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Persistence of the Cytomegalovirus Genome in Human Cells

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A small percentage of human fibroblast cells survived high-multiplicity infection by cytomegalovirus and were isolated as persistently infected cultures. Approximately 30% of the cells were in the productive phase of infection, since virus-specific structural antigens and virions were associated with these cells. The remaining cells contained neither viral structural antigens nor particles. Nuclear DNA from these nonproductive cells contained approximately 120 genome equivalents of viral DNA per cell as determined by reassociation kinetics. In situ hybridization confirmed that nuclei from nonproductive cells contained a significant amount of viral DNA that was distributed in most of these cells. Early virus-induced proteins and antigens were also detected. Nonproductive cells continued to grow, and there was a slow, spontaneous transition of some of these cells to productive viral replication. The majority of the viral DNA in nonproductive cells persisted with restricted gene expression. When infectious virus production was eliminated by growing the persistently infected cultures in the presence of anti-cytomegalovirus serum, approximately 45 genome equivalents of the viral DNA persisted per cell. The reassociation reaction approached completion. After removal of the antiserum and subculturing, infectious virus production resumed. Therefore, it was assumed that all sequences of the viral genome remained associated with these cells. Restriction of cytomegalovirus gene expression in persistently infected cell cultures is discussed.

Human cytomegalovirus (CMV), like the other herpesviruses (42), has a propensity for persistence in the host after primary infection. Persistent infection may last for years in the presence of a host immune response (26, 35, 53). In cell culture, persistent infection by a genital isolate of CMV has resulted in oncogenic transformation of these cells (13). Since CMV infections frequently follow immunosuppression and transplantation, it has been postulated that the virus is latent in a majority of the adult population (20, 26, 35, 53). The latency potential of CMV is supported by the isolation from infected individuals of cell lines that contain viral DNA but are free of detectable infectious virus or late viral antigens (22, 36).

The tendency of herpesviruses for persistent and latent infections may result from the restriction of viral replication in infected cells. Restriction of CMV replication and gene expression occurs in nonpermissive cells of animal origin. In these cells, early viral protein synthesis occurs, but viral DNA and late protein synthesis are not detectable (45). The viral genome can persist in nonpermissive cells for a long period of time and be recovered by fusion with permissive human cells (3). Another human herpesvirus, Epstein-Barr virus, can remain latent within lymphoid cells in the infected individual or in tissue culture (9). Early viral antigen synthesis (9) and limited virus-specific transcription (19) are detectable. The Epstein-Barr virus genome has been found in an episomal (27, 33) or integrated (1, 2) state. Both CMV-infected (48) and Epstein-Barr virus-infected (16) nonpermissive cells of human origin can be induced to produce infectious virus by treatment with halogenated pyrimidines.

This report describes the isolation and characterization of human fibroblast cells that survived infection with CMV and continued to grow. Evidence for persistence of the viral genome with limited viral gene expression is presented.

MATERIALS AND METHODS

Virus and cells. An established laboratory strain of human CMV (Towne) was plaque purified and passaged at low multiplicities in human fibroblast (HF) cells as previously described (45). Primary HF cell cultures were used for infection between passages 5 and 10. Cells persistently infected with CMV (HF_{CMV}) were isolated and subcultured in Eagle minimum essential medium containing 15% fetal calf se-

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rum (GIBCO, Grand Island, N.Y.) and 50 μg of aureomycin per ml (American Cyanamid Co., Lederle Laboratories, Pearl River, N.Y.). The amount of infectious virus was determined by assays for plaques (54) or tissue culture infective doses (11). Virus and cells were routinely tested for mycoplasm as previously described (43). Cell number was determined by dispersing cells in phosphate-buffered saline (PBS, pH 7.2) containing 0.5 mM EDTA and 0.05% trypsin and counting the cells on a hemacytometer. Cell viability was determined by erythrocin B dye exclusion.

The percentage of infectious cells was determined by washing monolayers three times with PBS containing 1 mM EDTA, dispersing the cells with PBS containing 0.5 mM EDTA and 0.05% trypsin, and counting the cells on a hemacytometer. Cell-free virus in the cultures was neutralized by anti-CMV serum as previously described (43). Cells were diluted in medium containing 2% anti-CMV serum and incubated at room temperature for 15 min before plating onto HF cell monolayers. Approximately 99% of the plaque-forming activity in an antiserum-treated cell suspension was trapped by a 0.45-μm filter and, therefore, was cell associated. Only 1% of infectious cell-free virus was trapped by a 0.45-μm filter.

Electron microscopy. Cells, grown on glass cover slips, were fixed for 15 min with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) containing 0.5 mM calcium chloride and postfixed with 1% osmium tetroxide in phosphate buffer for 15 min, 1% thiocarbohydrazide in phosphate buffer for 10 min, and 1% osmium tetroxide for 15 min as described by Kelly et al. (24). Fixed cells were dehydrated with ethanol and hydroxypropyl methacrylate (Polysciences, Warington, Pa.) by the method of Brinkley et al. (4) and then embedded in a mixture of Epon-Araldite (Ladd, Inc., Burlington, Vt.). Individual cell types in the embedding medium could be distinguished by light microscopy. Selected cells were thin sectioned parallel to the plane of the growth surface.

Antisera and immunofluorescence. Human convalescent sera specific for either immediate early and late antigens (M-19) or just late antigens (MIC) of CMV (28) were kindly provided by S. Michelson-Fiske, Unité de Virologie Médicale, Institut Pasteur, Paris. Rabbit neutralizing anti-CMV serum was prepared against the membrane antigens of purified virions and dense bodies of CMV as previously described (43). The rabbit antiserum was heated to 56°C for 30 min and absorbed with whole and lysed HF cells prior to use. Indirect immunofluorescence was performed on methanol-fixed cells by using standard methods (28).

Incubation with radioactive precursors. Cells were pulse-labeled with [^{35}S]methionine for 2- or 3-h periods as previously described (45).

Polyacrylamide gel electrophoresis. Equivalent amounts of radioactivity from cell lysates were analyzed by sodium dodecyl sulfate (SDS)-slab polyacrylamide gel electrophoresis in 9 or 10% gels by a modification (44) of the method of Laemmli (25). Molecular weights of viral polypeptides were estimated by the procedure of Weber and Osborn (52) using standard markers (43). After electrophoresis, gels were stained with Coomassie brilliant blue and destained as described by Fairbanks et al. (10). Autoradiography of dried gels was performed with Kodak Tri-X Ortho film at room temperature.

Cell separation. Cell cultures persistently infected with CMV were separated into nonproductive and productive cell fractions by Ficoll-Hypaque gradient centrifugation. After the cells were washed three times with ice-cold PBS, 0.25% trypsin in Hanks balanced salt solution was added and the cells were incubated at 37°C for 1 min. This step selectively dislodged many of the productively infected cells from the growth surface. The remaining mixture of cells was washed three times with PBS containing 1 mM EDTA and then treated with PBS containing 0.5 mM EDTA and 0.05% trypsin at 37°C for 5 min. Suspended cells were pelleted by centrifugation in ice-cold PBS containing 10% fetal calf serum. The cells were then layered onto a discontinuous gradient of 3.3% Ficoll (Sigma Chemical Co., St. Louis, Mo.)-3.6% Hypaque (Winthrop Laboratories, New York, N.Y.) to 8.3% Ficoll-9.0% Hypaque in PBS containing 1 mM EDTA and 1% fetal calf serum. Gradients were centrifuged at 400 × *g* for 10 min at 4°C. Cells producing infectious virus sedimented to the dense regions of the gradient, and nonproductive cells remained in the uppermost gradient fractions. Fractionated cells were washed twice with PBS at 4°C and then analyzed by SDS-polyacrylamide gel electrophoresis as described above or further fractionated as described below.

Extraction of whole cell or nuclear DNA. Nuclei were prepared from cells by the method of Penman (34). Whole cells or nuclei in 0.05 M Tris-hydrochloride (pH 8.1) containing 0.1 M NaCl, 0.01 M EDTA, and 0.4% SDS were treated with 600 μg of Proteinase K (predigested 1 h at 37°C, from Beckman, Palo Alto, Calif.) per ml for 1 h at 37°C. The concentration of SDS was increased to 1%, and the samples were deproteinized by extracting with phenol and chloroform-isoamyl alcohol (24:1). The samples were precipitated with 70% ethanol and dialyzed successively against 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 1 mM EDTA, 0.1× SSC containing 1 mM EDTA, and 0.02 M Tris-hydrochloride (pH 7.4) containing 1 mM EDTA. The RNA was degraded by adding RNase A (heated to 80°C for 10 min; Sigma) to a final concentration of 100 μg/ml. After 4 h at 37°C, the samples were deproteinized and dialyzed again as described above. The concentration of DNA was determined by the diphenylamine reaction according to Giles and Myers (17) using calf thymus DNA (Sigma) as a standard.

Preparation of radioactively labeled DNA. Purified adenovirus (18) or CMV (46) DNA was radiolabeled in vitro with [α-^{32}P]dCTP or [^{3}H]dTTP (Amersham, Arlington Heights, Ill.) by nick translation with *Escherichia coli* DNA polymerase I (Boehringer Mannheim, Chicago, Ill.) as described by Rigby et al. (37). The specific activity of the ^{32}P-labeled CMV DNA ranged from 6×10^7 to 10×10^7 cpm/μg, and the specific activity of the ^{3}H-labeled adenovirus type 2 or CMV DNA was 2×10^7 cpm/μg.

Reassociation kinetics. Reassociation of DNA was by the method of Schachat and Hogness (39). The reaction mixture consisted of 25 μg of cell DNA, 1 ng of ^{32}P-labeled CMV DNA, and 100 μg of calf thymus DNA in a total volume of 350 μl of 0.01 M Tris-

hydrochloride (pH 7.5) containing 1 mM EDTA. After the DNA was sonicated to an average size of 400 base pairs and denatured by boiling for 5 min, the temperature was equilibrated to 68°C and the reassociation reaction was initiated by adding 150 μl of 5 M NaCl in 0.01 M Tris-hydrochloride (pH 7.5) containing 1 mM EDTA. Samples of 50 μl were transferred to test tubes in a Dry Ice-propanol bath at various times. The extent of renaturation was assayed by determining the percent radioactivity resistant to the single-strand-specific S1 nuclease (Miles Laboratories, Elkhart, Ind.) as described by Sutton (49). Each 50-μl sample received 250 μl of 30 mM sodium acetate (pH 4.4) containing 0.5 mM $ZnCl_2$ and 50 μl (5,000 units) of S1 nuclease. After incubation at 37°C for 10 min, 20 μg of calf thymus DNA and 2 ml of ice-cold 10% trichloroacetic acid were added. The precipitates were collected by filtration onto Whatman GF/C glass fiber filters, and the acid-insoluble radioactivity was determined in a Packard liquid scintillation spectrometer. Under the above conditions, S1 nuclease digested 96 to 99% of the heat-denatured ^{32}P-labeled CMV DNA probe. Approximately 80% of nondenatured or completely renatured probe viral DNA was resistant to the enzyme. The fraction of probe viral DNA remaining single-stranded (C/C_0) at a given time was calculated as follows: $C/C_0 = [(cpm_t) - (cpm_i)]/[(cpm_f) - (cpm_i)]$, where cpm_t is the radioactivity resistant to S1 nuclease at a given time; cpm_i is the S1-resistant radioactivity in the fully denatured sample; and cpm_f is the S1-resistant radioactivity in either the native or fully renatured sample. Reassociation of randomly sheared viral DNA followed a modified second-order rate reaction according to the following equation (41): $(C_0/C)^{2.3} = 1 + k\,C_0t$. C_0 and C are the concentrations of single-stranded DNA at time $t = 0$ and t, respectively, and k is the second-order reaction rate constant.

In situ hybridization. Cell nuclei, squashed on slides as described by Gall and Pardue (12), were treated with 2× SSC containing 100 μg of RNase A per ml for 30 min at 37°C and then washed three times with 0.1× SSC. Nuclear DNA was denatured with 95% formamide in 0.1× SSC for 2 h at 65°C. After dehydration in ethanol, the slides were air dried and then treated for 5 min at 45°C with 2× SSC containing 50% formamide and 600 μg of calf thymus DNA per ml. The slides were washed three times with 0.1× SSC, and hybridization was initiated by adding 40 μl of 4× SSC containing 50% formamide, 9 μg of calf thymus DNA, and 2×10^5 cpm (10 ng) of ^3H-labeled CMV DNA that was sonicated to an average size of 400 base pairs and denatured by boiling for 5 min. The slides were incubated at 45°C for 60 h. ^3H-labeled adenovirus type 2 DNA was used as a control. After hybridization, the slides were washed for 30 min with 30 mM sodium acetate (pH 4.5) containing 0.1 M NaCl and 3 mM $ZnCl_2$ and then treated for 2 h at 45°C with 100 units of S1 nuclease per ml in the above buffer. The slides were washed overnight with six changes of the above buffer, dehydrated with ethanol, and air dried. The slides were coated with Kodak NTB-2 emulsion that was diluted 1:3 with 1% aqueous glycerol, dried, and treated with dioxane containing 0.2% PPO (2,5-diphenyloxazole) and 0.02% dimethyl POPOP [1,4-bis-(5-phenyloxazolyl)benzene], desiccated, and incubated at −70°C for 1 week. After the emulsion was developed, the slides were washed in dioxane and distilled water, air dried, and stained with Giemsa.

RESULTS

Isolation of HF cells persistently infected with CMV (HF_{CMV}). HF cells infected with CMV remain viable and continue to synthesize host-specific proteins even during the late phase of productive infection (44). To determine whether virus production could be sustained for an extended period of time, cells were infected at a multiplicity of 5 PFU per cell, and fresh medium was added every 4 days. High titers of infectious virus were produced for 3 weeks, during what is referred to as the productive phase of infection (Fig. 1). Productive infection appeared to involve the entire cell monolayer because of widespread viral cytopathic effect. Virus production declined at 3 weeks postinfection (p.i.) as the cell monolayer was destroyed (Fig. 1). After 4 weeks, foci of growing cells were observed and virus production increased with renewed cell growth (Fig. 1). This is referred to as the persistent phase of infection. Virus was detected through the transition from productive to persistent infection because productive infection did not subside abruptly and because of viral replication in outgrowing cells. Confluent persistently infected cells were subcultured every 2 weeks starting at 6 weeks p.i., and infectious virus continued to be produced throughout subculture (Fig. 1). HF_{CMV} cell cultures continued to grow until 25 weeks p.i. or more. Eventually crisis occurred and the cells could no longer be successfully subcultured. It is notable that no special handling was necessary to establish or to maintain HF_{CMV} cell cultures. For the experiments described below, HF_{CMV} cell passage levels of 5 to 12 were employed.

Characteristics of persistently infected cells. The HF_{CMV} cell cultures were studied by both light and electron microscopy. Two morphologically distinguishable cell types were observed. Approximately 30% of the HF_{CMV} cells contained typical cytoplasmic inclusions (Fig. 2A) and had a rounded cytopathology identical to productively infected HF cells (Fig. 2B). However, most of the cells were morphologically distinct from both productively infected (Fig. 2B) and uninfected (Fig. 2C) HF cells. These cells had a larger, cuboidal appearance (Fig. 2A). Fibroblastic cells were not apparent in HF_{CMV} cultures.

To determine the distribution of viral particles in the two cell types, HF_{CMV} cells were grown on glass cover slips, fixed, and embedded for electron microscopy. Since this procedure preserved the characteristic cell morphology, cells with a

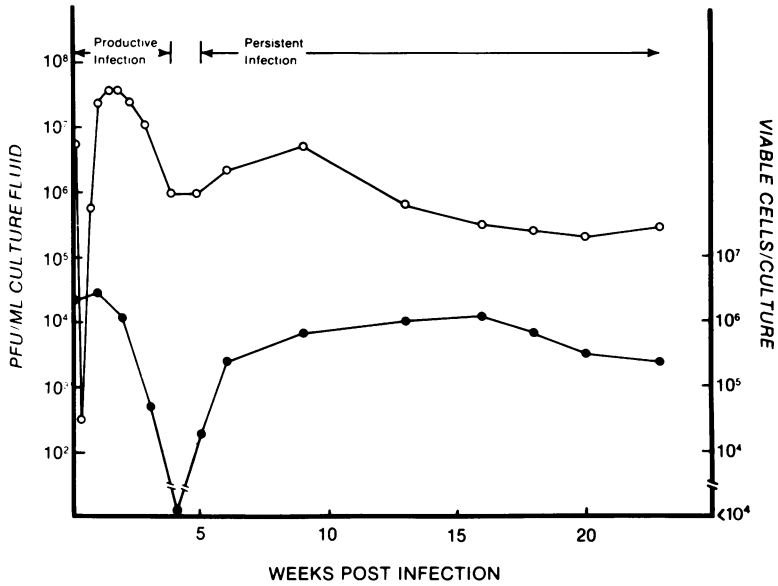

FIG. 1. *Infectious virus yield during productive and persistent infection. Outgrowing HF_{CMV} cells were subcultured at 2-week intervals starting at 6 weeks p.i. PFU per milliliter* (○) *and viable cell number per culture* (●) *were determined.*

rounded cytopathology or cells with the larger, cuboidal appearance were selected and thin sectioned for ultrastructural analysis as described in Materials and Methods. The rounded cell types contained viral nucleocapsids in their nucleus and both virions and dense bodies in the cytoplasm (Fig. 3A). Dense bodies are an aberrant morphogenic form of CMV (7, 40). Intracellular nucleocapsids, virions, or dense bodies were not detected in the larger, cuboidal cell type (Fig. 3B), but both virions and dense bodies were adsorbed to the cell surface (Fig. 3C). The rounded cell type appeared to be actively involved in virus production, whereas the cuboidal cell type appeared nonproductive. The ratio of productive to nonproductive cells did not change appreciably during passage of the HF_{CMV} cells.

Viral DNA sequences in persistently infected cells. To determine whether nonproductive HF_{CMV} cells contained viral DNA, they were separated from productive cells in Ficoll-Hypaque density gradients as described in Materials and Methods. Purified nuclei from nonproductive cells did not contain detectable infectious virus, as determined by plaque assay of 5×10^5 disrupted nuclei. In contrast, nuclei from productive cells had approximately 10^6 PFU per 5×10^5 disrupted nuclei. In addition, synthesis of late infected cell-specific polypeptides (ICSP) of CMV was used as a criterion for separation of nonproductive and productive cells. These results are described in the following section. Nuclear DNA was prepared from fractionated HF_{CMV} cells and analyzed by reassociation kinetics with ^{32}P-labeled CMV DNA. For comparison, nuclear DNA was also isolated from uninfected and late-phase productively infected cells. Reassociation kinetics followed the second-order reaction rate relationship described in Materials and Methods. The acceleration of the rate of reassociation was used to quantitate viral DNA sequences in infected cells (15). A reconstruction experiment was performed assuming a molecular weight of 1.47×10^8 for the CMV genome (14) and 3.92×10^{12} for the host cell genome (15). Known amounts of unlabeled CMV DNA, corresponding to 10 (0.01 μg), 30 (0.03 μg), 100 (0.1 μg), 300 (0.3 μg), and 1,000 (1 μg) genome equivalents per cell, were added to 25 μg of uninfected cell DNA and 5.7×10^4 cpm (1 ng) of ^{32}P-labeled viral DNA probe. The rate of reassociation was directly proportional to the amount of CMV DNA added, and, consequently, there was a linear relationship between the rate of reassociation and the genome equivalents of viral DNA per cell (Fig. 4A). Nuclear DNA from nonproductive as well as productive HF_{CMV} cells accelerated the rate of reassociation of the probe (Fig. 4B). Using the relationship between the rate of reassociation and genome equivalents per cell, nonproductive cell nuclei were estimated to contain 120 genome equivalents of CMV DNA per diploid complement of cell DNA. The reassociation reaction continued to completion, suggest-

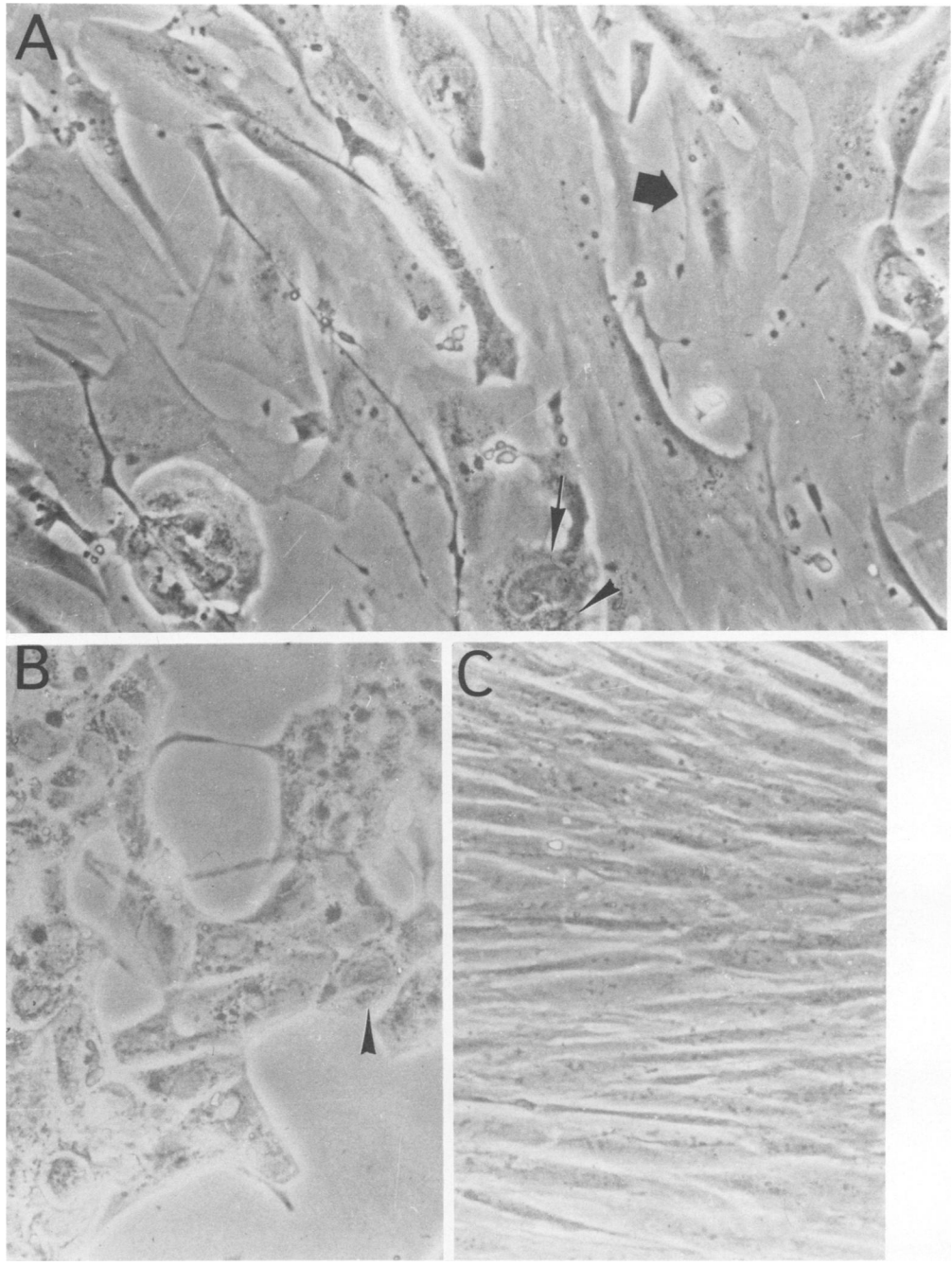

FIG. 2. *Phase-contrast photomicrographs of cells during productive and persistent infection. (A) Persistently infected cells; (B) productively infected cells at 72 h p.i.; (C) uninfected cells. The arrows designate the following: large arrow, cuboidal type cell; small arrow, rounded type cell; arrow head, cytoplasmic inclusion. ×600.*

ing that all CMV genome sequences were present in the nuclei from nonproductive HF_{CMV} cells (data not shown). Nuclear DNA from productive HF_{CMV} cells contained approximately 3,300 genome equivalents per cell, and nuclear DNA from infected cells at 72 h p.i. contained approx-

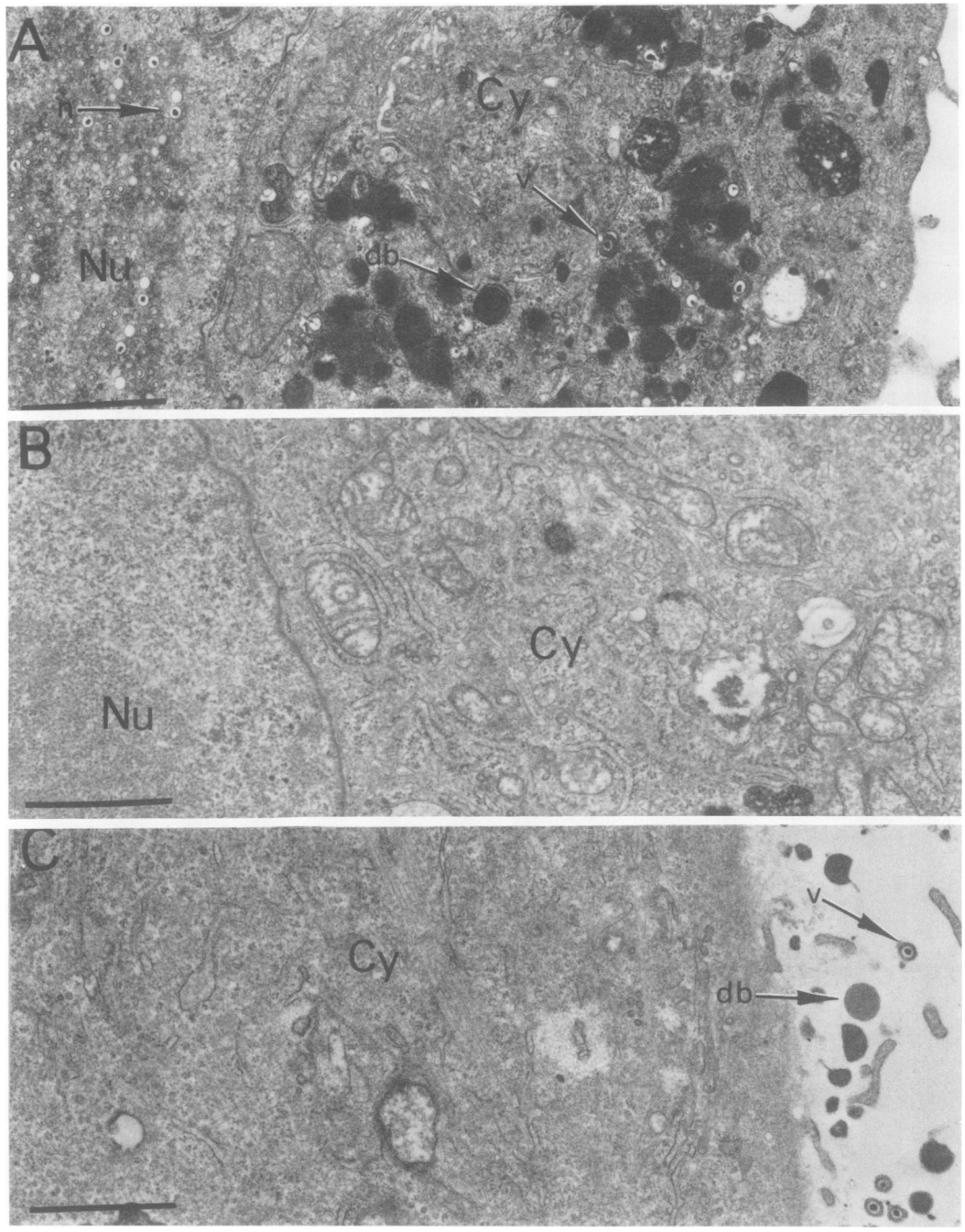

FIG. 3. *Electron micrographs of persistently infected cells. (A) Rounded cell type with viral nucleocapsids (n) in the nucleus (Nu) and virions (v) and dense bodies (db) in the cytoplasm (Cy). (B) Cuboidal cell type with no nucleocapsids, virions, or dense bodies. (C) Typical cuboidal cell with virions and dense bodies adsorbed to the cell surface. Bar, 1,000 nm.*

imately 2,100 genome equivalents of CMV DNA per cell.

To determine whether virus adsorbed to the cell surface contaminated the nuclear fraction of isolated nonproductive cells, HF cells were infected with 20 PFU per cell and maintained at 4°C to prevent viral penetration. The cells were subsequently removed from the growth surface

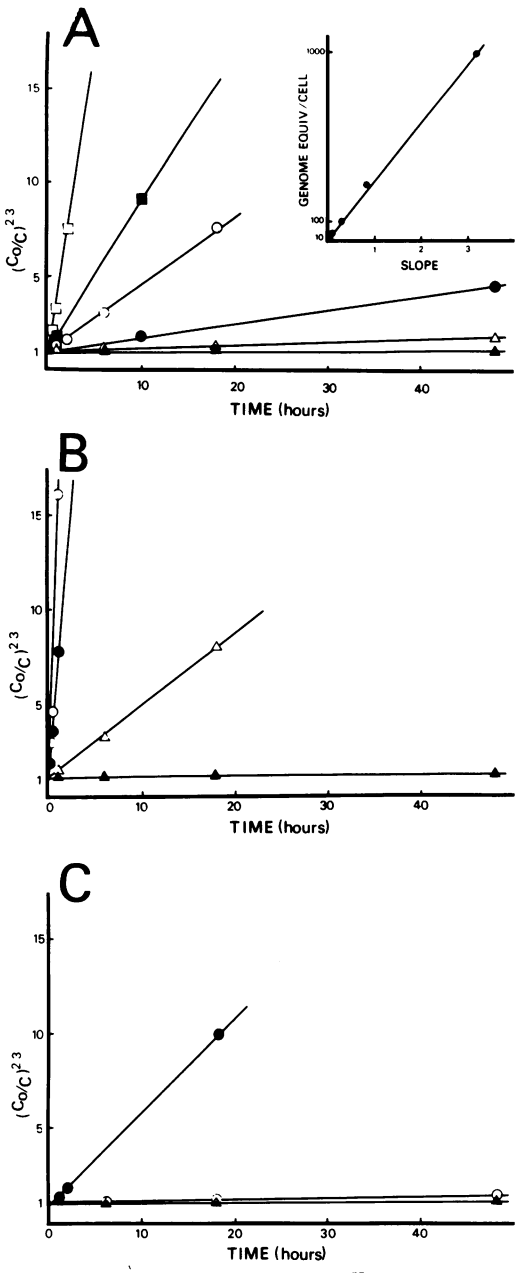

FIG. 4. *Reassociation kinetics of ^{32}P-labeled CMV DNA in the presence of DNA extracted from the nuclei of nonproductive or productive HF$_{CMV}$ cells fractionated as described in the text. (A) Reconstruction experiment; uninfected cell DNA (▲) and 10 (△), 30 (●), 100 (○), 300 (■), and 1,000 (□) genome equivalents of unlabeled CMV DNA per diploid cell complement of DNA. The inset is a standard curve illustrating the linear relationship between the slope of the reaction rate and the amount of CMV DNA added. (B) Nuclear DNA from uninfected cells (▲), nonproductive HF$_{CMV}$ cells (△), productive HF$_{CMV}$ cells (○), and late, 72 h p.i., productively infected cells (●). (C) Nuclear DNA from uninfected cells (▲) and cells with 20 PFU per cell (160 genome equivalents of CMV DNA per cell) adsorbed at 4°C (○). Whole-cell DNA from cells with 20 PFU per cell adsorbed at 4°C (●).*

with EDTA-trypsin solution as described in Materials and Methods. These cells adsorbed 160 genome equivalents of viral DNA per cell (Fig. 4C). After nuclei were purified and nuclear DNA was extracted, little or no acceleration of reassociation occurred, and less than two genome equivalents of viral DNA per cell remained (Fig. 4C). This represented less than 1% contamination. Therefore, the 120 genome equivalents of CMV DNA per cell in nonproductive HF$_{CMV}$ cell nuclei did not result from contamination by adsorbed virus.

To determine whether the CMV DNA associated with nonproductive HF$_{CMV}$ cells was evenly distributed or concentrated in a few cells, nuclei were also analyzed by in situ hybridization with ^{3}H-labeled CMV DNA as described in Materials and Methods. Nuclei from nonproductive HF$_{CMV}$ cells (Fig. 5A) contained two- to threefold more grains than uninfected cell nuclei (Fig. 5B). When 250 nuclei were analyzed, nonproductive cells contained a mean of 33 ± 6 grains and uninfected cells contained a mean of 15 ± 5 grains. The CMV DNA was distributed in most nonproductive cell nuclei, and these nuclei were free of contamination by productive HF$_{CMV}$ cell nuclei. Nuclei from productive HF$_{CMV}$ cells hybridized large amounts of ^{3}H-labeled CMV DNA and could be easily distinguished in an unfractionated HF$_{CMV}$ cell culture (Fig. 5C). In the control, the number of grains detected in nuclei from HF$_{CMV}$ cells hybridized with ^{3}H-labeled adenovirus type 2 DNA (Fig. 5D) was identical to nuclei from uninfected cells using ^{3}H-labeled CMV DNA. Therefore, the significant amount of CMV DNA probe hybridized to nuclei from nonproductive HF$_{CMV}$ cells was due to the presence of CMV DNA.

Virus-induced polypeptides in persistently infected cells. The pattern of polypeptide synthesis in [^{35}S]methionine-labeled HF$_{CMV}$ cell cultures was similar to that previously reported for the late phase of productive infection (44). Late ICSP 155, 120, 110, 105, 94, 83, 68, 61, 58, 54, 40, 37, 34, and 27 were detected (Fig. 6A). Since only productive HF$_{CMV}$ cells contained viral particles, it was assumed that the rounded cells were responsible for the synthesis of late ICSP in the persistently infected culture. To determine whether nonproductive cells synthesized ICSP of CMV, HF$_{CMV}$ cell cultures were labeled with [^{35}S]methionine and the nonproductive cells were separated from the

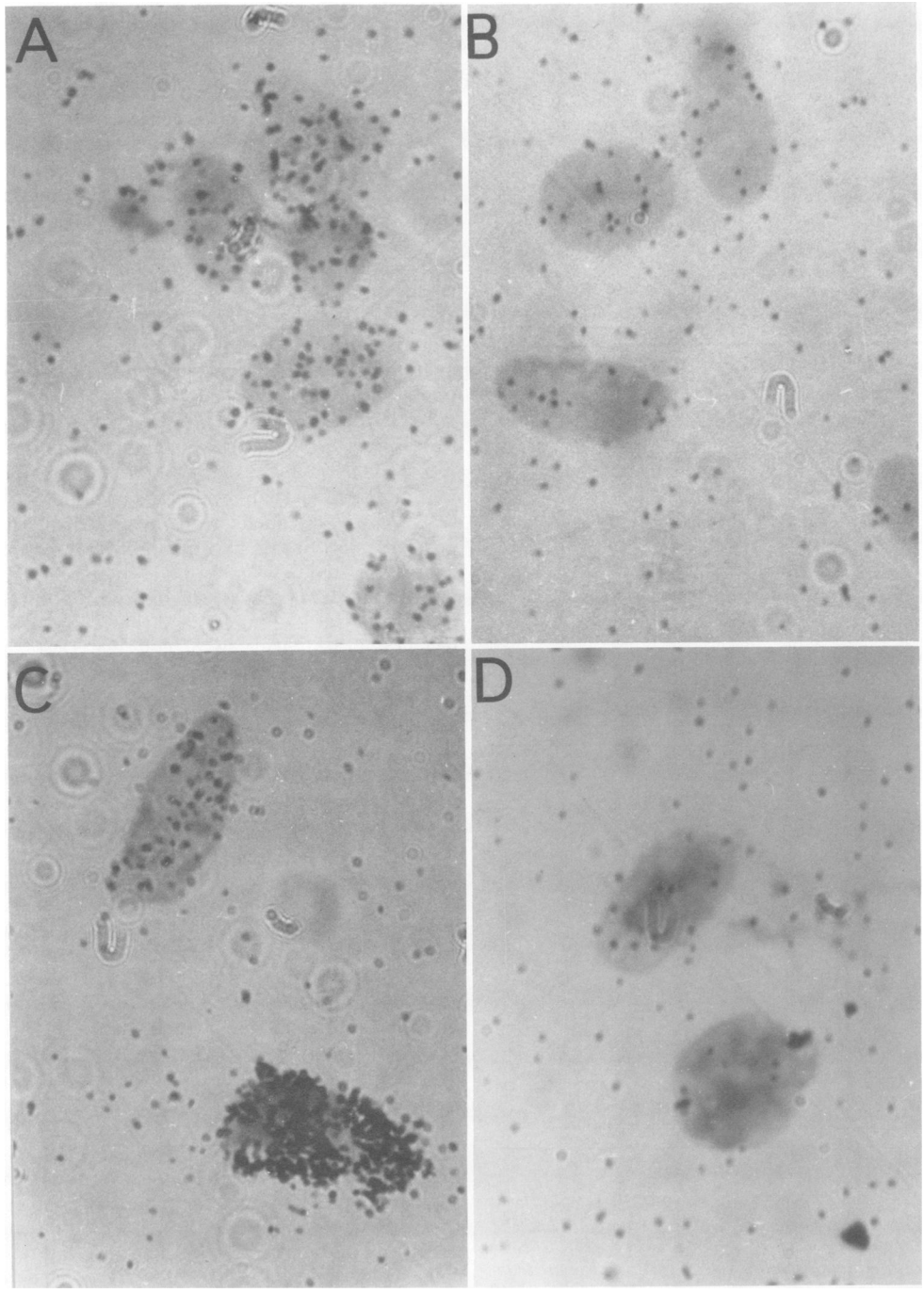

FIG. 5. *In situ hybridization to localize CMV DNA in nonproductive HF_{CMV} cells. Nuclei were prepared and in situ hybridization was as described in the text. (A) ^{3}H-labeled CMV DNA plus nuclei from nonproductive cells; (B) ^{3}H-labeled CMV DNA plus nuclei from uninfected cells; (C) ^{3}H-labeled CMV DNA plus nuclei from unfractionated HF_{CMV} cells; (D) ^{3}H-labeled adenovirus type 2 DNA plus nuclei from unfractionated HF_{CMV} cells.*

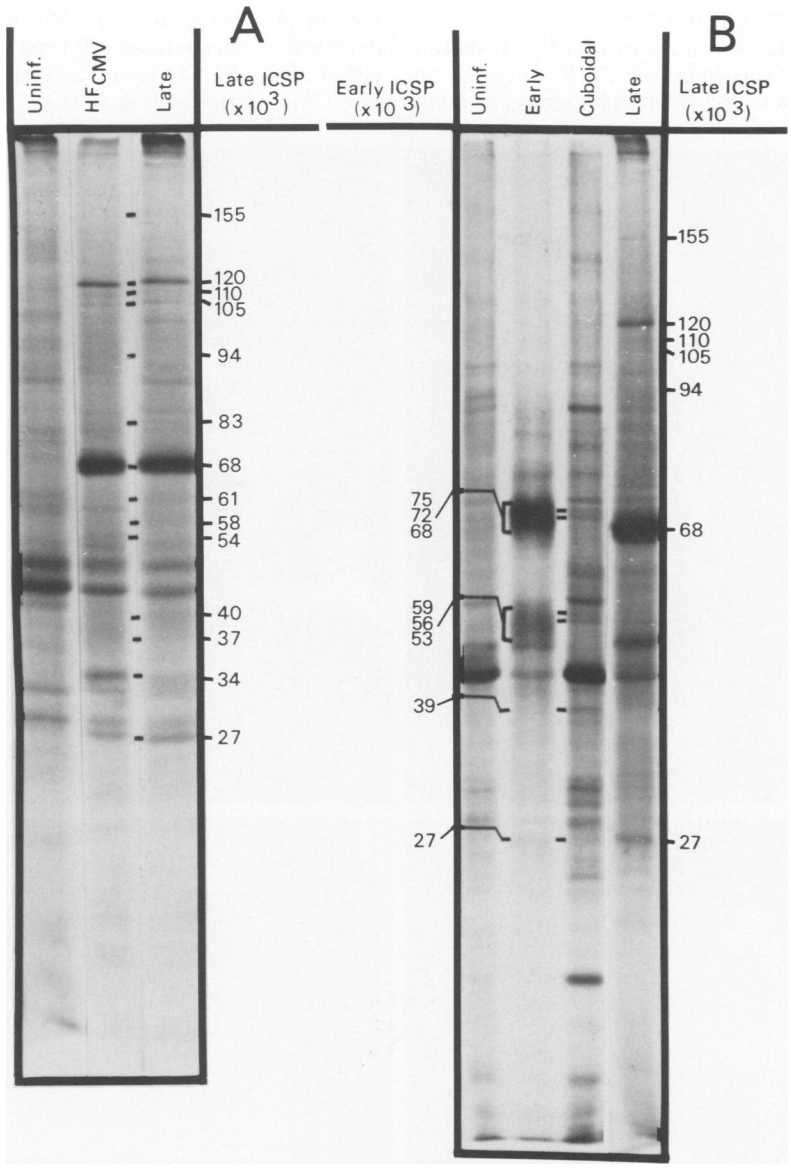

FIG. 6. *Autoradiogram of an SDS-polyacrylamide slab gel containing electrophoretically separated polypeptides synthesized in nonproductive or productive cells infected with CMV. Cell cultures were pulse-labeled with [^{35}S]methionine. Nonproductive and productive HF_{CMV} cells were fractionated as described in the text. (A) Comparison of the polypeptides synthesized in unfractionated HF_{CMV} cells and cells late in productive infection. (B) Comparison of polypeptides synthesized in nonproductive, cuboidal type cells and cells at early and late times during productive infection. ICSP are denoted by their apparent molecular weight ($\times 10^3$).*

productive cells in Ficoll-Hypaque density gradients as described in Materials and Methods. The incorporation of [^{35}S]methionine was approximately fivefold higher in the productive HF_{CMV} than in the nonproductive cells. Nonproductive cells did not contain detectable late ICSP (Fig. 6B). A majority of the polypeptides synthesized in nonproductive cells comigrated with those identified in uninfected cells, although a few polypeptides, not in uninfected cells, were synthesized in relatively low amounts. These polypeptides comigrated with the early ICSP of CMV previously described (45) and were designated as ICSP 75, 72, 59, 56, 39, and 27 (Fig. 6B). Therefore, the nonproductive HF_{CMV} cells appeared similar to cells in the early

phase of infection with CMV.

Viral antigens in persistently infected cells. The expression of CMV antigens in HF_{CMV} cells was investigated by indirect immunofluorescence. Structural CMV antigens were detected in the rounded cell type when treated either with a human serum capable of detecting late CMV antigens (Fig. 7A) or with a rabbit

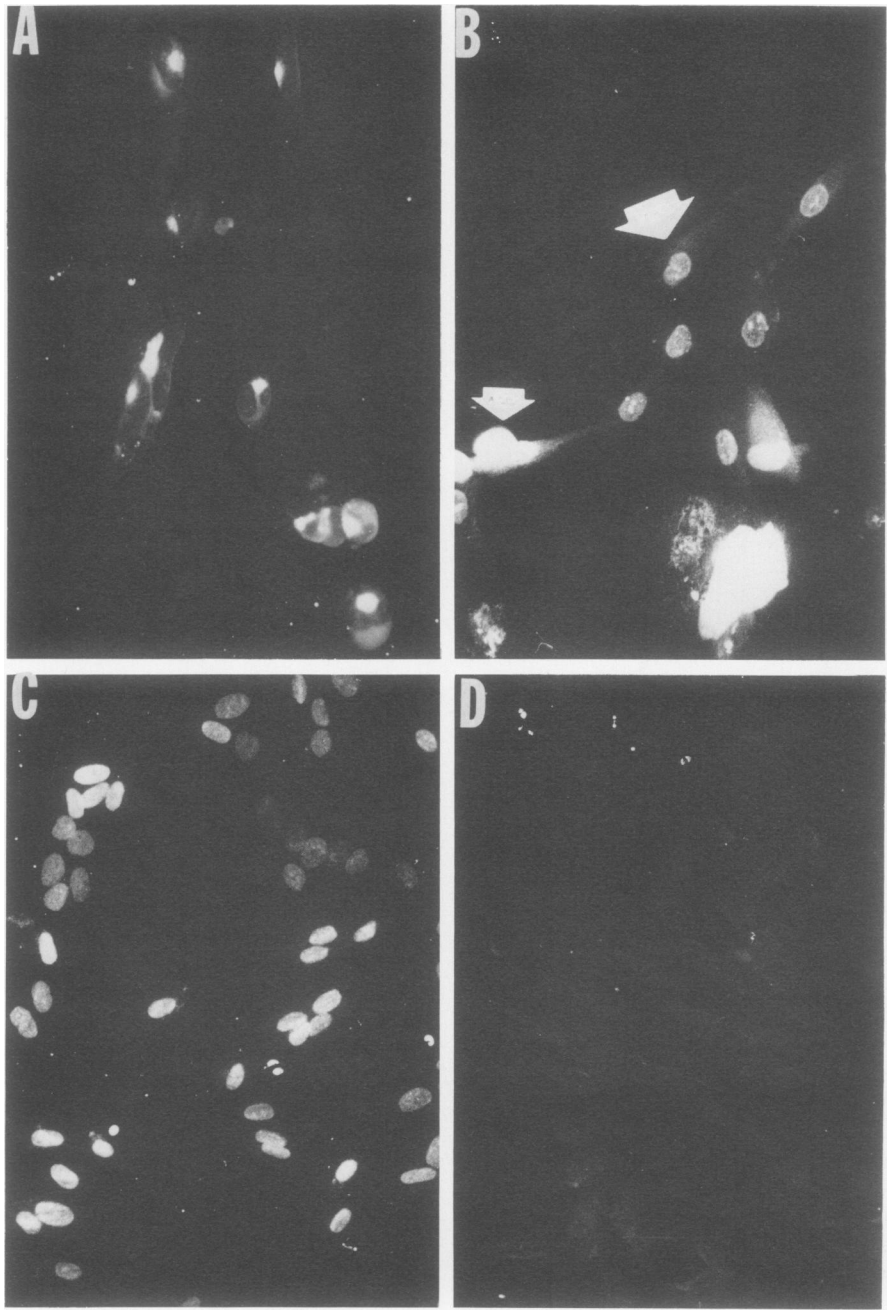

FIG. 7. *Immunofluorescence photomicrographs of viral antigens in HF_{CMV} cells. (A) Late antigen-positive serum. (B) Immediate early antigen-positive serum. (C) HF cells at 3 h p.i. with 20 PFU per cell treated with immediate early antigen-positive serum. (D) Uninfected HF cells treated with immediate early antigen-positive serum. Large arrow, nonproductive, cuboidal cell type; small arrow, productive, rounded cell type. ×480.*

serum prepared against virions and dense bodies of CMV (data not shown). The percentage of fluorescent cells correlated with the percentage of rounded cells in the culture and substantiated that they were involved in virus production. Structural CMV antigens were not detected in the nonproductive, cuboidal cell type. However, when the cells were treated with a human serum capable of detecting immediate early antigens of CMV as described by Michelson-Fiske et al. (28), a majority of cuboidal cells had fluorescent nuclei (Fig. 7B). The nuclear fluorescence was not as intense as that observed at 3 h p.i. during the early phase of viral replication (Fig. 7C), but it was clearly distinguishable from uninfected cells (Fig. 7D). Rounded, productive HF_{CMV} cells fluoresced intensely when treated with the serum detecting early antigens of CMV because this serum also reacts with late CMV antigens (28). The specificity of the detection of immediate early antigens in nonproductive HF_{CMV} cells was supported by the observation that a human serum detecting only late CMV antigens did not react with nonproductive cell nuclei. Thus, these results were consistent with the detection of early virus-induced ICSP synthesis in nonproductive cells and substantiated that these cells were held in the early phase of infection while continuing to grow.

Persistence of the viral genome in HF_{CMV} cells grown in the presence of anti-CMV serum. To further investigate the persistence of the CMV genome in human cells in the absence of infectious virus production, HF_{CMV} cells were subcultured in growth medium containing 2% neutralizing anti-CMV serum. This antiserum was prepared in rabbits against the antigens extracted by Triton X-100 from the membranes of purified virions and dense bodies (43). The antiserum specifically immunoprecipitates viral glycoproteins from infected cell extracts (44). The concentration of antiserum used to treat HF_{CMV} cell cultures reduced viral infectivity of 10^4 PFU/ml by 95% as determined by plaque reduction. The treated cells continued to grow and were subcultured six times during the period of treatment. The cultures produced high titers of infectious virus prior to treatment, and 100% of the cells were infectious (Fig. 8). Continued antiserum treatment during cell growth eliminated infectious virus production (Fig. 8). The treated cells returned to a fibroblastic morphology. After 15 weeks of treatment, no intra- or extracellular infectious virus was detected (Fig. 8). Infectious virus was also not detected when 5×10^5 treated cells were washed, collected, sonically disrupted, and analyzed by plaque assay (data not shown). It was assumed that residual antibody was not preventing detection of infectious virus because the above cell lysate did not neutralize 10^4 PFU/ml. After an additional 3 weeks of treatment, the cultures were again examined for infectious virus production as described above, and none was detected (Fig. 8). In addition, immediate early antigens of CMV were no longer detected in the cell nuclei (data not shown). To determine whether the CMV genome was still present in these cells, DNA was extracted and analyzed for viral sequences by DNA:DNA reassociation kinetics. There was a

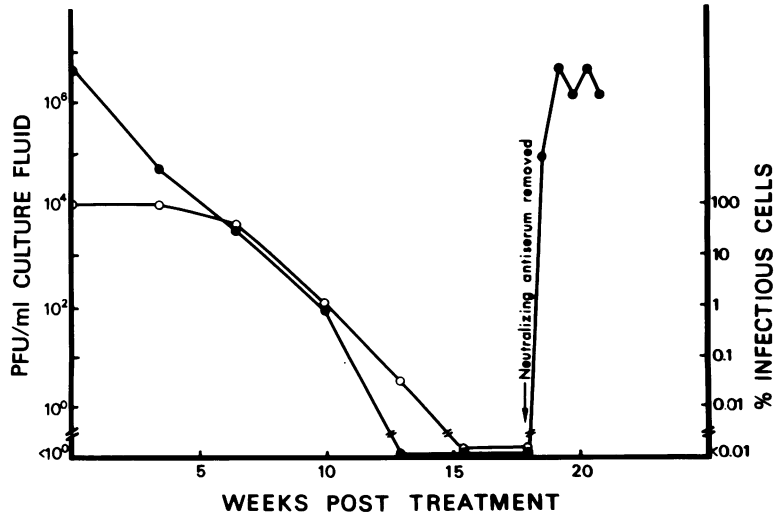

FIG. 8. *Effect of neutralizing anti-CMV serum on infectious virus production in persistently infected cells. HF_{CMV} cells were propagated in medium supplemented with 2% anti-CMV serum. The cells were subcultured six times during the period of treatment with antiserum. PFU per milliliter (●) and percent infectious cells (○) were determined.*

significant acceleration in the rate of reassociation of the ^{32}P-labeled CMV DNA probe (Fig. 9A). These cells contained approximately 45 genome equivalents of CMV DNA per cell. The reassociation of the probe continued essentially to completion, indicating that most or all sequences in the CMV genome persisted in treated cells (Fig. 9B). During antiserum treatment, there was a dramatic reduction in the amount of viral DNA associated with the cultures. Prior to antiserum treatment, the HF_{CMV} cells contained an average of 2,100 genome equivalents of CMV DNA per cell (Fig. 9A). After 6 weeks of antiserum treatment, approximately 680 genome equivalents per cell were present (Fig. 9A). Although the amount of viral DNA per cell was reduced significantly along with the elimination of infectious virus production, a substantial amount of viral DNA persisted throughout the long period of antiserum treatment.

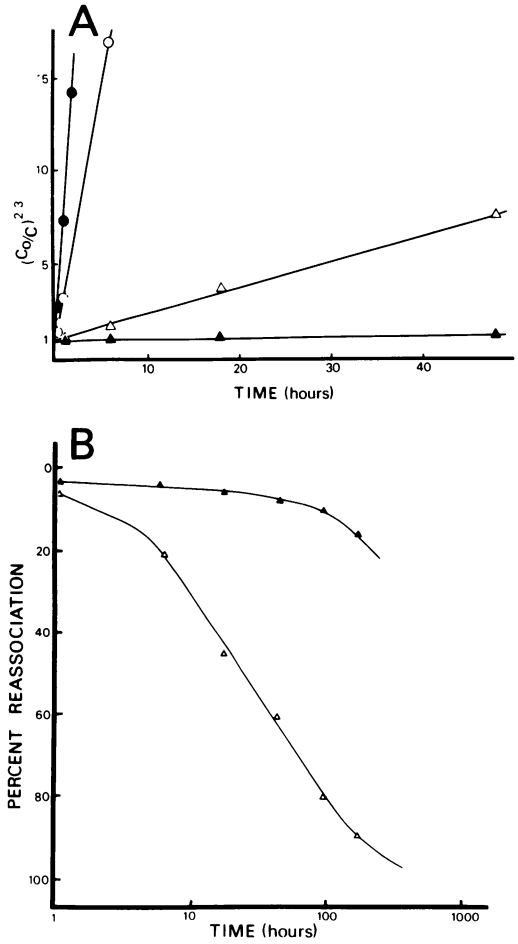

FIG. 9. *Reassociation kinetics of ^{32}P-labeled CMV DNA in the presence of DNA from antiserum-treated HF_{CMV} cell cultures. (A) Reassociation of the ^{32}P-labeled CMV DNA probe in the presence of DNA from uninfected cells (▲), HF_{CMV} cells before antiserum treatment (●), HF_{CMV} cells after 6 weeks of treatment (○), and HF_{CMV} cells after 18 weeks of treatment that had no detectable infectious virus (△). (B) The complete reassociation of the ^{32}P-labeled CMV DNA probe in the presence of DNA from HF_{CMV} cells that had no detectable infectious virus (△), compared with uninfected cell DNA (▲).*

Since all or most sequences of the CMV genome aparently persisted throughout antiserum treatment, the cultures were tested for renewed virus production after antiserum treatment ended. Antiserum was removed after 18 weeks and the cells were subcultured in regular growth medium. Both the productive, rounded and the nonproductive, cuboidal type cell were observed within 2 days. Fibroblastic cells were no longer apparent. Virus production was detected within 4 days and returned to normal levels within 8 days (Fig. 8). Because infectious virus production resumed after removal of the antiserum, it is proposed that the entire CMV genome persisted in these cells. The viral genome apparently remained in a latent state during antiserum treatment because neither infectious virus nor infectious virus-producing cells were detected by standard virological procedures.

DISCUSSION

This report demonstrates that human CMV can persistently infect human cells in culture. Nonproductive HF_{CMV} cells, which were the major cell type in persistently infected cultures, retained the virus in a nonreplicating state. These cells contained a significant amount of CMV DNA and had evidence of early, but not late, virus-induced proteins and antigens. The pattern of early virus-specific expression in nonproductive HF_{CMV} cells resembles the limited expression that occurs after infection of nonpermissive animal cells with CMV. Early viral proteins are synthesized, but viral DNA and late viral protein synthesis are not detectable (45). In contrast to the transient expression of early viral genes in nonpermissive cells, early expression was ongoing in the nonproductive HF_{CMV} cells. Unlike nonpermissive cells of animal origin, nonproductive HF_{CMV} cells retained the capacity for transition to virus production. A small number of these cells spontaneously converted to virus production while the remaining cells continued to grow. Therefore, the persistent infection was maintained because viral gene expression was restricted in the nonproductive HF_{CMV} cells and these cells continued to grow.

After infection of permissive cells with CMV, virus-specific expression is separated into early

and late phases by the onset of viral DNA replication at approximately 12 h p.i. (44, 45). During the early phase of infection, host cell-specific DNA, RNA, and protein syntheses are stimulated (44, 47, 51). When this stimulation is blocked chemically or by serum starvation, virus production is dramatically reduced or eliminated (8). These observations suggest that human CMV requires a host cell function to replicate. Another CMV, murine CMV, requires a function associated with the S-phase of the cell cycle to replicate (31) and cannot grow in cells arrested by serum starvation even though the viral DNA polymerase is present (32). Infected murine cells held in this manner continue to synthesize early virus-induced proteins, but late viral gene expression is not detectable (30). Thus, the physiological condition of the host cell may have an important role in mediating the outcome of CMV infection as well as other herpesvirus infections (38).

The cells persistently infected with CMV apparently arose from a small number of infected HF cells that did not proceed from early viral gene expression to viral DNA replication and late viral protein synthesis. The precise nature of this restriction on viral replication in the nonproductive HF_{CMV} cells is presently not known. Two observations suggest that low metabolic activity of the nonproductive HF_{CMV} cells may limit synthesis of a putative host cell protein necessary for CMV replication. (i) These cells incorporate relatively low levels of [^{35}S]-methionine. (ii) The generation time of these cells is four- to sixfold longer compared to uninfected cells. CMV replication may require host as well as viral proteins made de novo after infection. When proteins synthesized in the early phase of CMV infection are made nonfunctional by incorporation of amino acid analogs, viral DNA replication is inhibited and late virus-induced proteins are not detectable (45). Thus, in permissive cells, restriction of viral gene expression can be induced by factors that alter the function of host- or virus-specified proteins. The inability of the host cell to provide a needed protein(s) may be a point in the viral growth cycle that could encourage the viral genome to enter a latent state. Likewise, a cellular or viral restriction of the temporal sequence of viral gene expression may induce latency. The temporal regulation of herpes simplex virus RNA and protein synthesis is well established (6, 21, 23). Gene expression of both human and murine CMV is also temporally regulated in the appearance of virus-specific RNA (29) and virus-induced proteins (5, 45).

Growth of HF_{CMV} cells in the presence of CMV-neutralizing antiserum resulted in the elimination of virus-producing cells while a significant amount of viral DNA persisted in the treated cells. Because of the 2 to 3 weeks that a productive HF_{CMV} cell remains viable, a long period of treatment was necessary to eliminate detectable virus-producing cells from the cultures. Growth in higher concentrations of anti-CMV serum eliminates virus production more rapidly (unpublished data). Subculturing in the presence of a nonspecific rabbit serum did not have any effect on the HF_{CMV} cell cultures. Since infectious virus production was activated by removal of the antiserum, most of the resident viral genomes were complete and capable of full expression. Treatment with antiserum favored the growth of cells with restricted viral gene expression. These cells had a fibroblastic morphology. In addition, the immediate early antigens of CMV were no longer detectable. Presently, it is not known whether low levels of virus-specific RNA are present in these cells. Nevertheless, maintenance of the viral genome in persistently infected cells did not require the continued presence of infectious virus-producing cells. Similar observations have been reported with antiserum treatment of cells persistently infected with the BK papovavirus (50).

The mechanism of suppression of viral replication by antiserum is also not understood. Since the antiserum was prepared in rabbits against membrane antigens associated with purified virions and dense bodies (43) and was absorbed extensively with uninfected HF cells before use, it was assumed that antibody reacted with CMV-specific membrane antigens on the surface of the nonproductive as well as the productive HF_{CMV} cells. Antigens are detectable on the plasma membrane of productively infected cells by this antiserum during the early as well as the late phase of viral replication (46a). Treatment of viable, productively infected cells causes shedding of the antigen-containing membranes. The emergence of a fibroblastic cell as the predominant cell type in the antiserum-treated cultures suggested that the antiserum induced a change in the morphology of the nonproductive, cuboidal cells or allowed for the growth of a minor subpopulation of nonproductive, fibroblastic cells.

The outgrowth of persistently infected cells after infection with CMV is dependent upon the input multiplicity of infection. Persistent infection is favored at high input multiplicities when established CMV laboratory strains (Towne or AD_{169}) are employed (unpublished data). In contrast, fresh CMV isolates in low in vitro passages establish persistent infection after a low multi-

plicity of infection (13). Defective CMV develops after high-multiplicity infection (46) and is produced by HF_{CMV} cell cultures (unpublished data). Defective virions contain DNA molecules that have a specific structural rearrangement when compared to nondefective DNA by restriction enzyme analysis. The functional significance of defective viral DNA in persistent infection is being investigated.

Both cloned and uncloned HF cells yield comparable outgrowth of persistently infected cells at a similar input multiplicity of infection. Therefore, the HF_{CMV} cell cultures do not appear to arise from a genetically resistant subpopulation of HF cells. More than 20 independent isolates of HF_{CMV} cell cultures have been obtained in our laboratory from WI-38 and 12 different human foreskin fibroblast cell strains. In addition, the HF_{CMV} cultures do not have any detectable interferon, even though the cells are resistant to superinfection by CMV (unpublished data).

Although HF_{CMV} cells are not immortalized, this virus-cell interaction has some similarities of Epstein-Barr virus-producer cell lines (9). In both systems, a majority of cells repress viral replication while spontaneous activation to virus production occurs in a small percentage of cells. Since the Epstein-Barr virus genome resides as either integrated (1, 2) or episomal (27) viral DNA in the latently infected lymphoblastoid cells, it is possible that the latent CMV genome is maintained in a similar physical association with the host cell.

The predominant factor influencing restriction of CMV gene expression and latency remains to be investigated. The HF_{CMV} cultures described in this report provide a useful system for dissecting the molecular biology of restricted CMV gene expression and its relationship to viral persistence and latency.

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