Sequence of Protein Synthesis in Cells Infected by Human Cytomegalovirus: Early and Late Virus-Induced Polypeptides

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At least 10 distinct early virus-induced polypeptides were synthesized within 0 to 6 h after infection of permissive cells with cytomegalovirus. These virus-induced polypeptides were synthesized before and independently of viral DNA replication. A majority of these early virus-induced polypeptides were also synthesized in nonpermissive cells, which do not permit viral DNA replication. The virus-induced polypeptides synthesized before viral DNA replication were hypothesized to be nonstructural proteins coded for by the cytomegalovirus genome. Their synthesis was found to be a sequential process, since three proteins preceded the synthesis of the others. Synthesis of all early cytomegalovirus-induced proteins was a transient process; the proteins reached their highest molar ratios before the onset of viral DNA replication. Late viral proteins were synthesized at the time of the onset of viral DNA replication, which was approximately 15 h after infection. Their synthesis was continuous and increased in molar ratios with the accumulation of newly synthesized viral DNA in the cells. The presence of the amino acid analog canavanine or azetadine during the early stage of infection suppressed viral DNA replication. The amount of viral DNA synthesis was directly correlated to the relative amount of late viral protein synthesis. Because synthesis of late viral proteins depended upon viral DNA replication, the proteins were not detected in permissive cells treated with an inhibitor of viral DNA synthesis or in nonpermissive cells that are restrictive for cytomegalovirus DNA replication.

A knowledge of early viral gene functions is particularly important in understanding the virus-cell interaction. An early gene product of cytomegalovirus (CMV) is thought to stimulate the synthesis of cellular DNA, RNA, and protein in both permissive and nonpermissive cells at approximately 20 h after infection (10, 11, 34, 39). Stimulation increases the levels of host-specific enzymes associated with the S-phase of the cell cycle such as thymidine kinase (6). Stimulation apparently requires early CMV proteins because inactivation of the viral infectivity or inhibition of early viral protein synthesis prevents stimulation (9, 37, 39). An opposite view is that stimulation of cellular DNA synthesis occurs in abortively infected cells, whereas productive infection inhibits the synthesis of detectable amounts of cellular DNA, as does productive infection with other herpesviruses (3). The physiological state of the cells apparently plays a role in the modulation of expression of the viral genome (3). These observations suggest that the interaction between the early proteins of CMV and the host cell have important implications for control of the viral gene expression.

Human CMV, like the other members of the

herpes group of viruses, can exist in a latent state (29). The establishment of latent infection by CMV may be related to a failure in early viral gene functions. Since many of the diseases caused by CMV are considered to be associated with activation of virus from a latent state, the early viral gene functions and the sequence of viral gene expression have important roles in the activation of viral replication and in the nature of the diseases caused by this virus. The majority of the early proteins of CMV are presumably transported to the nucleus for involvement in viral DNA synthesis. A nuclear antigen(s) induced by CMV has been detected as early as 3 h after infection of human fibroblast (HF) cells (12). In addition, a CMV-induced nuclear antigen(s) has also been detected in CMV-transformed human cells (14). Transformation of nonpermissive (1) and permissive (13) cells by CMV may be associated with an early viral gene(s) that codes for an early protein as with other DNA tumor viruses (25, 38).

An approach to identifying early viral gene products is to analyze the early virus-induced proteins synthesized after infection. In the early phase of infection by CMV, 0 to 15 h post-

infection (p.i.), approximately 80% of the total proteins synthesized are host specific and 20% are viral (37). Consequently, detection of early virus-induced polypeptides, referred to as infected-cell-specific polypeptides (ICSP), is hindered by a high background of host cell protein synthesis. In this investigation, the synthesis of early CMV-induced proteins was enhanced relative to ongoing host cell protein synthesis by treatment with cycloheximide (CH) for various intervals followed by radioisotope pulse-labeling in hypertonic medium. This procedure has been shown to reduce host protein synthesis in several virus-cell systems (5, 17, 27, 33). This report describes three discrete phases of protein synthesis in CMV-infected cells. Major emphasis is on the immediate-early and early ICSP that are synthesized before the initiation of viral DNA synthesis. The immediate-early and early ICSP were detectable in both permissive and nonpermissive cells. Late ICSP were synthesized at increasing relative rates after initiation of viral DNA synthesis and were detectable only in permissive cells. This analysis of CMV ICSP synthesis indicates that CMV-induced immediate-early protein synthesis preceded early and viral DNA replication was required for late protein synthesis.

MATERIALS AND METHODS

Virus and tissue culture. The Towne strain of human CMV was propagated in HF cells as previously described (36). Plaque purification of CMV was performed by terminal dilution. Isolated plaques were picked with the aid of an inverted microscope and transferred to HF cell monolayers at a multiplicity of 10^{-5} PFU/cell. Virus, plaque purified in this manner three times, was inoculated onto monolayers of HF cells at a multiplicity of 0.001 PFU/cell. At 3 to 4 days after 100% cytopathic effect (CPE), infectious extracellular fluid was harvested and used for high-multiplicity infection. The amount of infectious virus was determined by assays for plaques (44) or tissue culture-infective doses (9). Virus and cells were routinely tested for mycoplasma contamination by methods previously described (36). Guinea pig embryo fibroblast (GPEF) cells were cultivated in Eagle minimal essential medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (Grand Island Biological Co.) and 50 μ g of aureomycin (American Cyanamid Co., Lederle Laboratories, Pearl River, N.Y.).

Chemicals. Phosphonoacetic acid (PAA; Bodman Chemical, Richmond, Va.) was prepared in distilled water at a concentration of 20 mg/ml, adjusted to pH 7.4 with 1 N NaOH, and sterilized by filtration. Canavanine and azetadine (Sigma Chemical Co., St. Louis, Mo.) were prepared in distilled water at a concentration of 10 mg/ml and sterilized by filtration.

Purified virions and dense bodies. Virions and dense bodies of CMV were radioactively labeled by adding [35 S]methionine (5 μ Ci/ml; 300 to 350 Ci/mmol;

Amersham/Searle, Arlington Heights, Ill.) at 36 h after infection with 1 to 4 PFU/cell. Virions and dense bodies were purified from the extracellular fluids as previously described (36).

Viral infection and incubation with radioactive precursors. Cells monolayered in 60-mm plastic petri dishes for 2 days were infected at input multiplicities of 10 to 30 PFU/cell. At 2 h p.i., or as indicated in specific experiments, 50 μ g of CH (Sigma) per ml was added to inhibit translation. After removal of the CH, the cells were washed four times with medium containing 5% the normal concentration of methionine. Then [35 S]methionine, 20 μ Ci/ml, in either isotonic (110 mM NaCl) or hypertonic (210 mM NaCl) medium containing 5% the normal concentration of methionine plus 10 μ g of actinomycin D per ml (Sigma) and 2% fetal calf serum was added. Actinomycin D was used to inhibit further transcription of the viral DNA.

PAGE. Equal volumes of cell lysates were analyzed by slab polyacrylamide gel electrophoresis (PAGE) in 9% gels as described previously (37), using a modification of the Laemmli method (24). Nucleic acids were solubilized, and the cells were prepared for sodium dodecyl sulfate (SDS)-PAGE as previously described (37). Thyroglobulin (160,000), beta-galactosidase (130,000), phosphorylase A (94,000), bovine serum albumin (68,000), ovalbumin (43,000), alpha-chymotrypsinogen (25,700), and cytochrome c (11,700) were used as standard markers for molecular weights. These markers were all obtained from Sigma. Molecular weights of the viral proteins were estimated by the procedure of Weber and Osborn (43).

After electrophoresis, gels were stained with 0.05% Coomassie brilliant blue in 25% isopropanol and 10% acetic acid by the method of Fairbanks et al. (7), destained with 10% acetic acid, and then dried with an SE540 slab gel dryer (Hoefer). Dried gels were exposed to Kodak Tri-X Ortho film at room temperature.

Radioactivity in individual proteins. Quantitation of individual proteins was obtained by cutting the bands out of the dried gel, treating the gel slices with Protosol and then Omnifluor (New England Nuclear, Boston, Mass.), and counting in a Packard liquid scintillation spectrometer as previously described (36). Radioactivity in the corresponding region of the uninfected cell sample was subtracted from the counts in each protein band cut out of the infected-cell sample.

DNA labeling and centrifugation. Separation of cellular from viral DNA was by equilibrium centrifugation by the method of Crouch and Rapp (2) with the following modifications. At 30 h p.i., 10 μ Ci of [3 H]thymidine per ml (15 to 30 Ci/mmol; Amersham/Searle) in medium containing 10% fetal calf serum was added. At 72 h p.i., the cell layer was rinsed three times with 0.001 M calcium acetate (pH 7.0) containing 0.15 M NaCl. The cells were scraped into $0.1\times$ SSC (0.0015 M sodium citrate-0.15 M NaCl, pH 7.0) containing 0.02 M EDTA and pelleted by centrifugation. The cells were suspended in $0.1\times$ SSC containing 0.02 M EDTA, 0.1% Sarkosyl NL30 (Geigy Chemical Corp.), and 0.1% Pronase (preincubated at 37°C for 2 h; Calbiochem, B grade) to solubilize the protein. After incubation at 37°C for 2 h, 0.05 ml of 3 H-labeled DNA was mixed with 6.5 ml of CsCl in $0.1\times$

SSC containing 0.02 M EDTA to give a final density of 1.700 g/ml and centrifuged at $64,500 \times g$ for 63 h at 17°C in a Beckman type 40 rotor, using an adaptor for 6.5-ml tubes. Fractions were collected from the bottom of the gradient onto filter paper disks (Whatman, 3MM). Radioactivity was counted in a Packard liquid scintillation spectrometer. Refractive indexes of periodic fractions were determined in a Bausch & Lomb refractometer to obtain the gradient densities. The relative amount of host cell or viral DNA synthesis was determined by calculating the percentage of radioactivity in the peak fractions.

Time course of viral DNA synthesis. The appearance of CMV-specific DNA was determined by DNA:DNA hybridization on nitrocellulose membranes. Monolayers of cells were infected as described above and pulse-labeled for 2 h at various intervals with 10 μ Ci of [*methyl*- 3 H]thymidine per ml (80 to 100 Ci/mmol; New England Nuclear Corp., Boston, Mass.). After the pulse, the cells were rinsed three times with 0.001 M calcium acetate (pH 7.0) containing 0.15 M NaCl, scraped into 0.02 M Tris-hydrochloride (pH 7.2), and pelleted by centrifugation. The cell pellet was suspended in 1 ml of 0.3 N NaOH, boiled in water for 10 min, cooled to 0°C, and neutralized with 1 ml of 0.01 M Tris-hydrochloride (pH 7.5) containing 0.3 N HCl. The DNA was extracted twice with an equal volume of distilled phenol pre-equilibrated with 0.01 M Tris-hydrochloride (pH 8.1) containing 0.1 M NaCl and 0.01 M EDTA. After extraction, sodium acetate was added to a final concentration of 2%. The DNA was precipitated with 2.5 volumes of ethanol at -20°C. After centrifugation in a Beckman SW56 rotor at $81,000 \times g$ for 20 min, the pellet was suspended in 200 μ l of 1.5 mM NaCl plus 0.15 mM sodium citrate (pH 6.8), heated for 15 min at 100°C, and then rapidly cooled to 0°C. After sonic treatment for 20 s to reduce the size of the DNA, 3×10^4 cpm from either uninfected or infected cells was added to 3 μ g of purified CMV DNA bound to nitrocellulose filters (Schleicher and Schuell, type B-6).

To prepare purified viral DNA, virions and dense bodies were purified away from any detectable host cell contaminants as previously described (36) and then treated with 1% Nonidet P-40 for 30 min at room temperature to solubilize the membranes. The resulting nucleocapsids were pelleted through a 20% sorbitol cushion by centrifugation in the Beckman SW27.1 rotor at $19,000 \times g$ for 60 min. After the pellet was suspended in 1% Sarkosyl in 0.1 M Tris-hydrochloride (pH 8.1) containing 0.1 M NaCl plus 0.01 M EDTA and incubated for 10 min at 37°C, 300 μ g of protease K was added that had been self-digested for 30 min at 37°C at a concentration of 10 mg/ml to remove any nuclease activity. After an additional 30 min at 37°C, the DNA was extracted three times with phenol and once with chloroform-2% isoamyl alcohol. After the DNA was dialyzed against 1 $\times$ SSC, cesium chloride (final density, 1.56 g/ml) and ethidium bromide (final concentration, 50 μ g/ml) were added. The mixture was centrifuged to equilibrium in an SW27.1 rotor at $95,000 \times g$ for 63 h at 17°C. The viral DNA-ethidium bromide complex banded at a density of 1.575 g/ml and was detected by fluorescence of the DNA-ethidium bromide complex upon exposure to UV light. Cellular

DNA-ethidium bromide complex, which bands at a lighter density, was not detected. Ethidium bromide was removed from the DNA by three extractions with *n*-butanol, and the CsCl was removed by dialysis against 0.1 $\times$ SSC containing 0.01 M EDTA. Viral DNA in 0.1 $\times$ SSC was denatured, diluted with 6 $\times$ SSC, and immobilized onto nitrocellulose filters as modified from the procedure of Green et al. (15). After immobilization of 3 μ g of DNA per filter, the filters were treated for 6 h at 60°C with 3 $\times$ SSC containing 0.02% Ficoll (Pharmacia, Piscataway, N.J.; average molecular weight, 400,000), 0.1% polyvinylpyrrolidone (Sigma; molecular weight, 360,000), and 0.1% bovine albumin by the Denhardt method (4). After the cells were washed with 6 $\times$ SSC, hybridization was allowed to occur at 60°C for 12 h. To solubilize single-stranded DNA, the filters were treated with the single-strand-specific nuclease S1 (Miles Laboratory, Kankakee, Ill.) by the method of Gubbins et al. (16). Finally, the filters were washed extensively with 3 mM Tris-hydrochloride (pH 9.4), dried, and counted. Immobilized viral DNA did not specifically bind 3 H-labeled DNA (3×10^4 cpm) from uninfected cells.

RESULTS

Early virus-induced protein synthesis in CMV-infected cells. Previous experiments with CMV-infected cells demonstrated that early ICSP were not clearly detectable by simply pulse-labeling with [35 S]methionine (37). Therefore, viral protein synthesis was enhanced relative to host by treating the cultures with CH (50 μ g/ml). The optimum time to add CH for detection of all the virus-induced polypeptides synthesized before viral DNA replication was 2 h p.i. Apparently, immediate-early proteins must precede early proteins as described below. After the CH was removed at various intervals up to 18 h, the cells were pulse-labeled with [35 S]methionine in isotonic medium. The criteria for identification of early ICSP was that they have either electrophoretic mobilities different from those of host cell polypeptides or an increased relative rate of synthesis compared with host cell proteins. Seven early ICSP were detected at approximately 6 to 8 h p.i., and they increased in their molar ratio until approximately 14 to 16 h p.i. (Fig. 1A). These ICSP were operationally defined as virus-induced polypeptides. They were not detected in cells inoculated with virus inactivated by UV irradiation (data not shown), and pretreatment with CH did not perceptibly affect the number of polypeptides detected in uninfected cells (Fig. 1A). Since stimulation of host cell protein synthesis in CMV-infected cells does not occur until approximately 20 h p.i. (37), the ICSP are postulated to be coded for by the viral genome. The early ICSP have been designated according to their apparent molecular weight $\times 10^3$ as ICSP 75, 72, 68, 59, 56, 53, and 39.

FIG. 1. Autoradiogram of an SDS-polyacrylamide gel slab containing electrophoretically separated polypeptides synthesized in CMV-infected cells during the early phase of infection. Infected (I) and uninfected (U) cells were treated with CH at 2 h p.i. CH was removed at various intervals until 18 h p.i. before pulse-labeling for 2 h with [35 S]methionine in isotonic medium as indicated above. ICSP are denoted by their apparent molecular weight ($\times 10^3$). Twice the concentration of protein was applied to the gel in B as compared with A.

To detect early ICSP present at low molar ratios, twice the concentration of protein was analyzed. At least three additional low-molecular-weight virus-induced polypeptides, ICSP 27, 21, and 19, were detected (Fig. 1B), but the resolution of the ICSP at high molar ratios was compromised. Some late ICSP, denoted by a dash in Fig. 1B, were synthesized when CH was added at 2 h p.i. rather than 0 h. These viral proteins will be described in more detail under "Sequence of early and late virus-induced protein synthesis."

To determine how long the early viral mRNA's were translatable, CH was removed at 18 h p.i., the cells were placed on media containing 10% fetal calf serum, and the cells were pulsed at various intervals. The synthesis of the early ICSP decreased rapidly in molar ratios (Fig. 1A). The synthesis of early ICSP 72 and 68

was still detected at 12 to 14 h (30- to 32-h pulse) after the removal of CH, but their synthesis was reduced considerably (Fig. 1A). These results suggested that the synthesis of the early proteins of CMV is a transient process.

The early ICSP, synthesized in cells after treatment with CH from 2 to 10 h p.i., were compared to the ICSP synthesized after viral DNA replication, i.e., at 72 h. To further reduce host cell protein synthesis, medium containing radioisotope was made hypertonic by adding NaCl to a final concentration of 210 mM. Ten early ICSP were detected according to the criteria described above and identified according to their apparent molecular weight ($\times 10^3$) (Fig. 2). Comparison of Fig. 1A and B with Fig. 2 shows that pulse-labeling in hypertonic medium after the removal of CH improved the detection of early ICSP of CMV and demonstrates that

FIG. 2. Comparison of electrophoretically separated polypeptides synthesized before and after viral DNA replication in CMV-infected cells. Infected (I) and uninfected (U) cells were either treated or not treated with CH at 2 h p.i. for various intervals. The CH was removed before pulse-labeling for 2 h with [35 S]methionine in hypertonic medium as indicated above. ICSP are denoted by their apparent molecular weight ($\times 10^3$). Host cell polypeptides are either labeled with the letter h or not labeled at all.

the majority of the early ICSP had electrophoretic mobilities and relative rates of synthesis different from those of the late ICSP. Since a

specific class of ICSP was synthesized early but not late in infection, the synthesis of CMV proteins might depend upon a control system associated with the replication of viral DNA.

Early virus-induced protein synthesis in the presence of an inhibitor of CMV DNA synthesis. The early viral polypeptides of DNA tumor viruses such as adenoviruses and papovaviruses have been defined as those polypeptides whose synthesis is not inhibited by an inhibitor of viral DNA synthesis (35, 41). PAA inhibits the synthesis of CMV DNA (21) and indirectly prevents the synthesis of the late ICSP of CMV (37). To test the effect of PAA on early CMV ICSP synthesis, infected cells were treated with CH from 2 to 10 or 2 to 14 h p.i. in the presence of PAA (100 μ g/ml) at a concentration that completely inhibits viral DNA synthesis (21). After removal of CH, cells were pulse-labeled for 2 h with [35 S]methionine in hypertonic medium and in the presence of PAA. Early ICSP were synthesized in cells treated with PAA or left untreated (Fig. 3). These polypeptides, ICSP 75, 72, 68, 59, 56, 53, 39, 27, and 21, appear to be true early ICSP since they were synthesized when viral DNA replication was inhibited. To examine the effect of PAA on late-ICSP synthesis, infected cells were treated with PAA and pulse-labeled from 72 to 74 h p.i. Late ICSP were not detected (Fig. 3). Even when PAA was added at 14 h p.i., after the completion of early-ICSP synthesis, late ICSP were not detected at 72 to 74 h p.i. (Fig. 3), suggesting a critical role for viral DNA replication in the synthesis of late gene products. In cells not treated with PAA, late ICSP were synthesized that had the same electrophoretic mobilities as the polypeptides associated with virions and dense bodies of CMV (Fig. 3), and they had high relative rates of synthesis as previously described (37). Hence, early ICSP of CMV were made in the presence of PAA. These polypeptides are probably the translation products of viral mRNA that is transcribed and translocated to ribosomes in the absence of viral DNA replication. The early ICSP detected under these conditions are summarized in Table 1 and are compared with the ICSP detected in nonpermissive GPEF cells as described below.

Synthesis of early virus-induced proteins in nonpermissive cells. In both permissive HF and nonpermissive GPEF cells, CMV induces the synthesis of a novel DNA polymerase (18, 20). It has been suggested that the DNA polymerase induced by CMV might be an early protein necessary for viral replication (18). These observations implied that early proteins of CMV are made in the nonpermissive cell. However, CMV DNA is not synthesized (34) nor is infectious

FIG. 3. Effect of an inhibitor of CMV DNA synthesis on early and late infected-cell-specific polypeptide synthesis. Infected (I) and uninfected (U) cells were either treated with PAA or left untreated. For early pulse periods, cells were treated with CH at 2 h p.i. CH was removed before pulse-labeling for 2 h with [35 S]-methionine in hypertonic medium as described above. Polypeptides associated with purified virions plus dense bodies were subjected to electrophoresis in parallel to infected cells pulsed at late periods. Apparent molecular weights ($\times 10^3$) of viral structural polypeptides and ICSP are indicated.

virus produced (8) in nonpermissive cells. Nevertheless, CPE are observed in nonpermissive cells, and these effects may be due to early CMV proteins. To determine whether early CMV ICSP are synthesized in nonpermissive cells, GPEF cells were infected and treated with CH from 2 to 10 or 2 to 14 h p.i. After the removal of the CH, the cells were pulse-labeled for 2 h with [35 S]methionine in the presence of hypertonic medium. Permissive HF cells were treated in a similar manner for comparison. Lysates of

infected HF and GPEF cells were subjected to electrophoresis in parallel. Many early ICSP were detected in nonpermissive cells: ICSP 75, 72, 68, 56, 53, 39, 27, and 19. They had high relative rates of synthesis compared with host cell proteins, and they had the same apparent molecular weights as those detected in permissive HF cells (Fig. 4). Early ICSP 59 and 21 were detected in permissive cells but not in nonpermissive cells. Additional ICSP were not detected in the nonpermissive GPEF cells, and late ICSP

TABLE 1. *Molecular weights of early CMV-infected cell-specific polypeptides in permissive HF cells treated with PAA or left untreated and in nonpermissive GPEF cells^a*

Mol wt ($\times 10^3$) ^b			
HF		HF (PAA treated)	GPEF
Immediate-early	Early		
75		75	75
72		72	72
68		68	68
	59	59	ND ^c
	56	56	56
	53	53	53
	39	39	39
	27	27	27
	21	21	ND
	19 ^d	ND	19

^a Infected cells were treated with cycloheximide (50 μ g/ml) 0 to 8 or 2 to 10 h p.i. and pulsed for 2 h with [³⁵S]methionine in hypertonic medium.

^b Apparent molecular weights were determined from a 9% SDS-polyacrylamide gel using the following standards: thyroglobulin (160,000), phosphorylase A (94,000), bovine serum albumin (68,000), ovalbumin (43,000), and cytochrome c (11,700).

^c ND, Not detected.

^d Occasionally not detectable.

were not detected. Some late ICSP were detected in permissive cells because CH was added at 2 h p.i. rather than 0 h, and these virus-induced polypeptides will be discussed below. Table 1 summarizes the early ICSP detected in permissive and nonpermissive cells in the absence of viral DNA replication. The majority of the early ICSP of CMV were synthesized in the nonpermissive cell.

To determine whether late ICSP of CMV were synthesized in nonpermissive GPEF cells during the late phase of infection, infected cells were pulse-labeled from 72 to 74 h p.i. with [³⁵S]-methionine. Cell lysates were subjected to electrophoresis in parallel to purified virions and dense bodies of CMV and to infected HF cells pulse-labeled at 72 to 74 h p.i. Even though many of the early ICSP of CMV were synthesized in both HF and GPEF cells, late ICSP were not detected in the nonpermissive GPEF cells (Fig. 5). The profile of polypeptides in the infected nonpermissive cell was identical to that of the uninfected cell (Fig. 5). Synthesis of late ICSP in HF cells was detected by their electrophoretic mobilities, which were similar to those of the polypeptides associated with purified virions and dense bodies, and by their relatively high rate of synthesis compared with host cell proteins (Fig. 5). Since nonpermissive cells do

not permit the synthesis of CMV DNA (34), these results support the contention that CMV DNA synthesis is required for the synthesis of the late ICSP.

Sequence of early and late virus-induced protein synthesis. The onset of early and late ICSP synthesis was investigated to determine whether a class of proteins is preferentially synthesized. It was necessary to treat the infected cells with CH for varying intervals to enhance for viral protein synthesis relative to host. Since treatment with CH delays viral mRNA translation, the preferential transcription and translation of unstable mRNA may go undetected in this type of experiment. Recognizing this limitation, infected HF cells were treated with CH and pulse-labeled for 2 h with [³⁵S]methionine in hypertonic medium. When cells were treated with CH from 0 to 8 h p.i. and pulsed from 8 to 10 h, ICSP 75, 72, and 68 were synthesized in high molar ratios compared with the other early ICSP (Fig. 6). These ICSP were hypothesized to be the immediate-early viral proteins. In infected cells treated with CH from 2 to 10 h p.i. and pulse-labeled from 10 to 12 h, ICSP 59, 56, 53, and 39 were also synthesized at high molar ratios (Fig. 6), and these ICSP are hypothesized to be early viral proteins. The synthesis of the immediate-early and early viral proteins decreased considerably when cells were treated with CH at 4 to 12 h p.i. and pulsed at 12 to 14 h. Last, when cells were treated with CH at 6 to 14 h p.i. and pulsed at 14 to 16 h, most immediate-early and early virus-induced proteins were absent, but late ICSP 115, 110, 105, 94, 83, 76, 68, 61, 49, 34, and 27 were detected. The rates of synthesis of these late ICSP continued to increase with time after infection (Fig. 6). These results substantiated the data in Fig. 1 indicating that early-CMV protein synthesis is a transient process. The data also imply that the synthesis of the immediate-early viral proteins is obligatory for the synthesis of the early viral proteins. The addition of CH at 2 h p.i. allowed for the detection of the early ICSP. In contrast, the addition of CH from 0 to 8 and pulse-labeling from 8 to 10 h p.i. allowed for the detection of only the immediate early ICSP. When CH was present from 0 to 8 h p.i. and the cells were pulsed 10 to 12 h p.i., both the immediate-early and the early ICSP were synthesized (unpublished data). The regulation of CMV protein synthesis is being investigated further.

During the synthesis of the immediate-early and early ICSP, there was little host cell protein synthesis under the conditions of the experiment. In contrast, after the synthesis of these ICSP, host protein synthesis was again detectable (Fig. 6). It is possible that the immediate-

FIG. 4. Autoradiogram of electrophoretically separated polypeptides synthesized in CMV-infected permissive HF cells and nonpermissive GPEF cells at early times after infection. Infected (I) and uninfected (U) cells were treated with CH at 2 h p.i. for various intervals. CH was removed before pulse-labeling for 2 h with [35 S]methionine in hypertonic medium as indicated above. ICSP are denoted by their apparent molecular weight ($\times 10^3$).

early and/or early ICSP are proteins that stimulate host cell macromolecular synthesis. Alternatively, the host cell may have recovered from the initial inhibitory effect of the viral inoculum on host-specific protein synthesis (37).

To correlate the synthesis of early and late

ICSP synthesis with viral DNA replication, radioactivity contained in individual polypeptide bands was subtracted from the corresponding uninfected cell values as described in Materials and Methods, and these data were related to the time course of CMV DNA replication in the

FIG. 5. Autoradiogram of electrophoretically separated polypeptides from CMV-infected permissive HF cells and nonpermissive GPEF cells synthesized at early and late times after infection. Cells were either treated with CH at 2 h p.i. or left untreated. The CH was removed before pulse-labeling for 2 h with [³⁵S]methionine in hypertonic medium as indicated above. Polypeptides associated with purified virions plus dense bodies were subjected to electrophoresis in parallel to infected HF cells pulsed from 72 to 74 h p.i. Structural viral polypeptides and ICSP are indicated according to their apparent molecular weight ($\times 10^3$).

infected cells as determined by DNA:DNA hybridization according to procedures described in Materials and Methods. Virus-induced polypeptides that could be cleanly cut from the gels were used in this analysis. The results (Fig. 7)

indicate that ICSP 75, 72, and 68 were the earliest of the virus-induced polypeptides, followed by ICSP 59 and 56. Subsequently, late ICSP 120, 110, 94, 83, and 68 were synthesized. The synthesis of the immediate-early and early ICSP

FIG. 6. Sequence of infected-cell-specific polypeptide synthesis in CMV-infected cells. Infected (I) and uninfected (U) cells were treated with CH for 8-h intervals or left untreated and then pulse-labeled for 2 h with [35 S]methionine as indicated above. Equal volumes of cell lysates were analyzed by slab gel electrophoresis and autoradiography as described in the text. ICSP are denoted by their apparent molecular weight ($\times 10^3$). Host cell polypeptides are either labeled with the letter h or not labeled at all.

preceded viral DNA replication, whereas the synthesis of late ICSP corresponded to the onset of viral DNA synthesis (Fig. 7). Whether ICSP 68 and 27 were the same protein synthesized early and late is not known. The immediate-early and early ICSP of CMV are listed in Table 1. These data indicate that virus-specific protein synthesis in CMV-infected cells is a sequential process that is partitioned into an early and a late phase, with the synthesis of late ICSP being closely associated with viral DNA replication. The reason for the slightly biphasic nature of viral DNA synthesis is not known. The cells for this experiment were infected with approxi-

mately 30 PFU/cell, and under these conditions the infection should have been synchronous. The cells were not synchronized for this experiment.

Relationship between the net amount of viral DNA synthesis and late ICSP synthesis. The appearance of early CMV-ICSP before viral DNA replication suggested that these proteins might have a functional involvement in the synthesis of viral macromolecules such as the viral DNA. If the early viral proteins and/or host-specific enzymes are nonfunctional, then viral DNA should not be synthesized. To test this hypothesis, CMV-infected cells were treated

FIG. 7. Time of appearance of CMV-induced proteins and of viral DNA in infected cells. Viral DNA synthesis was measured by DNA:DNA hybridization on nitrocellulose filters as described in the text. Levels of both early and late polypeptides synthesized at each time point were determined by scintillation counting of ^{35}S radioactivity contained in individual polypeptide bands of the SDS-polyacrylamide gels. Radioactivity present in the same regions of the corresponding uninfected cell sample was subtracted for each value, and the net amount was plotted.

from 0 to 30 h p.i. with one of the amino acid analogs canavanine and azetidine at concentrations of 100, 200, or 500 $\mu\text{g}/\text{ml}$. After removal of the amino acid analog, the infected cells were pulsed with [^3H]thymidine from 30 to 72 h p.i. or with [^{35}S]methionine from 72 to 74 h p.i. Viral DNA and host cell DNA in cellular extracts were separated by equilibrium centrifugation in CsCl , and the percentage of total viral or host cell DNA was calculated as described in Materials and Methods. Figure 8 illustrates the separation of viral DNA (1.717 g/ml) from host cell DNA (1.699 g/ml) by equilibrium centrifugation and the effect of the amino acid analogs from 0 to 30 h p.i. Canavanine at concentrations of 200 and 500 $\mu\text{g}/\text{ml}$ reduced viral DNA synthesis 23.5 and 41.1%, respectively, compared with the untreated control (Fig. 8). On the other hand, host cell DNA synthesis was not affected by 200 μg

of canavanine per ml but was reduced 37.6% by 500 $\mu\text{g}/\text{ml}$ (Fig. 8). With azetidine, concentrations of 200 and 500 $\mu\text{g}/\text{ml}$ reduced viral DNA synthesis 57.7 and 95.6%, respectively, compared with the untreated control, whereas host cell DNA synthesis was only reduced 6.3% by 200 $\mu\text{g}/\text{ml}$ and 52.0% by 500 $\mu\text{g}/\text{ml}$ (Fig. 8). The inhibition of viral and host-specific DNA synthesis in cells treated with the amino acid analog canavanine or azetidine indicated that the proteins required for viral and host cell DNA replication were not functioning. Assuming some association of viral DNA replication with the appearance of late ICSP, treatment with these analogs should indirectly affect the synthesis of the late ICSP. Figure 9 illustrates the effect of 100, 200, and 500 μg of either canavanine or azetidine per ml from 0 to 30 h p.i. on late ICSP synthesis at 72 to 74 h p.i. The late ICSP were

FIG. 8. Analysis of [^3H]thymidine-labeled DNA extracted from CMV-infected cells treated in the early phase of infection with the amino acid analog canavanine or azetadine. Cells were treated from 0 to 30 h p.i. with 200 or 500 μg of either canavanine or azetadine per ml or left untreated. After removal of the amino acid analogs, the cells were pulse-labeled from 30 to 72 h p.i. with [^3H]thymidine. Cell lysates were prepared, and equal volumes of DNA were centrifuged to equilibrium in CsCl as described in the text. The heavy DNA species is viral and the light species is host cell DNA. (A) Untreated; (B) 200 μg of canavanine/ml; (C) 500 μg of canavanine/ml; (D) 200 μg of azetadine/ml; (E) 500 μg of azetadine/ml.

identified by their electrophoretic mobilities and relative rates of synthesis as previously described (37). The relative amount of late ICSP synthesis decreased with increasing concentrations of the amino acid analog (Fig. 9), and consequently there appeared to be a direct relationship between the percentage of viral DNA synthesis and the relative amount of late ICSP synthesis. Viral DNA synthesis was only reduced 41.4% with 500 μg of canavanine per ml, and consequently late ICSP were detected (Fig. 9). In contrast, viral DNA synthesis was reduced 95.6% with 500 μg of azetadine per ml, and late ICSP were not detected (Fig. 9). At this high concentration of azetadine, both host cell DNA (see Fig. 8) and protein (Fig. 9) were still synthesized, albeit at lower relative concentrations. Hence, treatment of CMV-infected cells during the early stage of infection with amino acid

analogues suppressed viral DNA synthesis and, consequently, suppressed late ICSP synthesis. It is postulated that the immediate-early and early proteins of CMV and possibly host-specific enzymes such as thymidine kinase (6) are crucial for the replication of the viral DNA.

DISCUSSION

Although the functions of the early CMV proteins remain unknown, they have been implicated in the induction of CPE (9) and host cell macromolecular synthesis (10, 11, 34). In addition, a novel DNA polymerase found early in CMV-infected permissive or nonpermissive cells is considered to be an early viral protein (18, 20). Early protein(s) of CMV may also have DNA binding ability similar to that of certain virus-induced proteins of adenovirus (40) or herpes simplex virus (HSV) (28). Since an early anti-

FIG. 9. Effect of the amino acid analogs canavanine and azetadine on late ICSP synthesis. Cells were treated with amino acid analogs at 100, 200, or 500 µg/ml from 0 to 30 h p.i. or left untreated. Cells were pulse-labeled from 72 to 74 h p.i. with [35 S]methionine, and cell lysates were analyzed by slab gel electrophoresis and autoradiography as described in the text. ICSP are indicated according to their apparent molecular weight ($\times 10^3$). Host cell polypeptides are either labeled with the letter h or not labeled at all.

gen(s) of CMV is detectable both in the nucleus of productively infected cells (12) and in CMV-transformed cells (14), one or more of the early proteins of CMV may be sufficient to initiate and to maintain the transformed state. In this

investigation, the early ICSP of CMV were identified in infected cells according to the following criteria: (i) the virus-induced polypeptides had electrophoretic mobilities different from that of host cell polypeptides; (ii) after the removal of

CH, the polypeptides were synthesized in infected cells at relatively high rates compared with host cell proteins; (iii) the polypeptides were synthesized before viral DNA replication; and (iv) the polypeptides were synthesized in permissive cells treated with an inhibitor of viral DNA replication and in nonpermissive cells that do not permit viral DNA replication. Using one or more of these criteria, the polypeptides presented in Table 1 are considered to be early virus-induced polypeptides of CMV. At least 10 early ICSP of CMV were identified in permissive HF cells treated with CH before pulse-labeling in hypertonic medium. Six of these, ICSP 72, 68, 59, 56, 53, and 39, were synthesized at relatively high molar ratios and were easily identified in infected cells treated to inhibit viral DNA synthesis. All the early ICSP were positively identified in nonpermissive cells except ICSP 59 and 21. Since the early ICSP of CMV were synthesized at the same relative times and rates in both permissive HF and nonpermissive GPEF cells, they are hypothesized to be coded by the CMV genome. Although these results do not establish definitively the viral specificity of early ICSP, *in vitro* translation of early CMV proteins from infected-cell mRNA selected by hybridization to CMV DNA would establish the origin of these gene products. It is postulated that all 10 of the early ICSP described in the present study are coded by the CMV genome. In addition, two early glycoproteins, gp145 and -100, have been identified (37). They were detected at approximately 15 h p.i. and are synthesized in the presence of an inhibitor of viral DNA replication (37). Assuming no precursor-product relationships, the combined molecular weight of the early viral proteins and glycoproteins (7.3×10^5) corresponds to about 12% of the asymmetric coding capacity of a viral genome with an assumed molecular weight of 1.5×10^8 (23) and an average polypeptide molecular weight of 85,000.

The mechanism by which CH pretreatment and hypertonic conditions for pulse-labeling selectively suppressed host protein synthesis is not known. It has been suggested that these treatments may result in a more efficient initiation of viral mRNA translation and certain host mRNA relative to the majority of the host cell mRNA (26, 30). Early CMV-induced proteins were synthesized immediately after the removal of CH added at 0 or 2 h p.i., but these proteins were not detected in similarly treated uninfected cells. The initial rates of synthesis of many of the early ICSP increased with the length of the CH treatment. Some of the early ICSP synthesized immediately after the removal of CH have mRNA's that are stable for approximately 14 h. Stability of these early mRNA's is probably

crucial to the replication of the viral DNA and late genome expression. Nevertheless, the early viral mRNA's may undergo transient synthesis or a controlled translation, since the addition of CH at later times after infection resulted in the detection of relatively lower molar ratios of the early ICSP.

The sequence of CMV-induced early protein synthesis occurred in two stages. There were three immediate-early proteins synthesized before the rest of the early viral proteins. Pulse-chase experiments have indicated that immediate-early proteins are not precursors to the lower-molecular-weight early proteins (unpublished data). The data suggest that the immediate-early proteins are a class of proteins different from the early proteins. The control of the immediate-early proteins on sequential transcription and translation of the CMV genome is presently being investigated. The observations that the immediate-early and early ICSP were synthesized before viral DNA replication and that the majority of these early ICSP had electrophoretic mobilities and relative rates of synthesis different from those of the late ICSP suggest that most of the early virus-induced proteins are nonstructural proteins. Since addition of amino acid analogs, which cause improper folding of the polypeptide chains, suppressed viral DNA synthesis, one or more of the early virus-induced proteins and/or host-specific enzymes may be required for viral DNA replication. In these experiments, viral DNA synthesis was initiated at approximately 15 h p.i. The long delay in the initiation of CMV DNA synthesis compared with HSV appears to be related to the stoichiometry of the synthesis of the early viral proteins and the resumption of host-specific protein synthesis. We have previously demonstrated that approximately 40% of the total protein synthesis in the late phase of infection is host specific (37). This type of virus-cell interaction may promote the tendency of CMV to produce chronic and latent infections *in vivo*. The relationship between early viral protein synthesis and the propensity for CMV to cause chronic or latent infections is speculative. If early viral genome expression is affected by the physiological state of the cells, early viral proteins may be synthesized at relatively low molar ratios, and consequently replication of the genome will proceed at a slow rate. On the other hand, a failure of the immediate-early proteins to trigger further macromolecular synthesis may cause the CMV genome to enter a latent state.

The prototype of the herpesvirus classification group, HSV, replicates in both human and animal cells, and the replication process is complete within 18 h (31, 32). Cells infected with HSV

type 1 undergo a sequential synthesis of ICSP that is coordinately regulated. Nonstructural proteins are synthesized first and structural proteins are synthesized last, but the transition from one class of proteins to another does not require viral DNA synthesis (19). In contrast, early and late CMV genome expression was partitioned by the replication of the viral DNA. The initiation of CMV DNA replication heralds the onset of the late phase of the productive cycle. The relative amount of late genome expression was directly related to the amount of viral DNA synthesis. Consequently, nonpermissive GPEF cells, which are restrictive for CMV DNA replication (unpublished data), synthesized early but not late ICSP of CMV. The reason why only human cells support CMV DNA synthesis is not known. The block to CMV DNA synthesis in nonpermissive cells may be due to (i) nonfunctional early proteins, (ii) absence of a crucial early protein(s), or (iii) absence of a host cell factor necessary for CMV DNA replication.

Studies on the function of the early CMV proteins and their interaction with the host cell may provide insight into the nature of CMV latent infection (29) and cellular transformation (1, 13). It is postulated that early CMV genome expression occurs in cells destined to latency or transformation, but viral DNA replication and late genome expression is blocked. It is possible that the nature of the interaction between the early CMV proteins and the host cell plays an important role in these virus-cell relationships.

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ADDENDUM

Simultaneously with completion of this manuscript, a publication appeared indicating that CMV DNA synthesis began at 16 h p.i. and reached a maximum at 36 h. The rate of DNA synthesis then declined and was subsequently followed by a second increase. The authors hypothesized that the rate of CMV DNA synthesis is related to host cell DNA synthesis and the S-phase of the cell cycle. (S. C. St. Jeor and R. Hutt, *J. Gen. Virol.* 37:65-73, 1977).

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