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Human Cytomegalovirus Infection Inhibits G_1/S Transition

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Cell cycle progression during cytomegalovirus infection was investigated by fluorescence-activated cell sorter (FACS) analysis of the DNA content in growth-arrested as well as serum-stimulated human fibroblasts. Virus-infected cells maintained in either low (0.2%) or high (10%) serum failed to progress into S phase and failed to divide. DNA content analysis in the presence of G_1/S (hydroxyurea and mimosine) and G_2/M (nocodazole and colcemid) inhibitors demonstrated that upon virus infection of quiescent (G_0) cells, the cell cycle did not progress beyond the G_1/S border even after serum stimulation. Proteins which normally indicate G_1/S transition (proliferating cell nuclear antigen [PCNA]) or G_2/M transition (cyclin B_1) were elevated by virus infection. PCNA levels were induced in infected cells and exhibited a punctate pattern of nuclear staining instead of the diffuse pattern observed in mock-infected cells. Cyclin B_1 was induced in infected cells which exhibited a G_1/S DNA content by FACS analysis, suggesting that expression of this key cell cycle function was dramatically altered by viral functions. These data demonstrate that contrary to expectations, cytomegalovirus inhibits normal cell cycle progression. The host cell is blocked prior to S phase to provide a favorable environment for viral replication.

Human cytomegalovirus (CMV) is a betaherpesvirus that replicates productively in many differentiated cell types, including epithelial cells, endothelial cells, fibroblasts, and certain hematopoietic cell types (reviewed in reference 22). CMV replication is species specific, and primary human fibroblasts (HFs) are commonly used to support viral growth and to study the function of viral gene products. Low-passage HFs have also been the subject of many years of work on cell cycle regulation in response to growth factors (28), passage number (16), and genome stability (15). Recent analysis by Jault et al. (17) demonstrated a profound alteration of cyclin-dependent kinase activities in infected cells. Indeed, CMV infection has long been associated with the stimulation of host DNA, RNA, and protein metabolism in permissive as well as nonpermissive cells (2, 12–14, 40, 41, 44). More recently, these findings have been used to suggest a role for this virus in benign proliferative diseases such as atherosclerosis (21) and restenosis (39). However, in situ studies by DeMarchi and colleagues (8, 10) raised questions about the role of viral gene products in host cell stimulation because highly stimulated cells did not support viral replication. Viral gene expression and viral DNA replication occurred only in those cells that failed to activate cellular DNA replication (8, 10, 11).

To investigate the interaction between CMV and the cell cycle more closely, DNA content profiles and expression patterns of proliferating cell nuclear antigen (PCNA, a marker of G_1/S progression) and cyclin B_1 (a marker for G_2/M transition) in high and low serum conditions as well as in the presence of specific cell cycle inhibitors were analyzed on a single cell basis, using fluorescence-activated cell sorter (FACS) analysis and immunofluorescence. Contrary to expectations, CMV infection does not stimulate DNA synthesis in quiescent cells. Furthermore, infection does not allow serum-induced cell cycle progression beyond the G_1/S border.

Cell cycle progression and viral replication under low and high serum conditions. To determine whether CMV growth was dependent on cell cycle progression, the production of progeny virus and viral DNA was assessed in resting and serum-stimulated cells. HFs, arrested in the G_0 phase of the cell cycle by serum starvation for 72 h, were infected and placed in low (0.2%)- or high (10%)-concentration fetal bovine serum. Following infection with a multiplicity of infection (MOI) of 1, viral DNA and progeny accumulated in the supernatant of cells maintained in either low or high serum. At 120 h postinfection (p.i.), there was only a fivefold difference in progeny titers (data not shown). This and recent results by (19) demonstrate that viral replication was less dependent on serum stimulation than was previously recognized (9 and reviewed in reference 22).

To monitor cell cycle progression in CMV-infected cells, 5×10^5 confluent HFs (passage $\leq$15) were synchronized by serum starvation for 72 h prior to infection. This resulted in $\geq$90% of the cells being arrested in the G_0/G_1 phase of the cell cycle. Cells were infected (MOI of 6) as described previously (38), except that the inoculum, CMV strain AD169, was prepared from the supernatant of cells infected at an MOI of 0.01 and cultured in 0.2% serum. After a 1-h adsorption period in serum-free medium, the cells were maintained in either low (0.2%)- or high (10%)-concentration fetal bovine serum. Indirect immunofluorescence analysis to detect CMV nuclear antigens ($IE1_{491aa}$ and $IE2_{579aa}$) in infected cells confirmed that the infection was synchronous and showed that virus initiated infection in a high proportion ($\geq$90%) of cells (data not shown). Cells were harvested at various times p.i., and the DNA content in isolated nuclei was measured by propidium iodide (PI) staining and FACS analysis (36). Similar results were obtained by using either the Towne or AD169 strains of CMV. When cells were exposed to virus that had been neutralized by anti-glycoprotein B antibodies, FACS analysis revealed that cell cycle progression proceeded normally (data not shown). These experiments demonstrated that the observed alterations in cell cycle progression were not a consequence of contaminating factors in the virus stock but required viral entry.

Virus-infected cells held in low serum exhibited a G_0/G_1 peak at 24 h p.i. (Fig. 1D), but this peak broadened and

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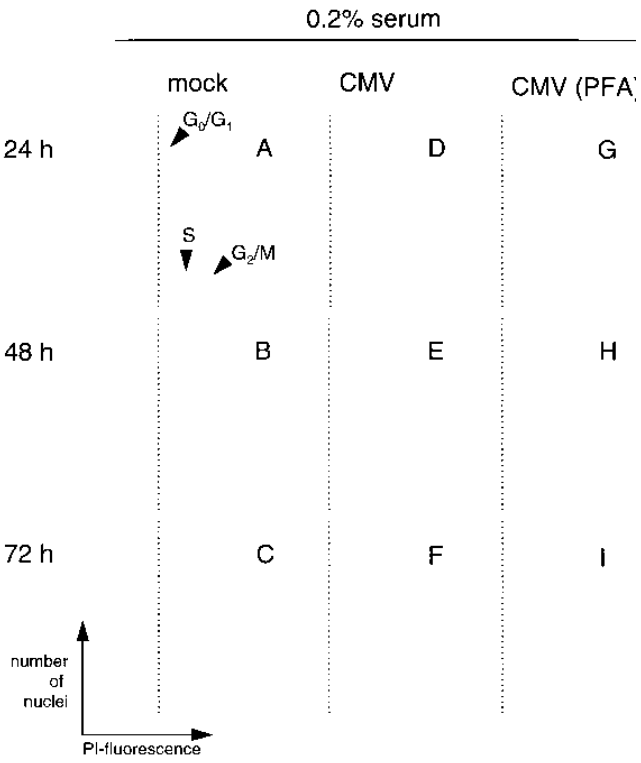

FIG. 1. DNA content analysis of CMV-infected (24, 48, and 72 h p.i.; panels D to I) and mock-infected (panels A to C) cells maintained in 0.2% serum. Panels G to I show virus-infected cells maintained in the presence of PFA at 200 μg/ml. Cells were processed as previously described (36). The relative DNA content depicted as PI fluorescence intensity and number of nuclei were determined by FACS analysis at 24, 48, and 72 h p.i. 20,000 events were evaluated for each analysis. Standard G_0/G_1, G_2/M, and S peaks are indicated in panel A.

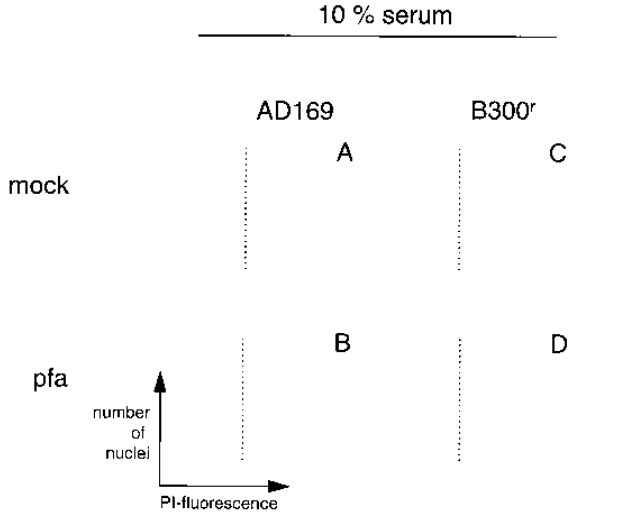

FIG. 2. DNA content analysis of cells at 72 h p.i. after infection with AD169 (A and B) or AD169-B300^r (C and D) strains of CMV maintained in 10% serum in the absence (A and B) or presence (C and D) of PFA at 200 μg/ml. Shown are the relative DNA content depicted as PI fluorescence intensity and number of nuclei as determined by FACS analysis.

became progressively more intense between 48 and 72 h p.i., consistent with the expected accumulation of viral DNA (Fig. 1E and F). Mock-infected cells maintained in low serum remained in G_0/G_1 at all times throughout the course of the experiment (Fig. 1A, B, and C). The single peak of fluorescence in infected cells at 24 h p.i. was broader than that in the mock-infected control. However, there was no indication of a significant S or G_2/M population in infected cells. This increase may be the result of input viral DNA binding to PI or of progression from G_0/G_1 toward the G_1/S border. The presence of 200 μg of phosphoroacetic acid (PFA) per ml, a specific inhibitor of viral but not cellular DNA replication (46, 47), abolished the increase in overall DNA content (Fig. 1G, H, and I), consistent with our interpretation that accumulating progeny viral DNA contributed substantially to the DNA content at late times. Greater than 4 N PI fluorescence has been observed up to 120 h p.i., and the PI intensity of infected cells at late times correlated with the size of the viral inoculum (data not shown). The CMV genome has 60% G+C content and is 2.3×10^5 bp in length. Approximately 10^3 to 10^4 genome equivalents have been detected in an infected cell (23). Thus, given the preference of PI to intercalate into G+C-rich regions of the DNA (33), the PI signal of accumulating virus progeny is in the range of the expected signal from cellular DNA (10^9 bp) and can account for the peak observed.

Morin et al. (24) showed that when 45 μg of PFA per ml was used, the overall DNA content increased at late times in CMV-infected HFs, despite a drastically reduced rate of thymidine incorporation into viral DNA. It is likely that under high MOI conditions the accumulation of viral DNA can be detected by FACS analysis even at greatly reduced rates of synthesis. To further characterize the contribution of viral DNA to the PI signal, we employed a mutant virus (B300^r) derived from AD169 which was highly resistant to PFA (42). Figure 2 shows that the increase in DNA content followed the growth properties and PFA sensitivity of the input viruses. The DNA content of wild-type infected cells was once again reduced by PFA (compare Fig. 2B and 2A) at 72 h p.i., whereas the DNA content of cells infected with the PFA-resistant mutant increased with a similar profile in the presence or absence of inhibitor (Fig. 2C and D). In summary, virus-infected cells differ from mock-infected, serum-stimulated cells in exhibiting a block in cell cycle progression and they differ from mock-infected, serum-starved cells because they exhibit a synchronous increase in DNA content at 48 h p.i., which correlates with the accumulation of viral DNA.

Cell cycle progression after serum stimulation and CMV infection. To determine whether normal cell cycle progression could be inhibited by CMV, DNA content profiles were recorded from virus- and mock-infected cells after serum stimulation. At 24 h after serum stimulation and infection, CMV-infected cells remained in the G_0/G_1 phase of the cell cycle (Fig. 3E), whereas mock-infected cells progressed to G_2/M (Fig. 3A), suggesting that CMV prevented cell cycle progression. At 72 h p.i., infected cells showed a DNA content profile similar to that of infected cells maintained in low serum (compare Fig. 1F with 3F). This demonstrated that after infection of quiescent cells CMV inhibited the cellular response to serum which was consistent with the failure of CMV-infected cells to grow exponentially when maintained in high serum (data not shown).

To determine whether cellular DNA synthesis contributed to the DNA content at all and to better locate the CMV-induced block of cell cycle progression, virus-infected cells were serum-stimulated in the presence or absence of hydroxyurea. At 1 mM, hydroxyurea arrests cells at the G_1/S border by inhibiting ribonucleotide reductase and preventing cellular DNA replication (3, 18). This concentration of hydroxyurea

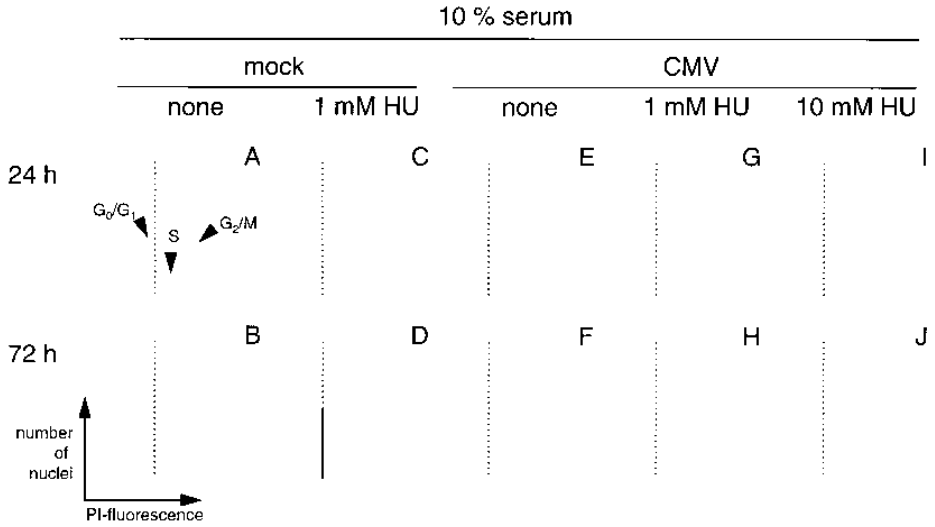

FIG. 3. DNA content analysis of cells from CMV-infected (E to J) and mock-infected (A to D) cells (24 and 72 h p.i.) maintained in 10% serum in the absence (A, B, E, and F) or presence (C, D, and G to J) of hydroxyurea (HU). Shown is the relative DNA content depicted as PI fluorescence intensity and number of RNase-treated cells as determined by FACS analysis. Standard G_0/G_1, G_2/M, and S profiles are indicated by arrowheads in panel A.

does not inhibit viral DNA replication (1). Serum-stimulated cells (Fig. 3C) held in 1 mM hydroxyurea remained at the G_1/S border, whereas untreated mock-infected cells progressed through the cell cycle by 24 h (Fig. 3A). Serum-stimulated virus-infected cells held in the absence (Fig. 3E) or presence (Fig. 3G) of the drug showed a DNA content profile identical to that of hydroxyurea-treated mock-infected cells (Fig. 3C), demonstrating that CMV-infected cells were arrested before the cell cycle stage (G_1/S) defined by this drug block.

At later times (72 h p.i.), the DNA content in virus-infected cells increased with similar pattern in untreated and hydroxyurea-treated cells (Fig. 3F and H). The DNA content of virus-infected cells was far greater than that of mock-infected cells (Fig. 3B and D) under either condition. Higher doses of hydroxyurea (10 mM), which are known to inhibit viral replication (1), blocked any increase in DNA content (Fig. 3J), consistent with our interpretation that the DNA content at 72 h p.i. was of viral origin. Mimosine, which at the dose used (0.1 mM) arrests cells at the G_1/S border by interfering with cellular ion metabolism (48), was used to confirm that infected cells did not proceed beyond the G_1/S border. Serum-stimulated mock-infected (Fig. 4B) and virus-infected cells held in the presence (Fig. 4D) or absence (Fig. 4C) of 0.1 mM mimosine exhibited a similar G_1/S block, whereas mock-treated uninfected cells progressed through the cell cycle in a 24-h period (Fig. 4A). These data demonstrate that the virus-induced block was similar to the mimosine-induced block. At 72 h, virus-infected cells in the presence or absence of the inhibitor showed increased DNA content compared to uninfected cells (Fig. 4E to H), since at these concentrations mimosine did not inhibit CMV

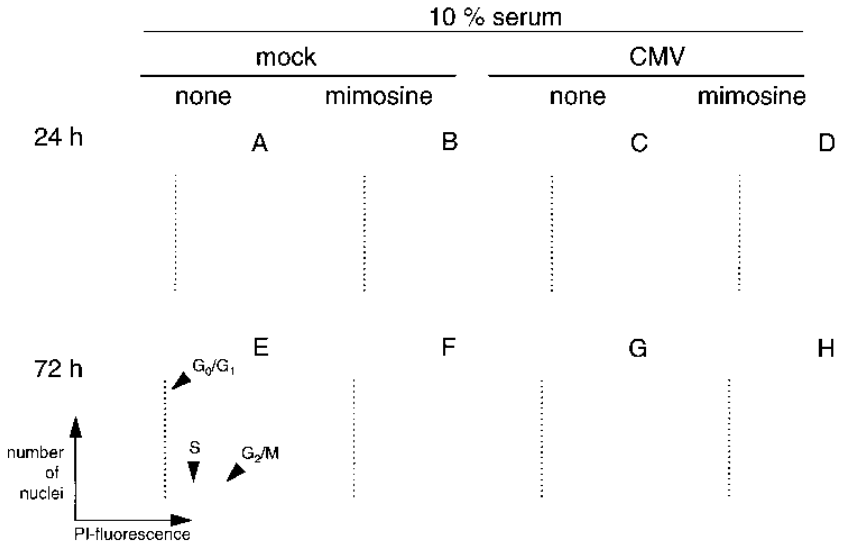

FIG. 4. DNA content analysis of cells from CMV-infected (C, D, G, and H) and mock-infected (A, B, E, and F) cells (24 and 72 h p.i.) maintained in 10% serum in the absence (A, E, C, and G) or presence (B, F, D, and H) of 0.1 mM mimosine. Shown are the relative DNA content depicted as PI fluorescence intensity and number of RNase-treated cells as determined by FACS analysis. Standard G_0/G_1, G_2/M, and S profiles are indicated by arrowheads in panel E.

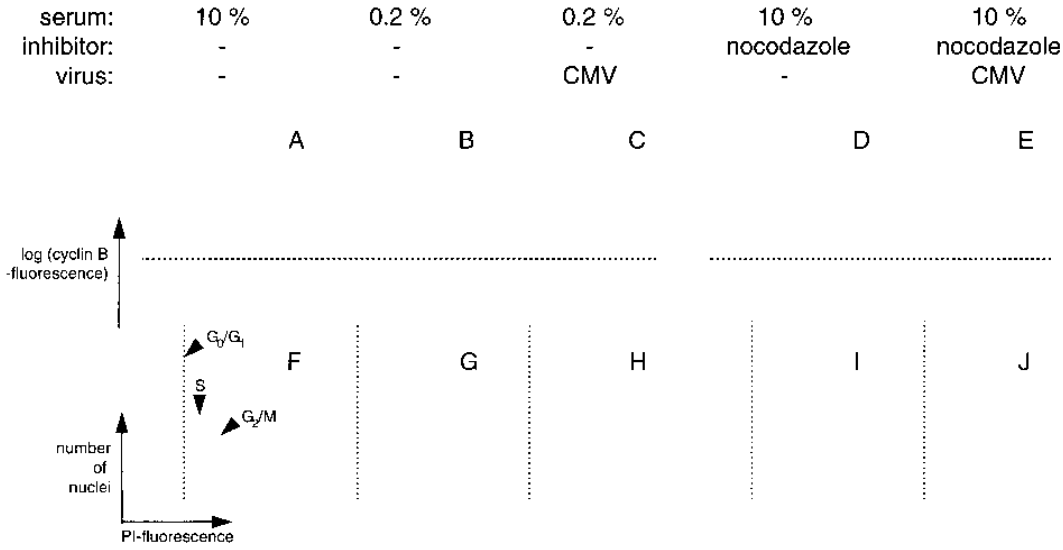

FIG. 5. Simultaneous FACS analysis of cyclin B_1 expression (A to E) or DNA content (F to J) in mock-infected (A, B, D, F, G, and I) or virus-infected (C, H, E, and J) cells maintained in 0.2% serum (B, C, G, and H) or serum stimulated in the absence (A and F) or presence (D, I, E, and J) of 40 ng of nocodazole per ml at 24 h p.i. The dotted line indicates background cyclin B fluorescence. In the top panels, the contour line density correlates with cell number. Standard G_0/G_1, G_2/M, and S profiles are indicated by arrowheads in panel F.

DNA replication (reference 7 and data not shown). These experiments show that the CMV-infected cells fail to exhibit a normal serum response and that viral replication proceeds independently of the presence or absence of two mechanistically different inhibitors of cell cycle progression.

To determine whether any quiescent (G_0) cells infected and maintained in low serum could escape the CMV-induced G_1/S block, the experiments were repeated in the presence of the microtubule inhibitor nocodazole which at 40 ng/ml slows the progression through G_2/M (50). In the presence of the drug, mock-infected cells accumulated in G_2/M at 24 h after serum stimulation (Fig. 5I) while CMV infected cells accumulated in G_1/S (Fig. 5J). Similar results were obtained by using colcemid as another inhibitor of G_2/M transition, and comparable levels of progeny virus were produced in the presence and absence of either inhibitor (data not shown). Colcemid or nocodazole at these concentrations induce a G_2/M cell cycle block that is not a result of the general inhibition of microtubule formation (6). These data demonstrate that CMV replication is independent of cell cycle progression. Cells infected at G_0 remain at the G_1/S border throughout infection.

Analysis of PCNA and cyclin B_1 expression during CMV infection. The inhibition in cell cycle progression in CMV-infected cells could have resulted from a virus-specific block, a cellular DNA damage response, or competition for cellular replication factors. In order to determine whether cell cycle-regulated gene expression in infected cells correlated with the failure to progress beyond G_1/S, we examined proteins that normally show cell cycle-regulated expression. Biochemical studies had shown that p53 was induced by CMV infection, suggesting a DNA damage response which normally coincides with G_1 arrest or apoptosis (5, 20) but also that cyclin A and cyclin B_1 expression were induced consistent with a G_2/M block (17). We extended these observations with immunofluorescence and FACS analysis to demonstrate that PCNA and cyclin B_1 levels, albeit induced, did not follow their normal expression patterns in infected cells.

In HFs, cyclin B_1 expression is induced in S phase, reaches maximal levels in G_2 phase, localizes to the nucleus in M phase, and is degraded prior to cell division (30). Cyclin B_1 degradation is necessary for cell division to occur (26). Cyclin B_1 kinase activity has been previously shown to be induced and to remain high at all times during viral infection (17), consistent with a cell cycle block prior to mitosis. We assessed cyclin B_1 levels and DNA content simultaneously by using FACS analysis. Figure 5F to J, depict the DNA content profiles, and panels A to E depict contour plots showing the relationship between cyclin B_1 expression and DNA content. Virus-infected cells held in low serum exhibited increased cyclin B_1 levels despite a G_1/S DNA content (Fig. 5C and 5H) at 24 h p.i. As expected, only the G_2/M fraction of mock-infected cells showed high cyclin B_1 levels at 24 h after serum stimulation, whereas the G_0/G_1 DNA content fraction of mock-infected, serum-stimulated cells exhibited low levels of cyclin B_1 (Fig. 5A and F) as did cells maintained in low serum (Fig. 5B and G). These results demonstrate that in virus-infected cells, cyclin B_1 expression is not correlated with the G_2/M phase but is induced in cells with a G_1/S DNA content. The virus-induced dysregulation of cyclin B_1 expression was exaggerated in nocodazole-treated cells. Virus-infected cells in the presence of the inhibitor showed a G_1/S DNA content (Fig. 5J) but high cyclin B_1 levels (Fig. 5E) similar to those in virus-infected serum-stimulated cells in the absence of nocodazole (Fig. 5C and H). Mock-infected serum-stimulated cells in the presence of nocodazole had an increased fraction of cells with a G_2/M DNA content compared to untreated cells (Fig. 5F and I) and increased cyclin B_1 expression (Fig. 5A and D) at 24 h after serum stimulation. This confirmed that CMV induces cyclin B_1 expression, but this induction occurred in cells exhibiting a G_1/S DNA content.

PCNA is a processivity factor of host cell δ polymerase (31), which exhibits a nuclear localization with dramatically increased levels in both early S phase and during DNA repair (37). CMV infection induced PCNA expression in low serum (Fig. 6C) at 24 h; however, at 48 h p.i. PCNA was seen in a punctate staining pattern in virus-infected cells (Fig. 6D) consistent with the formation of replication compartments (29). The PCNA staining pattern was nuclear based on counter-

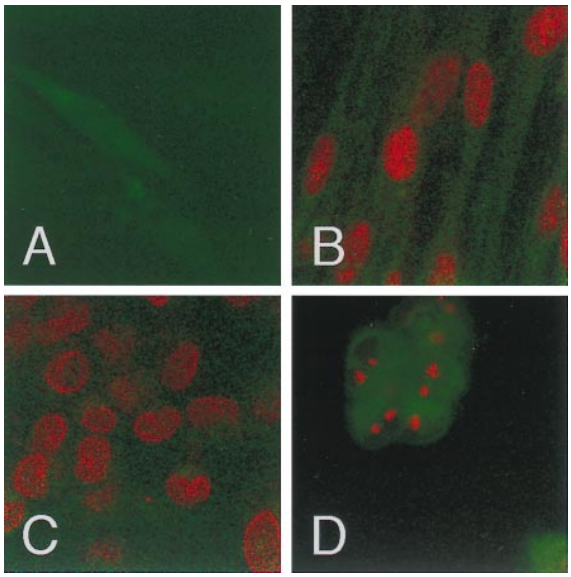

FIG. 6. Analysis of PCNA expression in mock-infected (A and B) or virus-infected (C and D) cells at 24 h p.i. (A, B, and C) and 48 h p.i. (D) maintained in low (0.2%, panels A, C, and D) or high (10%, panel B) serum by indirect immunofluorescence confocal microscopy with a biotinylated anti-PCNA monoclonal antibody (PC-10; Santa Cruz Biotechnology) followed by Texas red-conjugated streptavidin (Vector Laboratories) according to manufacturer's instructions. Colchicine-fluorescein isothiocyanate (FITC) (Molecular Probes) counter staining was used to indicate cell shape. In parallel experiments (not shown), FITC-conjugated antibody to CMV IE1/IE2 (810-FITC; Chemicon) was used to identify infected cell nuclei. Final magnification, ×600.

staining cells with either colchicine-fluorescein (to indicate the cytoplasm) or with an antibody directed against CMV nuclear antigens (IE1/IE2; data not shown). The cellular localization of PCNA in CMV-infected cells differed from that of mock-infected cells which showed no detectable PCNA signal when maintained in low serum (Fig. 6A) and diffuse nuclear staining 24 h after serum stimulation (Fig. 6B). Thus, despite induction, the unusual localization of PCNA suggested that its function was altered in virus-infected cells. This result was consistent with a virus-induced cell cycle arrest in which accessory cellular replication functions were induced but not functional in cellular DNA replication.

We have shown that CMV replication is complete regardless of serum conditions and that quiescent (G_0) cells infected with CMV do not proceed beyond the G_1/S border. Through the use of inhibitors for cell cycle progression (hydroxyurea, mimosine, nocodazole, and colcemid) or viral replication (PFA), we showed that the increase in DNA content in infected cells correlates with viral and not cellular DNA synthesis.

How can these findings be reconciled with earlier experiments showing that CMV stimulates cell DNA synthesis? Many of the earlier studies were carried out with lower virus inocula that failed to successfully initiate infection in all cells. This was best documented by the work of DeMarchi (8–10). Also, some of the data suggesting that CMV stimulates cell cycle progression was derived from nonpermissive cell systems rather than permissive HFs (14). The results of previous metabolic labeling experiments might be reconciled with a G_1/S block if the incorporation of metabolic precursors (12–14, 40, 41, 43, 44) represented incomplete DNA replication or extensive DNA repair. Consistent with this interpretation, an in situ analysis showed localization of bromodeoxyuridine incorporation (19, 29) and PCNA into a punctate pattern in virus-infected cells rather than diffuse staining seen in actively growing uninfected cells.

In addition to factors directly involved in cellular or viral replication, many regulatory functions are dysregulated in CMV-infected cells. Expression of many proteins such as DNA polymerase α, ornithine decarboxylase, thymidine kinase, dyhydrofolate reductase, and many transcription factors is induced during CMV replication (reviewed in reference 22). p53 is induced in CMV-infected cells, suggestive of a DNA damage response (25, 39). While this would normally lead to cell cycle arrest or apoptosis (5, 20), these pathways are apparently inhibited in infected cells (49). Furthermore, the late induction of cyclin A-dependent kinase activity in infected cells and the dysregulated expression of cyclin B_1 (17) suggest a complex interplay between CMV and the cell cycle machinery. This is consistent with a model in which CMV induces a cell cycle state that fosters viral proliferation, increasing the availability of replication factors and nucleotide pools, while at the same time abolishing competition by cellular polymerases, inhibiting cell division and apoptosis. The virus-induced cell cycle arrest likely depends on the state of the host cell at the time of infection insofar as cells infected in G_0 are arrested before the G_1/S border, whereas cells already in S or G_2 may proceed to the G_2/M border, as suggested by Jault et al. (17).

Small DNA tumor viruses like the adenoviruses, papillomaviruses, and simian virus 40 have evolved to induce the cellular replication machinery and depend on cellular enzymes for viral DNA replication (reviewed in references 27 and 45). In these viruses, expression of the viral regulatory proteins leads to cellular transformation in the appropriate settings. The herpesviruses, including CMV, appear less dependent on host enzymes for viral DNA replication (4, 35). Herpes simplex virus types 1 and 2 have evolved to shut down macromolecular host cell synthesis (32, 34), whereas CMV has often been held out as an exceptional herpesvirus that stimulates the host cell (2, 12, 13, 21, 39, 40, 41, 44); our work suggests that CMV replicates in resting cells without stimulating cell cycle progression. Instead of a broad shutdown of host cell proteins, CMV causes a specific cell cycle arrest at or before the G_1/S border. Cellular functions that normally associate with S phase (E2F, dihydrofolate reductase, PCNA, DNA polymerase α, and cyclin A) are induced, but cellular DNA replication fails to commence.

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