infected cells. Because others have shown that exposure to UV induces p53 protein MEFs (8, 9), we UV-irradiated p19ARF−/− MEFs and included the extract as a positive control. An abundant amount of p53 protein accumulated in p19ARF−/− MEFs at 24 h following a 60 J/mm² dose of UV radiation. Therefore, these findings suggest that the ability of IE1-72 to modulate p53 protein levels is not solely dependent on p19ARF and raises the possibility that IE1-72 acts through additional mechanism to alter p53 protein levels.

D. HCMV IE1-72 expression increases the levels of phospho-Ser18-p53 in cells

We found that IE1-72 can induce p53 protein in the presence or absence of p19ARF, implying that an additional mechanism exists by which IE1-72 affects p53 protein levels. In addition to the p19ARF/MDM2 pathway, stabilization of p53 protein levels can occur through the covalent modification of p53 (118). A well-studied model of this process is the up-regulation of p53 protein levels following exposure to either UV or ionizing radiation. DNA damage induces p53
Figure 4.3. IE1-72 induces p53 protein in the absence of p19\textsuperscript{ARF}.

Western blot analysis was performed on whole cell extracts from \( p19^{\text{ARF}/-} \) MEFs infected with either AdIE1-72 or control virus (MOI = 250). Cells were harvested at the indicated times p.i. Extracts from uninfected \( p19^{\text{ARF}/-} \) MEFs and \( p53^{-/-} \) MEFs were included as negative controls in our analysis. An extract from UV-irradiated \( p19^{\text{ARF}/-} \) MEFs infected with AdCon was included as a positive control. Endogenous p53 protein was detected using a commercially available anti-p53 polyclonal antibody. Actin levels were probed as a loading control.
phosphorylation at certain N- and C-terminal residues and as a result of these modifications the protein is stabilized (153). Phosphorylation at the Ser\textsuperscript{18} residue of mouse p53 (Ser\textsuperscript{15} on human p53) is a common event in response to DNA damage and enhances p53 stability (118). To test whether IE1-72 induces this modified form of p53, we expressed IE1-72 in WT MEFs and monitored the levels of phospho-Ser\textsuperscript{18}-p53 with a commercial antibody specific for this modified form of p53. As shown in Figure 4.4-A, an almost two-fold higher level of phospho-Ser\textsuperscript{18}-p53 protein was observed in extracts from wild-type cells expressing IE1-72 as compared to extracts from the control virus-infected cells. The levels of phospho-Ser\textsuperscript{18}-p53 following IE1-72 expression remained elevated throughout the time course of the experiment. We performed a similar experiment using p19\textsuperscript{ARF-/} MEFs to determine if p19\textsuperscript{ARF} was required by IE1-72 to increase the levels of the phospho-Ser\textsuperscript{18} form of p53. A greater increase in the levels of phospho-Ser\textsuperscript{18}-p53 was observed in the p19\textsuperscript{ARF-/} MEFs following IE1-72 expression (over twenty-fold as compared to the levels observed in the control virus-infected population), suggesting that the p19\textsuperscript{ARF}/MDM2 pathway was not required for this modification to p53 (Figure 4.4-B). Thus, it appears that in addition to affecting the p19\textsuperscript{ARF}/MDM2 pathway, IE1-72 may also influence p53 protein by inducing the phosphorylation of p53 at Ser\textsuperscript{18}. 
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Figure 4.4. IE1-72 expression increases phospho-Ser^{18}-p53 levels

Western blot analysis was performed on whole cell extracts from (A) WT MEFs and (B) p19^{ARF-} MEFs infected with either AdIE1-72 or a control virus (Adβgal) (MOI = 250). Cells were harvested at the indicated times p.i. Phosphorylated p53 at Ser^{18} was detected using a polyclonal antibody specific for this modified form of p53. Actin levels were detected as a loading control.
E. Phosphorylation of p53 at Ser\textsuperscript{18} following HCMV IE1-72 expression requires ATM

We found that IE1-72 expression in rodent fibroblasts induces the phosphorylation of p53 at Ser\textsuperscript{18}, which corresponds to Ser\textsuperscript{15} on human p53. Several kinases have been shown to phosphorylate p53 at Ser\textsuperscript{18} (or Ser\textsuperscript{15}) following DNA damage including the product of the ataxia telangiectasia mutated (ATM) gene, the ATM-Rad3-related protein (ATR), DNA protein kinase (DNA-PK), and the checkpoint kinase-2 protein (CHK2) (118). These kinases have been shown to be sensitive to caffeine (14, 125). Therefore, we determined if the levels of phospho-Ser\textsuperscript{18}-p53 induced by IE1-72 are affected by caffeine by culturing IE1-72-expressing WT MEFs in media containing increasing doses of caffeine. Consistent with the results observed from our earlier experiments using WT MEFs, in the absence of caffeine, IE1-72-expressing cells displayed a higher level of phospho-Ser\textsuperscript{18}-p53 relative to that seen in the control-virus infected cells (Figure 4.5) as shown previously (Figure 4.4-A). In the presence of caffeine, a dose-dependent decrease in the levels of phospho-Ser\textsuperscript{18}-p53 was observed in IE1-72-expressing cells. Specifically, the reduction in the levels of phospho-Ser\textsuperscript{15}-p53 observed following caffeine treatment was comparable to the decrease in phospho-Ser\textsuperscript{15}-p53 reported by others utilizing caffeine at similar doses to assess ATM and CHK2 activity (125). These results imply that a caffeine sensitive kinase phosphorylates p53 at Ser\textsuperscript{18} following IE1-72 expression. However, it is unclear whether caffeine treatment also mediated an effect on the
AdCon  AdIE1-72

p-Ser^{18}\cdot p53 - ( - ) 1 2.5 5 10  

Caffeine ( mM):

$\alpha$ -actin -

Figure 4.5. Phosphorylation of p53 at Ser^{18} following IE1-72 expression is sensitive to caffeine treatment

Western blot analysis was performed on whole cell extracts from early passage WT MEFs infected with either AdIE1-72 or a control virus (AdCon) (MOI = 250). Cells were cultured in the presence of increasing doses of caffeine for 6 h prior to harvesting at 24 h p.i. Phospho-Ser^{18} form of p53 was detected using a monoclonal antibody specific for this modification. Actin levels were probed as a loading control.
levels of endogenous p53 protein in the IE1-72-expressing cells since we did not examine total p53 protein levels in these cells.

Because we find that treatment with caffeine abrogates phosphorylation of p53 on Ser^{15} and that a caffeine sensitive kinase is required for this modification, we set out to identify the cellular kinase(s) responsible for phosphorylating p53 at Ser^{15} following IE1-72 expression. ATM is a primary signal transducer that is activated following DNA damage or double strand breaks (39). Recent studies have shown that the rapid phosphorylation of human p53 after ionizing radiation-induced damage requires ATM (10, 22, 107). To determine if ATM is required for p53 phosphorylation at Ser^{18} following IE1-72 expression, we utilized dermal fibroblasts from ataxia telangiectasia patients (AT) in our analysis. AT patients have an inactivated ATM gene and as a result, fail to execute many of the cellular responses to DNA damage including phosphorylating p53 at Ser^{15}, due to their inability to express a functional ATM protein. The levels of p53 phosphorylation following IE1-72 expression in AT fibroblasts were compared with the levels observed in age-matched, normal human dermal fibroblasts (WT) expressing IE1-72. IE1-72 expression in the WT fibroblasts resulted in almost a five-fold increase in the level of phospho-Ser^{15}-p53 as compared to the level observed in the control virus-infected WT fibroblasts (Figure 4.6). Concomitant with this observation, the level of total p53 was slightly elevated (1.6-fold) in the IE1-72-expressing WT fibroblasts as compared to the control virus-infected samples. These results are consistent with the results observed in Figures 3.10
Western blot analysis was performed on dermal fibroblasts isolated from a patient with ataxia telangiectasia (AT) or an age and gender-matched normal donor (WT) infected with either AdIE1-72 or a control virus (MOI = 250). Whole cell extracts were harvested at 24 h p.i. Phospho-Ser^{15} forms of p53 were detected using a polyclonal antibody specific for this p53 modification. p53 protein was detected using an anti-p53 polyclonal antibody. Actin levels were probed as a loading control.
and 4.4 where IE1-72 expression causes an increase in endogenous p53 levels and phospho-Ser$^{15}$-53 respectively, in p53$^{+/+}$ MEFs. Furthermore, the disparity seen between the change in phospho-Ser$^{15}$-p53 and total p53 levels following IE1-72 expression (4.8-fold vs. 1.6-fold, respectively) implies that the accumulation of phospho-Ser$^{15}$-p53 observed in the WT fibroblasts does not occur as a result of an increase in total p53 protein.

IE1-72 expression in the AT fibroblasts yielded different results. The level of phospho-Ser$^{15}$-p53 detected in the AT fibroblasts expressing IE1-72 was three-fold lower than that detected in the AdIE1-72-infected WT fibroblasts. Additionally, similar levels of phospho-Ser$^{15}$-p53 were observed in the AT fibroblasts infected with AdIE1-72 or AdCon (Figure 4.6). Moreover, IE1-72 expression had no effect on p53 levels in the AT fibroblasts since the level of total p53 detected in the AdIE1-72-infected fibroblasts was comparable to that observed in the control virus-infected fibroblasts. Taken together, these findings demonstrate that in the absence of ATM, IE1-72 fails to alter the levels of phospho-Ser$^{15}$-p53 as well as total p53.

F. HCMV IE1-72 expression promotes the nuclear retention of p53

We previously found that p53 localizes to the nuclei of IE1-72-expressing cells (Chapter III). One possible explanation for this observation is that IE1-72 inhibits the ability of p53 to shuttle in and out of nucleus from the cytoplasm. The p53 protein contains at least three nuclear localization sequences and one
nuclear export sequence (147, 155). These nuclear localization sequences are sufficient to mediate the shuttling of p53 from the cytoplasm into the nucleus and the nuclear export sequence can mediate the export of p53 from the nucleus. MDM2 can also promote export of p53 out of the nucleus (124, 150).

Recently, it was shown that phosphorylation of human p53 at the Ser\(^{15}\) residue enhances p53 nuclear accumulation by abrogating the ability of p53 to shuttle between the nucleus and the cytoplasm (169). Since IE1-72 expression induced the levels of phospho-Ser\(^{15}\)-p53, we asked if IE1-72 expression also interferes with p53 shuttling. To test this possibility, we generated heterokaryons in which p53-deficient SAOS-2 cells were infected with a recombinant adenovirus encoding a human p53 cDNA, Adp53, and in some instances, AdIE1-72. The infected SAOS-2 cells were then fused with \(MDM2^{+/+}/p53^{+/+}\) MEFs. The resulting heterokaryons were identified via staining for human Ku-86 and the location of p53 assessed by indirect immunofluorescence (Figure 4.7-A). Shuttling of p53 to the mouse nucleus was observed in the majority of heterokaryons formed from SAOS-2 cells expressing p53 alone (Figure 4.7-B). Co-infection with a recombinant adenovirus encoding an \(MDM2\) cDNA, AdMDM2, slightly diminished p53 nuclear shuttling within the heterokaryons whereas cells co-infected with both AdMDM2 and Adp19\(^{ARF}\) exhibited decreased p53 nuclear shuttling as reported previously (data not shown) (169).

In the presence of IE1-72, p53 nuclear shuttling occurred in only 25% of the heterokaryons formed from SAOS-2 cells co-expressing p53 and IE1-72
Figure 4.7
Figure 4.7. IE1-72 expression induces p53 nuclear retention

(A) SAOS-2 cells were co-infected with Adp53 (MOI = 20) and either AdIE1-72 or a control virus. Two hours after infection, cells were re-plated with MDM2+/−/p53−/− MEFs and cultured overnight at 37°C. The cells were then fused together by treatment with PEG and de novo protein synthesis was inhibited with cyclohexamide. Cells were fixed 1 h after fusing. Immunofluorescent detection of human Ku-86 (red) and p53 (green) was performed to identify the human mouse heterokaryons and the location of p53. (B-C) Human-mouse heterokaryons (N=20) were counted from each of the experiments. (D) SAOS-2 cells were infected with Adp53N/C (MOI = 20) and either AdIE1-72 or AdCon and the same procedure as described in (A) was used to assess p53 shuttling. The appearance of p53 protein in the mouse (Ku-86-negative) nuclei was scored as positive for shuttling. The averaged results from two separate experiments are shown in (B). The averaged results from three separate experiments and the standard deviation derived from the mean are depicted in (C and D). Numbers in parentheses indicate MOI of virus used.
(MOI = 100) as compared to heterokaryons containing only p53 (55%) (Figure 4.7-B). Infection of SAOS-2 cells with a higher dose of AdIE1-72 (MOI = 250) prior to fusion resulted in a more dramatic decrease in p53 nuclear shuttling as compared to heterokaryons that contained only transduced p53 (5% vs. 55%, respectively). Co-infection with a control recombinant adenovirus, AdCon, did not impede the ability of p53 to shuttle from human nuclei to mouse nuclei in heterokaryons because p53 nuclear shuttling occurred in an equal percentage of heterokaryons formed from SAOS-2 cells infected with only Adp53 (Figure 4.7-C). This finding demonstrates that the increased vector load did not affect shuttling.

We substituted Adp53 with Adp53N/C, a recombinant adenovirus that encodes a mutant form of p53 whose serine phosphorylation sites have been modified to alanine residues (8), to determine whether p53 phosphorylation is required by IE1-72 to disrupt p53 nuclear shuttling. As shown in Figure 4.7-D, IE1-72 expression had no effect on p53 nuclear shuttling since p53 shuttling was detected in an equal percentage of heterokaryons formed from SAOS-2 cells infected with Adp53N/C either alone or in combination with AdCon. Therefore, the nuclear accumulation of p53 following IE1-72 expression appears to be due at least in part, to the ability of IE1-72 to abrogate p53 nuclear shuttling and that p53 phosphorylation plays a critical role in this process.
G. Discussion

In the previous chapter, we reported that IE1-72 promotes nuclear p53 accumulation in cells. Because increases in p53 protein correlate with an increase in p53 activity (153), this IE1-72-mediated accumulation of p53 likely contributes to the inability of IE1-72 to induce S-phase and delay cell cycle exit in p53-expressing cells. However, the mechanism of p53 protein induction by IE1-72 was unclear. In this chapter, we present evidence that IE1-72 may induce p53 protein accumulation through two possible mechanisms: by inducing (1) p19ARF protein and (2) the phosphorylation of p53 at Ser\(^{15}\). Additionally, we demonstrate that p53 phosphorylation on Ser\(^{15}\) following IE1-72 expression requires ATM. Finally, we show that IE1-72 promotes p53 nuclear accumulation by abrogating p53 nuclear shuttling.

Accumulation of p53 protein occurs in response to different types of stress including DNA damage, hypoxia, and oncogene expression (153). As a consequence of this induction, p53 activity is enhanced leading to either growth arrest or apoptosis. To counter these p53-mediated effects, the small DNA tumor viruses express viral oncoproteins that inactivate p53 (108). By overriding the functions of RB proteins and blocking p53 activity, the small DNA tumor viruses generate an environment that is conducive to the replication of their viral DNA.

Analogous to the small DNA tumor virus oncoproteins, the HCMV IE proteins, IE1-72 and IE2-86, can interact with members of the RB family to
alleviate the RB-mediated repression of E2F-responsive promoters (43, 116). Additionally, IE2-86 interacts with p53 and can block p53 transactivation function (140, 152). As we have demonstrated in the previous chapter, IE2-86 expression is sufficient to induce quiescent cells to enter S-phase and delay exit from the cell cycle. Since IE1-72 also inactivates an RB family member, one might expect IE1-72 to mediate a similar effect as IE2-86. In contrast to this notion, IE1-72 expression is not sufficient to induce S-phase and delay cell cycle exit in cells containing wild-type p53. IE1-72 causes p53 protein accumulation, but does not block its activity. Thus, it appears that the inability of IE1-72 to induce proliferation in the presence of p53 is likely attributed to IE1-72 induction of p53 protein and its incapacity to inhibit p53 function.

We demonstrated by immunohistochemical staining and immunoblot analysis that IE1-72 expression modulates p53 protein levels. However, the molecular mechanism by which IE1-72 induces p53 protein was not known. Given that IE1-72 can co-activate numerous cellular promoters (59, 94), we examined its effect on p53 expression. We found that IE1-72 does not affect p53 mRNA levels. Our observation that IE1-72 does not alter p53 at the transcriptional level is consistent with an earlier report showing that IE1-72 fails to transactivate a transfected p53 promoter-reporter construct (103), and suggests that IE1-72 alters p53 proteins through a more indirect mechanism.

There is an existing precedent that p53 stability can be regulated at the posttranslational level. Normally, p53 exists at low levels in cells due to a short
half-life. The rapid turnover of p53 is attributed to MDM2, which facilitates p53 degradation by the proteosome (45, 46, 58, 63). The p19\textsuperscript{ARF} nucleolar protein can bind to MDM2 and inhibit its activity, thereby stabilizing p53 protein levels (75, 117, 158, 170). Numerous studies have shown that oncogenes such as E2F can stabilize p53 protein levels by inducing p19\textsuperscript{ARF} (12, 120). The p19\textsuperscript{ARF} promoter contains E2F-responsive elements and p19\textsuperscript{ARF} expression is activated by E2F (33). Additionally, both the adenovirus E1A protein and polyomavirus large T antigen require p19\textsuperscript{ARF} to induce p53 protein (31, 85). Therefore, it appears that p19\textsuperscript{ARF} serves as a key regulator of p53 protein in cells.

In light of the fact that p19\textsuperscript{ARF} governs p53 stability, we hypothesized that IE1-72 stabilizes p53 protein through p19\textsuperscript{ARF}. Although we did not assess the effect of IE1-72 on p19\textsuperscript{ARF} transcription, we examined p19\textsuperscript{ARF} protein levels in cells expressing IE1-72. We found that IE1-72 expression induces p19\textsuperscript{ARF} protein. This observation is consistent with the predicted outcome one might expect given that IE1-72 can relieve the p107-mediated repression of E2F-responsive promoters in cells (116). However, in contrast to the E1A protein and middle T antigen, our results demonstrate that IE1-72 can also modulate p53 protein levels in cells lacking p19\textsuperscript{ARF}. This suggests that IE1-72 can function in a p19\textsuperscript{ARF} independent manner to induce p53 and implies that IE1-72 operates through a separate pathway to modulate p53 levels.

It is well accepted that the covalent modification of p53 contributes to its stabilization and activation. In particular, the phosphorylation of p53 at certain N-
terminal serine residues significantly enhances p53 protein stability by disrupting the interaction between p53 and MDM2 (118). Studies of the cellular responses to ultraviolet or ionizing radiation indicate that phosphorylation of Ser\(^{15}\) on p53 (Ser\(^{18}\) on mouse p53) is a major event following DNA damage. This modification leads to the accumulation of p53 protein in the nuclei of cells by hindering p53 nuclear shuttling and likely enhances p53 protein stabilization by reducing the levels of cytoplasmic p53 protein (10, 22, 107, 169). In addition to ultraviolet or ionizing radiation, recent studies have shown that other events can induce the phosphorylation of p53 at this particular residue. For instance, targeting of the pRb protein by the polyomavirus large T antigen is required to induce p53 phosphorylation on Ser\(^{18}\) in polyomavirus-infected rodent fibroblasts (34).

Moreover, our laboratory and others have shown that the expression of certain E2F family members induces the phosphorylation of p53 at Ser\(^{18}\) (33, 120). These findings imply a link between the E2F proteins and p53 phosphorylation at Ser\(^{18}\) (Ser\(^{15}\) on human p53).

Given that IE1-72 expression can bypass p107 function, we hypothesized that IE1-72 induces p53 phosphorylation. Consistent with this supposition, our results show that IE1-72 expression induces the phosphorylation of p53 at Ser\(^{18}\) in MEFs. In addition to the wild-type MEFs, we examined the levels of phospho-Ser\(^{18}\)-p53 in p19\(^{ARF}\)-deficient MEFs expressing IE1-72. We observed a similar outcome in the p19\(^{ARF}\)-/- MEFs as compared to wild-type MEFs. These findings suggest that the IE1-72-mediated phosphorylation of p53 at Ser\(^{18}\) occurs
independent of p19ARF and most likely accounts for the increased levels of p53 protein observed in the p19ARF−/− MEFs following IE1-72 expression.

Besides affecting p53 protein levels, phosphorylation of p53 at Ser15 (the human equivalent to Ser18 on mouse p53) alters p53 nuclear shuttling (169). Our findings demonstrate that IE1-72 expression promotes the nuclear retention of p53. The inability of p53 to shuttle out of the nucleus in the presence of IE1-72 may account for the accumulation of p53 in the nuclei of IE1-72-expressing cells as described in the previous chapter. Moreover, the results from our experiments using p53N/C, a mutant form of p53 in which all of serine residues were changed to alanine residues, further supports the relationship between p53 phosphorylation and the regulation of p53 nuclear shuttling. Taken together, these observations suggest that phosphorylation of p53 at Ser15 contributes to the increase and to the nuclear accumulation of p53 in IE1-72-expressing cells.

IE1-72 has the ability to phosphorylate numerous cellular substrates including p107, p130, and the E2F proteins (1,2, and 3) (111). Although IE1-72 appears to exhibit kinase activity, p53 has not been demonstrated as a direct substrate (94, 111). This lack of evidence implies that another kinase is responsible for the phosphorylation of Ser18 on murine p53 and of Ser15 on human p53 following IE1-72 expression. Our results are consistent with this notion because we find that treating cells with caffeine following IE1-72 expression diminishes phosphorylation of p53 on Ser18. Furthermore, these findings imply that a caffeine-sensitive kinase phosphorylates p53 at Ser18.
Indeed, we find that ATM is required for phosphorylation of p53 on Ser\textsuperscript{15} (Ser\textsuperscript{18} on murine p53) because this modification does not occur in IE1-72-expressing cells from ataxia telangiectasia (AT) patients but does occur in IE1-72-expressing cells from age and sex-matched normal individuals. In the absence of ATM, IE1-72 also failed to alter total p53 levels suggesting that ATM plays an important role in the ability of IE1-72 to induce p53 protein levels in the WT fibroblasts. However, since we did not examine the effect of IE1-72 on p19\textsuperscript{ARF} protein in either AT or WT fibroblasts, we cannot rule out the possibility that p19\textsuperscript{ARF} contributes to part of the observed increase in p53 protein following IE1-72 expression in the WT cells. Furthermore, the results from our analysis with the WT cells suggest that the increase in phosho-Ser\textsuperscript{15}-p53 levels following IE1-72 expression is not due to an elevation in total p53 protein since the ratio of the fold-change in phosho-Ser\textsuperscript{15}-p53 to the fold-change in endogenous p53 was greater than 1:1.

ATM is a member of the phosphatidylinositol 3-kinase-like family and is a primary signal transducer activated following DNA damage or double stranded breaks (39). However, it is unclear how IE1-72 stimulates ATM activity. The activation of ATM occurs in response to defects in the G\textsubscript{1}/S, G\textsubscript{2}/M, and S-phase checkpoints (171). It is feasible that IE1-72 triggers ATM activity by modulating one of these checkpoints through the inactivation of p107 and induction of E2F activity. Additionally, IE1-72 has been described as being potentially mutagenic possibly by blocking apoptosis (90, 131). It is possible that IE1-72 expression
may act as a mutagen and somehow lead to DNA damage or activate components of the DNA damage pathway including ATM. Moreover, IE1-72 phosphorylates a number of cellular proteins (111). Therefore, it is conceivable that IE1-72 directly phosphorylates and induces ATM thereby causing the phosphorylation of ATM targets including p53. Finally, since ATM can phosphorylate and activate other DNA damage-inducible kinases such as CHK1 and CHK2, which also phosphorylate p53 at Ser\(^{15}\) (or Ser\(^{18}\)), it remains unclear whether ATM directly or indirectly mediates this modification in cells following IE1-72 expression.

HCMV induces p53 protein levels following infection in fibroblasts and smooth muscle cells (69, 104, 140). Although it is unclear which viral proteins are responsible for affecting p53 protein levels in infected cells, it appears that the HCMV IE proteins play a role in modulating p53. In support of this inference, the results presented in this chapter suggest that IE1-72 may be one of the proteins that cause p53 protein levels to increase following HCMV infection. Because IE1-72 is immediately expressed in cells following infection, the early increase in p53 protein that is observed in HCMV-infected cells (69, 104) may be attributed to IE1-72. Moreover, as demonstrated in the previous chapter, IE1-72 expression causes cells to undergo growth arrest in the presence p53 and this is likely due to the inability of IE1-72 to block p53 function. Thus, we speculate that IE1-72 may also play part in causing the HCMV-mediated growth arrest that is observed in infected cells.
Like IE1-72, IE2-86 expression also modulates p53 protein levels. IE2-86 stabilizes p53 protein by limiting its degradation (44). Additionally, IE2-86 can bind to p53 and inhibit its transactivation function by tethering a transcriptional repression domain to p53 (140, 152). Therefore, it appears that IE2-86 increases p53 protein levels in cells but can overcome the growth arrest response mediated by p53. This may explain why IE2-86 expression induces S-phase and delay cell cycle exit in p53 wild-type cells whereas IE1-72 cannot. Taken together, the expression of IE1-72 and IE2-86 causes an increase in p53 protein that may account for the elevated p53 protein levels observed in HCMV-infected cells and may contribute to the divergent effects mediated by HCMV on the host cell cycle.
CHAPTER V

Consequences of p53 protein accumulation following IE1-72 expression

1. HCMV IE1-72 expression induces p21 expression

To determine the consequences of the p53 protein induction following IE1-72 expression, we tested for the activation of a p53 transcriptional target. Because p53 transactivates the expression of the p21 promoter upon activation, we determined whether IE1-72 expression activates the p21 promoter in reporter assays. As shown in Figure 5.1, a 17-fold increase in p21 promoter-reporter activity was observed following IE1-72 expression as compared to control plasmid-transfected cells. We also examined p21 protein levels in p53+/+ MEFs infected with either AdIE1-72 or a control virus (AdCon). In contrast to our reporter assay results, IE1-72 expression caused a nominal increase in p21 levels relative to that observed in control virus-infected cells (Figure 5.2).

Additionally, we detected p21 protein in cells infected with AdIE1-72 or Adβgal and then performed immunohistochemical staining for p21 protein. We quantified the number of p21-positive cells in each population (Figure 5.3-A,B). Cells expressing IE1-72 exhibited a relatively higher percentage of p21-positive cells (>50%) as compared to both the mock-infected and control virus-infected cells where less than 30% of the population stained positive for p21 protein during a 48 h time course (Figure 5.3-A). The percentage of p21-positive cells
Figure 5.1. IE1-72 activates expression of a p21-promoter reporter construct.

Human lung fibroblasts were co-transfected with a p21-promoter reporter construct, pWAF-1-Luc, and either an IE1-72 cDNA expressing construct (pcDNA3-IE1-72) or a control plasmid (pcDNA3). A renilla luciferase plasmid was also co-transfected as a normalization control for transfection efficiency in our reporter assays. The averaged results from three separate assays and the standard deviation derived from the mean are depicted in the figure.
Figure 5.2. IE1-72 induces p21 protein.

Western blot analysis was performed on whole cell extracts from early passage WT MEFs infected with either AdIE1-72 or a control virus (AdCon) (MOI = 250). Cells were harvested at 24 h p.i. p21 protein was detected with an anti-p21 polyclonal antibody. Actin levels were probed as a loading control.
Figure 5.3
Figure 5.3. IE1-72 induces p21 protein accumulation.

Subconfluent cultures of REF52 cells were infected with either AdIE1-72 or a control virus (Adβgal) at the MOI indicated and then immunohistochemically stained for p21 protein at 24 and 48 h post-infection. Mock-infected cells were included as a negative control for our analysis to demonstrate the background levels of p21 protein in the cells. REF52 cells infected with Adp53 (MOI = 20) served as a positive control for p21 staining. The number of p21-positive cells within the population of REF52 cells was quantitated. A minimum of 300 cells was scored for each population. The results are presented as the percentage of p21-positive cells within the population and represent the averages from three separate analyses. Error bars represent the standard deviation derived from the mean. (B) Number of p21-positive cells within populations of p53−/− MEFs infected with either AdIE1-72 or a control virus (MOI = 250). p53−/− MEFs infected with Adp53 (MOI = 20) served as a positive control for p21 staining. The result from one representative experiment is shown.
observed following IE1-72 expression was similar to that in cells expressing p53, which served as a positive control for this analysis. To confirm if the induction of p21 protein following IE1-72 expression requires p53, we conducted a similar analysis in p53−/− MEFs. In the absence of p53, there were very few p21-positive cells observed following IE1-72 expression (Figure 5.3-B). As expected, p53 expression in the p53−/− MEFs caused an increase in the percentage of p21-positive cells. Taken together, these findings suggest that IE1-72 expression increases p21 levels through a p53-dependent mechanism.

2. HCMV IE1-72 expression induces quiescent cells to enter S-phase in the absence of p21

We previously demonstrated that IE1-72 is unable to promote S-phase entry in the presence of p53. Because p21 expression is activated by p53 and p21 is required for p53-mediated growth arrest, we determined whether p21 was necessary for the growth arrest phenotype induced by IE1-72 in wild-type cells by testing the ability of IE1-72 to induce S-phase entry in quiescent WT and p21−/− MEFs. We empirically optimized the conditions required to render the p21−/− MEFs and their WT counterparts into quiescence (data not shown). As shown in Figures 5.4A and B, culturing in 0.25% serum media for 48 h was sufficient to induce growth arrest in over 80% of the WT and p21−/− MEFs infected with the control virus. IE1-72 expression had no apparent effect on quiescent WT MEFs. In the presence of p21, only 5.5% of the IE1-72-expressing cells were in S-phase.
A.

B.
**Figure 5.4.** IE1-72 induces quiescent cells to enter S-phase in the absence of p21.

Early passage WT and p21-/- MEFs were cultured in low serum to induce growth arrest. Cells were then infected with AdlE1-72 or a control virus and maintained under low serum conditions. (A) At 24 h post-infection, the cells were harvested and stained with PI. Flow cytometry analysis was performed and the percentage of cells in S-phase was scored. Flow cytometry data is summarized as histograms from each of the different MEF populations. The average percentage of cells in S-phase from two separate experiments is represented as a histogram for each cell type. (B) Cells were labeled with BrdU for 12 h prior to harvesting at the indicated times post-infection. BrdU-positive cells were identified by immunohistochemical staining using an anti-BrdU monoclonal antibody and the number of BrdU-positive cells was scored. The averaged results from two separate experiments are presented as the percentage of cells that stained positive for BrdU incorporation. Error bars shown in (A) and (B) represent the standard deviation derived from the mean.
as compared to 6.8% observed in the control virus-infected cells (Figure 5.4-A). We infected cells with AdE2F1 as a positive control for our experiment and as expected, E2F1 expression induced the quiescent WT MEFs to enter S-phase as evidenced by the increased percentage of cells in S-phase (17%) relative to the control population.

In contrast, in the absence of p21, IE1-72 expression caused an increase in the percentage of S-phase cells as compared to the control virus-infected population (15.1% vs. 8.2%, respectively). As shown in Figure 5.4-A, following IE1-72 expression, the percentage of cells in S-phase was less than that observed in the E2F1-expressing p21/− MEFs (20.9%).

We also examined the levels of BrdU incorporated by quiescent WT and p21/− MEFs infected with AdIE1-72 or AdCon and then labeled with BrdU. As shown in Figure 5.4-B, there was a similar percentage of BrdU-positive cells observed in the IE1-72-expressing WT MEFs as compared to the control virus-infected cells at both time points examined (48h: 15.5% vs. 14.6%, and 72h: 17.0% vs. 13.4%, respectively). In contrast, IE1-72 expression in p21/− MEFs caused an increase in the percentage of BrdU-positive cells relative to the control population (48h: 34.5% vs. 22.1%, and 72h: 35.5% vs. 25.4%, respectively). Taken together, these results suggest that the inability of IE1-72 to induce quiescent cells into S-phase is due to the activation of the p53/p21 growth arrest pathway.
3. HCMV IE1-72 expression delays cell cycle exit in the absence of p21

Because IE1-72 expression was not sufficient to induce S-phase entry in the cells expressing p21, we wanted to verify if the presence of p21 prevents IE1-72 from delaying cell cycle exit following culture in reduced serum. To address this, we infected WT and p21<sup>−/−</sup> MEFs with AdIE1-72 or AdCon prior to serum withdrawal and analyzed for BrdU incorporation. IE1-72 expression failed to delay cell cycle exit in the WT MEFs (Figure 5.5). At 48 h, the percentage of BrdU-positive cells observed in the IE1-72-expressing cell population was similar to that of the control virus-infected cell populations (9.6% vs. 10.5%, respectively).

Contrary to the observations with the WT MEFs, IE1-72 expression delayed cell cycle exit in p21<sup>−/−</sup> MEFs following serum withdrawal. We observed a higher percentage of BrdU-positive cells amongst the IE1-72-expressing cells as compared to the control virus-infected cells such that almost 30% of the cells expressing IE1-72 stained positive for BrdU incorporation as opposed to less than 25% in the control population (35.1% vs. 22.2%, respectively). Additionally, WT and p21-deficient MEFs infected with AdE2F1 served as a positive control for our analysis and in both cell types; E2F1 expression delayed cell cycle exit in both cell types. Overall, these observations are consistent with the results from our cell cycle exit experiments in p53 wild-type and p53<sup>−/−</sup> MEFs and imply that the p53/p21 pathway mediates the growth arrest phenotype induced by IE1-72.
Figure 5.5. IE1-72 expression delays cell cycle exit in the absence of p21.

Early passage WT or p21-/- MEFs were infected with either AdCon or AdIE1-72 (MOI=250) and then cultured in media containing reduced serum. Cells were pulsed with BrdU for 12 h prior to harvesting at 48 h p.i. Cells were immunohistochemically stained for BrdU incorporation using an anti-BrdU monoclonal antibody and the number of BrdU-positive cells was scored. The result from one representative experiment is shown.
4. Discussion

In the previous chapter, we demonstrated that IE1-72 appears to modulate p53 protein levels by inducing p19\textsuperscript{ARF} protein and the phosphorylation of p53 at Ser\textsuperscript{18} (Ser\textsuperscript{15} on human p53). Here, we report that IE1-72 induces p21 protein in a p53-dependent manner and causes a p21-dependent growth arrest in cells following IE1-72 expression.

Due to the fact that increases in p53 protein correlate with an increase in its transcriptional activity, we examined the consequences of p53 induction following IE1-72 expression. We focused our analysis on p21 due to the fact that it plays a critical role as a downstream effector molecule in the p53-mediated growth suppressive pathway. Specifically, the p21 protein functions as a cyclin-dependent kinase inhibitor whose expression inhibits cell cycle progression (41, 57, 161). Because IE1-72 expression causes growth arrest in wild-type cells, we hypothesized that IE1-72 expression alters p21 protein levels analogous to that of p53 and that this contributes to the growth arrest phenotype observed in IE1-72-expressing cells.

We employed three different assays to assess IE1-72 effect on p21 expression. Consistent with our hypothesis, we observed that IE1-72 induces p21 protein expression. Although the levels of p21 protein detected in extracts from IE1-72-expressing MEFs do not reflect the increased level of p21-promoter activity observed following IE1-72 expression, the results from our
immunohistochemical analyses support the notion that IE1-72 influences p21 protein levels in a p53-dependent manner.

One possible explanation for the disparity between the results from our immunoblot analysis and our reporter assay is that p21 stability may be compromised in IE1-72-expressing cells. Support for this hypothesis stems from the fact that p21 levels fluctuate in HCMV-infected fibroblasts (27). HCMV induces a temporary increase in p21 protein during IE times that precedes a drop in p21 protein levels. The decrease in p21 protein following HCMV infection is caused by the virus-mediated increase in calpain activity, which enhances the degradation of p21 (27). While it is not apparent which HCMV protein is responsible for increasing calpain activity in HCMV-infected cells, it is possible that IE1-72 is the viral factor that promotes the calpain-mediated degradation of p21 following infection. In this manner, IE1-72 induces p21 protein but at the same time, may act to limit the levels of p21 protein in cells by facilitating its degradation. Moreover, our observation that IE1-72 induces p21 protein raises the possibility that IE1-72 is responsible for (or contributes to) the induction of p21 in HCMV-infected cells at IE times.

We extended our analysis to determine whether p21 plays a role in abrogating the S-phase-inducing effect of IE1-72 in wild-type cells. In cells lacking p53, IE1-72 expression induces S-phase, and this is likely due to the absence of a p53-mediated growth arrest response. Consistent with this observation, we find that IE1-72 also induces S-phase in cells lacking p21. Thus,
it appears that p21 also plays a role in blocking the ability of IE1-72 to promote S-phase entry in quiescent cells as well as to delay cell cycle exit. Therefore, we propose the following model in which IE1-72 induces p53 and this results in p21 protein induction. The end result to this chain of events is a p53-mediated growth arrest and this accounts for the growth arrest phenotype previously described for the IE1-72-expressing cells in Chapter III.
A. Thesis Overview

The initial goal of this study was to determine whether the HCMV IE proteins, IE1-72 and IE2-86, could modulate the cell cycle in a manner similar to the oncoproteins of the small DNA tumor viruses. There is existing evidence that the small DNA tumor viruses can alter the cell cycle to facilitate the efficient replication of their viral DNA. The ability of adenovirus, SV-40, and HPV to manipulate the cell cycle is dependent upon the expression of viral oncoproteins that neutralize the function of p53 and the RB family of proteins (108). Both HCMV IE1-72 and IE2-86 exhibit similar properties to the small DNA tumor virus oncoproteins in their ability to target RB family members. Binding of IE1-72 and IE2-86 to their respective RB target alleviates the repression of E2F-responsive genes and the growth suppressive effects mediated by pRb and p107 (43, 116). Additionally, IE2-86 binds to p53 and inhibits its transactivation function (16, 140, 152). Given the functional similarities between the HCMV major IE proteins and the small DNA tumor virus oncoproteins, we hypothesized that IE1-72 and IE2-86 expression alters cell cycle regulation in cells.

The data presented in this thesis demonstrate that expression of the HCMV IE proteins can indeed alter cell cycle control. Specifically, IE2-86 expression is sufficient to drive cells out of quiescence and into S-phase as well
as delay cells from exiting the cell cycle into $G_0$. IE1-72 can mediate a similar effect as IE2-86 but only in cells lacking either p53 or p21. IE1-72 induces the nuclear accumulation of p53 and this IE1-72-mediated induction of p53 protein correlates with its inability to induce S-phase entry and delay cell cycle exit in the presence of p53. The data also show that IE1-72 expression alters p53 protein levels by affecting the p19$^{ARF}$/MDM2/p53 pathway and by activating the ATM kinase, a component of the DNA damage response, to induce the covalent modification of p53, which also promotes p53 nuclear retention.

B. HCMV IE proteins

Our observation that IE2-86 induces cells to enter S-phase was contemporary to the findings reported by other groups (105, 160) and is consistent with the outcome one would expect from IE2-86 inactivating pRb protein. IE2-86 induces E2F activity as evidenced by the multitude of E2F-responsive genes that are activated in IE2-86-expressing cells (136). Among the genes that are activated following IE2-86 expression are cellular factors that regulate the cell cycle, function in nucleotide biosynthesis, and mediate the initiation of cellular DNA synthesis. Additionally, IE2-86 directly activates cyclin E gene expression (17), which is essential for progression of the cell cycle into S-phase. The IE2-86-mediated elevation in cyclin E levels and concomitant increase in cyclin E-cdk2 activity may enhance the phosphorylation of pRb and contribute towards the further induction of E2F activity. Thus, these findings
support the notion that IE2-86 promotes cell cycle progression towards the G1/S transition point and entry into S-phase.

Because IE1-72 interacts with p107 and can alleviate the p107-mediated repression of E2F-responsive promoters (116), one would predict that like IE2-86, IE1-72 should induce E2F activity. IE1-72 expression stimulates transcription from the promoters of several E2F-responsive genes such as DHFR and DNA polymerase α (59, 94). Therefore, it appears that IE1-72 can induce E2F activity; however, it is unclear whether IE1-72 can activate other E2F-responsive genes. Given the large number of genes that contain E2F-responsive elements in their promoter sequence, I speculate that IE1-72 affects the expression of many other E2F-specific genes. Moreover, there is a high probability that some degree of overlap exists between the E2F-responsive genes activated by IE1-72 and IE2-86. Hence, the ability of the HCMV IE proteins to induce E2F activity and the concomitant activation of factors associated with the cellular DNA replication machinery emphasizes their similarity to the small DNA tumor virus oncoproteins.

Since p107-E2F complexes accumulate at or near the G1/S boundary (177), one would hypothesize that the disruption of these complexes by IE1-72 should promote S-phase entry. IE1-72 can induce S-phase entry, but only in the absence of either p53 or p21. The fact that IE1-72 promotes S-phase entry in cells deficient for either protein underscores the inability of IE1-72 to block p53 activity, which in this case results in growth arrest. The importance of overcoming p53-mediated arrest is exemplified by our findings from the cell cycle
experiments conducted in WT cells expressing IE2-86. In the presence of p53, IE2-86 can induce S-phase entry and I suspect that this may be attributed in part to its capacity to inhibit p53 activity (140, 152). The ability of IE2-86 to block p53-mediated growth arrest most likely stems from the p53-repression domains present on IE2-86 (152). As shown in Figure 1.5, the p53-repression domains (aa 45 to 135; aa 290 to 579) contain sequences that originate from exon 5 of the IE2-86 transcript and are unique to IE2-86. This probably accounts for the divergent effects exhibited by each of the HCMV IE proteins on p53 activity where IE2-86 expression can overcome p53-mediated growth arrest whereas IE1-72 cannot. Overall, these findings imply that p53 acts as an important hurdle that must be bypassed in order to transit G1 to S-phase.

C. Proposed model for the effect of the HCMV IE proteins on cell cycle control

Based on the data presented in this thesis, I propose the following model to account for the effect of IE1-72 and IE2-86 on the cell cycle (Figure 6.1). Both IE1-72 and IE2-86 inactivate certain RB family members and consequently induce E2F activity. E2F induction results in the activation of S-phase genes and promotes the subsequent entry of cells into S-phase. As demonstrated in my thesis, IE1-72 expression promotes p53 protein accumulation and this leads to the induction of p21 protein. As a consequence of these events, IE1-72 is not able to induce S-phase entry. The IE1-72-mediated induction of p53 protein, coupled with the inability of IE1-72 to override p53 activity, effectively masks the
Figure 6.1. Model illustrating the effect of HCMV IE proteins on cell cycle control.
growth promoting effects of IE1-72 and may account for the growth arrest phenotype observed when IE1-72 is expressed in WT cells (Chapters III and V).

IE2-86 also promotes p53 protein accumulation. In contrast to IE1-72, IE2-86 can interact with p53 and down regulate its activity (140, 152). Moreover, a recent study showed that IE2-86 also binds to p21 and represses its cyclin-dependent kinase inhibitor activity (132). Thus, the ability of IE2-86 to block p53 function, as well as inhibit the activity of one of its downstream target proteins (i.e. p21), may allow the S-phase promoting activity of IE2-86 to take place unimpeded.

Our findings suggest that IE1-72 and IE2-86 can modulate the cell cycle towards an “S-phase-like” environment, however it is unclear why HCMV encodes two separate IE gene products to mediate this effect. One possible explanation is that HCMV expresses IE1-72 and IE2-86 in order to maintain an “S-phase-like” environment throughout the course of infection. Because IE1-72 is the predominant IE gene product that is initially expressed following infection, the expression of IE1-72 may promote an “S-phase-like” environment in the host cell during the early course (i.e. the first 24 h) of infection. However, since IE1-72 expression may also contribute to both the early increase in p53 protein following HCMV infection as well as the growth arrest that is observed in some HCMV-infected cells, the “S-phase-like environment” that is induced by IE1-72 may be negated. On the contrary, IE2-86, through its ability to block p53 and p21 function, may offset the growth arrest induced by IE1-72 and cause the
infected cells to maintain this “S-phase-like” state. Detectable levels of IE2-86 can be seen as early as 24 h post infection. Since IE2-86 expression is delayed relative to that of IE1-72, one would expect the “S-phase-like environment” mediated by IE2-86 to prevail during the latter course (> 24 h) of infection. Taken together, these events may account for the differential effects mediated by HCMV on the cell cycle.

D. Relevance to HCMV biology

1. HCMV and proliferative disease

Despite the evidence presented in this thesis that expression of the HCMV IE proteins can alter cell cycle control, HCMV does not appear to be oncogenic. However, the virus exhibits the capacity to transform certain types of cells in vitro (6, 15, 48). Although it is unclear how HCMV transforms these cells, it has been speculated that IE1-72 and IE2-86 play a role in this process by possibly causing genetic instability (90, 131). While there is no clear evidence that expression of the IE proteins causes DNA damage in vivo, HCMV can induce specific breaks on chromosome 1 at the 1q42 and 1q21 loci (42). This virus-induced damage is believed to occur while the cells are in S- or G2/M-phase. As demonstrated in my thesis, the IE proteins have the capacity to induce cells past the G1/S transition point. Moreover, I show that IE1-72 activates the ATM kinase, a primary signal transducer that is triggered following DNA damage or double strand breaks (39). Although it is unclear how IE1-72 induces ATM activity, the activation of this
kinase may signify DNA damage incurred by cells following IE1-72 expression. Thus, in addition to driving cells towards an "S-phase-like" environment, the IE proteins may inadvertently induce chromosome damage within the HCMV-infected cell.

There is evidence that implies a link between HCMV and the development of restenosis, a proliferative disorder linked to coronary angioplasty that is characterized by the overproliferation and increased migration of SMCs to the blood vessel injury site. HCMV seropositive patients are more likely to develop restenosis following balloon angioplasty as compared to HCMV seronegative patients (173). In addition, HCMV DNA and protein can be detected in SMCs grown from restenotic lesions. In fact, IE2-86 protein has been detected in lesions taken from restenosis patients (140). Our findings provide a possible mechanism to account for how the IE proteins may contribute to the overproliferation of SMCs that is characteristic of this proliferative disorder. Expression of the IE proteins in newly-infected SMCs or following HCMV reactivation in latently infected SMCs in vivo may promote deregulated proliferation of these cells by either inducing them to enter S-phase or by hindering their ability to undergo quiescence. In both instances, the SMCs would continue to proliferate, and this may subsequently lead to the over-accumulation of SMCs along the vessel wall indicative of restenosis.

Although the perturbation of cell cycle control appears to be one of the ways that HCMV may contribute towards the development of restenosis, other
virus-mediated events seem to play a role in this disorder. It is well accepted that the accumulation of SMCs along the vessel wall is attributed to complex mechanisms that include factors influencing both SMC proliferation and migration. HCMV induces the expression of several cytokines that may enhance the proliferation of SMCs. It has been suggested that HCMV may indirectly enhance SMC migration by increasing the expression of the PDGFβ receptor in infected SMCs (175). The virus also directly promotes SMC migration through the expression of the US28 gene product, an HCMV-encoded chemokine receptor (148). The expression of this chemokine receptor, coupled with the release of many chemokines that activate the Us28 protein, may induce the infected SMCs to migrate and localize to the site of injury along the vessel wall. Taken together, these observations provide a plausible link between HCMV and the development of restenosis and offer an explanation as to how the virus can contribute to both the overproliferation and accumulation of SMCs along the vessel wall.

2. Significance to HCMV DNA replication

   From the standpoint of HCMV infecting a cell, it is imperative to generate an environment that is conducive for viral DNA replication. Analogous to the small DNA tumor viruses, HCMV normally infects quiescent, non-cycling cells in vivo; cells that have a limited quantity of deoxyribonucleotides and co-factors available for the replication of viral or cellular DNA. This presents an unfavorable condition for viral DNA replication. To offset this situation, HCMV expresses
several proteins that can alter the cell cycle towards a more favorable environment, S-phase (Figure 6.2A; top panel). As demonstrated in my thesis, two of the HCMV IE gene products exhibit the capacity to alter cell growth control towards S-phase. In addition to IE1-72 and IE2-86, the ability of HCMV to stimulate the cellular replication machinery towards an S-phase-like environment may be due in part to the pp71 tegument protein, which can also induce cells to enter S-phase (74). One might predict that the presence of these viral proteins in HCMV-infected cells should promote cell cycle progression towards S-phase. Indeed, many of the events that are associated with the progression through G1 and entry into S-phase are observed in HCMV-infected cells. However S-phase, as measured by host DNA replication does not occur in these cells. Rather, S-phase progression, or at least cellular DNA replication, is interrupted and this may be due to the effect of viral proteins that have a negative impact on the host cell cycle (Figure 6.2A; bottom panel). As shown in Chapters III and IV, IE1-72 induces p53 and this appears to result in a p53-mediated growth arrest in IE1-72-expressing cells. In addition to IE1-72, the pUL69 protein induces cells to growth arrest in late G1 or alternately, specifically inhibits cellular DNA replication (89). Through this strategy, which may well involve other viral proteins, HCMV activates host cell factors that appear to facilitate the replication of the viral genome and also ensures that replication of viral DNA rather than cellular DNA occurs within the infected cell as has been observed (19, 35, 69, 88).
Figure 6.2. Model illustrating the effect of HCMV on the cell cycle.

(A) Schematic representation of the HCMV viral gene products and the host cell factors that they target. Also shown are the putative effects mediated by IE1-72, IE2-86, pp71, and pUL69 on the cell cycle to promote the transition from G0/G1 to G1 and into early S-phase. See text for details. (B) Graph representing the relative proteins levels (shown on the Y-axis) of HCMV IE1-72, IE2-86, pUL69, and pp71 that are observed during the course of an HCMV infection.
To ensure that an environment favoring viral DNA synthesis is maintained within the infected host cell, HCMV expresses multiple gene products that can alter the cell cycle at different times throughout the duration of the infection (Figure 6-2B). pp71 and pUL69 may be the first proteins to affect the host cell cycle during an HCMV infection. Both tegument proteins are released into the host cell following virus binding and entry. The pp71 protein may serve as the initial stimulus that drives the infected cell into S-phase whereas the pUL69 protein may counter this effect by causing the cell to arrest in early S-phase. Following virus entry, pp71 and pUL69 degrade and as a result, their opposing effects on the cell cycle wane during the immediate early phase of infection. However, IE1-72 expression also occurs at this time and this may prolong the block in the cell cycle due to the ability of IE1-72 to promote S-phase entry while inducing growth arrest. Subsequent to IE1-72, IE2-86 expression takes place and since IE2-86 can activate numerous factors required for DNA synthesis as well as drive cells into S-phase, one might expect cellular DNA replication to occur within the infected cell. Concomitantly, expression of the early gene product, pp71, also occurs during this time and this event may augment the S-phase promoting effect mediated by IE2-86 during the early phase of an HCMV infection. In spite of this, the continued expression of IE1-72 along with the early gene product, pUL69, may impede the putative S-phase progression mediated by IE2-86 and pp71 and cause an S-phase arrest in the host cell that persists through the early and late phases of infection. By this process, HCMV may
enhance the replication of its DNA by activating the host cell components needed for DNA synthesis while deterring cellular DNA replication.

It is clear that HCMV mediates divergent effects on the cell cycle (23, 74) and this may be attributed to the expression of the abovementioned viral gene products that can modulate the host cell cycle. HCMV infection causes human fibroblasts to arrest in what was thought to be late G₁ or G₂/M (19, 35, 69, 88). Although the virus induces what is characterized as a “G₁ arrest” in infected human fibroblasts, biochemically these cells exhibit hallmarks of S-phase that include pRb hyperphosphorylation, elevated levels of cyclin E, cyclin B, and PCNA, and increased cyclin E and cyclin A kinase activity (19, 35, 69). The expression of several S-phase genes including DHFR, DNA polymerase α, and topoisomerase II are also observed in HCMV-infected fibroblasts. In addition, recent evidence indicates that HCMV can induce S-phase in other cell types including monocytes and smooth muscle cells (132, 175), which are among the cells targeted by HCMV in vivo. Given the activation of S-phase associated factors and induction of S-phase entry in spite of the block in host cell DNA replication, it appears that HCMV may be inducing an “early S-phase arrest” rather than a G₁ arrest. By arresting cells during the stage of the cell cycle when the factors associated with DNA replication are at their peak, HCMV may provide a more favorable environment for viral DNA replication. In this manner, HCMV DNA replication may resemble that of the small DNA tumor viruses.
E. Viruses and the cell cycle

1. Small DNA viruses

Adenovirus and SV-40 serve as good paradigms for how the small DNA viruses can modulate the host cell cycle to achieve their overall goal: to replicate their viral DNA. Because both of these viruses lack accessory factors that support DNA replication, adenovirus and SV-40 virus have evolved a strategy to deregulate the normal control of S-phase in order to induce the cellular genes necessary for DNA replication (121).

2. Herpesviruses

Contrary to the small DNA viruses, the larger DNA viruses, such as the herpesviruses, appear to have a more complex relationship with the cell cycle. HSV, an alphaherpesvirus, induces cells to growth arrest and blocks cellular DNA synthesis (30, 40, 134). HCMV, which is a betaherpesvirus, mediates a divergent effect on the host cell cycle through its ability to activate factors necessary for DNA synthesis while blocking cell cycle progression and inhibiting host DNA replication. In contrast to HSV and HCMV, the absence of an efficient lytic replication system for the gammaherpesviruses, such as EBV, makes it difficult to fully assess the viral-mediated effects on the cell cycle. However, treatment of latent EBV-infected B-lymphocytes with lytic cycle-inducing agents induces cells to growth arrest (47, 76, 119). Moreover, each of the above-mentioned herpesviruses encodes a protein(s) that is capable of inhibiting the cell cycle. HSV expresses two proteins, ICP0 and ICP27, which inhibit the cell
cycle at G₁ (62, 86, 135). HCMV also expresses at least two gene products, IE1-72 (as shown in Chapters III and V) and pUL69 (89), that can cause cells to growth arrest. Expression of the EBV lytic protein, BZLF1, also blocks the cell cycle (25). Thus, it seems that inhibiting the host cell cycle is a significant step in herpesvirus replication.

One could argue that herpesvirus DNA replication is not cell cycle dependent because these viruses cause cells to growth arrest. Additional support for the notion that herpesvirus DNA replication is cell cycle independent stems from the fact that unlike the small DNA tumor viruses, the herpesviruses encode factors that support viral DNA synthesis. All of the herpesviruses express a large assortment of enzymes involved in nucleic acid metabolism including thymidine kinase, thymidylate synthetase, ribonucleotide reductase, and dUTPase as well as factors that play a role in viral DNA replication (DNA polymerase, helicase, and primase) (121). Experiments using specific HSV-deletion mutants provide additional evidence that viral DNA replication is not entirely dependent on the cell cycle. Infection with HSV mutants that fail to express ICP0, thymidine kinase, or ribonucleotide reductase fail to replicate in growth-arrested cells but can replicate to wild-type levels in cycling cells (21, 50, 68) implying that HSV replication is not dependent on the cell cycle.

Contrary to this argument, it seems that certain S-phase-associated factors are necessary for the replication of several herpesviruses. For example, viral DNA synthesis by HSV and HCMV is inhibited in the presence of
roscovitine, a Cdk inhibitor, or in cells expressing a dominant negative form of Cdk2 (18, 127) suggesting that Cdk2 activity is required by these viruses for DNA replication. However, this does not rule out the possibility that other Cdks may be necessary for HSV and HCMV DNA synthesis. In addition to the apparent requirement for Cdk2 activity, topoisomerase II may also needed for the efficient replication of some herpesviruses. Both topoisomerases I and II are required for EBV lytic replication (78). HCMV induces the expression of cellular topoisomerase II and the treatment of HCMV-infected cells with a topoisomerase II inhibitor blocks viral DNA replication (13). These findings suggest that topoisomerase II might be essential for both EBV and HCMV replication. In light of the cell cycle block mediated by the herpesviruses, it appears that these viruses require the activation of S-phase to efficiently replicate. Taken together, these findings suggest that for some herpesviruses such as HCMV and EBV, viral DNA replication may be cell cycle dependent.

Furthermore, targeting of the RB proteins and p53 by certain herpesviruses implies a link between viral DNA replication and the cell cycle. HCMV IE1-72 and IE2-86 can each interact with RB family members (43, 54, 116). IE2-86 can also bind to p53 and inhibit its ability to transactivate p53-responsive promoters (140, 152). Similarly, EBV encodes two IE gene products that target pRb and p53. The EBV BRLF1 protein binds to pRb and induces free E2F levels (166) and transient expression of BRLF1 is sufficient to induce S-phase in quiescent cells (149). The BZLF1 protein binds p53 and abrogates its
transactivation function (167). Alternatively, KSHV encodes a viral cyclin (vCYC) that associates with cellular CDK6 that can phosphorylate RB proteins as well as activate cyclin A expression (26, 38, 49, 84). In addition, the gammaherpesviruses express several latency-associated viral gene products that potently alter cell cycle regulation, which most likely contribute to the ability of these viruses to transform cells following infection (78). Overall, targeting and inactivation of the key cell cycle regulatory proteins by several herpesvirus proteins imply a connection between modulating the cell cycle and virus replication.

The replication of the small DNA tumor viruses such as adenovirus and SV-40, is dependent on their ability to induce quiescent cells to enter S-phase. Parvovirus, which is the smallest DNA animal virus known to man, only replicates when infected cells enter S-phase (106). These small DNA viruses utilize cellular factors that become or are active during S-phase. The replication of some large DNA viruses, such as the herpesviruses, appears to follow a similar pattern. As mentioned earlier, herpesvirus replication is dependent on the availability of certain S-phase associated factors in infected cells. Therefore, it appears that large DNA viruses, as represented by the herpesviruses, share an important trait with the small DNA viruses, that is the need to activate some components of the host cell replication machinery to ensure efficient virus replication.
F. Concluding remarks

The data presented in this thesis demonstrate how the HCMV IE proteins, IE1-72 and IE2-86, can alter cell cycle control. The contribution of HCMV IE proteins to the divergent effects of HCMV on the cell cycle has been addressed. In addition, the potential role of the IE proteins in the development of restenosis, a proliferative disorder that is linked to HCMV infection, has been described. Furthermore, evidence has been provided suggesting that like the small DNA viruses, some large DNA viruses require factors present during S-phase, to facilitate efficient virus replication. Because HCMV encodes gene products that can manipulate the host cell cycle in a multitude of ways including: promoting S-phase, inhibiting host DNA synthesis, and preventing infected cells from arresting in G₁ or undergoing apoptosis, I believe that HCMV serves as a good paradigm that represents the complex relationship between large DNA viruses, such as the herpesviruses, and the cell cycle.
Chapter VII

References


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