# Subversion of Cell Cycle Regulatory Pathways

**V. Sanchez, D. H. Spector(✉)**

**Contents**



**Abstract** Human cytomegalovirus (HCMV) has evolved numerous strategies to commandeer the host cell for producing viral progeny. The virus manipulates host cell cycle pathways from the early stages of infection to stimulate viral DNA replication at the expense of cellular DNA synthesis. At the same time, cell cycle checkpoints are by-passed, preventing apoptosis and allowing sufficient time for the assembly of infectious virus.

## The Host Cell Cycle

To understand the effects of HCMV on the host cell cycle, it is necessary to first present a brief overview of the phases of the cell cycle and the major regulatory proteins involved (see Fig. 1). The cell cycle is a finely orchestrated sequence of

D.H. Spector
Department of Cellular and Molecular Medicine and The Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA 92093-0712, USA
dspector@ucsd.edu

T. E. Shenk and M. F. Stinski (eds.), *Human Cytomegalovirus.*
*Current Topics in Microbiology and Immunology 325.*

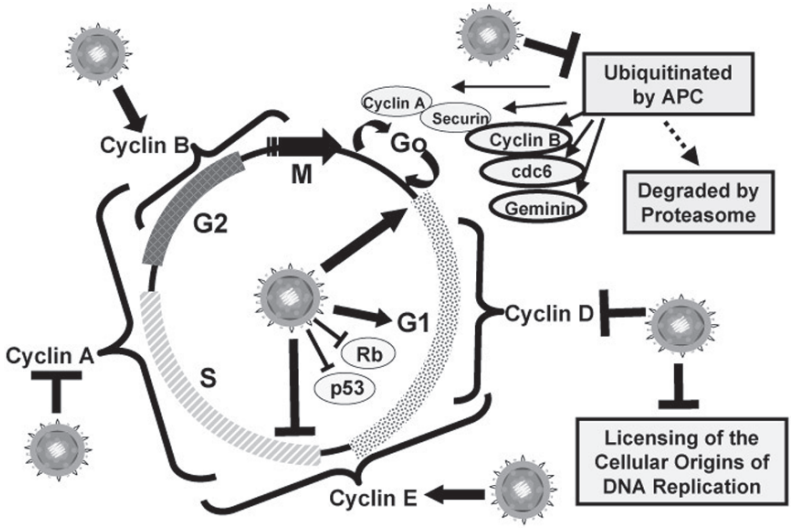


**Fig. 1** Effects of HCMV on the cell cycle. The cell cycle is a tightly regulated process through which the cell replicates its DNA and divides into two daughter cells. $G_0$ is the resting state, and cells are stimulated through growth signals to express cyclin D and enter $G_1$ phase. During $G_1$ phase, the anaphase-promoting complex (*APC*), an E3 ubiquitin ligase, remains active, and proteins that are not needed by the cell until later in the cell cycle are ubiquitinated and targeted for degradation by the proteasome. A prereplication complex (licensing) is established at cellular origins of DNA replication and cyclin E is induced. Replication of the cellular DNA in S phase requires cyclin A. $G_2$ phase follows S phase and marks the transition prior to cell division in M phase. Both cyclin A and cyclin B are required during the $G_2$/M period. The major guardians of the cell cycle are Rb and p53. Infection of cells during $G_0/G_1$ phase induces progression through $G_1$. However, the normal expression of the cyclin-dependent kinases is disrupted, and the cell cycle is blocked before the replication of cellular DNA. HCMV specifically inhibits the expression of cyclin D and cyclin A, but promotes expression of high levels of cyclin E and cyclin B. In infected cells, the activity of the APC is blocked and licensing of the cellular origins of DNA replication is inhibited. p53 is stabilized, but it cannot activate its target promoters, and the inhibitory activity of Rb on the E2F/DP transcription factors is relieved

events by which the cell prepares for division (for review see Sherr 1996, 2000). These events are regulated by heterodimeric kinases that consist of a regulatory cyclin subunit and a catalytic subunit, the cyclin dependent kinase (cdk). Multisubunit E3 ubiquitin ligases that target proteins for degradation by the proteasome also regulate cell cycle progression. A resting cell (in $G_0$ phase) can be induced to enter the cell cycle by a number of proliferative signals including growth factors and serum stimulation. Initially, there is activation of the expression of the D-type cyclins, which form kinase complexes with cdk4 or 6. The phosphorylation of substrates by the cyclin D/cdk4 and cyclin D/cdk6 kinases releases the quiescent cell from its resting state and permits entry into the $G_1$ phase of the cell cycle.

The $G_1$ phase is the period during which multiple transcription factors and genes encoding proteins involved in nucleotide metabolism and DNA replication are induced. In $G_1$ phase, there is a commitment to DNA synthesis and cell division if

the necessary requirements are met. DNA is replicated completely and only once during a single cell cycle (Fujita 1999; Diffley 2001; Lei and Tye 2001). During $G_1$ phase, prereplication complexes (pre-RC) assemble at the origins of DNA replication. The multisubunit origin recognition complex (ORC) is the first to bind to the DNA and serves as a nucleation point for the recruitment of other factors. Cdc6 and Cdt1 are recruited to the complex and facilitate the loading of the family of six MCM proteins (Maiorano et al. 2000; Nishitani et al. 2000, 2001; Rialland et al. 2002). Cyclin E expression is induced, and the active cdk2/cyclin E kinase promotes the transition into S phase. Cyclin E also facilitates the formation of pre-RC in a kinase-independent fashion through physical interaction with Cdt1 and the MCM proteins (Geng et al. 2003; Ekholm-Reed et al. 2004; Geng et al. 2007).

At the beginning of S phase, cyclin A accumulates and forms an active kinase complex with cdk2. Regulation of cyclin A occurs at both the protein and mRNA levels (Glotzer et al. 1991; Henglein et al. 1994; Desdouets et al. 1995; Schulze et al. 1995; Zwicker et al. 1995; Zwicker and Muller 1997; Bottazzi et al. 2001; Tessari et al. 2003). In S phase, cdk2/cyclin A and cdc7/Dbf4 complexes mediate the firing of DNA origins of replication and promote DNA replication (for review see Nishitani and Lygerou 2002; Machida et al. 2005). At the same time, Cdt1 is released from the replication complex, and its binding to the ORC is prevented by geminin, a protein that accumulates during S, $G_2$, and M phases. This ensures that DNA replication initiates at each origin only once and prevents polyploidy.

Following the complete replication of the parental chromosomes, cells enter $G_2$ and proteins involved in mitosis (M phase) begin to accumulate. Cdk1 and cyclin B play a major role at this point. Cyclin B first associates with cdk1 to form an inactive complex. At the $G_2$/M transition, dephosphorylation of cdk1 by cdc25 phosphatase induces the activation of the kinase, which in turn promotes the onset of mitosis (Millar and Russell 1992). Cdk1/cyclin B and cdk1/cyclin A complexes phosphorylate many substrates that facilitate condensation of the chromosomes, disassembly of the nuclear envelope, and modification of the cell's architecture to ensure that there is an ordered and even segregation of the chromosomes to the daughter cells. Inactivation of the cdk1 complexes occurs during mitosis by degradation of cyclins A and B through the ubiquitin-dependent proteolytic pathway involving the anaphase-promoting complex (APC) E3 ubiquitin ligase and the proteasome (Glotzer et al. 1991). The APC also ubiquitinates geminin and targets it for proteasome degradation as the cells exit mitosis, thereby allowing loading of the pre-RCs onto the chromatin of daughter cells during $G_1$ phase (McGarry and Kirschner 1998). This degradation of the cyclins and geminin continues until the onset of S phase (Brandeis and Hunt 1996).

Several checkpoints throughout the cell cycle ensure that progression will halt if there is DNA damage or aberrant spindle formation (for review see Lukas et al. 2004), thus protecting the integrity of the genome. The tumor suppressors p53 and the Rb family of pocket proteins (Rb, p107, and p130) are the best studied of these checkpoint sentinels. The Rb proteins, in their hypophosphorylated forms, bind to the E2F family of transcription factors, which then function as transcriptional repressors. Phosphorylation of the Rb proteins in late $G_1$ dissociates these complexes, allowing the E2F factors to activate transcription of multiple genes, many of which encode proteins required for DNA replication (Dyson 1998). p53

coordinates multiple cellular processes through its activity as a transcriptional activator and repressor in response to stress and growth factors (Vousden and Lu 2002; Slee et al. 2004). Phosphorylation of p53 controls its association with MDM2, which targets p53 for degradation by the proteasome (for review see Lavin and Gueven 2006). In response to DNA damage, nutrient deprivation, and other insults to the cell, p53 levels are stabilized. This can lead to the expression of the cdk inhibitor p21 as well as induction of several pro-apoptotic genes.

## The Effect of HCMV on the Cell Cycle

### *Cell Cycle Arrest and cdk Dysregulation*

Cells infected with HCMV in the $G_0/G_1$ phase of the cycle do not replicate their DNA and arrest in a pseudo-$G_1$ state that is distinguished by the expression of selected $G_1$-phase, S-phase and M-phase gene products (Jault et al. 1995; Bresnahan et al. 1996b; Lu and Shenk 1996; Dittmer and Mocarski 1997; Salvant et al. 1998; Wiebusch and Hagemeier 1999; Wiebusch and Hagemeier 2001; Challacombe et al. 2004; Hertel and Mocarski 2004) (see Fig. 1). The $G_1$/S- and $G_2$/M-phase cyclins E and B1, respectively, accumulate in infected cells with a concomitant increase in associated kinase activity (Jault et al. 1995; Wiebusch and Hagemeier 2001; Sanchez et al. 2003). In contrast, the steady-state levels of the S-phase cyclin A and $G_1$-phase cyclin D1 are reduced (Jault et al. 1995; Bresnahan et al. 1996b; Salvant et al. 1998; Wiebusch and Hagemeier 2001). The effects of the virus on cyclins E, A, and D1, are at the level of transcription, while accumulation of cyclin B1 is associated with increased stability of the protein (Salvant et al. 1998; Sanchez et al. 2003).

### *Checkpoint Control and DNA Damage*

The observation that HCMV infection during S phase induces breaks in chromosome 1 has prompted investigations into the effects of infection on DNA damage pathways (Fortunato et al. 2000). Infection of cells with HCMV causes significant changes in proteins involved in $G_1$ and S phase checkpoint control. For example, the tumor suppressors Rb, p130, and p107, which inhibit the expression of E2F-responsive genes and the activity of cdk2 kinase complexes, are maintained in an inactivate phosphorylated state in infected cells (Jault et al. 1995; McElroy et al. 2000). Interestingly, it has also been reported that HCMV-infected cells express high levels of the $p16^{Ink4}$ tumor suppressor (Noris et al. 2002; Zannetti et al. 2006). $p16^{Ink4}$ is a marker for senescence and activator of Rb through binding and inactivation of Cdk4 and Cdk6 kinases.

Early cell cycle studies demonstrated the stabilization of p53 in HCMV-infected cells; p53 was sequestered into viral replication centers, thus contributing to its inability to activate the expression of downstream cellular target genes such as p21 (Muganda et al. 1994; Bresnahan et al. 1996a; Fortunato and Spector 1998; Chen and Fang 2001). Later work by Rosenke and co-workers described the binding of p53 to viral promoters and showed that the DNA binding activity of the protein was required for sequestration into the viral replication center (Rosenke et al. 2006).

HCMV infection also activates the ATM and ATR kinases that regulate the double-stranded break and S phase checkpoints, respectively (Shen et al. 2004; Castillo et al. 2005; Gaspar and Shenk 2006; Luo et al. 2007). However, the initiation of a damage response does not appear to be necessary for the infection, as virus replicates normally in cells deficient in ATM (Luo et al. 2007). In addition, a fully functional damage response does not occur due to inefficient relocalization of all of the required proteins (Gaspar and Shenk 2006; Luo et al. 2007). For example, several proteins that are involved in nonhomologous end-joining (NHEJ) response appear to be completely excluded from the viral replication centers (Luo et al. 2007). This may be necessary to prevent the rejoining of ends that are generated during the replication of the virus.

## *Inhibition of Cellular DNA Replication*

In addition to downregulation of cyclin A observed during infection, the replication of cellular DNA is inhibited by several mechanisms during the licensing of the origins. Most importantly, the loading of MCM 2 and 7 (Biswas et al. 2003), as well as MCM 3, 4, 5, and 6 (Wiebusch et al. 2003), onto chromatin is inhibited in infected cells. This appears to be due to the premature accumulation of geminin, which, as discussed above, normally accumulates in S phase to ensure that there is no refiring of origins as the cells proceed through S and $G_2$/M.

## *Regulation of Cellular RNA Transcription and Protein Stability*

The advent of DNA microarrays provided a means to examine the global effects of HCMV infection on the accumulation of cellular RNAs (Zhu et al. 1997; Browne et al. 2001; Challacombe et al. 2004; Hertel and Mocarski 2004). While increases in the levels of RNA do not necessarily correlate with changes in the steady-state levels of protein, these analyses provide a foundation for defining infection-associated changes in transcription. The general picture that has emerged is that changes in the mRNA levels of key cell cycle proteins is mirrored in the corresponding protein levels, although there are several exceptions.

One example of an exception is where the accumulation of the protein is due to the effects of the infection on protein stability rather than transcript level. For example,

cyclin B1 accumulates in infected cells due to stabilization of the protein, with only minimal changes in RNA levels (Salvant et al. 1998; Sanchez et al. 2003). The degradation of cyclin B1 is mediated by the anaphase-promoting complex (APC), an E3 ubiquitin ligase. Interestingly, other substrates of the APC also accumulate in infected cells, including securin, geminin, Aurora B, and cdc6 (Biswas et al. 2003; Wiebusch et al. 2005). The increase in the steady-state levels of these proteins suggests that the APC is inactivated during infection. Data from our lab and others suggest that this dysregulation is due to the dismantling of the APC early in infection (Wiebusch et al. 2005; Tran et al. 2008).

## Importance of Subversion of the Cell Cycle for the Viral Infection

### *Cell Cycle Arrest*

A surprising observation regarding the kinetics of HCMV replication was that the initiation of the viral gene expression requires that the cells be in $G_0$ or $G_1$ at the time of infection. When cells are infected near or during S phase, many cells are able to pass through S phase and undergo mitosis prior to the synthesis of IE and early gene products (Salvant et al. 1998; Fortunato et al. 2002). The process of cell cycle arrest appears to be important for the early phase of infection, and proteins carried in the virus particle as well as those expressed at immediate early times contribute to this process (see Fig. 2).

#### Proteins in the Virus Particle

Two virion proteins, pUL69 and pp71 (pUL82), have been shown to modulate cell cycle progression (see the chapter by R. Kalejta, this volume). As components of the virion tegument, they can function as soon as the virus enters the cells. In the case of pUL69, overexpression of this protein stimulates accumulation of cells in $G_1$ phase of the cell cycle (Lu and Shenk 1999). In addition, cells infected with a virus lacking functional pUL69 do not efficiently undergo cell cycle arrest (Hayashi et al. 2000). This mutant does not replicate to wild type levels, but the growth defect may be attributed to other functions of pUL69.

The deletion of pp71 also creates a virus that is severely impaired for growth. The growth defect can be complemented by expression of the protein in trans (Bresnahan and Shenk 2000; Dunn et al. 2003). pp71 has been shown to interact with the cell growth suppressors Rb, p107, and p130 and to target hypophosphorylated forms of these pocket proteins for degradation by the proteasome (Kalejta et al. 2003). Consistent with this activity, pp71 expression in uninfected cells accelerates their progression through G1 phase, but does not change the overall doubling

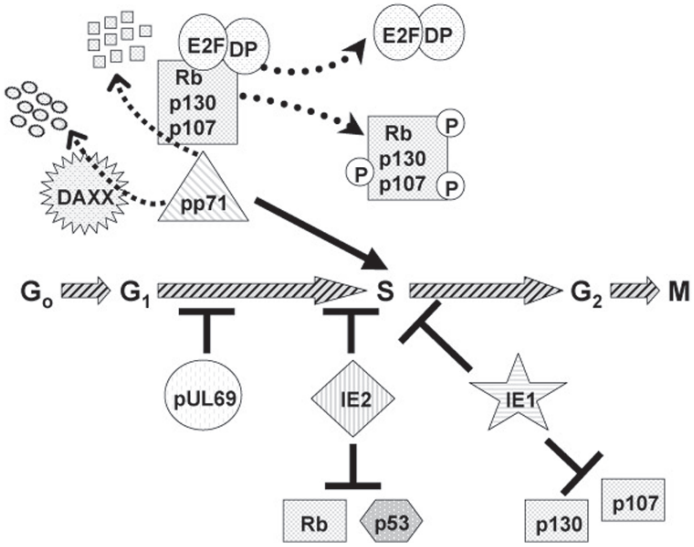


**Fig. 2** Modulation of the cell cycle by proteins in the virus particle and immediate early proteins. The tegument proteins pUL69 and pp71 can exert their effects on the cell cycle upon entry of the virus particle into the cell. pUL69 causes accumulation of cells in $G_1$ phase, while pp71 targets the hypophosphorylated forms of the Rb family of proteins (Rb, p107, p130) and the transcriptional inhibitor Daxx for proteasome-mediated degradation. This degradation of the Rb proteins along with their viral-mediated hyperphosphorylation lead to the release of the E2F/DP transcription factors, which activate many genes involved in DNA replication and promotes S phase. The IE1 protein blocks the activity of p130/p107, and IE2 interferes with at least some functions of Rb and p53. Both IE proteins can prevent cells from passage through S phase

time (Kalejta and Shenk 2003) . These results suggest that pp71 delivered to cells as part of the incoming virus particles may stimulate the cell cycle at the beginning of the infection before IE gene expression. The finding that pp71 also interacts with ND10-associated transcription repressor Daxx and promotes its proteasome-mediated degradation suggests an additional function for pp71 in initiating viral transcription at ND10 sites (Hofmann et al. 2002; Ishov et al. 2002; Marshall et al. 2002; Cantrell and Bresnahan 2005; Saffert and Kalejta 2006).

## Immediate Early Protein 1

HCMV encodes two IE proteins, IE1-72 and IE2-86, that have been shown to interfere with cell cycle progression in heterologous systems in the absence of infection. Transient expression of IE1-72 in asynchronously cycling cells stimulates their accumulation in the S and $G_2$/M phases of the cell cycle (Castillo et al. 2000). One possible explanation for this result is that it is due to the interaction of IE1-72 with

the pocket protein p107 (Margolis et al. 1995; Poma et al. 1996; Woo et al. 1997; Castano et al. 1998; Hansen et al. 2001; Zhang et al. 2003). IE1-72 alleviates p107-mediated repression of E2F-responsive promoters in transient transfection assays and thus may stimulate S-phase entry. Additionally, IE1-72 can reverse the inhibitory effects of p107 on cdk2/cyclin E kinase activity, which may also facilitate the $G_1$/S transition. It has been proposed that the formation of an IE1-72/p107 complex mediates these effects (Poma et al. 1996; Johnson et al. 1999; Zhang et al. 2003); however, the finding that IE1-72 can phosphorylate p107, p130, and E2F proteins (Pajovic et al. 1997) raises the possibility that some of the effects of IE1-72 on transcription and the cell cycle result from its reported kinase activity.

## Immediate Early Protein 2

Several studies have shown that transient expression of IE2-86 alters cell cycle progression, with a block at the G1/S boundary in a $p53^{+/+}$ cell or after entry into S phase in a p53 mutant cell (Murphy et al. 2000; Wiebusch and Hagemeier 2001; Noris et al. 2002; Wiebusch et al. 2003; Song and Stinski 2005) (see the chapter by M.F. Stinski and D.T. Petrik, this volume). In transient transfection assays, deletion of aa 451–579 abolished the ability of IE2-86 to induce G1 arrest in transient assays in U373 cells (Wiebusch and Hagemeier 1999). Perhaps the most convincing evidence that IE2-86 plays a role in cell cycle arrest is a recent study showing that a mutation of aa 548 of IE2-86 from Q to R results in a growth-impaired virus that does not inhibit cellular DNA synthesis or the cell cycle (Petrik et al. 2006). The observation that this mutant IE2-86 could still autoregulate the MIE promoter and activate viral early genes provides further evidence that efficient viral replication also requires the inhibition of host cell DNA synthesis.

Early observations showing that IE2-86 interacts with several proteins regulating the cell cycle, including Rb and p53, made it reasonable to link some of its functions to cell cycle arrest (Hagemeier et al. 1994; Sommer et al. 1994; Speir et al. 1994; Bonin and McDougall 1997; Fortunato et al. 1997). p53 levels are stabilized in infected cells but the expression of its target gene p21 is repressed (Muganda et al. 1994; Bresnahan et al. 1996b; Fortunato and Spector 1998; Chen et al. 2001). In transient expression and in vitro systems**,** IE2-86 interacts with the C-terminus of p53, and the binding of p53 to target promoters is inhibited (Speir et al. 1994; Bonin and McDougall 1997; Hsu et al. 2004). IE2-86 expression also inhibits the acetylation of p53 and of histones in proximity to p53-dependent promoters (Hsu et al. 2004), and thus IE2-86 may regulate expression of p53 target genes by multiple mechanisms. These effects on protein acetylation may result from downregulation of p300/CBP histone acetyl transferase (HAT) activity, which was detected in a complex with p53 and IE2-86 (Hsu et al. 2004). It is possible that the inhibition of p300/CBP HAT activity is p53-promoter-specific, as IE2-86 did not suppress histone acetylation globally. The biological relevance of these experiments must be considered with caution, given that none were performed in the context of the viral infection.

In permissive cells, the early phase of the infection is associated with the stimulation of many genes encoding proteins that are required for host cell DNA synthesis and proliferation (Hirai and Watanabe 1976; Estes and Huang 1977; Isom 1979; Boldogh et al. 1991; Wade et al. 1992; Browne et al. 2001). Many of these genes are regulated by the E2F/DP transcription factors, which are inhibited by complex formation with the Rb family of proteins. A role for IE2-86 has been suggested by work showing that there is an increase in the steady state levels of RNA from several E2F-responsive genes in human fibroblasts infected with an adenovirus expressing IE2-86 (Song and Stinski 2002). The key question, however, is what are the underlying mechanisms for the activation of these growth regulatory genes in the context of the infection?

Cyclin E expression is regulated by E2F, and the potential role of IE2-86 in its accumulation during the infection has been the focus of several studies (Bresnahan et al. 1998; McElroy et al. 2000; Wiebusch and Hagemeier 2001; Wiebusch et al. 2003). The majority of the experiments have used transient expression assays to examine the regulation of the cyclin E promoter driving a reporter gene. In one study, it was shown that IE2-86 could bind to sequences in the cyclin E promoter in vitro and could activate expression of a cyclin E promoter-driven reporter construct (Bresnahan et al. 1998). Work by Song and Stinski demonstrated that expression of IE2-86 induces synthesis of endogenous cyclin E mRNA, reinforcing the notion that IE2-86 expression upregulates cyclin E in infected cells (Song and Stinski 2002). In contrast, McElroy et al. reported that early viral gene expression, not IE2-86 expression, was necessary for accumulation of cyclin E protein (McElroy et al. 2000). These conflicting results suggest that cyclin E accumulation in infected cells is controlled by several pathways, and that the increase in cyclin E might be regulated at both the mRNA and protein level.

Besides E2F transcription factors, other proteins regulate the expression of cyclin genes. The finding that the architectural transcription factor HMGA2 (high mobility group AT-hook 2) regulates the transcription of cyclin A (Tessari et al. 2003) prompted our lab to determine whether HCMV affects the expression of this protein. HMGA proteins are referred to as architectural transcription factors because of their ability to organize the assembly of nucleoprotein structures (enhanceosomes), resulting in enhancement or repression of transcription. In the case of cyclin A, HMGA2 activates its expression through derepression of the promoter. Our studies showed that the transcription of the HMGA2 gene is specifically inhibited at the RNA level during the infection (Shlapobersky et al. 2006). To determine whether repression of HMGA2 was important for the HCMV infection, a recombinant virus expressing HMGA2 driven by the MIE promoter was constructed. High multiplicity infection with the HMGA2-expressing virus induced the synthesis of cyclin A mRNA and protein and inhibited virus replication. Furthermore, a role for IE2-86, but not IE1-72, in HMGA2 repression was suggested by additional experiments with HCMV recombinant viruses defective in either IE1-72 or IE2-86. We found that the IE1-72 deletion mutant virus CR208 (Greaves and Mocarski 1998) inhibits HMGA2 transcription. In contrast, in cells infected with an IE2-86 mutant virus lacking aa 136–290 (referred to as delta SX)

(Sanchez et al. 2002), HMGA2 expression is not significantly reduced, suggesting that IE2 is involved in the regulation of the HMGA2 promoter. Cyclin A transcription is also induced in cells infected with delta SX, although this effect is slightly delayed relative to HMGA2 expression. The mechanism involving the downregulation of HMGA2 RNA expression by IE2-86 has yet to be determined. An interesting possibility is that it is related to IE2 interaction with histone deacetylase (HDAC), as HMGA2 is an example of a gene that requires HDAC activity for its expression (Ferguson et al. 2003). Cyclin D1 expression also seems to require HDAC activity (Hu and Colburn 2005) and cyclin D1 and HMGA2 exhibit a similar pattern of expression in the wt and delta SX infected cells (R. Sanders, M. Shlapobersky, and D.H. Spector, unpublished data). Since the levels of IE2-86 are significantly reduced in cells infected with delta SX, there may be less inhibition of HDAC activity in these cells.

## *Checkpoint Control*

Recent studies have focused on the defining the roles of p53 and $p16^{Ink4}$ accumulation during infection. As described above, work from Rosenke and colleagues determined that p53 is bound to viral promoters during infection (Rosenke et al. 2006). This group has since shown that active p53 is required for the HCMV infection; p53-null cells infected with HCMV produced 10- to 20-fold less virus than controls (Casavant et al. 2006). The reduction in virus production was attributed to delays in viral DNA replication. Consistent with the binding of p53 to viral promoters, they also observed delays in the expression of early and late proteins in p53 null cells. Reintroduction of wild type but not mutant p53 partially rescued the phenotype, establishing a role for p53 in the viral lytic cycle.

A role for $p16^{Ink4}$ during infection was suggested by studies by Zannetti and coworkers, showing that there was a reduction in viral early and late gene expression, DNA synthesis, and production of extracellular virus in cells lacking functional p16 (Zannetti et al. 2006). In this case, $p16^{Ink4}$-null cells and cells containing reduced levels of the protein as a result of treatment with siRNAs were used. The authors proposed that the ras-p16-Rb pathway is activated during the early phases of infection in order to establish an environment favorable for viral replication; however, the hyperphosphorylation of Rb in infected cells and the induction of E2F-responsive genes suggest that other proteins may be targets of $p16^{Ink4}$ activity.

## *Role of Cyclin-Dependent Kinases in Viral Replication*

In order to establish the importance of cdk activity during infection, we and others have used cdk inhibitors such as the drug Roscovitine to treat infected cells (Bresnahan et al. 1997; Sanchez et al. 2004). This purine analog has been particularly

useful as it has high specificity for cdks 1, 2, 5, 7, and 9 and reversibly inhibits their activity by competing for binding of ATP (De Azevedo et al. 1997). While the inhibitor has provided several important insights, its broad effects on multiple cdks necessitates further experiments to determine the roles of individual cdk complexes during infection. Nevertheless, studies with such chemical inhibitors are a good starting place for understanding the impact of cellular kinase activity on virus replication.

Cdk activity appears to be required primarily at two distinct time intervals in the infection: one during the first 8 h and the second after the onset of viral DNA replication (Bresnahan et al. 1997; Sanchez et al. 2004; Sanchez and Spector 2006). Studies from our lab showed a significant decrease in the expression of select early and late genes and a reduction in viral DNA replication when the drug was added at the time of infection. Notably, processing of IE transcripts was altered in the presence of the drug. However, these effects on IE transcript processing and the inhibition of gene expression and viral DNA replication were not detected if addition of the drug was delayed until 6–8 h postinfection (p.i.). In contrast, a reduction of virus titer was observed even if the drug was not added until 48 h p.i.

The effects on IE gene expression when Roscovitine is added at the beginning of the infection were particularly intriguing and provided the impetus for further studies. IE1-72 and IE2-86 are alternatively spliced transcripts that share their first three exons and differ in their 3′ terminal exon, with the 3′ exon of IE2-86 being most distal from the start site of transcription. At early phases of the infection, IE1-72 RNA accumulates preferentially compared to IE2-86 RNA; however, as the infection progresses, more IE2-86 transcripts accumulate, while there is only a slight increase in IE1-72 transcripts. If Roscovitine is added at the onset of infection, IE2-86 RNA accumulation is favored at early phases and there is little increase in the level of IE1-72 transcript (Sanchez et al. 2004). In fact, there is a decrease in IE1-72 RNA relative to untreated control samples. Given that the polyadenylation signals for IE1-72 and splicing signals for IE2-86 are juxtaposed, the most likely explanation for these results is that the differential splicing and polyadenylation of the UL122–123 transcript is altered by treatment with the drug. This results in enhanced utilization of the 3′ splice acceptor site that generates the IE2-86 transcript and a decrease in the cleavage/polyadenylation of the IE1-72 transcript. Consistent with this hypothesis, the differential processing of the IE UL37 RNAs, which also have signals for polyadenylation and splicing in close proximity, is similarly affected by cdk inhibition.

The requirement for cdks at later times in the infection is directly related to formation of infectious viral particles. In a recent paper, we showed that addition of Roscovitine at 24 h p.i., after the onset of viral DNA synthesis, results in a 1- to 2-log decrease in viral titer (Sanchez and Spector 2006). There is a corresponding decrease in the amount of viral DNA detected in the supernatant from drug-treated cells, indicating that there is a defect in the production or release of extracellular particles. Consistent with this result, we observed changes in the expression, post-translational modification, and localization of virion structural proteins in Roscovitine-treated cells. The levels of the IE2-86 and pp150 (UL32) proteins

were significantly reduced, but there was not a corresponding decrease in the mRNAs. The pUL69 matrix protein accumulated in drug-treated cells and the protein was present in a hyperphosphorylated form. pUL69 localized to intranuclear aggregates that did not overlap with viral replication centers. The matrix protein pp65 was also retained in the nucleus (Sanchez et al. 2007). In contrast, the levels of capsid, envelope, and other tegument proteins were only moderately affected in that their expression was slightly delayed. Although preliminary, our recent data with the specific inhibitor of cdk1, RO-3306 (Vassilev et al. 2006), indicate that only some of these effects are due to inhibition of cdk1. We find that the titers are significantly reduced and pUL69 accumulates in a hyperphosphorylated form, but the expression of the IE2-86 and UL32 proteins is not significantly affected (V. Sanchez and D.H. Spector, unpublished data). Thus, there may be a later role for cdk2, cdk7, and cdk9.

Although limited, there have been some attempts to try to decipher the contributions of specific cdks during the infection by expressing dominant-negative forms of the catalytic subunits in infected cells. Work by Bresnahan and colleagues showed a decrease in the levels of some capsid proteins in infected cells transfected with a dominant-negative cdk2, suggesting that the activity of cdk2/cyclin E complexes is essential for viral replication (Bresnahan et al. 1997). In contrast, preliminary work by Hertel et al. suggests that cdk1 activity is dispensable for the infection, given that virus titer was not markedly reduced in cell cultures transduced with a retrovirus expressing a GFP-tagged dominant negative cdk1 (Hertel et al. 2007). The only effect that the dominant negative cdk1 had was to inhibit a pseudomitosis phenotype that they observed in some cells infected with a variant of the HCMV strain AD169 (Hertel and Mocarski 2004). As noted above, however, our preliminary results with a specific inhibitor of cdk1 suggest that this kinase is required for production of infectious virus. Experiments involving expression of siRNAs directed against the individual cdks will likely provide more insight into their individual and combined roles in the infection.

## *Role of Cyclin-Dependent Kinases in Viral RNA Processing*

Our hypothesis for the altered pattern of expression for the IE1-72/IE2-86 and UL37 IE RNAs in the presence of Roscovitine is based on the phosphorylation of the C-terminal domain (CTD) of the large subunit of RNA polymerase II (RNAP II) by cdk7/cyclin H and cdk9/cyclin T. Cdk7/cyclin H is responsible for activating cdks1, 2, and 4 and is also a part of TFIIH, which phosphorylates the carboxyl terminal domain (CTD) of the large subunit of RNA polymerase II within the 52 repeats of the heptapeptide YSPTSPS. Cdk9/cyclin T (P-TEFb) also phosphorylates the CTD. The current consensus is that transcription and RNA processing are integrated events whereby the differential phosphorylation of the CTD repeats at Ser 2 and Ser 5 defines its affinity for various transcription factors, kinases, and RNA processing factors. When the transcription initiation complex is formed on a

promoter, the CTD of RNAP II is unphosphorylated (the polymerase is designated RNAP IIa). Cdk7 with cyclin H and MAT1 primarily phosphorylates Ser 5 on the CTD (the polymerase is now designated RNAP IIo), which leads to the recruitment of the RNA capping enzymes. Further phosphorylation of the CTD Ser 2 residues by cdk9/cyclin T occurs upon entry to elongation and is associated with recruitment of the cleavage/polyadenylation and splicing machinery.

Previously, our lab showed that infected cells contain more cdk7, MAT-1, cdk9, and cyclin T1 at 24 h p.i. than the uninfected cells, and the abundance and activity of these proteins increase as the infection proceeds (Tamrakar et al. 2005). All of MAT-1 is complexed with cdk7, although free cdk7 is also present, and most of cdk9 and cyclin T1 are in complex. In accord with the increase in the activity of the cdk9 and cdk7 kinases, an increase in the phosphorylation of the RNAP II CTD, particularly on the Ser 2 and Ser 5 residues of the heptad repeats, was also noted. By immunofluorescence analysis, it was observed that cdk7 and hypophosphorylated RNAP II localize to replication centers. In contrast, cdk9 and ser2-phosphorylated RNAP II are distributed in a punctate pattern throughout the nucleus with some concentration at the periphery of the viral replication centers. Similarly, ser5-phosphorylated RNAP II appears in clusters at the rim of the viral replication centers. These results suggest that at late times in the infection, HCMV may commandeer the RNA polymerase machinery, with viral RNA synthesis initiated within the replication center and active viral transcription occurring at the periphery.

Our lab has also demonstrated that addition of the cdk inhibitor Roscovitine at the time of infection results in decreased CTD phosphorylation in the infected cells and a decrease in the level of the hypophosphorylated RNAP II in both infected and mock-infected cells (Tamrakar et al. 2005). Consistent with our previous results regarding the effect of the cdk inhibitors on the processing and accumulation of the HCMV IE1/IE2 and UL37 IE transcripts, the decrease in CTD phosphorylation does not occur if the drug is added after 8 h p.i. One clue to explain this restricted interval in which cdk activity is required may be found in the differential localization of cdk9 and cdk7 at the beginning of the infection. Upon cell entry, incoming HCMV genomes localize near ND10, where viral IE transcription begins (Ishov and Maul 1996; Ishov et al. 1997) (see the chapter by G. Maul, this volume). Following translation, the IE1-72 and IE2-86 proteins return to nucleus and concentrate near the ND10. IE1-72 mediates the dispersal of ND10 associated proteins and it also disperses, while IE2-86 persists at the site (Kelly et al. 1995; Korioth et al. 1996; Ahn and Hayward 1997; Ishov et al. 1997; Ahn et al. 1998). We find that as early as 4 h p.i., several proteins involved in RNA transcription, including cdk9, cdk7, and Ser2-phosphorylated RNA polymerase II, colocalize with IE2-86 in distinct aggregates (referred to as viral transcriptosomes) adjacent to the ND10 that are undergoing dispersal (Tamrakar et al. 2005) (see Fig. 3). However, if Roscovitine is added at the beginning of the infection, IE2-86 is found in the transcriptosome, but cdk7 and cdk9 are not recruited (Kapasi and Spector 2008). In contrast, both cdks colocalize with IE2-86 in the transcriptosome if Roscovitine is added after 8 h p.i. These results suggest that the formation of a distinct viral transcriptosome at the

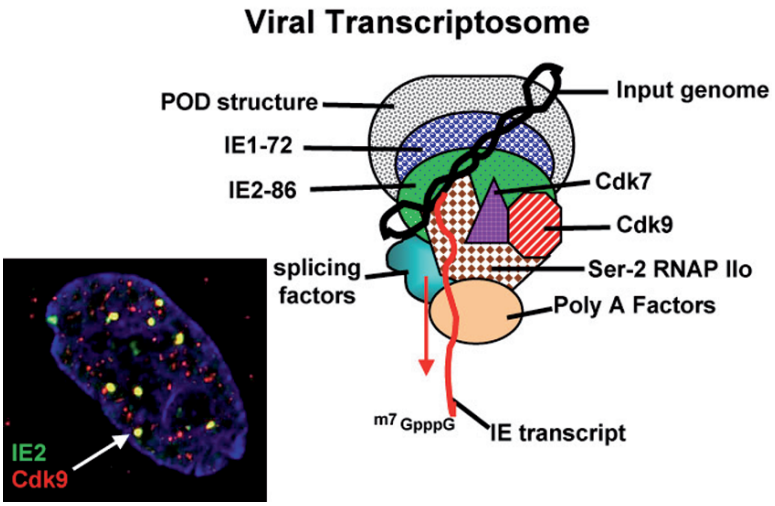


**Fig. 3** Model of the viral transcriptosome formed at the beginning of the infection. The input genome that is deposited at the POD structure functions as the template for IE RNA synthesis. Cellular hypophosphorylated RNAP IIa is recruited to the site along with cdk9 and cdk7, which hyperphosphorylate RNAP IIa to the transcriptionally active RNAP IIo that serves as a platform for RNA processing enzymes. The IE transcripts are synthesized and translated into the IE1-72 and IE2-86 proteins, which return to this nuclear body. IE1-72 causes POD dispersal and it also disperses, while IE2-86 remains at the established transcription site, referred to as the transcriptosome. The inset is an infected cell nucleus at 8 h p.i. and shows the accumulation of both cdk9 and IE2 at several transcriptosomes

beginning of the infection serves as a specialized site for recruitment of cellular and viral proteins necessary for viral transcription and replication. The correct phosphorylation of the RNAP II CTD at these sites is essential for accurate processing of the IE transcripts and for transcription of early genes, and it appears that the required level of phosphorylation is established within the first 8 h.

## Perspectives

For more than a decade, we have known that HCMV dramatically alters cell cycle regulatory pathways, leading to cell cycle arrest. These alterations begin as soon as the viral particle enters the cell, but sustained effects require early viral gene expression. The molecular mechanisms underlying the viral-mediated effects operate at multiple levels, including altered RNA transcription, changes in the levels and activity of cyclin-dependent kinases as well as other cellular kinases involved in cell cycle control, modulation of protein stability through targeted effects on the ubiquitin-proteasome degradation pathway, and movement of

proteins to different cellular locations. A key question regarding all of these effects is which ones are actually important for viral replication? For example, are high levels of cyclin B needed for productive infection or is this simply a side effect of altering the ubiquitin-proteasome pathway to allow accumulation of some other cellular or viral protein that is required? Future experiments involving knockdown of gene expression through the use of siRNAs or induced overexpression with lentiviral vectors should provide some insight into these questions. It is clear, however, that the virus depends on the host cell being in the $G_0/G_1$ phase to initiate the infection and subverts some G2/M phase activities of the cell for later stages of replication. At this point, only a few viral genes, primarily input virion proteins and IE gene products, have been implicated in these changes. It is critical to identify the other genes, particularly those expressed during the early phase just prior to initiation of viral DNA replication. It also should be recognized that most of the studies to date have been done in fibroblasts, and it will be important to examine the effects of the viral infection on host cell regulatory pathways in other relevant target cells such as endothelial cells and monocytes.

## References

Ahn JH, Hayward GS (1997) The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated nuclear bodies at very early times in infected permissive cells. J Virol 71:4599–4613

Ahn JH, Brignole ER, Hayward GS (1998) Disruption of PML subnuclear domains by the acidic IE1 protein of human cytomegalovirus is mediated through interaction with PML and may modulate a RING finger-dependent cryptic transactivator function of PML. Mol Cell Biol 18:4899–4913

Biswas N, Sanchez V, Spector DH (2003) Human cytomegalovirus infection leads to accumulation of geminin and inhibition of the licensing of cellular DNA replication. J Virol 77:2369–2376

Boldogh I, AbuBakar S, Deng CZ, Albrecht T (1991) Transcriptional activation of cellular oncogenes *fos*, *jun* and *myc* by human cytomegalovirus. J Virol 65:1568–1571

Bonin LR, McDougall JK (1997) Human cytomegalovirus IE2 86-kilodalton protein binds p53 but does not abrogate G1 checkpoint function. J Virol 71:5831–5870

Bottazzi ME, Buzzai M, Zhu X, Desdouets C, Brechot C, Assoian RK (2001) Distinct effects of mitogens and actin cytoskeleton on CREB and pocket protein phosphorylation control the extent and timing of cyclin A promoter activity. Mol Cell Biol 21:7607–7616

Brandeis M, Hunt T (1996) The proteolysis of mitotic cyclins in mammalian cells persists from the end of mitosis until the onset of S phase. EMBO J 15:5280–5289

Bresnahan WA, Shenk TE (2000) UL82 virion protein activates expression of immediate early viral genes in human cytomegalovirus-infected cells. Proc Natl Acad Sci U S A 97:14506–14511

Bresnahan WA, Boldogh I, Ma T, Albrecht T, Thompson EA (1996a) Cyclin E/CDK2 activity is controlled by different mechanisms in the G0 and G1 phases of the cell cycle. Cell Growth Differ 7:1283–1290

Bresnahan WA, Boldogh I, Thompson EA, Albrecht T (1996b) Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1. Virology 224:156–160

Bresnahan WA, Boldogh I, Chi P, Thompson EA, Albrecht T (1997) Inhibition of cellular CDK2 activity blocks human cytomegalovirus replication. Virology 231:239–247

Bresnahan WA, Albrecht T, Thompson EA (1998) The cyclin E promoter is activated by human cytomegalovirus 86-kDa immediate early protein. J Biol Chem 273:22075–22082

Browne EP, Wing B, Coleman D, Shenk T (2001) Altered cellular mRNA levels in human cytomegalovirus-infected fibroblasts: viral block to the accumulation of antiviral mRNAs. J Virol 75:12319–12330

Cantrell SR, Bresnahan WA (2005) Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication. J Virol 79:7792–7802

Casavant NC, Luo MH, Rosenke K, Winegardner T, Zurawska A, Fortunato EA (2006) Potential role for p53 in the permissive life cycle of human cytomegalovirus. J Virol 80:8390–8401

Castano E, Kleyner Y, Dynlacht BD (1998) Dual cyclin-binding domains are required for p107 to function as a kinase inhibitor. Mol Cell Biol 18:5380–5391

Castillo JP, Yurochko A, Kowalik TF (2000) Role of human cytomegalovirus immediate-early proteins in cell growth control. J Virol 74:8028–8037

Castillo JP, Frame FM, Rogoff HA, Pickering MT, Yurochko AD, Kowalik TF (2005) Human cytomegalovirus IE1-72 activates ataxia telangiectasia mutated kinase and a p53/p21-mediated growth arrest response. J Virol 79:11467–11475

Challacombe JF, Rechtsteiner A, Gottardo R, Rocha LM, Browne EP, Shenk T, Altherr MR, Brettin TS (2004) Evaluation of the host transcriptional response to human cytomegalovirus infection. Physiol Genomics 18:51–62

Chen J, Fang G (2001) MAD2B is an inhibitor of the anaphase-promoting complex. Genes Devel 15:1765–1770

Chen Z, Knutson E, Kurosky A, Albrecht T (2001) Degradation of p21cip1 in cells productively infected with human cytomegalovirus. J Virol 75:3613–3625

De Azevedo WF, Leclerc S, Meijer L, Havlicek L, Strnad M, Kim SH (1997) Inhibition of cyclin-dependent kinases by purine analogues: crystal structure of human cdk2 complexed with roscovitine. Eur J Biochem 243:518–526

Desdouets C, Matesic G, Molina CA, Foulkes NS, Sassone-Corsi P, Bréchot C, Sobszak-Thepot J (1995) Cell cycle regulation of cyclin A gene expression by the cyclic AMP transcription factors CREB and CREM. Mol Cell Biol 15:3301–3309

Diffley JF (2001) DNA replication: building the perfect switch. Curr Biol 11:R367–R370

Dittmer D, Mocarski ES (1997) Human cytomegalovirus infection inhibits G1/S transition. J Virol 71:1629–1634

Dunn W, Chou C, Li H, Hai R, Patterson D, Stolc V, Zhu H, Liu F (2003) Functional profiling of a human cytomegalovirus genome. Proc Natl Acad Sci U S A 100:14223–14228

Dyson N (1998) The regulation of E2F by pRB-family proteins. Genes Dev 12:2245–2262

Ekholm-Reed S, Mendez J, Tedesco D, Zetterberg A, Stillman B, Reed SI (2004) Deregulation of cyclin E in human cells interferes with prereplication complex assembly. J Cell Biol 165:789–800

Estes JE, Huang E-S (1977) Stimulation of cellular thymidine kinases by human cytomegalovirus. J Virol 24:13–21

Ferguson M, Henry PA, Currie RA (2003) Histone deacetylase inhibition is associated with transcriptional repression of the Hmga2 gene. Nucleic Acids Res 31:3123–3133

Fortunato EA, Sommer MH, Yoder K, Spector DH (1997) Identification of domains within the human cytomegalovirus major immediate-early 86-kilodalton protein and the retinoblastoma protein required for physical and functional interaction with each other. J Virol 71:8176–8185

Fortunato EA, Spector DH (1998) p53 and RPA are sequestered in viral replication centers in the nuclei of cells infected with human cytomegalovirus. J Virol 72:2033–2039

Fortunato EA, Dell'Aquila ML, Spector DH (2000) Specific chromosome 1 breaks induced by human cytomegalovirus. Proc Natl Acad Sci U S A 97:853–858

Fortunato EA, Sanchez V, Yen JY, Spector DH (2002) Infection of cells with human cytomegalovirus during S phase results in a blockade to immediate-early gene expression that can be overcome by inhibition of the proteasome. J Virol 76:5369–5379

Fujita M (1999) Cell cycle regulation of DNA replication initiation proteins in mammalian cells. Front Biosci 4: D816–D823

Gaspar M, Shenk T (2006) Human cytomegalovirus inhibits a DNA damage response by mislocalizing checkpoint proteins. Proc Natl Acad Sci U S A 103:2821–2826

Geng Y, Yu Q, Sicinska E, Das M, Schneider JE, Bhattacharya S, Rideout WM, Bronson RT, Gardner H, Sicinski P (2003) Cyclin E ablation in the mouse. Cell 114:431–443

Geng Y, Lee YM, Welcker M, Swanger J, Zagozdzon A, Winer JD, Roberts JM, Kaldis P, Clurman BE, Sicinski P (2007) Kinase-independent function of cyclin E. Mol Cell 25:127–139

Glotzer M, Murray AW, Kirschner MW (1991) Cyclin is degraded by the ubiquitin pathway. Nature 349:132–138

Greaves RF, Mocarski ES (1998) Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus *ie1* mutant. J Virol 72:366–379

Hagemeier C, Caswell R, Hayhurst G, Sinclair J, Kouzarides T (1994) Functional interaction between the HCMV IE2 transactivator and the retinoblastoma protein. EMBO J 13:2897–2903

Hansen K, Farkas T, Lukas J, Holm K, Roonstrand L, Bartek J (2001) Phosphorylation-dependent and -independent functions of p130 cooperate to evoke a sustained G1 block. EMBO J 20:422–432

Hayashi ML, Blankenship C, Shenk T (2000) Human cytomegalovirus UL69 is required for efficient accumulation of infected cells in the G1 phase of the cell cycle. Proc Natl Acad Sci U S A 97:2692–2696

Henglein B, Chenivesse X, Wang J, Eick D, Bréchot C (1994) Structure and cell cycle-regulated transcription of the human cyclin A gene. Proc Natl Acad Sci U S A 91:5490–5494

Hertel L, Mocarski ES (2004) Global analysis of host cell gene expression late during cytomegalovirus infection reveals extensive dysregulation of cell cycle gene expression and induction of pseudomitosis independent of US28 function. J Virol 78:11988–12011

Hertel L, Chou S, Mocarski ES (2007) Viral and cell cycle-regulated kinases in cytomegalovirus-induced pseudomitosis and replication. PLoS Pathog:e6

Hirai K, Watanabe Y (1976) Induction of α-type DNA polymerases in human cytomegalovirus-infected WI-38 cells. Biochim Biophys Acta 447:328–339

Hofmann H, Sindre H, Stamminger T (2002) Functional interaction between the pp71 protein of human cytomegalovirus and the PML-interacting protein human Daxx. J Virol 76:5769–5783

Hsu C-H, Chang MDT, Tai K-Y, Yang Y-T, Wang P-S, Chen C-J, Wang Y-H, Lee S-C, Wu C-W, Juan L-J (2004) HCMV IE2-mediated inhibition of HAT activity downregulates p53 function. EMBO J 23:2269–2280

Hu J, Colburn NH (2005) Histone deacetylase inhibition down-regulates cyclin D1 transcription by inhibiting nuclear factor-kappaB/p65 DNA binding. Cancer Res 3:100–109

Ishov AM, Maul GG (1996) The periphery of nuclear domain 10 (ND10) as site of DNA virus deposition. J Cell Biol 134:815–826

Ishov AM, Stenberg RM, Maul GG (1997) Human cytomegalovirus immediate early interaction with host nuclear structures: definition of an immediate transcript environment. J Cell Biol 138:5–16

Ishov AM, Vladimirova OV, Maul GG (2002) Daxx-mediated accumulation of human cytomegalovirus tegument protein pp71 at ND10 facilitates initiation of viral infection at these nuclear domains. J Virol 76:7705–7712

Isom HC (1979) Stimulation of ornithine carboxylase by human cytomegalovirus. J Gen Virol 42:265–278

Jault FM, Jault J-M, Ruchti F, Fortunato EA, Clark C, Corbeil J, Richman DD, Spector DH (1995) Cytomegalovirus infection induces high levels of cyclins, phosphorylated RB, and p53, leading to cell cycle arrest. J Virol 69:6697–6704

Johnson RA, Yurochko AD, Poma EE, Zhu L, Huang E-S (1999) Domain mapping of the human cytomegalovirus IE1-72 and cellular p107 protein–protein interaction and the possible functional consequences. J Gen Virol 80:1293–1303

Kalejta RF, Shenk T (2003) The human cytomegalovirus UL82 gene product (pp71) accelerate progression through the G1 phase of the cell cycle. J Virol 77:3451–3459

Kalejta RF, Bechtel JT, Shenk T (2003) Human cytomegalovirus pp71 stimulates cell cycle progression by inducing the proteasome-dependent degradation of the retinoblastoma family of tumor suppressors. Mol Cell Biol 23:1885–1895

Kapasi AJ, Spector DH (2008) Inhibition of the cyclin-dependent kinases at the beginning of the human cytomegalovirus infection specifically alters the levels and localization of the RNA polymerase II carboxyl-terminal domain kinases cdk9 and cdk7 at the viral transcriptosome. J Virol 82:394–407

Kelly C, Driel RV, Wilkinson GW (1995) Disruption of PML-associated nuclear bodies during human cytomegalovirus infection. J Gen Virol 76:2887–2893

Korioth F, Maul GG, Plachter B, Stamminger T, Frey J (1996) The nuclear domain 10 (ND10) is disrupted by the human cytomegalovirus gene product IE1. Exp Cell Res 229:155–158

Lavin MF, Gueven N (2006) The complexity of p53 stabilization and activation. Cell Death Differ 13:941–950

Lei M, Tye BK (2001) Initiating DNA synthesis: from recruiting to activating the MCM complex. J Cell Sci 114:1447–1454

Lu M, Shenk T (1996) Human cytomegalovirus infection inhibits cell cycle progression at multiple points, including the transition from G1 to S. J Virol 70:8850–8857

Lu M, Shenk T (1999) Human cytomegalovirus UL69 protein induces cells to accumulate in $G_1$ phase of the cell cycle. J Virol 73:676–683

Lukas J, Lukas C, Bartek J (2004) Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time. DNA Repair 3:997–1007

Luo MH, Rosenke K, Czornak K, Fortunato EA (2007) Human cytomegalovirus disrupts both ataxia telangiectasia mutated protein (ATM)- and ATM-Rad3-related kinase-mediated DNA damage responses during lytic infection. J Virol 81:1934–1950

Machida YJ, Hamlin JL, Dutta A (2005) Right place, right time, and only once: replication initiation in metazoans. Cell 123:13–24

Maiorano D, Moreau J, Mechali M (2000) XCDT1 is required for the assembly of pre-replicative complexes in *Xenopus laevis.* Nature 404:622–625

Margolis MJ, Panjovic S, Wong EL, Wade M, Jupp R, Nelson JA, Azizkhan JC (1995) Interaction of the 72-kilodalton human cytomegalovirus IE1 gene product with E2F1 coincides with E2F-dependent activation of dihydrofolate reductase transcription. J Virol 69: 7759–7767

Marshall KR, Rowley KV, Rinaldi A, Nicholson IP, Ishov AM, Maul GG, Preston CM (2002) Activity and intracellular localization of the human cytomegalovirus protein pp71. J Gen Virol 83:1601–1612

McElroy AK, Dwarakanath RS, Spector DH (2000) Dysregulation of cyclin E gene expression in human cytomegalovirus-infected cells requires viral early gene expression and is associated with changes in the Rb-related protein p130. J Virol 74:4192–4206

McGarry TJ, Kirschner MW (1998) Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell 93:1043–1053

Millar JBA, Russell P (1992) The cdc25 M-phase inducer: an unconventional protein phosphatase. Cell 68:407–410

Muganda P, Mendoza O, Hernandez J, Qian Q (1994) Human cytomegalovirus elevates levels of the cellular protein p53 in infected fibroblasts. J Virol 68:8028–8034

Murphy EA, Streblow DN, Nelson JA, Stinski MF (2000) The human cytomegalovirus IE86 protein can block cell cycle progression after inducing transition into the S phase of the cell cycle. J Virol 74:7108–7118

Nishitani H, Lygerou Z (2002) Control of DNA Replication. Genes Cells 7:523–534

Nishitani H, Lygerou Z, Nishimoto T, Nurse P (2000) The Cdt1 protein is required to license DNA for replication in fission yeast. Nature 404:625–628

Nishitani H, Taraviras S, Lygerou Z, Nishimoto T (2001) The human licensing factor or DNA replication cdt1 accumulates in G1 and is destabilized after initiation of S-phase. J Biol Chem 276:44905–44911

Noris E, Zannetti C, Demurtas A, Sinclair J, De Andrea M, Gariglio M, Landolfo S (2002) Cell cycle arrest by human cytomegalovirus 86-kDa IE2 protein resembles premature senescence. J Virol 76:12135–12148

Pajovic S, Wong EL, Black AR, Azizkhan JC (1997) Identification of a viral kinase that phosphorylates specify E2Fs and pocket proteins. Mol Cell Biol 17:6459–6464

Petrik DT, Schmitt KP, Stinski MF (2006) Inhibition of cellular DNA synthesis by the human cytomegalovirus IE86 protein is necessary for efficient virus replication. J Virol 80:3872–3883

Poma EE, Kowalik TF, Zhu L, Sinclair JH, Huang E-S (1996) The human cytomegalovirus 1E1-72 protein interacts with the cellular p107 protein and relieves p107-mediated transcriptional repression of an E2F-responsive promoter. J Virol 70:7867–7877

Rialland M, Sola F, Santocanale C (2002) Essential role of human CDT1 in DNA replication and chromatin licensing. J Cell Sci 115:1435–1440

Rosenke K, Samuel MA, McDowell ET, Toerne MA, Fortunato EA (2006) An intact sequence-specific DNA-binding domain is required for human cytomegalovirus-mediated sequestration of p53 and may promote in vivo binding to the viral genome during infection. Virology 348:19–34

Saffert RT, Kalejta RF (2006) Inactivating a cellular intrinsic immune defense mediated by Daxx is the mechanism through which the human cytomegalovirus pp71 protein stimulates viral immediate-early gene expression. J Virol 80:3863–3871

Salvant BS, Fortunato EA, Spector DH (1998) Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription. J Virol 72:3729–3741

Sanchez V, Spector DH (2006) Cyclin-dependent kinase activity is required for efficient expression and posttranslational modification of human cytomegalovirus proteins and for production of extracellular particles. J Virol 80:5886–5896

Sanchez V, Clark CL, Yen JY, Dwarakanath R, Spector DH (2002) Viable human cytomegalovirus recombinant virus with an internal deletion of the IE2 86 gene affects late stages of viral replication. J Virol 76:2973–2989

Sanchez V, McElroy AK, Spector DH (2003) Mechanisms governing maintenance of cdk1/cyclin B1 kinase activity in cells infected with human cytomegalovirus. J Virol 77:13214–13224

Sanchez V, McElroy AK, Yen J, Tamrakar S, Clark CL, Schwartz RA, Spector DH (2004) Cyclin-dependent kinase activity is required at early times for accurate processing and accumulation of the human cytomegalovirus UL122–123 and UL37 immediate-early transcripts and at later times for virus production. J Virol 78:11219–11232

Sanchez V, Mahr JA, Orazio N, Spector DH (2007) Nuclear export of the human cytomegalovirus tegument protein pp65 requires cyclin-dependent kinase activity and the Crm1 exporter. J Virol 81:11730–11736

Schulze A, Zerfass K, Spitkovsky D, Middendorp S, Berges J, Helin K, Jansen-Dürr P, Henglein B (1995) Cell cycle regulation of the cyclin A gene promoter is mediated by a variant E2F site. Proc Natl Acad Sci U S A 92:11264–11268

Shen YH, Utama B, Wang J, Raveendran M, Senthil D, Waldman WJ, Belcher JD, Vercellotti G, Martin D, Mitchelle BM, Wang XL (2004) Human cytomegalovirus causes endothelial injury through the ataxia telangiectasia mutant and p53 DNA damage signaling pathways. Circ Res 94:1310–1317

Sherr CJ (1996) Cancer cell cycles. Science 274:1672–1677

Sherr CJ (2000) The Pezcoller Lecture: Cancer cell cycles revisited. Cancer Research 60:3689–3695

Shlapobersky M, Sanders R, Clark C, Spector DH (2006) Repression of HMGA2 gene expression by human cytomegalovirus involves the IE2 86-kilodalton protein and is necessary for efficient viral replication and inhibition of cyclin A transcription. J Virol 80:9951–9961

Slee EA, O'Connor DJ, Lu X (2004) To die or not to die: how does p53 decide? Oncogene 23:2809–2818

Sommer MH, Scully AL, Spector DH (1994) Trans-activation by the human cytomegalovirus IE2 86 kDa protein requires a domain that binds to both TBP and RB. J Virol 68:6223–6231

Song Y-J, Stinski MF (2002) Effect of the human cytomegalovirus IE86 protein on expression of E2F responsive genes: a DNA microarray analysis. Proc Natl Acad Sci U S A 99:2836–2841

Song YJ, Stinski MF (2005) Inhibition of cell division by the human cytomegalovirus IE86 protein: role of the p53 pathway or cyclin-dependent kinase 1/cyclin B1. J Virol 79:2597–2603

Speir E, Modali R, Huang E-S, Leon MB, Sahwl F, Finkel T, Epstein SE (1994) Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 265:391–394

Tamrakar S, Kapasi AJ, Spector DH (2005) Human cytomegalovirus infection induces specific hyperphosphorylation of the carboxyl-terminal domain of the large subunit of RNA polymerase II that is associated with changes in the abundance, activity, and localization of cdk9 and cdk7. J Virol 79:15477–15493

Tessari MA, Gostissa M, Altamura S, Sgarra R, Rustighi A, Salvagno C, Caretti G, Imbriano C, Mantovani R, Sal GD, Giancotti V, Manfioletti G (2003) Transcriptional activation of the cyclin A gene by the architectural transcription factor HMGA2. Mol Cell Biol 23:9104–9116

Tran K, Mahr JA, Choi J, Teodoro JG, Green MR, Spector DH (2008) Accumulation of substrates of the anaphase-promoting complex (APC) during human cytomegalovirus infection is associated with the phosphorylation of cdh1 and the dissociation and relocalization of the APC subunits. J Virol 82:529–537

Vassilev LT, Tovar C, Chen S, Knezevic D, Zhao X, Sun H, Heimbrook DC, Chen L (2006) Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. Proc Natl Acad Sci U S A 103:10660–10665

Vousden KH, Lu X (2002) Live or let die: the cell's response to p53. Nature Rev 2:594–604

Wade M, Kowalik TF, Mudryj M, Huang ES, Azizkhan JC (1992) E2F mediates dihydrofolate reductase promoter activation and multiprotein complex formation in human cytomegalovirus infection. Mol Cell Biol 12:4364–4374

Wiebusch L, Hagemeier C (1999) Human cytomegalovirus 86-kilodalton IE2 protein blocks cell cycle progression in $G_1$. J Virol 73:9274–9283

Wiebusch L, Hagemeier C (2001) The human cytomegalovirus immediate early 2 protein dissociates cellular DNA synthesis from cyclin dependent kinase activation. EMBO J 20:1086–1098

Wiebusch L, Asmar J, Uecker R, Hagemeier C (2003) Human cytomegalovirus immediate-early protein 2 (IE2)-mediated activation of cyclin E is cell-cycle-independent and forces S-phase entry in IE2-arrested cells. J Gen Virol 84:51–60

Wiebusch L, Bach M, Uecker R, Hagemeier C (2005) Human cytomegalovirus inactivates the G0/G1-APC/C ubiquitin ligase by Cdh1 dissociation. Cell Cycle 4:1435–1439

Woo MS, Sanchez I, Dynlacht BD (1997) p130 and p107 use a conserved domain to inhibit cellular cyclin-dependent kinase activity. Mol Cell Biol 17:3566–3579

Zannetti C, Mondini M, Andrea MD, Caposio P, Hara E, Peters G, Gribaudo G, Gariglio M, Landolfo S (2006) The expression of p16INK4a tumor suppressor is upregulated by human cytomegalovirus infection and required for optimal viral replication. Virology 349:79–86

Zhang Z, Huong S-M, Wang X, Huang DY, Huang E-S (2003) Interactions between human cytomegalovirus IE1-72 and cellular p107: functional domains and mechanisms of up-regulation of cyclin E/cdk2 kinase activity. J Virol 77:12660–12670

Zhu H, Cong JP, Shenk T (1997) Use of differential display analysis to assess the effect of human cytomegalovirus infection on the accumulation of cellular RNAs: induction of interferon-responsive RNAs. Proc Natl Acad Sci U S A 94:13985–13990

Zwicker J, Muller R (1997) Cell-cycle regulation of gene expression by transcriptional repression. Trends in Genetics 13:3–6

Zwicker J, Lucibello FC, Wolfraim LA, Gross C, Truss M, Engeland K, Muller R (1995) Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression. EMBO J 15:4514–4522