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

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Human cytomegalovirus infection in the presence of the cyclin-dependent kinase (cdk) inhibitor roscovitine leads to changes in differential splicing and the polyadenylation of immediate early IE1/IE2 and UL37 transcripts (V. Sanchez, A. K. McElroy, J. Yen, S. Tamrakar, C. L. Clark, R. A. Schwartz, and D. H. Spector, J. Virol. 78:11219-11232, 2004). To determine if this was associated with specific phosphorylation of the C-terminal domain (CTD) of the RNA polymerase II (RNAP II) large subunit by cdk7/cyclin H and cdk9/cyclin T1, we examined the expression and localization of these kinases and the various phosphorylated forms of RNAP II. Infection resulted in increased RNAP II CTD phosphorylated on serines 2 and 5 and increased levels of activity of cdk7 and cdk9. At early times, cdk9 localizes with input viral DNA, and aggregates of cdk9 and cdk7 and a subset of Ser2-phosphorylated RNAP II colocalize with IE1/IE2 proteins adjacent to promyelocytic leukemia protein oncogenic domains. Later, cdk9 and Ser2-phosphorylated RNAP II form a nuclear punctate pattern; cdk7 resides in replication centers, and Ser5-phosphorylated RNAP II clusters at the peripheries of replication centers. Roscovitine treatment leads to decreased levels of hyperphosphorylated RNAP II (RNAP IIo) in infected cells and of hypophosphorylated RNAP II in mock-infected and infected cells. The RNAP IIo decrease does not occur if roscovitine is added 8 h postinfection, as was previously observed for processing of IE transcripts. These results suggest that accurate IE gene expression requires specific phosphorylation of the RNAP II CTD early in infection.

Human cytomegalovirus (HCMV), a herpesvirus, is an obligate parasite whose life cycle requires an intricate set of interactions between the virus and the host that optimize the environment for viral replication and assembly (for a review, see reference 14). Intertwined with this subversion of the host cell is a defined temporal order of viral gene expression that has been loosely divided into three phases—immediate early (IE), early, and late. The IE gene products are synthesized soon after infection and rely primarily on host factors for their expression, although proteins carried in the viral particle clearly contribute to the process. Several of the viral IE proteins serve as essential transactivators of the next class of gene products, the early genes. Included in the early class are those viral proteins required to “activate” the cell to a metabolic state most conducive for viral DNA synthesis and those proteins involved in the actual replication process itself. Late genes, which constitute the majority of the viral genome and encode primarily structural and maturation proteins, are transcribed in abundance only after the onset of viral DNA replication.

Upon infection, the viral DNA enters the nucleus and a subset of HCMV genomes are deposited at nuclear structures referred to variously as nuclear domain 10 (ND10) structures

or promyelocytic leukemia protein (PML) oncogenic domains (PODs), where viral RNA synthesis begins (21, 22). The HCMV tegument protein pp71 interacts with POD-associated Daxx, which may contribute to the initiation of transcription at POD sites (9, 20, 23, 28). The proximity of the PODs to the spliceosome assembly factor SC35 domains may aid in rapid expression of IE genes, which are often multiply spliced (22). A major region of viral IE transcription includes two genetic units, IE1 and IE2 (for reviews, see references 15 and 31). The predominant IE RNA (IE1) consists of four exons; a single open reading frame (ORF) (UL123) initiates in exon 2 and specifies a 72-kDa nuclear protein designated IE1-72. The IE2 gene product, IE2-86 (ORF UL122), is an 86-kDa protein that is encoded by an alternatively spliced RNA that contains the first three exons of IE1 and a different terminal exon. Another region of IE gene expression is UL36-38, which includes at least five transcripts directed by three promoters (1, 26, 49, 50). One of the promoters directs the synthesis of several spliced 3.2- to 3.4-kb RNAs (UL37 and UL37_M ORFs) that are present in small amounts only at IE times as well as an abundant 1.7-kb unspliced RNA that encodes the UL37 exon 1 (UL37X1) gene product.

Newly synthesized IE1-72 and IE2-86 localize to the PODs. While the punctate pattern of the IE2-86 protein persists, at 3 to 6 h postinfection (p.i.), both IE1-72 and POD-associated proteins become dispersed throughout the nucleus (5, 22, 24, 25). Several studies have shown that IE2-86 is able to localize to the PODs in the absence of IE1-72 but is not able to disrupt

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them (4, 5, 22). IE1-72 is required for disruption of the PODs, but since an IE1 deletion mutant virus (CR208) replicates well at high multiplicity (18, 19, 32), this event is not essential. It appears that even after IE1-72 has caused the dispersal of the PODs, these locations remain important for viral replication. The UL112-113 early gene proteins appear to colocalize with IE2-86 at the peripheries of the original POD sites beginning at about 6 h p.i., and these go on to form sites of viral DNA replication (3, 35). By 48 h p.i., there is a high level of viral DNA synthesis in the replication centers, and the majority of the viral genome is being transcribed.

Viral transcription is directed by the cellular RNA polymerase II (RNAP II), a multisubunit complex. The largest subunit of RNAP II contains a C-terminal domain (CTD), which in human cells consists of 52 repeats of the consensus heptapeptide sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. The CTD is differentially phosphorylated primarily at the serine 2 and serine 5 positions, and the level of phosphorylation varies considerably during the transcription cycle (for reviews, see references 27, 30, and 37). Hypophosphorylated RNAP II (RNAP II_a) is recruited to the initiation complex, and the CTD is then phosphorylated to a hyperphosphorylated form (RNAP II_o). The phosphorylation of the serines in position 5 by cdk7/MAT-1/cyclin H (a component of the basal transcription factor complex TFIID) is followed by the phosphorylation of the serines in position 2 by cdk9/cyclin T (also referred to as positive transcription elongation factor B [P-TEFb]), which is associated with the commitment of the RNAP II complex to elongation. RNAP II_a and II_o forms are in a dynamic equilibrium such that RNAP II_o needs to be dephosphorylated to the II_a form in order to engage in another round of transcription. The primary phosphatase is Fcp1, which is highly conserved and can remove phosphates from the CTD of RNAP II_o when it is engaged in transcription or free. Recently, other phosphatases have been discovered, including the small CTD phosphatases and Ssu72.

The prevailing hypothesis is that the CTD plays a regulatory role in all steps of transcription by serving as the binding domain and transporter of factors involved in RNA synthesis initiation, elongation, 5' capping, splicing, and cleavage/polyadenylation (for reviews, see references 8, 30, 34, and 38). It is believed that the differential recruitment and binding of specific factors that are involved in these processes are significantly influenced by the pattern of phosphorylation of the various Ser2 and Ser5 residues (and possibly by the ubiquitylation, glycosylation, and phosphorylation of other residues) within the 52 repeats. For example, the phosphorylation of the CTD at Ser5 is associated with the recruitment of the mRNA capping enzymes, and the phosphorylation of the CTD at Ser2 is linked to the recruitment of 3' RNA processing factors.

In a recent study, we used the drug roscovitine, which is a specific inhibitor of the cyclin-dependent kinases (cdk's) 1, 2, 5, 7, and 9 (7, 10, 13, 29, 45, 51), to examine the role of these kinases in viral replication (44). We found that addition of the drug at the beginning of the infection resulted in changes in the accumulation and processing of IE transcripts and inhibition of the expression of selected viral early gene products, viral DNA replication, and late gene expression. Roscovitine specifically affected the differential splicing and polyadenylation of the RNAs from both the IE1/IE2 and UL37 genes. The relative position of the sequences used for processing the HCMV un-

spliced UL37X1 RNA and spliced UL37 IE RNAs (47) is very similar to the position of the region between UL123 exon 4 and UL122 exon 5, which are used for the alternative cleavage/polyadenylation and splicing that generates the IE1-72 and IE2-86 RNAs, respectively. For both regions, the signals on the RNA for cleavage/polyadenylation overlap those for the downstream 3' splice acceptor site, with the cleavage/polyadenylation site being preferentially used to generate either IE1-72 or UL37X1 RNAs at IE times. However, in the presence of roscovitine, there was greater utilization of the downstream 3' splice acceptor site, yielding higher levels of the IE2-86 and spliced UL37 RNAs and corresponding lower levels of the IE1-72 or UL37X1 RNAs. We also showed that when roscovitine was added after the first 4 h of infection, the effects on IE gene expression were no longer observed. When it was added after 6 h, viral replication proceeded through the late phase but the viral titer remained low.

One possible explanation for the altered pattern of viral RNA processing was that the effects of the cdk inhibitor were related to the phosphorylation of the CTD of the large subunit of RNAP II by cdk7/MAT-1/cyclin H and cdk9/cyclin T. A recent paper showing that HCMV induces an intermediate form of phosphorylated RNAP II supports this idea (6). Those authors proposed that the CTD might be phosphorylated by the HCMV-encoded kinase UL97. However, their data showed that although the CTD can serve as a substrate for UL97 *in vitro*, RNAP II does not appear to be phosphorylated by this kinase *in vivo*.

In this study, we examine the effect of HCMV infection on the expression, activity, and localization of cdk7/MAT-1/cyclin H, cdk9/cyclin T1, and several forms of the large subunit of RNAP II at both early and late times during the infection. We show that during the course of the infection, there is an increase in cdk7 and cdk9 protein levels and kinase activity and in the amount of RNAP II that is phosphorylated on serine 2 and serine 5 within the CTD. At 48 h p.i., cdk7 and hypophosphorylated RNAP II localize to replication centers, cdk9 and Ser2-phosphorylated RNAP II are distributed in a punctate pattern throughout the nuclei, and Ser5-phosphorylated RNAP II appears in clusters at the peripheries of the viral replication centers. At early times, cdk9 localizes with input viral DNA. In addition, aggregates of cdk9 and cdk7 and a subset of Ser2-phosphorylated RNAP II colocalize with IE1/IE2 proteins adjacent to the PODs. 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. In accord 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. These results suggest that the phosphorylation of the CTD is essential at early time points of the infection and that the required level of CTD phosphorylation for IE gene expression is established within 8 h.

MATERIALS AND METHODS

Cell culture and virus. Human foreskin fibroblasts (HFF) were obtained from the University of California, San Diego, Medical Center and cultured in Earle's minimal essential medium supplemented with 10% heat inactivated fetal bovine serum, 1.5 µg/ml amphotericin B, 2 mM L-glutamine, 100 U/ml penicillin, and

100 µg/ml streptomycin. All reagents were from Invitrogen (Carlsbad, CA). Cells were kept in incubators maintained at 37°C with 7% CO₂. The Towne strain of HCMV was obtained from the American Type Culture Collection (VR 977) and propagated as previously described (48).

Cell synchronization and infections. HFF (passage numbers 15 to 20) were synchronized in G₀ phase by allowing them to grow to confluence as previously described (42). Three days after confluence, the cells were trypsinized, replated at a lower density to allow progression into the cell cycle, and infected at a multiplicity of infection (MOI) of 3 to 5 with HCMV Towne or mock infected with tissue culture supernatants. At 6 h postplating/postinfection, the inoculum was replaced with fresh medium. At designated times p.i., 20 µM roscovitine (Sigma Aldrich, St. Louis, MO) was added to the medium. For experiments extending beyond 24 h, medium was replaced with fresh drug-containing medium at 24 h p.i. and 48 h p.i. The roscovitine stock solution was in dimethyl sulfoxide. Control samples were treated with appropriate volumes of dimethyl sulfoxide. At various times p.i., cells were washed with phosphate-buffered saline (PBS), scraped or trypsinized, and processed as described below.

Antibodies. We used cdk7 monoclonal antibody (MAb) MO-1 (BD Pharmingen, La Jolla, CA); ARNA3 (Chemicon, Temecula, CA); CH16.0, UL44, and UL57 MABs (Goodwin Institute, Plantation, FL); cdk7 polyclonal Ab sc-529, cdk9 MAB sc-13130, cdk9 polyclonal Ab sc-484, MAT-1 polyclonal Ab sc-6234, MAT-1 MAB sc-13142, cyclin H MAB sc-1662, cyclin T1 polyclonal Ab sc-10750, β-catenin MAB sc-7963, and PML MAB sc-966 (Santa Cruz Biotechnology, Santa Cruz, CA); H5, H14, and 8WG16 MABs (Covance, Berkeley, CA); β-actin MAB (Sigma Aldrich); cyclin T1 MAB (Novocastra, Newcastle upon Tyne, United Kingdom); rat anti-BrdU (Accurate Chemicals & Scientific Corp., Westbury, NY); goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) and goat anti-rabbit IgG-HRP (Calbiochem, San Diego, CA); goat anti-mouse IgM-HRP, donkey anti-rat IgG-fluorescein isothiocyanate (FITC), goat anti-rabbit IgG-Cy3, and normal rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA); normal mouse IgM, IgG, IgG1, and IgG2b (Zymed, San Francisco, CA); and FITC- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse IgG1, IgG2a, and IgG2b (Southern Biotech, Birmingham, AL).

Western blots and immunoprecipitations. For Western blot analyses, cells were lysed in Laemmli reducing sample buffer (50 mM Tris, pH 6.8, 0.2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, 50 mM leupeptin, 100 mM pepstatin, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate, 5 mM β-glycerophosphate). The lysates were then sonicated briefly to shear the DNA, and protein content was determined by Bradford assay. Proteins from an equivalent number of cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were stained with amido black to assess protein loading in each lane. The blots were blocked with 5% nonfat dried milk in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) and incubated with primary antibodies diluted in blocking solution as follows: cdk7 MAB, 1:500; cdk9 MAB, 1:250; MAT-1 MAB, 1:500; cyclin T1 MAB, 1:250; CH16.0 MAB, 1:14,000; RNAP II MAB ARNA3, 1:300; CTD Ser2 MAB H5, 1:500; CTD Ser5 MAB H14, 1:1,000; CTD MAB 8WG16, 1:500; β-actin MAB, 1:10,000; and β-catenin MAB, 1:500. The β-actin MAB and β-catenin MAB were used as controls for protein loading. Following three washes with TBST, blots were incubated with horseradish peroxidase-conjugated immunoglobulin-specific antibodies and diluted 1:2,000 (anti-IgG) or 1:10,000 (anti-IgM). After the washing in TBST, proteins were detected using SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) according to the manufacturer's instructions.

For immunoprecipitations (IP), 0.8 to 1.2 µg of polyclonal antibody was coupled per µl of protein A-Sepharose beads with dimethyl pimelimidate dihydrochloride. Cells were lysed in immunoprecipitation buffer (100 mM NaCl, 20 mM Tris-HCl [pH 8], 1 mM EDTA, 0.5% IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO), 1× protease inhibitor cocktail [Roche Chemicals, Indianapolis, IN], and the phosphatase inhibitors 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, and 5 mM β-glycerophosphate). Empirically determined amounts of antibody-coupled beads (2 to 8 µg antibodies per IP) were used for immunoprecipitation of the various proteins. Lysates and antibody-coupled beads were incubated for 4 h or overnight at 4°C. Immunocomplexes were washed in IP buffer and used in kinase assays or boiled in reducing sample buffer prior to polyacrylamide gel electrophoresis and Western blotting.

Bacterial production of GST-CTD. Bacteria harboring a glutathione *S*-transferase (GST)-CTD expression vector were a gift from William Dynan, and the protein was produced as previously described with minor modifications (36). Briefly, bacterial cultures were grown in 5 liters of LB-ampicillin. At an optical density at 600 nm of 0.4 to 0.6, GST-CTD expression was induced with 0.5 mM

IPTG (isopropyl-β-D-thiogalactopyranoside) at 30°C for 16 h. The cell pellet was collected by centrifugation at 4,000 × g. The cells were lysed in 20 ml lysis buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 100 mM KCl, 1% Triton X-100, 20 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A, 100 µg/ml lysozyme) and incubated on ice for 30 min; DNA was sheared by sonication for 2 min in 30-second pulses. The lysate was centrifuged at 6,800 × g, and the supernatant was incubated with glutathione Sepharose beads (Amersham Pharmacia, Uppsala, Sweden) for 6 h at 4°C. The beads were washed three times with wash buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5% IGEPAL CA-630, and 1× protease inhibitor cocktail). The final wash was with kinase reaction buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.5 mM dithiothreitol). GST-CTD was eluted with 50 mM glutathione solution in kinase reaction buffer (pH 8.0). The purified protein was quantified by silver staining of a polyacrylamide gel and was stored in aliquots at -80°C.

Kinase assays. Kinase reactions were carried out with 0.1 µg of GST-CTD per reaction. The kinase complexes were immunoprecipitated by incubating cell lysates with 4 to 6 µg of polyclonal antibody for 4 h at 4°C. The reaction mix (130 µl), consisting of kinase reaction buffer, GST-CTD, 50 µM ATP, and 12 µCi of [γ -³²P]ATP, was added to the Sepharose beads with the immunocomplexes. Reactions were carried out at 37°C for 30 min with mixing of the beads at frequent intervals to prevent settling. The reactions were stopped by the addition of 4× Laemmli buffer and boiled for 5 min. The entire contents of the reactions except the beads were loaded onto an 8% polyacrylamide gel and separated by electrophoresis. The gel was fixed for 5 min, dried, and exposed to X-ray film.

Immunofluorescence. Cells were infected as described above with HCMV Towne or the IE1 deletion virus CR208 (a gift from Edward Mocarski) or mock infected and plated on sterile glass coverslips. At specified time points, the coverslips were washed in PBS and fixed either with ice-cold methanol for 10 min or with ambient-temperature 2% paraformaldehyde solution in PBS for 20 min. Cells were processed as described before (43). Paraformaldehyde-fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min or treated with -20°C acetone for 2 min. Following the washes, the cells were blocked in 10% normal goat serum in PBS for 30 min. Specific antibodies diluted in blocking solution were used to stain the cells. The dilutions were as follows: cdk7 MAB, 1:50; cdk9 MAB, 1:50; cdk9 polyclonal Ab, 1:50; MAT-1 MAB, 1:50; PML MAB, 1:25; CH16.0 MAB, 1:1,000; RNAP II MAB ARNA3, 1:50; CTD phospho-Ser2 MAB H5, 1:50; CTD phospho-Ser5 MAB H14, 1:100; CTD MAB 8WG16, 1:50; UL44 MAB, 1:1,000; and UL57 MAB, 1:500. As controls, the slides were dually stained with each specific antibody and a purified normal mouse IgG, mouse IgM, or rabbit IgG. After three washes with PBS, the coverslips were incubated with an FITC- or TRITC-conjugated, goat anti-mouse isotype-specific secondary antibody diluted 1:50 or a goat anti-rabbit IgG-Cy3 secondary antibody diluted 1:600 and Hoechst dye. Coverslips were washed three times and mounted with SlowFade mounting solution (Molecular Probes, Eugene, OR) onto glass slides. Images were captured using a Nikon Eclipse E800 microscope and Photometrics CoolSnap fx charge-coupled-device camera with Metamorph software. Confocal images were captured at the UCSD Cancer Center Digital Imaging Shared Resource using a Deltavision microscope and SoftWorx software.

BrdU-labeled virus production, infection, and immunofluorescence. Production of BrdU-labeled HCMV Towne virus and infections were performed as previously described (41). Briefly, confluent G₀-phase-synchronized HFFs were trypsinized and infected at an MOI of 0.05 with HCMV Towne. The medium was replaced the next day. At day 6 (~90% cytopathic effect), the medium was replaced with fresh medium containing 10 µM BrdU (Sigma), and the cells were protected from light thereafter. On day 8, additional BrdU was added to the medium, and the viral supernatant was harvested the following day. The titer of the virus was determined by a standard plaque assay.

Two hours prior to infection, confluent G₀-phase-synchronized HFFs were trypsinized and seeded onto coverslips. The cells were incubated on ice 30 min prior to being infected at an MOI of 3 with the BrdU-labeled Towne HCMV or tissue culture supernatant. The cells were incubated on ice for an additional 30 min before the medium was replaced and cells were transferred to 37°C. Immunofluorescence was performed as described above with the following modifications. After completion of the staining for cdk9, cells were denatured with 4 N HCl for 10 min, followed by three 5-min washes in PBS. The cells were blocked with 10% normal donkey serum in PBS for 30 min. The BrdU-specific antibody was diluted in blocking solution at 1:50. A nonspecific rat IgG was used as a control. The FITC-conjugated donkey anti-rat secondary antibody was diluted 1:200 in blocking solution that contained Hoechst dye.

FIG. 1. Mobility of RNAP II changes during the infection along with the increases in Ser2 and Ser5 phosphorylation of the CTD. G_0 -synchronized cells were released into G_1 , infected with HCMV Towne at an MOI of 5 or mock infected, and harvested at the time intervals indicated. Total cell lysates from an equal number of cells were loaded on 6% polyacrylamide gels and transferred to nitrocellulose membranes in buffer containing 0.1% SDS. Western blotting was carried out using different antibodies against RNAP II. ARNA3 recognizes the region of RNAP II outside of the CTD, 8WG16 is specific for unphosphorylated CTD and the CTD that is phosphorylated only on Ser5, and H5 and H14 are specific for the CTD phosphorylated on Ser2 and Ser5, respectively. β -Actin was used as a loading control.

RESULTS

HCMV infection is associated with increased RNAP II CTD phosphorylation. To assess the effect of the HCMV infection on the pattern of phosphorylation of the CTD of the largest subunit of RNAP II, we used Western blot analysis with monoclonal antibodies that recognize different phosphorylated forms of the CTD (Fig. 1). The hyperphosphorylated form (IIo) with the majority of the phosphorylation on serines 2 and 5 within the CTD repeats migrates on gels at 240 kDa, while the hypophosphorylated form (IIa) migrates at approximately 220 kDa. With the antibody ARNA3, which detects both forms of RNAP II, we detected a modest increase in the amount of the hyperphosphorylated protein at the early time points in the infected cells (8 and 12 h p.i.). In the viral samples, there was also a diffuse signal between the two major forms of RNAP II that likely corresponds to the intermediate form of phosphorylated RNAP II reported by Baek et al. (6). At 48 h p.i., an increase in the accumulation of all forms was observed in the infected cells. The difference is somewhat greater than that represented in the figure, as the β -actin control shows that the 48-h viral sample is slightly underloaded on the gel. Using the antibody 8WG16, which detects the hypophosphorylated RNAP IIa and the protein with phosphorylation of Ser5 (but not Ser2) within the CTD, the pattern of accumulation of RNAP IIa in the infected cells was comparable to that observed with the ARNA3 antibody. With this antibody, the intermediate forms were readily seen at 8 h p.i. in the infected cells, although a low level could also be detected in the mock samples.

To further define the nature of the hyperphosphorylated RNAP IIo, we analyzed the cell lysates by immunoblotting with the antibody H14, which detects primarily phosphorylation of Ser5 within the CTD repeats, and with the antibody H5, which has greater specificity for the CTD that is phosphorylated on

Ser2. In accord with the above results, there was an increase in the amount of the Ser5-phosphorylated RNAP IIo and the faster-migrating intermediate species in the infected cells at 8 h p.i. In the case of the Ser2-phosphorylated RNAP IIo, a significant increase in the viral sample was most apparent at 48 h p.i. Taken together, these results suggest that there were increases in both the hypophosphorylated and hyperphosphorylated RNAP II forms as well as in the intermediate forms in the infected cells.

Localization of differentially phosphorylated forms RNAP II in infected and mock-infected cells. RNAP II assembles in the transcription initiation complex in its hypophosphorylated form. The phosphorylation of the serines at position 5 within the CTD is associated with the recruitment of the enzymes involved in the addition of the 7-methyl G cap to the RNA and initiation of transcription. Commitment of the RNAP II complex to elongation follows phosphorylation of the serines at position 2 within the CTD, which is also associated with the recruitment of factors involved in 3' RNA processing. Since the alteration of host protein localization is one of several means by which HCMV furthers its replication at the expense of the host cell, we examined the distribution of the various phosphorylated forms of RNAP II in the infected cells. We were particularly interested in the localization of RNAP II relative to the viral replication centers since high levels of viral RNA are synthesized after the onset of viral DNA synthesis. In addition, the above results showed that the levels of phosphorylated RNAP II were significantly higher in the infected cells at 48 h p.i. than in the mock-infected cells.

To visualize both the viral DNA replication centers and RNAP II, the cells were dually stained at 48 h p.i. with antibodies specific for the different forms of RNAP II as well as with antibodies to the viral DNA replication proteins UL44 and UL57. With the antibody ARNA3, which detects all species of RNAP II, staining in the infected cells was concentrated in the replication center, with a small amount visible in the nucleus outside of the replication center (Fig. 2, panels 1 to 3), while in the mock-infected cells, the staining was diffuse throughout the nucleus (panel 4). In contrast, the confocal images revealed that the RNAP II recognized by the antibody 8WG16 (hypophosphorylated RNAP II and RNAP II with only Ser5 phosphorylated) was localized in the infected cells at the periphery of the replication center in small aggregates (panels 5 to 7) and more diffusely distributed with a punctate appearance in the mock-infected cells (panel 8). The pattern of staining with the H14 antibody (specific for the Ser5-phosphorylated CTD) resembled the 8WG16 staining in that the localization was at the periphery of the replication center in the infected cells (panels 9 to 11) and more diffusely distributed and punctate in the mock-infected cells (panel 12). This was expected since 8WG16 recognizes both hypophosphorylated CTD and a subset of Ser5-phosphorylated CTD that is not phosphorylated at Ser2. However, with H14, some staining in the region of the nucleus distant from the replication center was also visible in the infected cells, which may represent RNAP II that has the CTD phosphorylated at both Ser5 and Ser2. The H5 antibody showed that the Ser2-phosphorylated form of RNAP II was distributed throughout the nucleus, with a punctate appearance in both the infected (panels 13 to 15) and mock-infected (panel 16) cells. Panels 17 to 23 are controls in which the infected cells were stained with a specific antibody

FIG. 2. Localization of RNAP II in HCMV-infected cells. G_0 -synchronized cells that were released into G_1 and infected with HCMV at an MOI of 5 or mock infected were seeded onto glass coverslips. At 48 h p.i., cells were washed with PBS and fixed in either paraformaldehyde or ice-cold methanol. Paraformaldehyde-fixed cells were permeabilized with 0.1% Triton X-100 or with ice-cold acetone. Cells (acetone-treated) were immunostained with the monoclonal antibody ARNA3 (IgG1) or 8WG16 (IgG2a). Cells permeabilized with Triton X-100 were stained with H5 (IgM) to detect the RNAP IIo with the CTD phosphorylated on Ser2, and the methanol-fixed cells were stained with H14 (IgM) to detect the RNAP IIo with the CTD phosphorylated on Ser5. Cells were costained with a RNAP II antibody and either UL44 (IgG1) or UL57 (IgG2a), both of which localize to the viral replication center. Specific antibodies were detected with FITC- or TRITC-conjugated isotype-specific secondary antibodies. Nuclei were stained with Hoechst dye. For immunostaining controls, one of the specific antibodies in the pair was replaced with a nonspecific immunoglobulin (purified mouse IgM or IgG). The corresponding isotype-specific secondary antibodies matching those in the costaining were then used. Except for those marked M48h, all confocal images are of virus (V) at 48 h p.i. The images are confocal optical sections of 0.2 microns. Magnification, $\times 600$.

FIG. 3. The cdk inhibitor roscovitine affects the virus-induced phosphorylation state of RNAP II. HCMV-infected (lanes V) or mock-infected (lanes M) cells were treated with 20 μ M roscovitine at the time of infection or at 8 h p.i. and were harvested at different time points. Total cell lysates from an equal number of cells were run on 6% gels and transferred to nitrocellulose membranes in buffer containing 0.1% SDS. The membranes were subjected to Western blotting using the ARNA3 antibody, which detects all forms of RNAP II. +* indicates that roscovitine was added at 8 h p.i. The membrane stained with amido black is shown below each Western blot to show the protein loading.

along with a nonspecific IgG or IgM antibody that matched the second antibody used in the dual staining. Taken together, these results suggest that in the infected cells, the initiation of viral (and possibly cellular) transcription, as indicated by Ser5 phosphorylation of RNAP II, may occur in clusters at the peripheries of the replication centers and be physically separate from the major region of viral DNA synthesis.

Treatment of cells with the cdk inhibitor roscovitine leads to a loss of the phosphorylated forms of RNAP II in infected cells. The above results showed that there was an increase in the phosphorylation of the RNAP II CTD as the infection progressed. Using the cdk inhibitor roscovitine at a concentration (20 μ M) that inhibits cdk7 and cdk9, the two primary kinases involved in phosphorylating the CTD, we looked for any changes in the pattern of CTD phosphorylation. Western blot analysis with the antibody ARNA3, which detects all forms of RNAP II, revealed that in the presence of roscovitine, there was a marked loss of the hypophosphorylated Ila form of RNAP in the mock-infected

FIG. 4. The cdk inhibitor roscovitine affects virus-induced phosphorylation of Ser5 within the RNAP II CTD. Infected (lanes V) and mock-infected (lanes M) cells were treated with roscovitine, and lysates were prepared, subjected to gel electrophoresis, and transferred to nitrocellulose membranes for Western blotting as described in the legend for Fig. 3. The membranes were probed with the antibody H14, which is directed against RNAP Ilo with the CTD phosphorylated on Ser5. +* indicates that roscovitine was added at 8 h p.i. The lower portion of the blot was probed for β -catenin as a protein loading control.

lysates at 8 h p.i. (Fig. 3). In contrast, in the infected cells, the Ila form showed little change in the presence of roscovitine, while there was a detectable, albeit modest, loss of the hyperphosphorylated RNAP Ilo. At 12 h p.i., the effect of roscovitine on RNAP II can be clearly seen in the infected cells, as evidenced by the decreased levels of both the Ila and Ilo forms.

The observed loss of RNAP Ilo in the infected cells was confirmed by Western blot analysis of the lysates with the antibodies that detect primarily either Ser5- or Ser2-phosphorylated RNAP Ilo (Fig. 4 and 5, respectively). Roscovitine had no significant effect on the levels of the hyperphosphorylated RNAP Ilo in the mock-infected cells. In contrast, roscovitine primarily prevented the virus-associated increase in Ser5-phosphorylated RNAP Ilo that was observed by 8 h p.i., but the level was not significantly lower than that in the mock-infected cells in the presence or absence of roscovitine. A different pattern was observed for the Ser2-phosphorylated RNAP Ilo. In this case, the level of the Ser2-phosphorylated RNAP Ilo in the infected cells treated with roscovitine was significantly lower than that observed in the mock-infected cells in the absence or presence of roscovitine. It should be noted that at the 24-h p.i. time point the lanes contain-

FIG. 5. The cdk inhibitor roscovitine affects the virus-induced phosphorylation of Ser2 within the RNAP II CTD. Infected (lanes V) and mock-infected (lanes M) cells were treated with roscovitine, and lysates were prepared, subjected to gel electrophoresis and transferred to nitrocellulose membranes for Western blotting as described in the legend for Fig. 3. The membranes were probed with the antibody H5, which is directed against RNAP IIo with the CTD phosphorylated on Ser2. +* indicates that roscovitine was added at 8 h p.i. The lower portion of the blot was probed for β -catenin as a protein loading control.

ing the viral samples in Fig. 4 and 5 are underloaded relative to the mock-infected samples.

In our previous study (44), we found that the effect of roscovitine on viral gene expression depends on the time of addition. When added at the time of the infection, there were marked changes in the accumulation and processing of the IE transcripts and inhibition of early gene expression. However, by delaying the addition of the drug until 8 h p.i., these effects on viral gene expression were abrogated and the infection proceeded normally until late times. We reasoned that if the

FIG. 6. Levels of cdk-activating kinase (CAK) components and P-TEFb increase during HCMV infection. Upon release from G₀, synchronized HFFs were infected with HCMV Towne (lanes V) at an MOI of 5 or mock infected (lanes M). Cells were harvested at the indicated time points. Lysates from an equal number of cells were loaded onto gels, electrophoresed, and subjected to Western blotting with antibodies against cdk7, MAT-1, cyclin H, cyclin T1, and cdk9. β -Actin was used as a protein loading control.

phosphorylation of the RNAP II CTD and the effects of roscovitine on the phosphorylation were related to the effects on viral transcription, then a delay in the addition of roscovitine should prevent the loss of the hyperphosphorylated RNAP IIo. Figures 3 to 5 show that this is what occurs. Notably, at 24 h p.i., the levels of the hyperphosphorylated RNAP IIo in the infected cells that were treated with roscovitine at 8 h p.i. were comparable to those in the untreated cells. Interestingly, at 24 h p.i., there were still lower levels of the hypophosphorylated RNAP IIa in both the infected and mock-infected cells regardless of the time of addition of roscovitine, although the effect was greater in the cells that had been in roscovitine for a longer period of time.

The kinase activity and amount of cdk7, cdk9, and associated proteins are upregulated in infected cells. The changes in the phosphorylation of RNAP II and the effect of roscovitine in the infected cells suggested that the viral infection might also affect the levels, activity, or localization of cdk7 and cdk9. cdk7 is found in association with cyclin H or in a complex with cyclin H and MAT-1 to form the cdk-activating kinase. These three proteins are also part of the multisubunit complex TFIIH, which is an RNAP II transcription initiation factor. To assess the effect of the infection on cdk7 and the proteins that associate with this kinase, cells that were synchronized in G₀ were released into G₁ at the time of infection and then harvested at various times p.i. By Western blot analysis, we found that an increase in the amount of cdk7 could be detected between 8 and 24 h p.i. in the infected cells, and this level continued to increase as the infection progressed (Fig. 6). MAT-1 and cyclin H displayed a similar trend, although the increase in MAT-1 was greater than that of cyclin H.

FIG. 7. The levels of cdk7/MAT-1 and cdk9/cyclin T1 complexes and the kinase activities of cdk7 and cdk9 increase during the HCMV infection. G₀-synchronized HFFs were mock infected (lanes M) or HCMV infected (lanes V) and harvested at 48 h p.i. (A) Lysates from an equal number of cells were immunodepleted of cdk7 or MAT-1 using rabbit polyclonal antibodies. The immunoprecipitates and immunodepleted lysates were subjected to gel electrophoresis and Western blotting with cdk7- or MAT-1-specific monoclonal antibodies. A control immunoprecipitation with normal rabbit IgG was also included. (B) Equal numbers of mock-infected or HCMV-infected cells were lysed and immunodepleted of cdk9 or cyclin T1 using rabbit polyclonal antibodies. Western blotting of the immunoprecipitates and the depleted lysates was carried out using monoclonal antibodies against cdk9 and cyclin T1. A control immunoprecipitation with normal rabbit IgG was also included. The filled arrowhead indicates the 62-kDa band recognized by the cyclin T1 monoclonal antibody, and the open arrowhead indicates the 55-kDa band recognized by the cdk9 antibody. (C) Equal numbers of mock-infected or HCMV-infected cells were lysed and immunoprecipitated with cdk7 or cdk9 polyclonal antibodies or normal rabbit IgG coupled to the agarose beads. Purified GST-CTD (0.1 μ g) was used as the substrate in each reaction as described in Materials and Methods. A mixture of 2 μ Ci of [γ -³²P]ATP, kinase assay buffer, and substrate was added to the immunocomplex, followed by incubation at 37°C. The protein was separated by electrophoresis and exposed to X-ray film. The faint band that appears in the IP of the viral lysate at 48 h p.i. with the normal rabbit IgG is nonspecific and does not run at the same position as the phospho GST-CTD.

Western blot analysis of the lysates revealed that the pattern of cdk9 accumulation in the infected cells was similar to that of cdk7. An increased amount of cdk9 was visible in the infected cells at 24 h p.i., and the level continued to rise throughout the infection. We also noted that the cdk9 antibody detected a band of 55 kDa at later times p.i., which likely corresponds to the isoform of cdk9 described by Shore et al. (46). The level of cyclin T1, another component of P-TEFb, was also greater in virus-infected cells at 24 h p.i. and continued to increase as the

infection progressed. In accord with previously published studies (16, 17), cdk9 and cyclin T1 did not cycle in the mock-infected cells, and the level of cyclin T1 remained low in the mock-infected cells throughout the time course. The antibody to cyclin T1 also detected a faster-migrating protein of 62 kDa that disappeared from infected cells by 24 h p.i. It is possible that this smaller form is cyclin T2a (11).

To assess whether the increased cdk7 and MAT-1 remained in association with each other during the infection, we per-

formed IP experiments with antibodies to the individual proteins. Lysates of the infected and mock-infected cells were prepared at the 48-h p.i. time point and immunoprecipitated with cdk7 antibody or with control IgG coupled to protein A-Sepharose beads. The immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with antibodies to both cdk7 and MAT-1. The Western blot of the cdk7 immunoprecipitate (Fig. 7A) shows that both MAT-1 and cdk7 are in a complex, and there appears to be no free MAT-1 in the post-IP supernatant. As expected, neither protein was immunoprecipitated with the control IgG. In a reciprocal experiment, the immunoprecipitation of MAT-1 also brings down cdk7 from the lysates of both mock- and virus-infected cells that were obtained at 48 h p.i. However, cdk7 is still present in the MAT-1-depleted lysate, suggesting that cdk7 is present in excess. Taken together, these data suggest that MAT-1 is not found in a free form in either infected or mock-infected cells. In these experiments, we could not assess whether cyclin H was present in the complexes, as the antibody has low affinity in immunoprecipitation experiments, and there is a high level of background staining of the Western blot following immunoprecipitation with either MAT-1 or cdk7.

In a similar manner, we also examined whether cdk9 and cyclin T1 were in a complex. Immunoprecipitation of lysates obtained at 48 h p.i. with an antibody to cdk9 (Fig. 7B) demonstrated that cdk9 is associated with cyclin T1 in both mock- and virus-infected cells. Likewise, antibody to cyclin T1 coprecipitated cdk9 in lysates from both mock-infected and infected cells. The data also showed that the increased levels of the proteins in the infected cells coincided with an increase in the amount of the complex. However, the presence of a small amount of cyclin T1 and cdk9 in the post-IP lysates of infected cells indicated that a minor population of each protein was not present in the complex. This may be due to the presence of free cdk9 or cdk9 that associates with cyclins T2a, T2b, and K. The unidentified protein of 55 kDa that is recognized by cdk9 antibody was also coprecipitated by cyclin T1. Therefore, this protein is likely to be a higher-molecular-weight form of cdk9. Similarly, cdk9 coprecipitated the smaller protein (62 kDa) that was recognized by anti-cyclin T1 antibody by Western blotting. This form disappeared in the infected cells as early as 24 h p.i. and therefore was not seen in the cdk9 IP of viral lysates.

To determine whether the increase in the protein level of the CTD kinases that began at 24 h p.i. corresponds to their activity, bacterially expressed GST-CTD was used as a substrate for *in vitro* kinase assays with lysates obtained at 48 h p.i. Figure 7C shows that in accord with the increased levels of cdk7 and cdk9 in the infected cells at 48 h p.i., there was also a significant increase in the kinase activities.

Cdk7 and MAT-1 are localized primarily in replication centers, and cdk9 is distributed throughout the nuclei of infected cells. Since the above results showed that there was an increase in both the number of complexes containing cdk7 and MAT-1 and the associated kinase activity, we wanted to determine if their localization was also altered by the HCMV infection. Visualization of the immunofluorescence by confocal microscopy of vertical optical sections documented that cdk7 and MAT-1 were uniformly distributed in the nuclei of mock-infected cells (Fig. 8A,

panels 1 to 3), while at 48 h p.i., they accumulated primarily in the viral replication centers of infected cells (panels 5 to 7). The increased intensity of the cdk7 and MAT-1 signals in the infected cells correlated with the Western blot results. The presence of cdk7 and MAT-1 in the replication centers was confirmed by dual staining of the slides with antibodies to cdk7 or MAT-1 and the viral replication protein UL44 or UL57, respectively (panels 8 to 10 and 11 to 13, respectively). However, the cdk7 and MAT-1 staining was not limited to the replication centers, as both proteins were also observed in the nuclear region at the peripheries of the replication centers.

The distribution of cdk9 in the infected cells differed from that of cdk7. Costaining with UL44 showed that the majority of the protein was distributed uniformly throughout the nucleus and punctate in appearance, although some of the protein did localize to the replication centers (panels 14 to 16). This was similar to the distribution of RNAP II with Ser2 phosphorylation of the CTD (Fig. 2, panels 13 to 15). Although the staining for cdk9 in the mock-infected cells was fainter, it was similar to that seen in the infected cells (for visualization, the photo in panel 4 is overexposed). As controls, the slides were dually stained with each specific antibody and a nonspecific antibody that matched the isotype of the second antibody used in the dual staining (Fig. 8B, panels 1 to 6).

Cdk7 and cdk9 kinase activities are elevated at early times in the infection. As shown above, the level of phosphorylated RNAP II was greater in the infected cells at 8 h p.i. than in the mock-infected cells. However, at this time point the amount of cdk7 and cdk9 in the infected cells was comparable to that in the mock-infected cells. To determine whether the kinase activity was higher in the infected cells at this early time point, we immunoprecipitated the lysates with antibody to cdk7 or cdk9 and assayed the phosphorylation of bacterially expressed GST-CTD in an *in vitro* kinase assay. Figure 9A shows that the kinase activities of both cdk7 and cdk9 were higher in the infected cells. The Western blot of the immunoprecipitates confirmed that the levels of cdk7 and cdk9 were equivalent in the mock-infected and infected cells at this time (Fig. 9B).

Ser2-phosphorylated RNAP II, cdk9, and cdk7 colocalize with the IE1/IE2 proteins at the beginning of the infection. Ishov et al. (22) previously showed that some HCMV input genome is deposited at a subset of the PODs and that HCMV IE transcription originates at these sites. Their work also demonstrated that there is a differential localization of the IE1 and IE2 proteins, with the IE1 protein initially localizing to most PODs and IE2 juxtaposed to only a subset of the PODs. All domains that had IE2 protein also had IE transcripts. Although IE1 then disrupted the PODs at approximately 3 to 4 h p.i., IE2 protein remained at these sites.

In order to determine if transcriptionally active RNAP II is being recruited to and accumulates at the immediate early sites of viral gene transcription, we examined the distribution of RNAP II with the CTD phosphorylated on Ser2 (H5 Ab) relative to the IE proteins (Fig. 10). CH16.0, an antibody that detects both IE1 and IE2, was used to track IE protein localization. Confocal microscopy revealed that a subset of Ser2-phosphorylated RNAP II localized near the IE protein aggregates (Fig. 10A, panels 1 to 3). Within a single visual plane, the majority of the IE aggregates were localized adjacent to the punctate spots containing RNAP II with the Ser2-phosphorylated

FIG. 8. Localization of cdk7, MAT-1 and cdk9 in HCMV-infected cells. (A) G_0 -synchronized cells were released in G_1 , infected with HCMV at an MOI of 5 or mock infected, and seeded onto the glass coverslips. At 48 h p.i., cells were washed with PBS, fixed in paraformaldehyde, permeabilized, and immunostained with monoclonal antibodies against cdk7 (IgG2b), MAT-1 (IgG1), or cdk9 (IgG2b) and a viral replication center protein UL44 (IgG1) or UL57 (IgG2a) as described in Materials and Methods. Specific antibody staining was detected with FITC- or TRITC-conjugated isotype-specific secondary antibodies. (B) For controls, one of the specific antibodies of the pair was replaced with purified normal mouse IgG, and the corresponding pair of isotype-specific secondary antibodies was used. Nuclei were stained with Hoechst dye. Except for those marked M48h, all of the images are of infected cells at 48 h p.i. All of the images except image 4 in panel A are confocal optical sections of 0.2 μ m. Magnification, $\times 600$.

FIG. 8—Continued.

CTD, and occasionally they were partially or completely colocalized (panel 3). In the mock-infected cells, the Ser2-phosphorylated RNAP IIo appeared as punctate spots throughout the nucleus, and as expected, IE1/IE2 staining was not detected (Fig. 10A, panel 4).

Since the above results showed that at 8 h p.i., there was an increase in the kinase activities of cdk7 and cdk9, we were interested in determining whether these kinases accumulated at the sites of HCMV IE transcription. At 2 h p.i., the IE proteins localized to few defined spots (panel 5). As the infection proceeded (3 to 8 h p.i.), the IE1/IE2 staining became more dispersed throughout the nucleus, with several large IE protein aggregates (panels 8 and 12). In mock-infected cells, cdk9 and cdk7 were found throughout the nucleus (Fig. 10A, panels 11 and 15, respectively). In the infected cells, nuclear aggregates appeared when stained for either kinase. Confocal microscopy of infected cells that were costained with antibody against IE1/IE2 and a rabbit anti-cdk9 antibody revealed a colocalization of the IE1/IE2 spots with small cdk9 aggregates as early as 2 h p.i. (Fig. 10A, panels 5 to 7). Both the IE protein spots and cdk9 aggregates increased in abundance and size as the infection progressed (panels 8 to 10). In order to determine if cdk7 is also present at the IE protein spots, cells that were costained with antibodies against IE1/IE2 and cdk7 were visualized by confocal microscopy. In a single visual plane, almost all of the IE protein spots were partially or completely colocalized with the concentrated cdk7 spots (Fig. 10A, panels 12 to 14). The concentrated cdk7 spots that colocalized with the IE protein spots were not as big as the cdk9 aggregates but became more abundant as the infection progressed.

To obtain additional evidence that components of the host cell transcriptional machinery accumulate at sites of IE transcription, we examined whether the input viral genome colocalized with the cdk9 aggregates. Cells were infected with BrdU-labeled HCMV Towne and visualized by dual immunostaining with a rat anti-

FIG. 9. cdk7- and cdk9-associated kinase activities towards the RNAP II CTD are increased in infected cells at early times. Cells infected with HCMV (lanes V) or mock infected (lanes M) were harvested at 8 h p.i. (A) Equivalent numbers of cells were lysed and immunoprecipitated with cdk7 or cdk9 polyclonal antibodies, or normal rabbit IgG coupled to the agarose beads. Purified GST-CTD (0.1 μ g) was used as the substrate in each reaction as described in Materials and Methods. A mixture of 2 μ Ci of [γ - 32 P]ATP, kinase assay buffer, and substrate was added to the immunocomplex, followed by incubation at 37°C. The electrophoresed protein was exposed to an X-ray film. (B) Western blots of the corresponding immunoprecipitates probed for cdk7 or cdk9 with monoclonal antibodies.

BrdU antibody and a cdk9 antibody. By confocal imaging, a clear colocalization between the viral genome and the cdk9 aggregates was observed (Fig. 10A, panels 16 to 18). In a single visual plane, there were some viral genomes that did not colocalize with the cdk9 aggregates, but these were usually outside of the nucleus. No BrdU signal or cdk9 aggregates were detected in mock-infected cells (panel 19). It should be noted that the diffuse background nuclear staining for cdk9 and the Hoechst dye seen in panels 17 to 19 are the result of an added HCl denaturation step during the immunostaining. Figure 10B shows immunostaining controls where one of the specific antibodies in the dually stained panels shown in Fig. 10A was replaced with purified mouse IgM, rat IgG, rabbit IgG, or isotype-specific mouse IgG.

cdk9 localizes adjacent to sites that correspond to the PODs in infected cells prior to dispersal. To confirm that the above results were consistent with the previous studies showing that the PODs are the sites of viral IE transcription, the localization of the cdk9 aggregates in relation to the PODs was examined by confocal microscopy (Fig. 11). Cells were costained with the rabbit anti-cdk9 antibody and an anti-PML MAb, a standard marker for the PODs. In mock-infected cells, the PML staining displayed a prominent speckled pattern in the nucleus, and costaining with cdk9 showed that cdk9 spots were seemingly randomly oriented with respect to the PODs (panels 1 to 4). The trend in the infected cells was that the cdk9 aggregation and the maintenance of PML at the PODs are mutually exclusive (panels 5 to 7). The cell on the left in panels 5 to 7 shows the dispersal of PML and the presence of cdk9 aggregates, and the cell on the right shows the typical PML speckles marking the position of the PODs and the absence of cdk9 aggregates. In the rare case that an infected cell exhibited some cdk9 aggregation before dispersal of the PML, the majority of the cdk9 aggregates were not colocalized at the remaining PODs, although some cdk9 did appear to be adjacent to the PODs. A representative example of this is shown in panels 8 to 11.

One explanation for the above observations was that the cdk9 aggregates are located at PODs, but those PODs are preferentially disrupted in the infected cell. Another possibility was that cdk9 aggregation cannot occur at sites where the PODs are still intact. To distinguish between these possibilities, cells were infected with HCMV CR208, a mutant virus that does not express the IE1-72 protein and hence does not disrupt the PODs. The CR208-infected cells maintained the PODs with a distribution similar to that in mock-infected cells, and dual staining showed that each cdk9 aggregate localized directly adjacent to one or more PODs (panels 12 to 15). Antibody staining controls are shown in panels 16 and 17. The

fact that the cdk9 aggregates still formed in the CR208-infected cells implies that POD dispersal is not a requirement for cdk9 aggregation during the infection, and the localization pattern suggests that the cdk9 aggregates form at sites where POD structures previously existed.

DISCUSSION

The current model of transcription is that synthesis and processing of pre-mRNA are temporally and functionally coupled (for reviews, see references 8, 27, 30, 34, 37, and 38). The CTD on the large subunit of RNAP II plays an important role in providing access to the nascent RNA for different factors involved in the processing of mRNA, and there is increasing evidence that specificity is associated with differential phosphorylation on serines 2 and 5 of its heptapeptide repeats. Phosphorylation of Ser5 on the CTD occurs near the promoter and is involved in the recruitment of capping enzymes. In contrast, phosphorylation of Ser2 appears to occur at a site downstream of the promoter after the initiation of transcription and is associated with the binding of the 3' RNA processing machinery. In this study, we examined the effect of HCMV infection on CTD phosphorylation and the kinases involved at both early and late times. We found that the levels of cdk7 and cdk9, as well as other components of the kinase complexes, MAT-1/cyclin H and cyclin T1, respectively, are upregulated during the infection. In addition, there was a corresponding increase in the kinase activities of cdk7 and cdk9, as measured by the phosphorylation of GST-CTD *in vitro*. The relative amount of phosphorylated CTD was also elevated in the infected cells.

Interestingly, the infected cells do not show a noticeable increase in the amount of cdk7 or cdk9 until 24 h p.i. However, their *in vitro* activities, as well as the level of the hyperphosphorylated form of the RNAP II CTD, increase at earlier times. This suggests that the initial increased kinase activity may be due to other virus-induced changes. It is possible that a viral protein functions as a regulatory subunit for either of these kinases or that the virus induces an association of the kinases with another cellular protein to facilitate the phosphorylation of the RNAP II CTD during the infection. This is consistent with the recent observation that the RNAP II CTD is phosphorylated in herpes simplex virus 1-infected cells by a complex that contains cdk9 and ICP22 (12). Alternatively, the virus may induce relocalization and/or recruitment of cdk7 and cdk9 to RNAP II. Our data showing that concentrated aggregates of these kinases form in infected cells at the site of IE transcription support this hypothesis.

FIG. 10. Ser2-phosphorylated RNAP IIo, cdk7, and cdk9 localize with IE protein domains. (A) G₀-synchronized cells were released into G₁ and infected at an MOI of 3 or 5 or mock infected and seeded onto coverslips. The cells were washed with PBS and fixed with either 2% paraformaldehyde or ice-cold methanol between 2 to 8 h p.i. Cells were coimmunostained with the appropriate combinations of CH16.0 MAb (IgG1) to detect IE1/IE2, H5 MAb (IgM) to detect Ser2-phosphorylated RNAP IIo, and cdk9 rabbit polyclonal antibody, cdk7 MAb (IgG2b), and BrdU rat polyclonal antibody. Specific antibodies were detected with FITC- or TRITC-conjugated isotype-specific anti-mouse Ab, goat anti-rat FITC-IgG, or goat anti-rabbit Cy3-IgG secondary antibodies. Nuclei were stained with Hoechst dye. (B) For immunostaining controls, one of the specific antibodies in the pair was replaced with a nonspecific immunoglobulin (purified mouse IgM, isotype-specific mouse IgG, rabbit IgG, or rat IgG). The corresponding isotype-specific secondary antibodies matching those in the costaining were then used. Arrows denote examples exhibiting some degree of colocalization: a, adjacent; pc, partial colocalization; c, completely colocalized. All the images are 0.2- μ m sections that were obtained by confocal microscopy. Magnification is $\times 1,000$ under oil immersion.

FIG. 11. Cdk9 localizes adjacent to sites that correspond to the PODS in infected cells prior to their dispersal. G_0 -synchronized cells were released into G_1 and infected with HCMV Towne or HCMV CR208 virus at an MOI of 5 or mock infected and seeded onto coverslips. The cells were washed with PBS and fixed with 2% paraformaldehyde at 8 h p.i. Cells were coimmunostained with the appropriate combinations of PML MAb (IgG1) and cdk9 rabbit polyclonal antibody. Specific antibodies were detected with goat anti-mouse isotype-specific FITC-conjugated or goat anti-rabbit Cy3 secondary antibodies. Nuclei were stained with Hoechst dye. For immunostaining controls, one of the specific antibodies in the pair was replaced with a normal isotype-specific mouse IgG1 or rabbit IgG. The corresponding isotype-specific secondary antibodies matching those in the costaining were then used. Panels 4, 11, and 15 are insets depicting an enlarged view of the region within the white box from the previous panel. All of the images are 0.2- μ m sections that were obtained by confocal microscopy. Panels 5 to 7 and 8 to 11 are different cells from the same experiment. Magnification is $\times 600$ under oil immersion for panels 5 to 7 and $\times 1,000$ under oil immersion for all other panels. Inset panels 4, 11, and 15 were magnified 200% using Adobe Photoshop v. 7.0.

Previously, we found that infection of cells in the presence of the cdk inhibitor roscovitine altered the pattern of processing for both the UL122-123 and UL37 transcripts (44). Normally, the first cleavage/polyadenylation site is preferentially used to

generate IE1-72 or UL37X1 RNAs, but in the presence of roscovitine, there appeared to be suppression of the first cleavage/polyadenylation site and enhanced utilization of the adjacent downstream 3' splice acceptor site. As a result, there were

lower levels of the IE1-72 or UL37X1 RNAs and higher levels of the IE2-86 and spliced UL37 RNAs. We proposed that these changes were related to the phosphorylation of the RNAP II CTD, primarily by cdk7/cyclin H and cdk9/cyclin T1 (44). In support of this hypothesis, we show here that when roscovitine is present at the beginning of the infection, the level of CTD phosphorylation is significantly decreased in the infected cells, most notably the phosphorylation of Ser2; there is no change in the mock-infected cells. For the Ser5-phosphorylated form, it appears that inhibition of cdk primarily prevents the increase observed in the infected cells, at least during the first 12 h. In the case of the Ser2-phosphorylated form, the levels are actually lower in the infected cells than in the mock-infected cells. One possibility for this result is that the virus either brings into the cell or activates a phosphatase that preferentially removes the phosphate from Ser2 within the CTD. In the absence of roscovitine, the action of cdk9/cyclin T1 may counter this dephosphorylation. It is tempting to speculate that the reduction in Ser2 phosphorylation of the CTD is associated with decreased recruitment of the cleavage/polyadenylation factors, but further experiments will be required to determine if this is the case.

The effects of roscovitine on the hypophosphorylated RNAP II also differ in the infected and mock-infected cells. Here, there is a more rapid decrease in the mock-infected cells than in the infected cells during the first 8 h, although by 12 h, the levels are comparable. This loss of the hypophosphorylated RNAP IIa in the presence of roscovitine may be related to some inhibitory effect on transcription, as others have observed a similar loss of RNAP IIa when cells are exposed to UV light, α -amanitin, or actinomycin D (33, 39, 40). It is possible that the rate of degradation of the hypophosphorylated RNAP II in the infected cells is comparable to that in the mock-infected cells, but the dephosphorylation of the Ser2-phosphorylated form by a viral-infection-associated phosphatase initially compensates for this. Alternatively, inhibition of the cdk's may lead to differential degradation of the hypophosphorylated and hyperphosphorylated forms of RNAP II in the infected and mock-infected cells. These two possibilities are not mutually exclusive, and there may be some combination of enhanced phosphatase activity and altered stability. In-depth studies of protein stability and assays of phosphatase activity may help resolve these possibilities, and these experiments are in progress.

In accord with our previous study of the effect of roscovitine on IE gene expression (44), the decrease in CTD phosphorylation does not occur if the drug is added after 8 h p.i. There are several possibilities for this result. One explanation is that the phosphorylation of RNAP II necessary for programmed viral IE gene expression is complete by 8 h p.i., and thus inhibitors of cdk7 and cdk9 no longer have an effect. Alternatively, the kinases may no longer be accessible to the inhibitor. In addition, if there is a virus-associated phosphatase that makes the infected cells more sensitive to cdk inhibition, it may be present for only a limited period of time at the beginning of the infection. All of these possibilities are currently being investigated.

Taken together, the studies presented here suggest that the *in vivo* increase of CTD phosphorylation is due to the upregulated cdk7 and cdk9 kinase activities. However, although these two kinases are believed to be responsible for most of the CTD

FIG. 12. Model for an IE transcription site in HCMV-infected cells. The input genome that is deposited at the POD structure functions as a template for IE RNA synthesis. Cellular hypophosphorylated RNAP IIa is recruited to the site and is phosphorylated by cdk7 and cdk9 to the hyperphosphorylated and transcriptionally active RNAP IIo. The IE transcripts move towards the SC35 domains, probably for further processing. The newly synthesized major IE proteins also localize to this region. IE1-72 eventually causes POD dispersal, but the IE2-86 protein remains at the established transcription site. The color key to structures is shown at the right.

phosphorylation, it remains possible that other kinases are also involved in CTD phosphorylation in the infected cell. These results also do not preclude the possibility that there is cdk-dependent modification of other proteins that have an impact on the posttranscriptional processing of the IE transcripts. Multiple factors are involved in the splicing and cleavage/polyadenylation of RNA transcripts, and a change in the abundance, activity, or localization of any of the factors due to alteration of their phosphorylation state could affect the balance of transcripts produced. For example, it has recently been shown that the splicing factor ASF/SF2 is hypophosphorylated and presumably less active early in the infection (2).

In addition to identifying specific components of the host transcriptional machinery that are upregulated during the infection, we also determined the nuclear localization of these components at times of viral IE and late transcription. Ishov et al. (22) provided a model for the IE transcription environment based on their results showing that the viral input genome, IE transcripts, and IE proteins all localize at the PODs and that the IE transcripts move towards the spliceosome assembly factor domains. They hypothesized that either the incoming viral genome is required to localize at preexisting transcriptional environments in the host cell or essential transcription factors must be recruited to the input viral genome. Our data suggest that both events take place. Figure 12 summarizes the findings from Ishov et al. (22) with respect to the IE transcription environment and extends their model based on the results of our studies. We find that the transcription elongation factor cdk9 forms aggregates that colocalize with the IE proteins as early as 2 h p.i. As the infection proceeds during the next few hours, the aggregates of cdk7 and cdk9 increase in size, indicating that both CTD kinases are further recruited to these transcription sites. Since continued IE tran-

scription requires recruitment of the CTD kinases to the sites of viral transcription within the first hours of infection, it is reasonable to assume that the required phosphorylation of RNAP II for synthesis of the IE RNAs may be complete by 8 h p.i. This would also be consistent with the finding that the IE protein spots are localized near the Ser2-phosphorylated RNAP II.

At later times in the infection, multiple copies of the viral DNA are synthesized and there is abundant late gene transcription. It was therefore of interest to determine the relative locations of the viral DNA replication proteins, host cell CTD kinases, and RNA polymerase. We find that cdk7 is localized primarily within the replication center, although some kinase appears at the periphery of the replication center. Interestingly, with the antibody (ARNA3) that is directed against the body of the large subunit of RNAP II outside of the CTD (and thus detects both the hypophosphorylated and hyperphosphorylated forms of the CTD), RNAP II shows a distribution similar to that of cdk7. Yet, the antibody (8WG16) that is directed against the hypophosphorylated CTD, as well as the forms of the RNAP II CTD that are phosphorylated on Ser5 but not Ser2, detects primarily RNAP II in aggregates at the periphery of the replication center. This may be due to the relative affinity of the antibodies in the immunofluorescence assay. We also cannot rule out the possibility that ARNA3 detects RNAP II that is heavily phosphorylated on a residue different than Ser2 or Ser5 (or modified in other ways) and that this form preferentially localizes at the replication centers. However, it is likely that the antibody 8WG16 detects Ser5-phosphorylated CTD, as a similar pattern in the infected cell was observed with the antibody (H14) that detects the Ser5-phosphorylated CTD, irrespective of the phosphorylation of Ser2. In contrast, the RNAP II with the CTD phosphorylated on Ser2 was distributed throughout the replication center and nucleus. One interpretation of these results is that the initiation of the high level of late gene transcription on the viral DNA, as indicated by the clusters of Ser5-phosphorylated RNAP II, occurs at a site that is physically separate from the replication center where the viral DNA is being synthesized. These may also be sites of initiation of host cell RNA transcription. Commitment to elongation then occurs at domains distal to these aggregates.

The studies presented here provide another example of the ingenious ways that CMV subverts the host cell for its own needs. Deciphering the mechanisms governing the coupling of RNA transcription and processing and the role of differential CTD phosphorylation in the regulation of the various steps involved in mRNA synthesis is an active area of research. An understanding of how the virus commandeers the transcriptional machinery not only may advance our knowledge of viral pathogenesis but also may help elucidate the mechanisms underlying regulated gene expression.

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